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TITLE:  Tumor Suppressors and Breast Cancer: Molecular Interaction of Retinoblastoma Protein (Rb) with a New Rb-binding Protein (RIZ)

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Cancer arises from an accumulation of multiple mutations that may occur in oncogenes, tumor suppressor genes or DNA repair genes. Tumor suppressors control cell cycle and growth and mutations or alterations in these suppressors can be associated with the uncontrolled growth of malignant tumors. In this project, tumor suppressors were studied highlighting a new protein called RIZ. The goal is to use x-ray crystallography to study the molecules. The results will be important to understanding the role of the new regulator protein RIZ in tumorigenesis in breast cancer. This IDEA project focused on the first steps in the process, i.e. production, purification and crystallization of the proteins. Notable progress was made in identifying the PR domain in RIZ that is directly linked to tumor suppression. PR is underexpressed in breast cancer. Feasibility for structural studies of this new protein motif (PR) was established.
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INTRODUCTION

In this project, funded as an IDEA award, we have targeted proteins that influence cell cycle and growth. The study addresses issues for direct prevention of breast cancer to provide an understanding of processes such as cell proliferation and the role of tumor suppressors in the onset of breast cancer. Cell cycle and growth are critical for normal human health. Cancer arises from the accumulation of multiple mutations and these mutations can arise in oncogenes, tumor suppressor genes or DNA repair genes. Alterations in tumor suppressor genes are associated with a variety of cancers. A model proposing that both normal alleles of a tumor suppressor gene must be mutated for loss of function and tumorigenesis (15) has been generally confirmed by the identification of more than a dozen tumor suppressors. In this study, we target two proteins that may function as tumor suppressors in breast cancer. One is the well-studied retinoblastoma (Rb) gene product (6, 8, 25) and the other is a newly identified Rb-binding protein RIZ that is just now being characterized (11, 16, 26). RIZ is a member of a recently described family that are involved in human cancers in a yin-yang fashion (12). The RIZ gene is located on human chromosome 1 in the 1p36 region (3) where deletion (5) or loss of heterozygosity (1, 18, 9, 19, 20) has been reported in breast carcinoma. There may be as many as four loci for tumor suppressors on chromosome 1p for breast cancer (20). RIZ is a 250 kDa protein that contains zinc finger domains, a retinoblastoma (Rb)-binding motif, a domain (AR) homologous to the E1A oncoprotein, GTPase and SH3 domains and an interesting new segment in the N-terminal sequence called the PR domain.

![Diagram](image_url)

Figure 1. Schematic map of the human RIZ1 protein.

The PR domain is located in the amino-terminal sequence of RIZ (see Fig. 1). The RIZ gene normally encodes two gene products: the full-length protein (~280 kDa) and a shorter product (~250 kDa) called RIZ1 and RIZ2 respectively. RIZ2 lacks the PR domain but the rest of the sequence is identical to RIZ1 (17). Cloning of RIZ by Dr. Huang led to the identification of a new protein motif (2, 10) found in a family within the Krüppel-like zinc finger genes that includes the MDS1-EVI1 leukemia gene and the PRD-BF1 or BLIMP1 transcriptional repressor involved in B cell maturation (4, 13, 24). The MDS1-EVI1 gene also gives rise to a product lacking PR, the EVI1 oncogene (4). A sequence alignment of the PR domains in several related proteins is presented in Fig. 2.
Figure 2. Alignment of PR domain sequences. Identical (black background) or similar (gray background) residues are highlighted. Dashes indicate sequence gaps. Conserved blocks with high homology are indicated.

By direct comparison of gene products that bear or lack the PR domain, the suggestion is that proteins containing the PR domain are tumor suppressors. Significantly, when our collaborator Dr. Huang generated mice lacking RIZ1 using gene targeting technology, the incidence rate for tumors was remarkable, i.e., 67% (8 of 12 mice). Following up on the conclusion from these results that loss of RIZ1 predisposes for tumor formation, RIZ1 expression was tested in human tumor tissues and tissue-derived cell lines (7). The results showed that RIZ1 expression is decreased or lost in human breast cancer, neuroblastoma or lung cancer cells whereas expression of RIZ2 is normal. The fact that underexpression of RIZ1 may be common in human breast tumors is a new observation that may represent an innovative tool for diagnosis or possibly treatment. In this project, we focus on the structure of the PR domain to gain insight into the molecular features of this new protein motif.

**BODY**

As stated in the original application, the long-term goal of this project is to generate three-dimensional crystal structures of tumor suppressors that play a role in the development of breast cancer. Not all proteins can be crystallized. However, this IDEA project was funded to support an intense effort to produce soluble proteins and complexes of the tumor suppressors and then to crystallize the subject proteins. There is seldom a format to support this stage of the efforts in a crystallographic study. Consequently, some projects fall because funding is
not available to support the pilot studies. Here, with funding from USAMRMC, we have devoted considerable resources to the effort, to maximize the prospects for success. Success will ultimately be measured by production of crystals, and time-lines vary from original projections when soluble proteins are produced. Also, at early stages in structural investigations with newly discovered factors, the biological relevance and functional role of the protein is being characterized by our collaborators while the structural studies are being initiated. In some cases, the direction and emphasis for structural data becomes focused as the recombinant constructs are being designed and priorities are set based on the latest discoveries in the cell biology collaborative investigations. In this study, for example, the discovery that the PR domain is vital for the role of RIZ as a tumor suppressor led us to use the limited resources in the IDEA award primarily for study of the PR domain. The structural work is therefore timely and appropriate for what we now recognize as critical for activity of RIZ. Progress to date is outlined in the following sections.

