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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The overexpression or amplification of the AIB1 gene is observed in the majority of primary breast tumors, and is also correlated with a large percentage of hereditary and sporadic ovarian carcinomas. The AIB1 protein is a member of the steroid receptor coactivator family of transcriptional regulatory proteins that contain a conserved C-terminal histone acetyltransferase domain, and an N-terminal bHLH-PAS domain. We hypothesize that the HAT and bHLH-PAS domains of AIB1 play critical roles in mediating elevated transcriptional activation levels in AIB1-mediated cancers. The goal of this proposal is to determine the three-dimensional structure of the HAT and bHLH-PAS domains of AIB1 to facilitate the structure-based design of small molecule inhibitors to specifically target AIB1 for therapeutic application. To date, we have prepared several HAT and bHLH-PAS -containing AIB1 recombinants. Each of the HAT-containing domains, either alone or as GST fusion proteins, are unstable suggesting that the HAT domain is not correctly folded and may require association with another protein factor for AIB1-mediated HAT activity. We have overexpressed and purified to homogeneity two N-terminal bHLH-PAS-containing domains that migrate as apparent monomers on gel filtration chromatography. Crystallization efforts to these bHLH-PAS-containing AIB domains are underway to facilitate an X-ray crystal structure determination.

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(5) INTRODUCTION

Gene amplification is a common cause of increased gene expression in human breast cancer (Ingvarsson, 1999; Schwab, 1998). Commonly amplified chromosomal regions located within 17q12, 8q24 and 11q13 encode the proteins Her-2/neu, c-myc and cyclin-D1, respectively, and have been extensively studied both *in vitro* and *in vivo* (Ingvarsson, 1999; Schwab, 1998) In addition to these chromosomal regions, the long arm of chromosome 20 (20q) has received considerable attention since it encodes a gene termed AIB1 (amplified in breast cancer-1) (Guan et al., 1996). The overexpression or amplification of the AIB1 gene is observed in the majority of primary breast tumors (Anzick et al., 1997), and is also correlated with a large percentage of hereditary and sporadic ovarian carcinomas (Tanner et al., 2000). The AIB1 protein is a member of the steroid receptor coactivator family of transcriptional regulatory proteins that contain a conserved C-terminal histone acetyltransferase domain, and an N-terminal bHLH-PAS domain (Leo and Chen, 2000). We hypothesize that the HAT and bHLH-PAS domains of AIB1 play critical roles in mediating elevated transcriptional activation levels in AIB1-mediated cancers. The goal of this proposal is to determine the three-dimensional structure of the HAT and bHLH-PAS domains of AIB1 to facilitate the structure-based design of small molecule inhibitors to specifically target AIB1 for therapeutic application.

The specific technical objectives of this proposal are to: (1) Clone for overexpression and purify recombinant bHLH-PAS and HAT domains of AIB1 (tasks 1-4), (2) Characterize the respective functions of the recombinant bHLH-PAS and HAT domains *in vitro* (tasks 5-6), (3) Prepare crystals of a functionally active bHLH-PAS and HAT domain protein construct suitable for structure determination using X-ray crystallography (tasks 7-9), and (4) Determine the X-ray crystal structure of the bHLH-PAS and HAT domains of AIB1 using X-ray crystallography (tasks 10-15). We expect that these studies will provide a scaffold for the design of AIB1-specific inhibitory molecules that will be useful for the treatment of AIB1-mediated breast and ovarian cancer.

(6) BODY

Over the last year we have made significant progress towards achieving the overall goal of the proposal. In summary, we cloned for overexpression two constructs of AIB1 containing the N-terminal terminal bHLH-PAS domain fused to an N-terminal 6xHis fusion (task 1). The shortest AIB1 protein construct, His-AIB1(1-326), contains the complete bHLH, PAS-A and PAS-B domains only, and the longer AIB1 construct, His-AIB1(1-405), also contained a ~65-residue C-terminal region that is highly conserved among the Steroid Receptor Coactivator proteins. Both proteins are highly soluble when overexpressed in bacteria induced at 15°C and can be purified to homogeneity using a combination of Ni-chelate chromatography, Q-Sepharose ion exchange and gel filtration chromatography (Superdex-200) (task 2). Gel filtration chromatography shows that the proteins comigrate with globular protein standards that corresponding to a monomer molecular weight of the AIB1 protein (Fig. 1). The His-AIB1(1-405) protein construct was also subjected to dynamic light scattering analysis, revealing a hydrodynamic radius corresponding to a protein molecular weight of 52 KDa, agreeing well with the predicted mass for the monomer of 46 KDa (task 5). Both proteins have been concentrated

to ~20 mg/ml and stored at -70°C prior to crystallization trials. The protein yield is about 10 mg of purified protein form 1 liter of bacterial culture. A non-His-tagged version of the shorter AIB1 protein construct, [AIB1(1-326)}], was also overexpressed in bacteria and purified to homogeneity using a combination of Q-Sepharose ion exchange, Cebricon Blue affinity and gel filtration (Superdex-200) chromatography (task 4), although the protein yield was considerably lower (~ 1mg/ml of purified protein from 1 liter of bacterial culture).

