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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. Initial purification and expression of a SWI/SNF functional core and the SNF2H containing NURF complex were also unsuccessful. The conserved core ATPase domain was identified in two more primitive organisms. A recombinant homologous ATPase domain was expressed, purified, and small needle-like crystals were grown. Optimization and refinement of these initial crystals is underway to obtain X-ray diffraction quality crystals for structural determination of this homologous archael ATPase domain.				
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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 majority of work completed within the previous year has been focused on the first task outlined in the approved statement of work: the determination of the X-ray crystal structure of the ATPase domain of BRG1. Bacterial expression, multiple attempted purification schemes, and refolding experiments, of the ATPase catalytic core domain of BRG1 failed to produce significant amounts of pure, soluble protein for crystallization. A pure, soluble, recombinant ATPase domain of BRG1 was finally prepared with insect cells using a baculovirus expression system and subsequently purified by FLAG-affinity chromatography coupled with gel filtration. Crystallization factorial screens (11, 12) utilizing the hanging drop method were set up with protein alone, in the presence of ATP, or in the presence of a stable non-hydrolyzable analogue, ATP- γ -S at 20 °C and 4 °C. In the previous report, it was noted that based upon the ratio of clear drops to those that showed precipitate in the initial crystallization experiments that the concentration of the protein must be greater than 2.0 mg/ml. No crystals were observed from the newly attempted crystallization screens and the concentration of the protein could not be increased past 3.0 mg/ml due to the limiting nature of the aggregation rate of the protein during purification.

Another approach to the completion of the first task involved the identification of a conserved ATPase domain in a more primitive organism state. The ATPase domain of BRG1 is highly conserved and two archaeal homologues were identified using a BLASTP search in conjunction with a SMART (Small Molecular Architecture Research Tool) analysis (13, 14). The top three matches were: a SWI/SNF helicase from *methanosarcina mazei* (Accession number: Q8PWU7), a SNF2 helicase from *methanosarcina acetivorans* (Q8TU84), and two hypothetical proteins SSO1653 and SSO1655, that together form a conserved BRG1-like ATPase domain, from the thermostable archaea, *sulfolobus solfataricus* (Q97XQ5 and Q97XQ7). The proteins from *methanosarcina mazei*, denoted **MOATP** (for abbreviation purposes only), and from *sulfolobus solfataricus*, denoted **SSOATP**, were chosen as viable targets because both of their genomic DNA were commercially available.

The ATPase domain of MOATP and SSOATP were identified from sequence homology comparison with BRG1 (Fig. 1). Constructs for the ATPase domain of MOATP were designed to include amino acid residues 550 to 1089 with a N-terminal cleavable histidine tag spanning the previously defined ATPase domain. The ATPase domain has been cloned into a T7 expression vector and transformed into a competent bacterial host, BL21(DE3)*, with this vector for overexpression in bacteria. A six-liter, 15 °C bacterial growth, followed by nickel affinity, ion exchange, and size exclusion chromatography yielded approximately 0.5 milligrams of pure protein. This protein fraction was concentrated, using centrifugal membrane devices from Millipore, to a concentration of 1.9 mg of protein/ml at greater than 95% purity by SDS-PAGE analysis (Fig. 2).

The recombinant ATPase domain from MOATP was assayed for ATPase activity and chromatin remodeling ability in two experiments. The recombinant ATPase domain of MOATP exhibits intrinsic DNA-stimulated activity (Fig. 3), but not nucleosome-stimulated ATPase activity as compared to recombinant full-length SNF2H, a human homologue to BRG1 (15). The recombinant ATPase domain does not display chromatin-remodeling activity as evidenced by a negative result on a Sal I nucleosome accessibility assay (16).

Initial crystallization (11, 12) factorial screens utilizing the hanging drop method were set up at 20 °C with and without ATP- γ -S. After 4-5 weeks small needle-like crystals were observed from two subsequent conditions (Figs. 4 & 5). Initial optimization of these conditions for precipitant,

yielded no better quality crystals. The length of time for crystal nucleation and the ratio of clear drops to those that showed precipitate in the initial crystallization screens, suggest that the protein concentration needs to be subsequently increased by at least a factor of two.

The expression and purification strategy outlined above proved to be limiting in the amount of pure, soluble protein that could be produced. To increase the amount of purified protein, the ATPase domain of MOATP was cloned into another T7 expression vector that replaced the N-terminal histidine tag with a N-terminal FLAG affinity tag. Initial purification by FLAG affinity and size exclusion chromatography yielded between 1 to 2 milligrams of pure, soluble protein increasing the yield four-fold over that of the Histidine-tagged construct. Additional crystallization trials at 3.7 mg of protein/ml are underway.

A single construct for the ATPase domain of SSOATP were designed to include residues 227 to 788 of hypothetical protein SSO1653 and residues 6 to 124 of hypothetical protein Sso1655 with a N-terminal cleavable histidine tag spanning the previously defined ATPase domain. The purification and subsequent crystallization of this thermostable protein has been undertaken.