**TASK 1: To generate and crystallize stable complexes of the “pocket domain” of the retinoblastoma protein bound to the AR E1A-like domain of the Rb-binding protein RIZ (Months 1-24)**

Crystals of the Rb “pocket” domain were obtained in space group $P6_22$ with $a=b=110.2\AA$, $c=154.6\AA$, $\gamma=120^\circ$, and the crystals diffract to high resolution (2Å). At the time of the submission of the original application, although these crystals were in hand, the crystal structure of another modified fragment of the Rb “pocket” was determined in another laboratory (16). Our goal instead, is to crystallize the Rb “pocket” domain in complex with the AR domain from RIZ. The AR domain of RIZ was cloned as a fusion protein with glutathione-S-transferase. The Rb protein encompassing the full “pocket” domain was cloned in the pET expression system. The GST-AR fusion protein contains a site that can be cleaved by thrombin to release the AR domain. The free AR fragment was soluble after release from the GST fusion partner. To begin the structural studies, the purification protocols were scaled up. The affinity purification of the GST-AR domain was not straightforward. An interfering substance from the bacterial culture was generated when the production was scaled up to the multi-liter level. After much headache and struggle, we identified the interfering “substance” as RNA. The contaminating RNA fragments inhibit binding affinity of the GST-AR protein to the glutathione resin and yields of purified fusion protein from the lysate are reduced. We have developed protocols to use RNase during the purification steps to remove the RNA, thus eliminating a significant hurdle in the protein production. The AR protein is thus ready for crystallization trials.
TASK 2: To solve the crystal structure of the Rb “pocket” domain/RIZ AR domain complex (Months 12-24)
This part of the project is dependent on the successful completion of the work outlined in Task #1.

TASK 3: To characterize the protein-protein interactions of the PR domain of RIZ (Months 1-14).
The work in this task is linked to the work in Task #4 since both Tasks rely on the successful production of soluble protein.

TASK 4: To crystallize the PR domain of RIZ alone or in complex with the interacting region of RIZ oligomerization (Months 6-24).
As shown in Figure 2, there are now several members of the PR family that have been recognized by our collaborator, Dr. Huang. The PR domain is a segment of ~150 residues in the amino-terminal sequence of RIZ that represents a new protein motif. When the PR domain is present, the full-length protein functions as a tumor suppressor but when PR is lost due to truncation at the amino-terminus, abnormal cell proliferation and tumorigenesis may ensue. There is strong sequence homology within the family. For RIZ1, Dr. Huang has demonstrated in the past year that loss of the PR domain predisposes for tumor formation and this phenomenon, evident in underexpression of RIZ1 containing the PR domain, is common in breast cancer. This is a new observation. In response to this new insight, we set our focus on the structure of the PR domain to reveal the molecular features of this new protein motif. The PR domain may represent an innovative tool for the diagnosis (or treatment) of breast cancer.

The PR domains from four molecules shown in Figure 2 were cloned as GST-fusion proteins, expressed in large scale bacterial culture and purified by affinity chromatography on glutathione-agarose columns. GST-fusion partners were released from the PR domains by thrombin digestion. The resulting products were purified by additional ion-exchange steps. To ensure success with this new and uncharacterized motif, several PR domains were selected as candidates for crystallization. The purified domains were tested for solubility by concentration and evaluated by dynamic light scattering. This method measures the aggregation state of the proteins and can be used as a positive indicator for successful crystallization. Samples that are monodisperse in solution are likely to crystallize.

The first success was with the PR domain of the BLPR protein from PRDI-BF1. This domain was entered into crystallization trials. Small crystals formed readily overnight from solutions to which a mixture of jeffamine and ammonium sulfate precipitants had been added. Hundreds of tiny needles formed. These needles were used in seeding trials to attempt to convert the small crystals into single large crystals. With time, single block-shaped or hexagonally shaped crystals
grew slowly in the vapor diffusion drops. Technically, these crystals are not optimal since they grew only after several months’ incubation and the reproducibility of their production was difficult to control. Moreover, the small block crystals were tested for diffraction at the Stanford Synchrotron Laboratory (SSRL). Diffraction was not suitable to pursue structure solution.