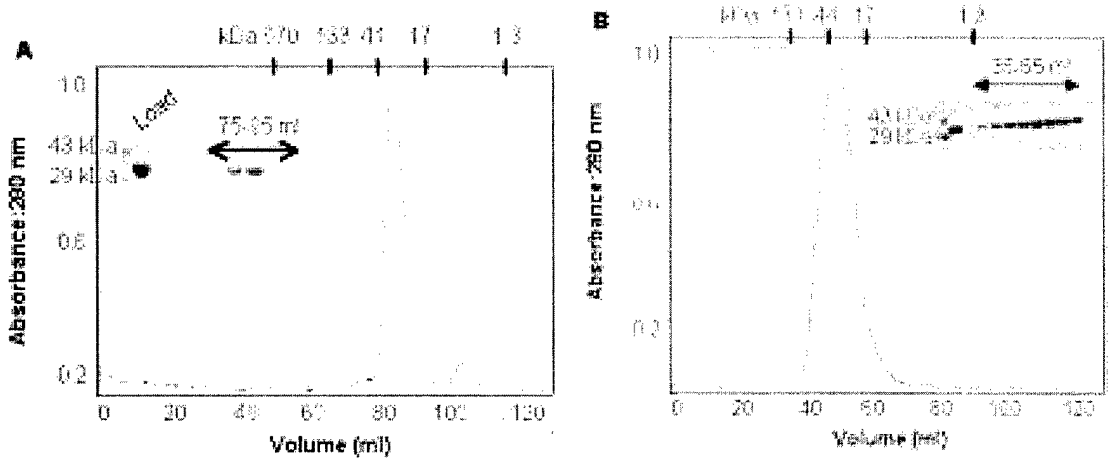

Figure 1. Purification of theAIB1 protein constructs. (A) His-AIB1(1-326) was purified using a combination Ni-chelate affinity, Q-Sepharose anion exchange and gel filtration chromatography. The final gel filtration chromatograph on a Superdex-200 gel-filtration FPLC column is shown with the corresponding peak fractions illustrated on the embedded SDS-PAGE gel. (B) As in A except that the purification of AIB1(1-405) is shown.

Although sufficient quantities of AIB1(1-326) could not be obtained for crystallization efforts, both His-AIB1(1-326) and His-AIB1(1-405) were obtained in suitable quanities for crystallization efforts. Both protein constructs were subjected to extensive crystallization efforts using several different crystallization screens [(Hampton Research Protein Screens I and II and Wizard Screens 1 and 2 (Emerald Biosystems)] at both 18 °C and 4 °C (Task 7). No suitable crystals have yet been obtained, as the majority of the crystallization conditions resulted in the formation of amorphous precipitate.

In order to facilitate the structure determination of the C-terminal HAT domain of AIB1, we cloned for overexpression in bacteria residues 975-1412 of AIB1 [AIB1(975-1412)], both as N-terminal 6xHis-tagged and N-terminal GST-tagged fusion proteins. Both protein constructs expressed to high levels in bacteria that were induced at 15 °C, but were found exclusively in the inclusion bodies of the bacteria. We conclude that this HAT domain is inherently unstable and may require association with a cofactor protein for protein stability and HAT activity.

In the coming year we will concentrate our efforts on crystallizing the bHLH-PAS domain of AIB1 and initiate structure determination using X-ray crystallographic techniques (tasks 8-11). In the event that the N-terminal 6xHis-tag of the AIB1 bHLH-PAS constructs are preventing crystallization we will use site directed mutagenesis to introduce TEV protease sites between the 6xHis-tag and bHLH-PAS region. This will allow us to take advantage of the His-

tag for protein purification purposes and then to remove it to facilitate crystallization. If these protein constructs do not crystallize, we will subject the protein constructs to limited proteolysis to identify smaller globular domains that may be more emendable for crystallization. Once suitable crystals are obtained (task8) we will (1) Characterize the diffraction properties of cryogenic crystalline samples of the AIB1 bHLH-PAS (task9), (2) Collect and process a native cryogenic crystallographic data set for crystals of the bHLH-PAS domains of AIB1 (task 10), and (3) Prepare heavy atom/MAD derivatives for crystals and collect X-ray data from the heavy atom – derivatized crystals. This information will be used to determine the three-dimensional structure of the bHLH-PAS domain of AIB1 (task 12).

