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Mutations or changes in normal cellular genes are generally linked to human cancer. Multiple gene changes involving at least two types of cancer genes, protooncogenes and tumor suppressor genes, are required for the clonal expansion of a malignant cell. The RIZ gene plays an important role in human cancer and more particularly in breast cancer. RIZ is the founding member of the PR-domain family of zinc finger genes. Two protein products are produced from the RIZ gene which differ by the presence or the absence of the PR domain : RIZ1 and RIZ2. RIZ1 is commonly lost or underexpressed in tumors whereas RIZ2 is always present. RIZ1 is a tumor suppressor whereas RIZ2 is not.

In this project, we are trying to characterize the structural and the functional role of the RIZ1-PR and/or related domains using protein crystallography. Our goal is to understand the tumor-suppression mechanism of RIZ1 and PR-proteins in breast cancer. This will provide us new tools for possible treatment in breast cancer.

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INTRODUCTION

The retinoblastoma interacting zing finger (RIZ) gene was isolated originally in a functional screen for proteins that bind to the retinoblastoma (Rb) tumor suppressor (1). RIZ maps to the human chromosome band 1p36.23 (2,3). This region commonly undergoes deletions, rearrangements or loss of heterozygozyty in tumors including human mammary cancer (4).

RIZ is the founding member of the PR-domain family zinc finger genes. It contains an Rb-binding motif called the AR domain similar to the E1A viral oncoprotein (5), eight zinc finger motifs, some GTPase and SH3 domains and a PR domain at its N-terminus (6, 7, 8, 9). The RIZ gene normally produces two different protein products, RIZ1 and RIZ2, which respectively differ in length by the presence or the absence of the PR domain. RIZ2 is produced by an internal promoter (7). RIZ1 is a tumor suppressor whereas RIZ2 is not. RIZ1 expression is commonly lost in human breast cancer tumors as well as in several other types of tumor examined (9). In contrast, RIZ2 is uniformly expressed in all cases examined. So there may be a specific negative selection for RIZ1 versus RIZ2 in tumors.

We are focusing on the PR domain of RIZ1 which plays a role in tumorigenesis in breast cancer. The PR domain is composed of approximately 100 amino-acids and is conserved in a subfamilly of kruppel-like zinc finger genes. Other members of the PR familly include the MDS1-EVI1 breakpoint gene involved in human leukemia and the PRDI-BF1 or BLIMP1 transcription repressor, which can active B-cell maturation (10-13). Different observations suggest that PR genes or the PR-containing product of these genes are the negative regulators of cell growth and tumorgenesis. A common function of the PR domain is to mediate protein-protein interaction. The PR domain of RIZ1 is a protein–binding interface (6). Binding is mediated by residues conserved among different PR domains suggesting that similar functions may be shared among them.

The PR domain was found to be homologous to the SET domains (6) which have recently been shown to mediate protein-protein interaction too (14, 15). PR and SET domains are important in tumor suppression and define a new class of tumor suppressor genes (16). Furthermore, PR proteins may function in chromatin-mediated control of gene expression as inferred from the homology with SET proteins (6).

Now, we have successfully produced milligram amounts of pure RIZ1-PR domain and this protein has been introduced into crystallization trials in order to solve its structure. Our long term goal is to determine the crystal structure of this domain in order to provide us a new, innovative tool for diagnostic purposes or possible treatment in breast cancer.

BODY

Task 1 : To construct a stable RIZ PR domain.

a) Computer modeling/threading:

The PR domain shares sequence homology with the SET domain (6).

```
                              BOX A                                        BOX B

SET CONTAINING
SUV39HI   253   GRGWGVRTLEKIRKNSFVMEYVGEIITSEE-(24)-VYTVDAAYY--GNISHFVNHSCDPNLQVYNVFID-
EZH2      621   VAGWGIFIKDPVQKNEFISEYCGEIISQDE-(21)-DFVVDATRK--GNKIRFANHSVNPNCYAKVMMVNG
MLL1      3838  IHGRGLFCKRNIDAGEMVIEYAGNVIRSIQ-(22)-EV-VDATMH--GNRARFINHSCEPNCYSRVINIDG
PR CONTAINING
BLIMP1    60    EEVIGVMSKEYIPKGTRFGPLIGEIYTNDT-(19)-HHFIDGFNEEKSNWMRYVNPAHSPREQNLAACQN-
RIZ1      39    KTRIGVWATKPILKGKKFGPFVGDKKKRSQ-(16)-WMCIDATDPEKGNWLRYVNWACSGEEQNLFPLEI-

                              BOX C

SET CONTAINING
SUV39HI   253   (7)-RIAFF-ATRTIRAGEELTFDYNMQVDPVD
EZH2      621   --DHRIGIF-AKRAIQTGEELFFDYRYSQADAL
MLL1      3838  --QKHIVIF-AMRKIYRGEELTYDYKFPIEDAS
PR CONTAINING
BLIMP1    60    --GMNIY-FYTIKPIPANQELLVWYCRDFAERL
RIZ1      39    --NRAIY-YKTLKPIAPGEELLVWYNGEDNPEI
```

In a comparison between the sequences, the Box C region is the most conserved. The main differences lie in the boxes A and B in which the PR domain has several conserved motifs that are not found in SET.

