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13. ABSTRACT (Maximum 200 Words)

Mutations in the tumor suppressor gene, BRCA1 account for 45% of families with a high incidence of breast cancer and the majority of families with high incidences of both breast and ovarian cancers. Recent data has shown BRCA1 to be associated to be associated with a human SWI/SNF complex, serving to link breast cancer to chromatin remodeling (3). Current evidence points to the idea that BRCA1 works through SWI/SNF; therefore a molecular understanding of the SWI/SNF complex and other human chromatin remodeling complexes will offer insight into the biology of BRCA1. The central catalytic ATPase subunit of SWI/SNF is BRG1; the central catalytic subunit of a related human chromatin remodeling complex, NURF, is SNF2H. Initially, crystallization and X-ray structural determination of the core ATPase domain, in addition to the full-length proteins was undertaken unsuccessfully. The core domains and the full-length proteins behave poorly in solution and proved unsuitable for formation of successful crystals. Currently, purification of the entire SWI/SNF and NURF complexes, as well as functional cores of these complexes is underway. This will alleviate the stability problems, and offer more structural and mechanistic insight into how chromatin remodeling complexes interact with BRCA1.

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Introduction:

Breast cancer is the most common malignancy in women in the Western world (1). BRCA1 is a tumor suppressor gene linked to familial breast and ovarian cancers (4, 5). Current evidence has led to the hypothesis that BRCA1 may function as a transcriptional regulator (2). The strong correlation of cancer-associated mutations and its loss of function phenotype in recent experiments, strengthen the idea that the role of BRCA1 in transcription is physiologically relevant during the development of disease (2). Furthermore, BRCA1 has been linked to chromatin remodeling complexes, specifically through the stable association of the BRCA1 protein with BRG1, the catalytic subunit of the SWI/SNF chromatin remodeling complex (3). This recent evidence that implicates BRCA1 as a component of a human SWI/SNF chromatin-remodeling complex implicate chromatin-remodeling complexes in the pathogenesis of breast and ovarian cancers (3).

Chromatin remodeling is associated with gene expression and remodeling and the subsequent assembly in an energy dependent manner of both activating and repressive proteins in the nucleosomal infrastructure. The mistargeting of these enzymes contributes to human developmental abnormalities and tumorigenesis, illustrating the role of chromatin remodeling complexes in human disease (6). Regulation of gene expression occurs in the context of chromatin whose structure inhibits transcription at various levels, including activator binding, preinitiation complex formation, and transcriptional elongation (7). In the past five years, numerous ATP-dependent chromatin-remodeling complexes have been isolated and characterized.

All of the ATP-dependent chromatin-remodeling complexes contain an ATPase subunit that belongs to the SNF2 superfamily of proteins. Enzymes that resemble SWI2/SNF2 in their ATPase domains form a distinct subfamily within the family of nucleic-acid-stimulated DEAD/H ATPases (8). The SWI2/SNF2 subfamily can be further divided into at least four groups of enzymes, according to the domains flanking their ATPase domains, biochemical properties, and mechanisms of nucleosome remodeling. BRCA1 has been found to be associated with a human SWI/SNF (hSWI/SNF) complex, which contains either BRG1 or hBRM as the central ATPase (8). The SWI/SNF complexes contain eight or more subunits, and each contain a DNA-dependent ATPase homologous to that of yeast SWI2/SNF2. It has been demonstrated that an efficient ATP-dependent remodeling complex can be constituted using a subset of the proteins found in the human SWI/SNF complex, specifically BRG1, BAF170, BAF155, and INI1 with BRG1 as the central ATPase (8).

A second class of ATP-dependent chromatin remodeling complexes is the ISWI-based complexes, which contain 2-4 subunits including the central ATPase. The ISWI group includes yeast ISWI1 and ISWI2, *Drosophila* NURF, CHRAC, and ACF, and human WCRF/ACF, CHRAC, and RSF complexes (9). A four-subunit human NURF complex with the protein SNF2H as the central ATPase has recently been identified (data not published). It has only recently been demonstrated that the central ATPase subunits of both SWI/SNF and ISWI complexes can alter chromatin structure in the absence of any remaining subunits (10).

Taken together, chromatin-remodeling complexes, such as SWI/SNF and NURF play critical roles in gene regulation. Current evidence points to the idea that BRCA1 works through SWI/SNF, SWI/SNF and NURF complexes are related functionally and compositionally, and therefore a molecular understanding of the SWI/SNF and NURF complexes will offer insight into the biology of BRCA1.