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 sub complex, identified by Kingston (17) has been attempted unsuccessfully. BRG1 is homologous to SNF2H, the catalytic ATPase of the human ISWI complex, NURF, first identified by the Shiekhatar group at the Wistar Institute (15). The catalytic ATPase SNF2H is surrounded by two subunits: BPTF and Rbap48. Co-expression of the three proteins and subsequent FLAG affinity purification does not yield a stable recombinant complex, because BPTF is proteolytically digested during expression in the Sf9 insect cells. It is hypothesized that the addition of the other two subunits to the catalytic ATPase to form a complex might alleviate the stability and aggregation problems encountered in the purification of full-length SNF2H.

Figures:

BRG1	694	-----SDDVSEVLAHITENKCDVDDYGG-----
MOATP	550	-----SDGNVFG-----
SSO1653	347	DYKISIGDKDISEEFMFLVSENITVHLGGNLEIIEKSLQKIKDLYIKSKKIDKID
BRG1	719	---VSQLARGTOSYYAVAHEVTERVQKQSALMNEVLEKQYIKGLEIVSYYANNING
MOATP	558	---LNATGWIGELISLADKIGFELPAPITGFSGLRPYCFRSGWLAFFRQWIKGA
SSO1653	407	ILRESLLEADIEINDELDPLEGNKSEQLPEYILTKANLRPPYQIKGSEWRFRNKLSEFI
BRG1	776	IADENGLGFTVDTTIALIT-----YLMHKRIN-----VLEIVPI
MOATP	612	LADDMGLGFTVDTTIALIHDLEQAKEKAEKIEEPAEEIIEKVDLRKAPKIVLIVCFI
SSO1653	466	LADDMGLGFTVDTTIAVFS-----LAKRENELT-----SMLICPI
BRG1	813	ITSLWAYFLDVAISVVKVSKKSPAAFRFVPCLRSGKINVDITTEYITKKEIIRAK
MOATP	672	GVNHHKKLAEFTHELSVAHHHCH-SRKKSEEFKEAMNHAIVISSISAVQPELRFLEH
SSO1653	502	AVIKKVEEELDFAPHRFAFEEH-----LRSEHLELDIHLITTYVALLPITRLEH
BRG1	873	LRWPTMIVDLCHEMIDHHCITOVINTHYVPRILLTGTLEQIFPELWALINELLTH
MOATP	731	LRAGVLDKACHITHNEETFOAPAAAR-ALVDYRLALTGTVEHINAGDLWITDFLHPGF
SSO1653	554	LEWYVVEDEACHITHEFCITHEFAVK-EKKKYFHALTGTTEHNFVDLWISITFLHPGI
BRG1	933	FKQCTIEQWVNAIFAMTIEKVDLNEEETILIIIRIEKVRFPFLRPLF--TEVEAQLPF
MOATP	790	LRSGQGFHRNFITFOAERIKESA-----EELIEITGPFILRPLITITSLISLDF
SSO1653	613	LRGYVEEFSKATPKK-SNNEK-----EELHAIHSEFILRPTTYEPAIINDLPI
BRG1	991	VEYVHKDYSALRVLSHQAQGVLLTQSEKDKGKGGTKTLMNHLIQEKIHHFY
MOATP	841	MEMKTYGTLKEQASLAAVLEDREAREAEKEDGCEK---HILGALSRLQVQHHPV
SSO1653	663	IEETNVYGNLITPEQANIKAEVENLFINIL-VVIEIFPK---MILSTLLRFPQIVDHPV
BRG1	1051	MEQIIEEFSEHLGFTGGIVQGLDLYRASGFELDRILPKLRATNHEMLIFCMTSMT
MOATP	898	QPLDNDSLIPG-----SGLARITTEIEVLEINTEPAIMFTQFAEIKH
SSO1653	719	ILKGGCQVLR-----SARVIMITMIEEALDESDIARFTQFVDKIH
BRG1	1111	IMEYFAYRG-FKYLRIDTTKAEFGMFKTINPGSEYRTEFLSFRAGGLRLHQAL
MOATP	942	MYSEHIQASFGCEVLEFLHSGVPHQPIRMIEKRFQPKYEYETFLSFRAGGTLLHQAL
SSO1653	762-788 (6)	IEENIEKELMTEVPPLYEELSKIEEDTISSEFN-NPSKRFIILRAGGFRLHLSAI
BRG1	1170	TITLPI SDWHHQLLACQPAHFICQNEVRLSICQVNSVEEFLAAAKYRINVDQKVI
MOATP	1002	RVHFEDRWNNPAVENADTDPARIGKKNVHHFICAGTLEEKIEDEIEERKQVAENV
SSO1653	39	RVHFEDRWNNPAVEDADTDPVPRIGCTENIVVHRLISVGTLEERIQDLARFSLFKLIT
BRG1	1230	QAMFDQKSSCHERRAFQALHEHEQDESRHCTGSGSASFHTAPPAGVNPDL
MOATP	1062	GIGEDVITELSTDE---SKDILARPEVGE-----
SSO1653	99	SGESVITELSTDE---LRKVLEISVGGY-----

Figure 1: Sequence Alignment of the ATPase domain of BRG1 with the archaeal homologues from *methanosarcina mazel* and *sulfolobus solfataricus*.

