Phosphorylation of INI1 Protein *in vitro*

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Abstract

SWI/SNF mutations occur in twenty percent of all human cancers (Kim *et al.* 2014). Integrase Interactor 1 (INI1) is a key component of the SWI/SNF remodeling complex that acts as a tumor suppressor, and mutations or deletions of this gene are linked to atypical teratoid/rhabdoid tumors (Brenca *et al.* 2013). A basic knowledge of INI1 and its cellular mechanisms is crucial to understanding the protein's role in the cell. In this study, we focus on the phosphorylation of INI1 protein to determine which protein kinase interacts with the protein in vitro. To date, one protein kinase (PKA) has been tested in a kinase assay and five others will be tested in the future. Though the results of the PKA reaction are inconclusive, further kinase assays will be performed to ascertain whether or not this kinase successfully phosphorylates INI1.

Keywords: Phosphorylation, INI1, in vitro PKA, at/rt

Introduction

INI1, also referred to as SMARCB1, SNF5, and BAF47, is a tumor suppressor gene located in the SWI/SNF chromatin remodeling complex on chromosome 22q11.2. Inactivation of the INI1 gene due to de novo truncations or deletions is found in approximately 70% of atypical teratoid/rhabdoid tumors (at/rt), a type of pediatric cancer located in the central nervous system (Judkins *et al.* 2005). Treatments for these tumors are ineffective, and children diagnosed with at/rt usually have a survival time of under 1 year (Chen *et al.* 2005). Little is known about the function of INI1 within the cell, and understanding its basic cellular mechanisms could reveal more about its influence on tumor development.

Studies have revealed DNA binding properties of SWI/SNF that are linked to the regulation of gene expression. INI1 is one of the subunits of SWI/SNF that binds to DNA in region 105-186 of the protein and may affect the transcriptional factors associated with protein expression (Das *et al.* 2013). Within the DNA binding domain is a phosphorylation site at serine residue 129 (Matsuoka 2007). The reversible phosphorylation of a protein by a protein kinase influences several eukaryotic cellular mechanisms including metabolism, signaling, and transcription (Yoshizaki *et al.* 2015). It is unknown which protein kinase interacts with INI1, but identification of the kinase could reveal information about the protein's role within both normal and cancerous cells. Furthermore, the phosphate group could impact the DNA binding mechanisms of INI1 in region 105-186.

The objective of this study is to identify which protein kinase interacts with INI1 in vitro. The protein was purified, and data from bioinformatics sites were combined to narrow down which protein kinases most likely phosphorylate INI1. Protein kinase A (PKA) was tested in a kinase assay, and other protein kinases will be tested in the same manner to determine which phosphorylates INI1 at serine residue 129.

Methods

Protein Purification

A vector encoding INI1-243, which includes base pairs 105-186, and kanamycin resistance was introduced to BL-21 *Escherichia coli* cells through heat shock treatment. Once the cells were placed in 800 mL of Laurel Broth and incubated at 37° C overnight, 2.4 mL kanamycin was added to the media. The cells were tested by ultraviolet visible (UV) spectroscopy to ensure an optical density of 0.6 at 600 λ. The protein was expressed by Isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were centrifuged inside a GSA rotor for the DuPont Sorvall RC-5B Refrigerated Superspeed Centrifuge at 16000 rpm to form a pellet.

Pellets were suspended in high salt Lysis extraction buffer (10 mM Tris pH 7.5, 0.5 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.1% NP-40, 10 mM imidazole, protease inhibitors) and sonicated for 2 minutes to break open the cells. The sample was centrifuged at 13000 rpm in an SA-600 rotor for 20 minutes and the supernatant was collected. The