Body:

The first task outlined in the approved statement of work was *the determination of the X-ray crystal structure of the ATPase domain of BRG1*. The first step to completing this goal was the purification of the recombinant ATPase domain of BRG1, after overexpression in *E. coli.* Clones for BRG1 and a BRG1 mutant were obtained from Ramin Shiekhattar (The Wistar Institute). The ATPase domain of BRG1 was identified from sequence homology comparison. Constructs for the ATPase domain were designed to include amino acid residues 759-1258 with a N-terminal histidine tag spanning the previously defined ATPase domain. The mutant contains lysine to arginine, and threonine to serine mutations at positions 798 and 799, respectively. This mutation abolishes ATP hydrolysis (11). The ATPase domain has been cloned into a T7 expression vector and transformed into a competent bacterial host, BL21 (DE3)/LysS, with this vector for overexpression in bacteria (12). Initial test inductions at 37 °C followed by cell disruption show that the protein was found almost exclusively in the insoluble pellet. Low temperature growths of many proteins often produce soluble protein, however a 15 °C growth of the ATPase domain of BRG1 did not enhance solubility. Expression in various bacterial cell lines and at different temperatures were attempted and failed to produce any significant increases in soluble protein. The insoluble pellet was resolubilized in 6 M urea and 6 M guanidine HCl, followed by either dialysis (to remove the denaturant) or direct application to a nickel-agarose affinity column, and subsequent washings to remove the denaturant. The protein remained insoluble. GST-fusion proteins were produced in the hope of enhancing solubility, and the protein was coexpressed in the presence of molecular chaperones (GroEL/ES, dnaK/dnaJ, and grpE), but the solubility was not enhanced (13).

A soluble ATPase domain of BRG1, was finally prepared with insect cells using a baculovirus expression system. The Kingston group at Harvard Medical School provided us with baculovirus containing the recombinant BRG1 ATPase domain from residues 728-1258. The virus was subsequently amplified and expressed in Sf9 insect cells and purified by FLAG affinitiy chromatography to greater then 90% by SDS PAGE. The production of the BRG1 ATPase was scaled up to a three-liter suspension, which yielded between 2-4 mg of purified protein after FLAG affinity purification. As an additional purification step, the purified protein was applied to an analytical Supadez-200 size exclusion column. Upon binding to this column, more then 90% of the protein aggregated, resulting in a dilute, impure sample unsuitable for crystallization trials. Increasing the ionic strength of the protein solution (by increasing salt concentration), and addition of ATP, decreased the aggregation rate. The protein was then concentrated, using centrifugal membrane devices from Millipore, to a concentration between 1.0 to 3.4 mg to protein/ml at greater than 95% purity by SDS-PAGE analysis (Fig. 1).

Initial crystallization factorial screens (14, 15) utilizing the hanging drop method were set up at 20 °C in the presence of ATP or a stable non-hydolyzable analogue, ATP-γ-S. Two different concentrations were used, and based upon the ratio of clear drops to those that showed precipitate, the concentration of protein must be greater then 2.0 mg/ml, for successful crystallization. To date, no crystals have been obtained. Additional crystallization trials at concentration of 2.0 mg/ml are directly underway.

An additional task, defined under the first goal was: *the purification of full-length BRG1 and limited proteolysis experiments, to again obtain enough protein to set up crystallization trials*. The Kingston group of Harvard Medical School provided baculovirus containing full-length recombinant BRG1 from residues 1-1647 with a N-terminus FLAG tag. After time course experiments, were used to identify the optimal expression conditions, the full-length recombinant BRG1 was expressed in Sf9

insect cells in three-liter suspensions for 48 hours. The full-length protein is purified by FLAG-affinity chromatography, followed by gel filtration. The intact protein is expressed less well than the recombinant BRG1 ATPase domain, and is less stable upon application to a size exclusion column. Insufficient amounts of protein are obtained for crystallization trials. Limited proteolytic digests of the full-length protein to define a proteolytic resistant core ATPase domain has not yet been undertaken.

Due to the difficulty encountered with both the full-length protein and the ATPase domain of BRG1, *crystallization of the full-length human SNF2H protein, the central ATPase of the ISWI class of ATP-dependent remodelers, was undertaken* in concert with the BRG1 crystallization. The Shiekhattar group at the Wistar Institute provided FLAG-tagged full-length SNF2H recombinant baculovirus. After optimization of expression conditions, production was scaled up to a 3-liter suspension, and approximately 6 mg of 90% pure protein was obtained (Fig. 2). Gel filtration chromatography was undertaken but protein aggregation again postponed any crystallization attempts.

Limited proteolysis using a battery of non-specific proteases was applied to FLAG-affinity purified full-length SNF2H, to identify stable domains of the protein. Limited proteolysis by tryptic digest resulted in a stable C-terminus SANT containing domain identified by mass spectrometry. Constructs for this stable C-terminus domain were constructed to include amino acids 762-1052 with a cleavable N-terminal histidine tag. The construct was purified by a combination of nickel-affinity, ion exchange, and size exclusion chromatography and initial factorial crystallization screens were positive. Unfortunately, two other groups in collaboration have already determined the crystal structure of this C-terminal domain of SNF2H *(in print)*.

The second task defined under the initial statement of work was: *determine the structure of a SWI/SNF complex containing the catalytic ATPase.* The preparation and purification of a functional BRG1 core SWI/SNF subcomplex, identified by Kingston (16), has been attempted. It has been demonstrated that the addition of INI1, BAF155, and BAF170 to BRG1 increases remodeling activity to a level comparable to that of the whole SWI/SNF complex (16). BRG1 has been co-expressed in Sf9 insect cells, with INI1, BAF155, and BAF170, but only BRG1 appears to be expressed. Expression conditions and the state of the recombinant baculoviruses for these three subunits need to be optimized before expression and purification at a scale suitable for crystallization can proceed.