Very recently, some SET domain structures were able to be found in the literature (17-22). Generally speaking, the SET domain is rich in β-conformation : it consists of several small β-sheets, each containing a few short strands. The SET domain is most characterized by turns and meandering loops. The C-terminal region is a knot-like structure : the C-terminal segment passes through a loop. This particular structure is the most highly conserved sequence motif in the SET family and must certainly play a critical role in binding and catalysis. The structurally conserved core of the SET domain appears to be made up of two discontinuous parts of the primary sequence. These two parts are interrupted by a highly variable segment which differs in every structure.

We tried to build a homology model of our RIZ1-PR domain based on the different structures of SET domains and the general shape is the same, but we expect that the real structure of the PR domain will certainly be different in some points from the SET domain. This difference should be seen, more particularly, in the variable segment, because the sequence similarity between PR and SET domains is typically 20-30% amino acid identity. This percentage corresponds to the limit for a homology model validation.

b) Protein expression and purification

The GST-fusion construct of the PR sequence of RIZ1 was prepared by our collaborator Dr Shi HUANG at the Burnham Institute. After purification by affinity chromatography on a glutathione-agarose column and by ion-exchange chromatography on a Q-Sepharose column, we have obtained a good amount of protein to induce crystallization trials.

Task 2 : To crystallize the RIZ PR domain.

a) Crystallization trials :

We started crystallization trials as soon as we had purified the protein. We used first pre-made crystallization kits from Hampton-Research (Crystal screens I and II) and Emerald Biostructure (Wizard I and II) or our own crystallization systems (changing temperature, concentration...). Up to this time we have not be able to detect any crystals.

In order to increase the chances to crystallize the protein, we sent a sample of our protein to a facility for high throughput screening testing ~1500 conditions. For the moment we don't have the result of these searches.

More of that, the SET domain structures were done in presence of co-factors : the S-adenosylmethionine (SAM) and the S-adenosylhomocysteine (AdoHcy) (17-22). So we will try to crystallize the RIZ1 PR domain in presence of these two co-factors. They could help to stabilize the protein structure using its natural ability for methylation.

Task 3 : To solve the crystal structure of RIZ PR domain.

This part depends on the task 2, so we did not begin this part.

Task 4 : To solve the crystal structure of related PR domains.

We are working too on two other proteins: the BLIMP-PR from the same family as the RIZ1-PR domain and a longer construct of the RIZ1 protein. This last protein is particularly of interest because nobody has done a structure of this type untill right now. All the constructs were produced by the Dr Shi HUANG laboratory.

a) Protein expression, purification, characterization

- BLIMP-PR called BLPR

The PR domain of BLIMP has been made as a GST-fusion protein. The overexpression and purification of this protein is already done and we have this protein at a 25 mg/ml concentration.

- RIZ1 protein

A GST-fusion construct was prepared. The purification of the longer RIZ1 construct was difficult because of the degradation of the protein by a protease during the process and particularly during the thrombin cutting. After some analysis of the degradation products by mass spectroscopy and amino-acid analysis, we have discovered that the protein was degradated between the PR and the AR domains. The PR domain obtained in this way is not very well folded as shown by NMR and light scattering experiments.
So we asked Dr Shi Huang to prepare a His-tagged RIZ1 in order to avoid the degradation during the purification steps. This time, the process does not include a thrombin cutting. But the result was the same and the yield of the overexpression was lower.

The next step will be to produce a GST-fusion mutated RIZ1. The mutations that we plan to do correspond to the amino-acid where the degradation occurs. But we will also mutate other residues in this region. Some activity tests will be done in order to ensure that the mutations do not affect the activity of RIZ1 concerning the methylation of the Histone 3. We expect to avoid the RIZ1 degradation and to be able to obtain a good quantity of the RIZ1 protein to put it in the crystallization trials.

b) Crystallization trials

Right now, as we have the BLPR protein in good quantity, we will try to crystallize it with and without the two co-factors SAM and AdoHcy. We will try the Hampton screening and the Emerald Biostructure kits and more particularly the conditions which contain a high salt concentration as it worked for the other SET domains. We will try the high throughput screening too.

KEY RESEARCH ACCOMPLISHMENTS

- Molecular modeling of the RIZ1-PR domain
- Overexpression and purification of the RIZ PR domain used for crystallization trials
- Overexpression and purification of the BLPR domain

REPORTABLE OUTCOMES

None

CONCLUSIONS

During the first 10 months of our research, we have successfully engineered two PR domains belonging to the same family of protein : the RIZ1-PR domain (163 amino acids) and the BLPR protein (198 amino acids). These two proteins were or will be introduced to crystallization trials which will allow us to determine their 3D structures. Concerning the 3D structure, we have built a homology model of the RIZ1- PR domain. Then we have designed a mutated construct of a longer segment of the RIZ1 protein which hopefully will lead us to succeed in its structural studies.

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