Expression and Purification of the Tumor Suppressor INI1

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Abstract

The absence or mutation of a chromatin remodeling protein called INI1, part of the SWI/SNF complex, is linked to the formation of a pediatric cancer called Atypical Teratoid Rhabdoid tumors (AT/RT) which produces tumors on the brain and spinal cord of infants. Due to the low survival rate of this condition and the lack of research on INI1, researchers want to learn all that they can about this protein in hopes to find a cure. We intend to determine the structure of INI1 bound to chromatin using X-ray crystallography and determining if and where INI1 binds to DNA. In preparation for this work, I have expressed and isolated multiple batches of full length INI1 along with five other truncations (1-186, 186-385, 1-104, 259-385, 31-186). To express and isolate the protein and protein fragments, I used heat shock transformation, inoculation, induction, sonication, Ni-resin affinity chromatography, and elution and then visualized the resulting product using SDS-PAGE gel electrophoresis. All of the protein and protein fragments are now at least 50% isolated. Future projects will include further isolating the samples, determining the domains of INI1 which bind to chromatin using gel shift assay, and determining INI1's structure using X-ray crystallography.

Keywords: INI1, Atypical Teratoid Rhabdoid tumors (AT/RT), expression, isolation, X-ray crystallography

Introduction

Atypical teratoid rhabdoid (AT/RT) tumors are deadly forms of pediatric cancer. The tumors attack the central nervous system and usually develop either in the brain or in the spinal cord. As of now, there is no known cure for this disease, which results in a 10% survival rate in children under three and those who do survive suffer from severe developmental defects (Kim *et al.* 2014). Versteege *et al.* (1998) described defining characteristics of AT/RT tumors to try to find a cure. They discovered that when looking at the karyotype of the AT/RT tumor cells, unlike normal cancer cells whose chromosomes are completely scrambled, AT/RT patients have a normal karyotype apart from the deletion or mutation of one gene. The absence or mutation of the gene that codes for INI1 protein, a protein that is known to be part of the chromatin remodeling complex SWI/SNF, is believed to be linked to the development of AT/RT tumors (Versteege *et al.* 1998).

In early 2000, a team of researchers set out to determine if SWI/SNF complexes and INI1 are essential for embryotic development and to explore the role of INI1 in the formation of cancer (Klochendler-Yeivin *et al.* 2000). The researchers mutated the INI1 gene to make it dysfunctional and inserted the mutated gene into embryonic stem cells of mice. All of the mutated embryos died, while all of the wild type embryos survived. They concluded that INI1 must be essential for the growth of embryonic cells and its loss results in cell death (Klochendler-Yeivin *et al.* 2000). They also concluded that the widespread expression of INI1 in the natural embryos suggests that SWI/SNF chromatin remodeling activity may be essential to early development of life (Klochendler-Yeivin *et al.* 2000). Previous studies showed that the mutation or absence of the INI1 protein correlates strongly to the AT/RT pediatric cancer (Sevenet *et al.* 1999). The team monitored 124 mice with a heterozygous INI1 mutation gene for potential development of tumors and found that all of the mutated mice formed AT/RT tumors, while none of the wild type mice formed tumors. These data provide direct evidence that loss of INI1's function results in tumor formation in mice, suggesting that INI1 acts as a tumor suppressor gene because tumors only appeared when the protein was removed (Klochendler-Yeivin *et al.* 2000).

When packaged into chromosomes, chromatin is wrapped around structural proteins (histones) to form nucleosomes. For the transcription enzymes to transcribe properly, the nucleosomes must be removed. It is now known that the SWI/SNF complex's job involves chromatin remodeling where it unwraps the chromatin from the structural proteins and opens the chromatin so that the transcription enzymes can work effectively (Tang *et al.* 2010).