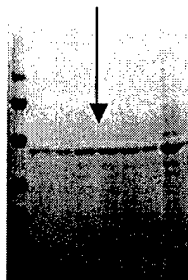


Figure 2: purified recombinant ATPase domain of MOATP after gel filtration (63 kDA)

Figures (con't)

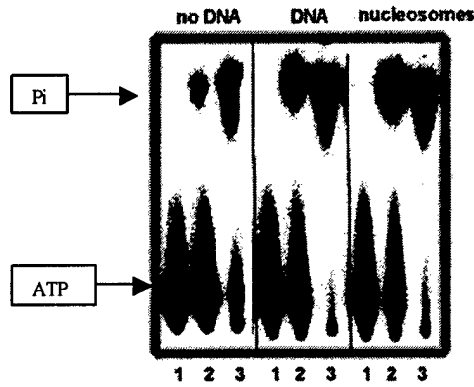


Figure 3: MOATP exhibits DNA-stimulated ATPase activity. # 1 denotes buffer alone (negative control); # 2 is MOATP; # 3 is recombinant full-length SNF2H (positive control).

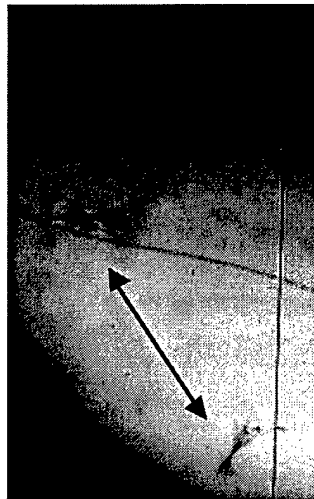


Figure 4: Initial Crystals of MOATP from the following condition: 0.04 M Mg Acetate, 0.05 M Cacodylate pH 6, 30% MPD.

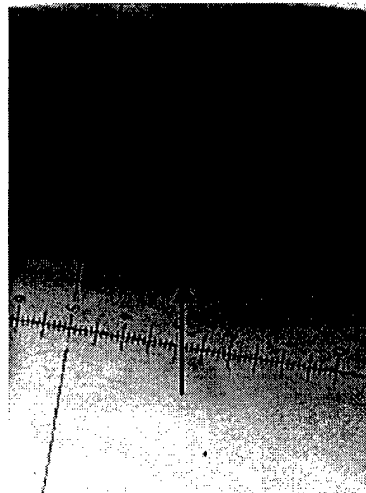


Figure 5: Initial Crystals of MOATP from the following condition: 0.2 M KCl, 0.01 M Mg Chloride, 0.05 M Hepes pH 7, 20% 1,6 Hexanediol.

Key Accomplishments:

- ✍ Identification of two archaeal homologues to the ATPase domain of BRG1 from *methanosarcina mazei* and *sulfolobus solfataricus*.
- ✍ Bacterial expression and purification of an extended ATPase core domain from *methanosarcina mazei* spanning residues 550 to 1089.
- ✍ This extended ATPase core domain from *methanosarcina mazei* displays intrinsic DNA-dependent ATPase activity as evidenced by a performed ATPase activity assay.
- ✍ Factorial crystallization screens of the purified extended ATPase domain from *methanosarcina mazei* yielded preliminary needle-like crystals.
- ✍ Cloning of a homologous extended ATPase domain from the thermostable archaea, *sulfolobus solfataricus*, into a T7 expression vector.
- ✍ Expression and purification of 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.

Conclusions:

The determination of the crystal structure of the ATPase domain of BRG1 and the full-length protein has proved difficult. Neither the ATPase domain nor the full-length protein could be expressed and purified in significant amounts for crystallization. Numerous purification and expression strategies were employed to no avail. The purification and crystallization of a related ATPase family member, SNF2H, was also attempted with the same negative result. To address this problem it was reasoned that 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, and a SNF2H containing NURF complex was attempted. Initial attempts at expression and purification of these complexes were unsuccessful.

An alternative attempt to determine the crystal structure of the ATPase domain of BRG1 has been undertaken with a promising degree of success. Two archaeal homologues of the extended ATPase core domain of BRG1 have been identified, and cloned into T7 expression vectors. The ATPase domain from the protein MOATP, endogenous to the archaea *methanosarcina mazei*, has been expressed and purified by a combination of affinity, ion exchange, and size-exclusion chromatography. Initial crystallization factorial screens yielded small-needle like crystals in more than one crystallization condition, suggesting that diffraction-quality crystals can be obtained in the near future. ATPases assays were performed with the recombinant ATPase domain from BRG1, and the recombinant protein demonstrated intrinsic DNA-dependent ATPase activity.

Abbreviations and Acronyms:

ATP: adenosine tri-phosphate

BL21 (DE3)*: 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

MOATP: an arbitrary abbreviation for a SWI/SNF helicase prtein from *methanosarcina mazei*.

SSOATP: an arbitrary abbreviation for a hypothetical SWI/SNF helicase protein from *sulfolobus solfataricus*.

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