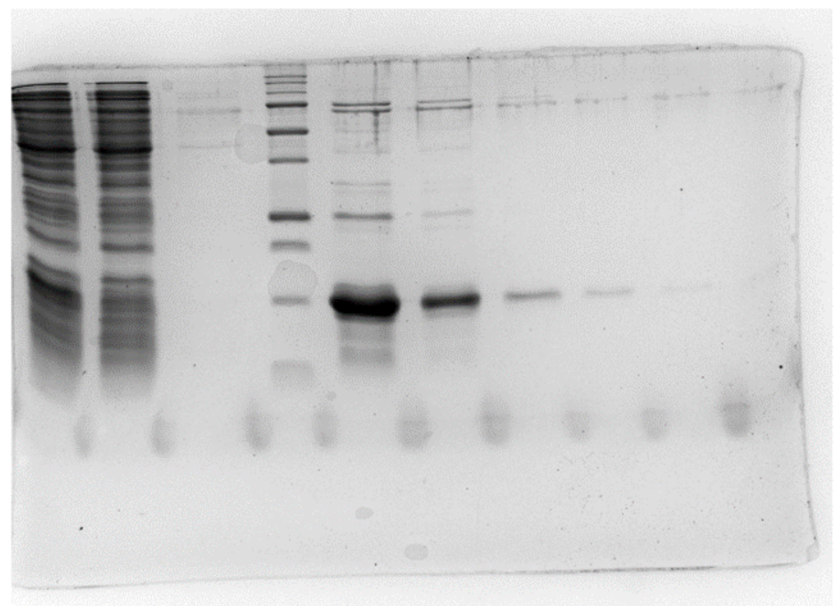

Figure 2 *- The dark bands in lanes 5-10 indicate INI1-243. The protein has a molecular weight of 9.4 kD and the bands are located just below the 15 kD mark on the corresponding molecular weight standard. Therefore, INI1 was successfully isolated*

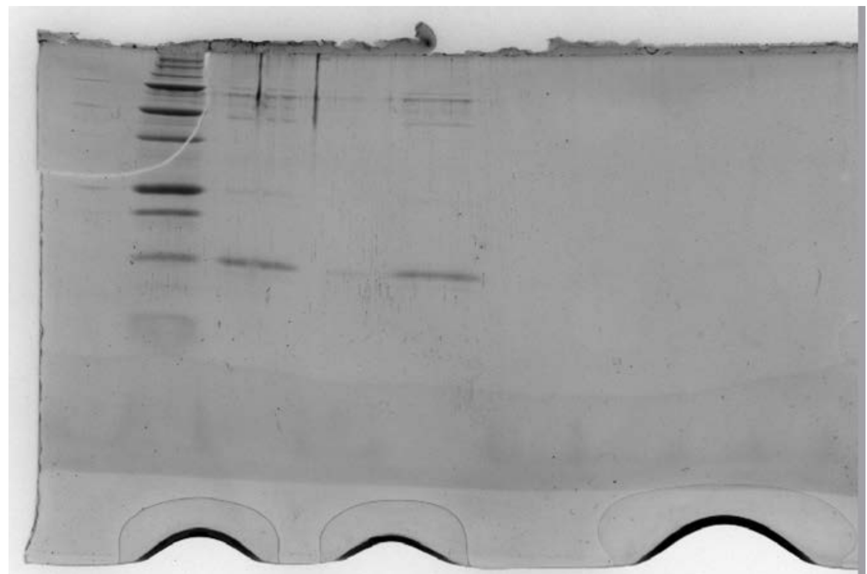

Figure 1 *- The potentially phosphorylated sample is in lane 3 while the negative control is in lane 5. The band of protein in lane 3 appears slightly higher than that in lane 5. This could be due to a phosphorylation or the position of the gel. Another kinase assay will need to be performed to obtain clear results.*

supernatant was rocked over nickel-resin at 4º C for 2 hours and run through a cylinder with glass wool to rid of extra proteins expressed by the *E. coli* cells. The protein was washed with a high salt wash buffer (10 mM Tris pH 7.6, 0.5 M NaCl, 10% glycerol, 15 mM imidazole, 0.2% NP-40, 1.0 mM DTT, 0.5 mM PMSF) then eluted with elution buffer (10 mM Tris pH 7.5, 200 mM NaCl, 10% glycerol, 250 mM imidazole, 0.1% NP-40, 1.0 mM DTT, 0.5 mM PMSF) and collected in six 1 mL fractions.