Past studies of INI1 have shown what INI1 affects but not what its function is. We hypothesize that INI1 escorts SWI/SNF to the specific locations of DNA that are relevant to the function of the SWI/SNF complex. The loss of INI1 results in the SWI/SNF complex not finding the proper location on DNA, making the cell dysfunctional and cancerous. The goal of our project is to determine the structure of INI1 and determine its binding domain(s). By doing this, we can determine if and how INI1 aids in the transcription process which can lead to helping find a cure for AT/RT tumors. This past semester, we have been expressing and isolating full length INI1 in anticipation of determining INI1's structure and we have been expressing and purifying truncations of INI1 in anticipation of determining INI1's binding regions.

Methods

Plasmids

A set of SKB3 vectors that include the entire coding region for INI1 or a truncation of INI1 with a His-tag (six histidine codons in a row) were used. Previously in the Ruhl lab, these were cloned and sequenced to ensure that the right gene or truncation is present and that the his-tag, start codon, and stop codon are also present.

Transformation

First, the SKB3 vector with the insert was put on ice while allowing the BL21 *Escherichia coli* cells to thaw. 20 µL of the BL21 *E. coli* cells were mixed with 2 µL of SKB3 vectors in a chilled tube. These solutions were incubated on ice for 30 minutes then placed in a 42° C water bath for exactly 30 seconds. Afterwards, the tubes were immediately put the tube on ice again for a minute and thirty seconds. Next, 180 µL of SOC broth was added to the tubes and incubated in a 37° C water bath for one hour. All of the resulting solution was added to 5 ml of Luria broth media with Kanamycin (10 mg/ml) and then grown in a 37° C shaker at 250 RPM overnight.

Inoculation and Induction

One mL of the overnight bacteria culture was dispensed into 400 mL of Luria broth media with Kanamycin (10 mg/ml) that was shaking at 250 RPM at 37° C environment in a New Brunswick Scientific C24 Incubator Shaker®. Then, 1 mL of sample was transferred to a cuvette the OD was read in a Beckman Coulter DU®730 spectrometer. A starting OD reading between 0.05 - 0.07 was ideal. Once in the range, the bacteria were incubated under the same conditions until the OD reached 0.6. At that point, 400 µL of IPTG were added to induce the bacteria which were then incubated to produce protein while staying at 250 RPM and remaining in a 37° C environment for two hours.

Once the two hours were complete, the samples were aliquoted into two centrifuge bottles and were spun down for five minutes at 5000 RPM (4068 RCF) using a Sorvall® RC-5B Refrigerated Superspeed Centrifuge with a GSA rotor. The supernatant was poured off and the pellets were stored at -80° C.

Sonication

Ten mL of high salt extraction buffer pH 7.5 (10 mM Tris pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 10% glycerol, 10 mM imidazol, 0.1% NP 40) were added to each pellet and were vortexed using a Fisher Scientific Vortex Genie 2® until no pieces were visible then were poured into two 30 mL beakers and were put on ice. Next, each of the beaker's contents were sonicated for two minutes at 20% of full power using a Fisher Scientific Sonic Dismembrator® FB-120 model for ten seconds on and ten seconds off. After ensuring that the procedure worked by observing a layer of foam at the top of the sample, the contents were poured into a centrifuge tube. Centrifuge tubes and their contents were balanced until they weighed about the same. The centrifuge tubes were put in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge with the SA600 rotor for 10 minutes at 15,000 RPM (32,571 RCF). The supernatant and an extract sample were stored at -80° C.

Ni-Resin Affinity Chromatography

In a 2 mL microfuge tube, 200 µL of Ni-NTA resin and 1 ml of equilibration buffer pH 7.5 (10 mM Tris pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 10% glycerol, 0.1% NP 40) was added and centrifuged using a VWR Micro 1814 centrifuge® for five minutes at 4,000 RPM (1,300 RCF) in 2.8° C. After the five minutes, the supernatant was pipetted off and another 1 mL of equilibration buffer was added. This process was repeated two more times for a total of three rounds of equilibration buffer spin downs. After three cycles the resin was kept on ice until needed.