To address the question of reproducibility and to produce crystals that diffract, we initiated a new approach to improve the protein interactions within the crystal lattice. The predicted secondary structure of BLPR was evaluated and the sequence of the domain was analyzed in a hydrophilicity plot. This analysis, when correlated with secondary structure prediction, can predict regions of the sequence that may be located on the surface of the protein. These analyses were used to identify residues that could be involved in crystal contacts within the crystal lattice. Eight residues were marked by these methods that may be surface residues and the residues were changed using the Quikchange™ method for site-directed mutagenesis. Residues selected included single cysteines, charged residues that appear in clusters or hydrophobic residues that fall in regions that are otherwise highly hydrophilic. Substitutions of alanine or small hydrophilic residues were made singly at each of the eight sites. The mutant proteins were then cloned as GST-fusion proteins. The mutants each differ from wild-type by one residue. Each of these proteins was purified by affinity chromatography sequentially, and each of the mutant proteins was tested for crystallization. However, no crystals were obtained from these trials.

During the final year, we designed a new protein construct of the RIZ PR domain that is shorter and corresponds to predicted secondary structural features. PR domains have strong homology with the SET domain (11), a region of 130 amino acids that is conserved in proteins that modulate gene regulation by binding to chromatin. The design was based on molecular modeling that would predict the structure of PR based on homology with SET domains. This effort was initiated to broaden our search for a stable domain suitable for crystallographic studies. These is no known structure for SET domains, however it has been noted recently that SET domains share strong sequence homology with methyltransferases (22). While there are no crystal structures reported for lysine methyltransferases, several structures of the related arginine methyltransferases are available in the PDB database. The class of AdoMet-dependent methyltransferases share a conserved catalytic domain structure (21). This core contains four short β-strands arranged in a parallel order flanked on either side by α-helices. Modeling suggested that PR residues would assume a similar configuration except that the β-strands would be arranged in an antiparallel fashion, as seen in the PRMT3 arginine methyltransferase (27). Further search of the PDB database used PR sequences with different lengths and a selection process that was optimized to maintain predicted secondary structural elements. The program 3D-PSSM (14) produced a comparatively meaningful integrated
predicted structure for RIZ PR based on structural homology with the PTB domain of insulin receptor substrate-1 (PDB accession code 1irs). This domain also consists of short β-strands and flanking α-helix as shown in Figure 3.

Figure 3. Schematic diagram illustrating the folding pattern predicted for RIZ PR domain based upon threading analyses.

Figure 4: SDS-PAGE analysis of the purification of RIZ PR domain. Molecular weight markers are shown on the left. The PR sample is shown in the far right lane (the upper band is residual GST, which is removed by ion-exchange chromatography).

Figure 5. Results of isoelectric focusing of the PR domain produced from the new shortened construct. The pI is 5.4, a value that is close to the calculated isoelectric point.
Based on this model, a new construct for RIZ PR was generated as a GST fusion protein and also as a protein with a hexahistidine tail, to permit affinity purification of two similar fusion proteins. The new ‘trim’ construct lacks residues at the amino- and carboxyl-termini. These termini most likely had an inherent unstructured flexibility that inhibited crystallization. All indications are that the new shorter construct is improved. The protein has been purified and the contaminating substance previously carried along in the purification has been eliminated (see Figure 4). The protein product is soluble and homogeneous with respect to isoelectric point (see Figure 5). The purified protein is also monodisperse when tested by dynamic light scattering (as compared to the previous sample that was polydisperse and seriously aggregated at certain pH ranges). Homogeneity and monodispersity are strong indicators that the protein is well-folded and structurally suitable for crystallization. This sample is far superior to any PR samples that we have produced in the earlier stages of this project. The protein has been introduced into crystallization trials.

**KEY RESEARCH ACCOMPLISHMENTS**

- Crystallization of Rb "pocket domain."
- Purification of four GST-PR domain fusion proteins.
- Crystallization of wild-type PDF1-BF1 PR domain.
- Generation of 8 mutant PDF-BF1 PR domain proteins.
- Design of a ‘trim’ construct of RIZ PR with much-improved physical properties.

**REPORTABLE OUTCOMES**

None

**CONCLUSIONS**

The most notable progress to date has tested the feasibility for crystallization and structure solution of the interesting PR domain of RIZ family proteins. The clue to the design of the optimal construct came with the discovery that RIZ PR is closely and functionally related to a methyltransferase. The results are significant since in many cases, defining the stable functional domain is key to the ultimate crystallization of the target protein. This study has established that it is feasible to generate milligram quantities of soluble protein from the RIZ tumor suppressor. In the long term, as this project expands beyond the scope of feasibility studies, atomic models generated from the planned crystallographic
studies will provide critical insight into the structural and functional roles of PR domain in malignancy. Because RIZ is a newly characterized factor, the structural data generated will be novel in the search for a biochemical understanding of malignant transformation in breast cancer. RIZ or the interacting surface between Rb and RIZ may be innovative targets for drug regulation of cell cycling. Further, the new PR domain itself may be an entirely novel candidate for a drug since this motif is needed for tumor suppressor activity of RIZ. When this domain is missing, the propensity of development of tumors is greatly increased. It has been suggested that the PR domain may function in regulating chromatin-mediated gene expression. The molecular models that will ultimately be produced in this study will provide unparalleled molecular detail of the interacting proteins that can serve as the framework to understand the role of these tumor suppressors in breast cancer. In particular, the structures of the RIZ PR domain may suggest other functional roles for this interesting protein. The translational potential for this new regulator of tumorigenesis in breast cancer is real.

REFERENCES

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