Purification of nuclear extract from the Ini-I11 cell line to isolate BRG1 containing complexes, characterization to identify BRCA1 association, and subsequent limited proteolysis experiments have not yet been attempted.

In concert with the attempted crystallization of related SNF2H, the *production and purification of SNF2H-containg ISWI complex* is in its initial stages of development. The Shiekhattar group at The Wistar Institute has identified a novel human ISWI complex homologous to the *Drosophila* NURF complex. The catalytic ATPase is SNF2H surrounded by two subunits: BPTF and Rbap48. Currently protein co-expression time trial experiments in Sf9 insect cells, after infection with recombinant baculoviruses for each of these three subunits has been undertaken. It is hypothesized that the addition of the other two subunits to the catalytic ATPase to form a complete complex may alleviate the stability and aggregation problems encountered in the purification of full-length SNF2H.

Figures:

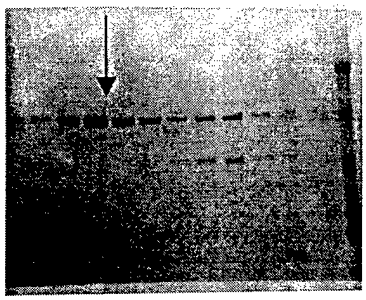

Figure 1: purified recombinant BRG1 ATPase domain after gel filtration (78 kDa).

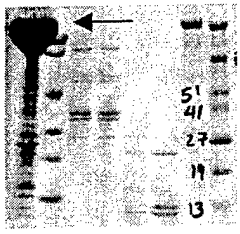

Figure 2: purified recombinant full-length SNF2H (122 kDa).

Key Accomplishments:

- Bacterial expression and multiple attempted purification schemes of the ATPase catalytic core domain of BRG1. These included temperature studies, refolding experiments and coexpression of molecular chaperones.
- Expression and purification of an extended ATPase core domain and full-length BRG1 produced from insect cells, using a baculovirus system.
- Factorial crystallization screens of the extended BRG1 ATPase core domain expressed and purified from insect cells.
- Attempted preparation of a catalytically functional 4-subunit BRG1 containing SWI/SNF complex.
- Expression and purification of the SNF2H protein and the SNF2H-containing NURF complex in insect cells using a baculovirus system. SNF2H is a related ATPase associated with the ISWI family of chromatin remodeling complexes.
- Limited proteolysis experiments of full length SNF2H resulted in the identity of a stable C-terminal SANT domain. This C-terminal construct was designed, overexpressed and purified from *E. coli*, and subsequent crystallization trials were undertaken.

Conclusions:

The determination of the crystal structure of the ATPase domain of BRG1 has proved difficult. The initial construction and overexpression of the ATPase domain of BRG1 in *E. coli* proved unsuccessful because the construct was not soluble. Refolding of the insoluble construct failed, as did attempts at enhancing solubility through protein fusion partners, and coexpression with molecular chaperones. Switching to a baculovirus expression system alleviated the solubility problems, but introduced new problems. An extended BRG1 ATPase domain could be expressed and purified by FLAG affinity chromatography to near homogeneity, but upon concentration and application to gel filtration chromatography, aggregation and instability resulted. Initial crystallization trials were attempted unsuccessfully to this point. The full-length protein was expressed using the baculovirus system with the same outcome as stated for the ATPase domain of BRG1. The purification and crystallization of a related ATPase family member, SNF2H, was also attempted in the baculovirus expression system with the same result. Limited proteolysis experiments of SNF2H did result in a stable C-terminus domain, but unfortunately another group has determined the crystal structure of this domain. It appears from all above-mentioned work, that these central catalytic ATPases when singularly expressed and purified are not well behaved in solution outside their natural physiological environments.

To address this problem, these ATPase proteins must be expressed within a context that lends stability and allows manipulation conducive to crystallization experiments. For this reason, expression and purification of the functional core of SWI/SNF, containing BRG1, BAF170, BAF155 and INI1 is currently being attempted. In concert with this, the expression and purification of the SNF2H containing NURF complex is ongoing. In the future, expression of the entire SWI/SNF complex, characterization of BRCA1's interaction with BRG1-containing complexes and any possible interaction with SNF2H containing complexes need to be examined, in addition to the ongoing structure determination.

Abbreviations and Acronyms:

ATP: adenosine tri-phosphate

BL21 (DE3)/LysS: competent bacterial cell type

BRCA1: tumor suppressor gene and translated protein

BRG1: brahma-related protein-1

FLAG: affinity tag

INI-I11: human HeLa cell line

ISWI: imitation switch

RSC: remodel the structure of chromatin

SF9: insect cell-line used in baculovirus expression system.

SDS-PAGE: Sodium dodecyl sulfate polyacrylimide gel electrophoresis

SWI/SNF: switch/sucrose non-fermenting

SNF2H: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5. The gene name is SMARCA5.

HBRM: human brahma protein

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