To mix the resin with the protein sample, some of the protein sample was pipetted into the resin, and then some of the resin/sample mixture was pipetted back into the conical tube and this process was repeated until little to no resin was present in the resin tube. To allow the his-tag to bind to the resin, the mixture was put on a rocker in a refrigerated area for two hours.

Table 1: Running Gel Preparation		
Substance	Amount for a 12% gel prep (INI1 full length)	Amount for a 15% gel prep (truncations of INI1)
30% acrylamide	9.00 ml	11.00 ml
1.5 M Tris pH 8.8	5.60 ml	5.60 ml
Deionized H2O	7.60 ml	5.60 ml
10% SDS	225 µl	225 µl
TEMED	11.25 µl	11.25 µl
APS*	112.5 µl	112.5 µl
*APS is made by dissolving 0.1 grams of Ammonium Persulfate into 1 ml of deionized water		

Table 2: Stacking Gel Preparation	
Substance	Amount
30% acrylamide	1.89 ml
1.5 M Tris pH 8.8	2.49 ml
Deionized H2O	10.2 ml
10% SDS	150 µl
TEMED	15 µl
APS (same solution made for running gel prep)	150 µl

To prepare the column, a pinch of glass wool was inserted into a clean Kontes Flex-Column™. Then, to wash the column, at least 10 mL of high salt wash buffer pH 7.5 (10 mM Tris pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 10% glycerol, 15 mM imidazol, 0.2% NP 40) was run through it.

In a refrigerated room, after two hours on the rocker, the resin/sample mixture was poured into the column and was watched to make sure that a layer of resin was forming on top of the glass wool. To get as much protein as possible, poured some of the flow through was poured back into the original container and was swirled to wash the remaining resin off the sides and then was poured it back into the column. Afterwards, a flow through sample was taken and stored at -80° C. Next, using a transfer pipet, high salt wash buffer was layered into the column carefully to ensure that the resin was not disturbed. The column washed overnight and a sample of the wash that ran through the column was collected in the morning and stored it in the -80° C freezer. Six 1 mL samples were eluted with elution buffer pH 7.5 (10 mM Tris pH 7.5, 200 mM NaCl, 10% glycerol, 250 mM imidazol, 0.1% NP 40) and stored the samples at -80° C.

SDS Acrylamide Gel Electrophoresis

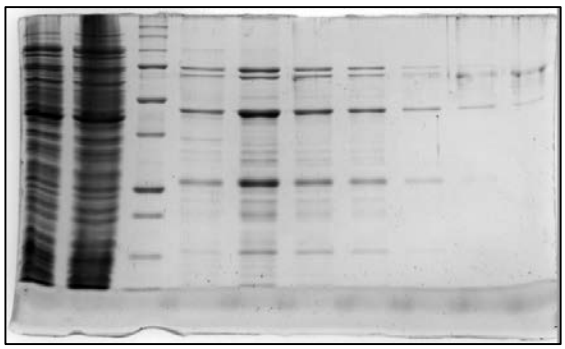

Figure 1| *Full length INI1 SDS PAGE gel. Lane 1: Extract; Lane 2: Flow through; Lane 3: Standard Ladder; Lane 4-10: Fractions 1-6. This sample is about 50% purified.*

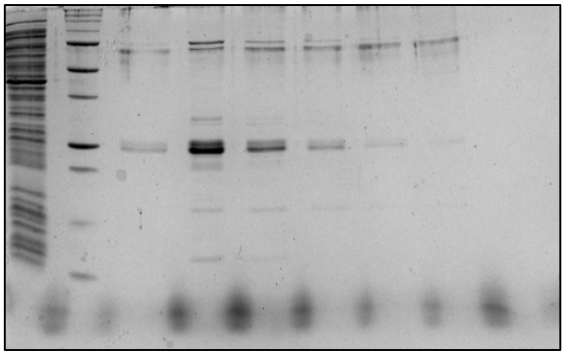

Figure 2| *1-186 INI1 truncation SDS PAGE gel. Lane 1: Flow through; Lane 2: Standard Ladder; Lane 3-9: Fractions 1-6. This sample is about 90% purified.*

To visualize the protein collected, SDS-PAGE gels were utilized. Full-length INI1 used 12% gels while the truncations of INI1 used 15% gels (Table 1). After making the stacking gel (Table 2), the stacking gel solution was pipetted onto the running gel until it was overflowing. A comb was inserted while the gel polymerized.