<u>*SDS Page*</u>

A 15% resolving gel was poured using 30% acrylamide, 1.5 M Tris pH 8.8, 10% SDS, TEMED, and APS. The separating gel was made using 30% acrylamide, 1.0 M Tris pH 6.8, 10% SDS, TEMED, and APS. Protein fractions were prepared with 4X

sample buffer (0.25 M Tris-Cl pH 6.8, 2% SDS, 10% mercaptoethanol, 20% glycerol, 0.01% Bromophenol Blue) and 10 µL were loaded into the gel. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run at 150 V and the gel was stained with Thermo Scientific Gel CodeTM Blue Safe Protein Stain.

Kinase Assay

Results from KinasePhos and PhosphoNet were used to determine which kinases should be used in a kinase assay. 1 µL Protein Kinase A, 1 µL 2 mM ATP, 1 µL 10X reaction buffer (25 mM Tris pH 7.5, 10 mM MgCl2, 2 mM DTT, 0.1 mM EDTA, and 0.01% NP-40), and 1 µL ultrapure H2O were combined for a total volume of 10 µL. A negative control was also made, substituting the ATP for water. Each sample was incubated at 30º C for 30 minutes, and the reaction was stopped with the addition of 2.5 µL of 10% SDS and 4 µL of 4X sample buffer. The samples were boiled at 100º C for approximately 3 minutes and loaded into a 15% SDS-PAGE. The gel was stained with the aforementioned staining product.

Progress to Date

Lane one of the gel contained 25 µL of the protein extract from the *E. coli* cells, lanes 2-3 contained 25 µL of the flow through from the wash, and lane 4 contained 10 µL of a molecular weight standard for INI1-243. Lanes 5-6 were loaded with 10 µL of each fraction from the elution. INI-243 was calculated to have a molecular weight of 9.4 kD, and bands from the sample fractions were below the corresponding 15 kD standard mark, so it is safe to assume that INI1-243 was successfully isolated (see figure 1).

Bioinformatics sites KinasePhos, at 90% prediction specificity, and PhosphoNet were used to determine the protein kinases most likely to phosphorylate INI1. Sequences in INI1 were compared to sequences from other proteins that have been phosphorylated experimentally. The top kinases predicted to phosphorylate INI1 are as follows: PKA, protein kinase B, protein kinase C, protein kinase G, calmodulin-dependent protein kinase 2, and cyclin-dependent kinase 9. These kinases will be tested individually in a kinase assay, and another SDS-PAGE will be run to determine any shift in molecular weight of the protein.

The acrylamide gel contained 10 µL of a molecular weight standard in lane 2, 10 µL of the test sample in lane 3, and 10 µL of the negative control in lane 5. The band of protein would be slightly more than 9.4 kD if the phosphorylation reaction was successful, and the band appeared to have shifted somewhat (Figure 2). However, the shift only differs from the negative control by a very small margin and could have been caused by the position of the gel during imaging. There is not enough evidence to draw definite conclusions about the phosphorylation of INI1-243 by PKA.

Discussion

Due to the inconclusive results of the kinase assay with PKA, another assay will be run with an 18% gel to give a better indication of whether or not the molecular weight has changed. The assay will include another negative control, substituting PKA for water, and histones will be used as a positive control. When loading the gel, one lane will contain the sample while another lane will contain both the sample and a negative control to help identify two distinct bands. If two bands are visible in the gel, we can conclude that the phosphorylation reaction was successful.

The five remaining protein kinases will be incorporated into a kinase assay with INI1-243 and an appropriate buffer following the protocol above. These kinase assays will be run on an 18% gel as well to determine a change in the molecular weight of the protein. Once a molecular weight is detectable, the sample will be analyzed by mass spectrometry to determine the site of the phosphorylation group.

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