In the coming year we will also prepare other C-terminal protein constructs of AIB1 that may be more amenable for biochemical and structural analysis. The protein construct we had initially prepared, harboring residues 975-1412 was insoluble and we propose that this might be due to the poly-glutamine linker region within residues 1239-1266 of the protein. To get around this potential problem, we will prepare two subdomains of this C-terminal AIB1 protein, initially as 6xHis-tagged fusion proteins. Specifically, we will prepare His-AIB1(975-1239) and His-AIB1(1266-1412). If we are able to purify these proteins to homogeneity, we will assay them for HAT activity both separately and in combination (tasks 5-6). We will then initiate crystallographic efforts for these protein domains (tasks 7-9).

(7) KEY RESEARCH ACCOMPLISHMENTS

- Cloned and overexpressed in bacteria several different AIB1 protein constructs including the bHLH-PAS domains containing constructs His-AIB1(1-326) and His-AIB1(1-405); and the HAT domain containing AIB1 constructs 6xHis-AIB1(975-1412) and GST-AIB1(975-1412).
- Purification to homogeneity of the His-AIB1(1-326) and His-AIB1(1-405) protein constructs.
- Demonstration that the His-AIB1(1-326) and His-AIB1(1-405) protein constructs are monomeric in solution.
- Initiated crystallization efforts for the His-AIB1(1-326) and His-AIB1(1-405) protein constructs

(8) REPORTABLE OUTCOMES

None

(9) CONCLUSIONS

During the first year of the funding period we have successfully carried out tasks 1-4 of the proposal. Specifically, we have prepared 6xHis-tagged AIB1 protein constructs containing the bHLH-PAS domain and crystallization efforts are underway. While we will continue our crystallization efforts (task 7), we will also prepare comparable protein constructs without the 6xHis-tag to facilitate crystallization for X-ray structure determination. We will then employ

standard X-ray crystallographic procedures to determine the structure of the bHLH-PAS domain of AIB1. The initial C-terminal HAT domain constructs of AIB1 that we prepared was not amenable to biochemical and structural analysis. In the coming year, we will also prepare new C-terminal constructs of AIB1 that may be more amenable to biochemical and structural analysis (tasks 8-12).

Since the AIB1 protein is amplified or overexpressed in the majority of breast tumors, and since the high conservation of the bHLH-PAS and HAT domains implies functional importance, these AIB1 protein domains are highly relevant target for the development of inhibitory compounds that may provide effective therapeutics for the treatment of breast cancer. The structure of the bHLH-PAS and HAT domains will allow us to use structure-based strategies to design "lead" compounds to associate with the protein via its site of action and thereby inhibit the protein function. This lead compound can then be optimized for increased protein binding affinity, specificity and cell delivery properties using a combinatorial chemistry approach (Beeley and Berger, 2000; Kirkpatrick et al., 1999; Leach et al., 2000; Roe et al., 1998). Such compounds may have clinical applications towards the treatment of AIB1-mediated cancers.

(10) REFERENCES

Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science *277*, 965-968.

Beeley, N., and Berger, A. (2000). A revolution in drug discovery. Combinatorial chemistry still needs logic to drive science forward. BMJ *321*, 581-582.

Guan, X.-Y., Xu, J., Anzick, S. L., Zhang, H., Trent, J. M., and Meltzer, P. S. (1996). Hybrid selection of transcribed sequences from micrdissected DNA: Isolation of genes within an amplified region at 20q11-q13.2 in breast cancer. Cancer Res *56*, 3446-3450.

Ingvarsson, S. (1999). Molecular genetics of breast cancer progression. Semin Cancer Biol *9*, 277-288.

Kirkpatrick, D. L., Watson, S., and Ulhaq, S. (1999). Structure-based drug design: Combinatorial chemistry and molecular modeling. Comb Chem High Throughput Screen *2*, 211-221.

Leach, A. R., Bryce, R. A., and Robinson, A. J. (2000). Synergy between combinatorial chemistry and de novo design. Journal of Molecular Graphics & Modelling *18*, 358-367.

Leo, C., and Chen, J. D. (2000). The SRC family of nuclear receptor coactivators. Gene *245*, 1-11.

Roe, D. C., Kick, E. K., Skillman, A. G., Liu, G., and Ellman, J. A. (1998). Combining structure-based drug design with combinatorial chemistry. Abstr Pap Am Chem Soc *216*, 011-COMP.

Schwab, M. (1998). Amplification of oncogenes in human cancer cells. Bioessays *20*, 473-479.

Tanner, M. M., Grenman, S., Koul, A., Johannsson, O., Meltzer, P., Pejovic, T., Borg, A., and Isola, J. J. (2000). Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. Clin Cancer Res *6*, 1833-1839.

(11) APPENDICES

None