To prepare the samples, 19 µL of the sample (elution tubes 1-6, extract, flow through, or wash), 5 µL of 10% SDS (28.83 g/L), and 8 µL of 4X were mixed together. The 4X was prepared by mixing 90 µL of 4X Sample Buffer pH 6.8 (0.25 M Tris-Cl pH 6.8, 10% SDS, 50% glycerol, 0.04% Bromophenol blue, deionized H2O) with 10 µL of β-mercaptoethanol. After the sample solution is made, the samples were boiled for one minute then pulsed in a VWR Micro 1814 centrifuge®.

For the standard ladder, 10 µL of Biorad's© Unstained Protein Ladder was loaded into the gel. When the gel was ready, 1X SDS Running Buffer pH 7.5 (192 mM Glycine, 25 mM Tris, 0.1% SDS) was poured around it. The gel was run at 150V until the blue stain reached the bottom of the gel. Once completed, the gel was washed with deionized H2O then stained overnight in Thermo Scientific GelCode™ Blue Safe Protein Stain.

Progress to Date

As of now, all six batches of full length INI1 and all of the INI1 truncations have been run on a gel and are at least 50% purified. INI1 contains roughly 1155 base pairs which translates to 385 amino acids. We can safely assume that the dark line visible at roughly 47,000DA is INI1 (Figure 1).1-186 INI1 truncation contains 558 base pairs which translates to 558 amino acids. We can safely assume that the dark line visible at about 22,000DA is 1-186 (Figure 2). Since there are other impurities in the lane, we must further purify the samples. All successful gels looked similar to Figures 1 and 2.

Discussion

So far, we have all of the full length INI1 samples and truncation of INI1samples (1-186, 186-385, 1-104, 259-385, 31-186) at least 50% purified. In regards to full length INI1, to conduct X-ray crystallography, we will need about 15 mg of 85% purity INI1. As of right now, we do not have enough to move on so more expressions and isolations are necessary. Therefore, the next step is to further purify all of the samples using additional sequential chromatography techniques including size exclusion chromatography and ion exchange chromatography. Next, we will use the truncations of INI1 to determine what specific domain of INI1 binds to DNA using gel shift assays. Determining INI1's binding domain(s) can help deduce INI1's function. Lastly, since INI1's structure has yet to be determined by modern research, we plan to determine it through X-ray crystallography which will allow us to "take a picture" of INI1. If we determine that INI1 does bind to DNA, we would want to preform X-ray crystallography while INI1 is attached to DNA so we can physically see what binding domain INI1 is using.

References

Kim, K. H., and C. W. M. Roberts. 2014. Mechanisms by which SMARCB1 loss drives rhabdoid tumor growth. *Cancer Genetics* 207:365-372.

Klochendler-Yeivin, A., L. Fiette, J. Barra, C. Muchardt, C. Babinet, and M. Yaniv. 2000. The murine SNF5/INI1 chromatin remodeling factor is essential for embryonic development and tumor suppression. *EMBO Reports* 1:500–506.

Sevenet N., Sheridan, E., Amram, D., Schneider, P., Handgretinger, R. and Delattre, O. 1999. Constitutional mutations of the hSNF5/INI1 gene predispose to a variety of cancers. *American Journal of Human Genetics* 65:1342–1348.

Tang, L., E. Nogales, and C. Ciferri. 2010. Structure and function of SWI/SNF chromatin remodeling complexes and mechanistic implications for transcription. *Progress in Biophysics and Molecular Biology* 102:122–128.

Versteege, I.N. Sevenet, J. Lange, M.F. Rousseau-Merck, P. Ambros, R. Handgretinger, A. Aurias, and O. Delattre. 1998. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* 394:203-206.