STRUCTURE DETERMINATION OF THE FCS DOMAIN OF POLYCOMB REPRESSIVE COMPLEX 1 FROM *DROSOPHILA MELANOGASTER*

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ABSTRACT

Polycomb group (PcG) proteins play an important regulatory role in gene expression. These proteins are crucial in stem cell regulation and normal development in all organisms. The FCS domain is part of a multimeric polycomb protein complex called Polycomb repressive complex 1 (PRC1). Based on previous studies, the domain has been hypothesized as a link binding PRC1 to polylinker DNA. This protein/DNA interaction allows other proteins to associate with the repression complex, creating a higher order repressed chromatin structure. This study seeks to determine the structure of the FCS domain via protein crystallization that utilizes a fusion protein construct. The fusion protein methodology attaches the FCS domain to maltose binding protein (MBP), promoting crystallization. The FCS-MBP protein was over-expressed and purified to more than 95% purity followed by observation of FCS-MBP protein crystals. This study determined an adequate expression system, purified the FCS-MBP protein, and observed and refined crystal growth in the FCS-MBP construct. Further optimization of the crystals is required to analyze and determine the FCS domain structure.

INTRODUCTION

Gene expression is the process by which genetic material is used as a template for the manufacturing of gene products including proteins. Proteins play an integral part in every functional cell and, as expected, gene expression is a complex and highly regulated process. Regulatory processes monitor genetic expression according to environmental factors and cellular signals, thereby allowing cells to express a unique combination of genetic products to suit functional needs. Each cell possesses its own individual function that contributes to the needs of an organism. A mutation that alters genetic expression or diverts a cell from its expected purpose often affects an organism adversely. Similarly, organisms are affected unfavorably by additional information such as extra chromosomes. These changes disrupt the delicate balance of genetic information essential for normal function and generally result in unwanted consequences such as cancer or cellular/organismal death.

Gene silencing, a mechanism exhibited by all cells, functions to maintain the genetic balance in organisms. Proteins assist in the silencing process by a variety of mechanisms, many of which are still speculative. Recently much work has been done on regulatory proteins known as Polycomb proteins. Studies have shown that Polycomb proteins play an integral part in dosage compensation in X-linked genes in humans (1). Also, more than 150 genes involved with cell growth and proliferation have been identified that may be subjected to Polycomb protein repression (2). It is because of their important regulatory role in cellular development, stem cells, and cancer that much attention has been directed towards better understanding Polycomb proteins (3,4).

Polycomb group (PcG) proteins arise from conserved DNA sequences found in all organisms and known as Polycomb group genes. PcG proteins aggregate into large multiprotein complexes that work on chromatin, creating higher order structures silencing targeted genes over

many mitotic divisions. Two multimeric complexes, Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2), have been most abundantly studied because of their intrinsic collaborative behavior. PRC1 and PRC2 are composed of several individual PcG proteins, all contributing to the overall function of the repressive complexes. PRC2 core members include the enhancer of zeste [E(z)], extra sex combs (Esc), the suppressor of zeste 12 [Su(z)12], and P55 (5). PRC1 contains the core proteins, polyhomeotic (Ph), posterior sex combs (Psc) (Bmi-1 in humans), RING1, and polycomb (Pc) (10,11).

PRC2 possesses methyltransferase activity attributed to the SET domain within E(z) with specificity for K9 and K27 of histone 3 (6-9). This histone methytransferase activity establishes a binding site for PRC1 recruiting the repression complex to the targeted gene. PRC1 is then responsible in compacting the targeted genes, as marked by PRC2, creating a repressed chromatin structure (12). Although known to exhibit these actions, the exact mechanisms involved in the formation of the repressed chromatin structure are undiscovered. PRC1 has been known to inhibit chromatin remodeling enzymes which could be attributed to a possible mechanism (13). It has also been observed that PRC1 is coupled with transcription factors such as TBP, TFIIF, and TFIIB resulting in an alternative method of repression (14, 15). Thanks to the seemingly multiple capabilities of PRC1 one can isolate functional domains, determine the structure of such sequences, and piece together the individual proteins to propose a mechanism that explains PRC1's unique abilities.

This study focuses on a 30 amino acid sequence called the FCS domain located in various subunits in PRC1. The FCS domain is a conserved sequence in all organisms. It is named appropriately after the homologous sequence of phenylalanine (F), cysteine (C), and serine (S) amino acids prevalent in all variations of the FCS domain. Because of the arrangement of the amino acids, the sequence is assumed to exhibit a Zn-ribbon fold. This Zn-binding domain has been unappreciated in the past, but with recent evidence of the FCS domain's unique activities, the FCS domain has proven to be essential to the overall repressive function of PRC1. Interestingly the FCS domain is able to exhibit non-sequence-specific binding to DNA via the conserved first two cysteine residues (16). The zinc finger motif of TFIIA is known to bind to RNA and DNA (17). The sequences of the FCS domain are homologically similar to those of the zinc finger motif of TFIIA. Because of these similarities, a proposed FCS Zn-ribbon motif is possible; however, the actual FCS structure is still unknown. Based on recent studies, one may hypothesize that the FCS domain is a link binding PRC1 to polylinker DNA, allowing other proteins to associate with the repression complex creating the higher order repressed chromatin structure.

Although much literature defends this hypothesis, there is no physical evidence of the actual mechanism used by FCS in gene silencing. Structural analysis of a protein often gives insight into the behavior of a molecule. Proposed models provide bases for understanding mechanisms, functions, and interactions of biomolecules. Indeed, structures have been an essential tool for understanding molecules, from Rosalind Franklin's X-ray crystallography, the foundation for Watson and Crick's DNA structure, to recent discoveries about the mechanisms of C-RING1B of PRC1 (5).

Because of the importance of understanding the structure of the FCS domain, various human and *Drosophila* FCS domains were studied. Preliminary studies of the FCS domain by Professor Chongwoo Kim and his lab showed that the NMR was an inadequate technique for determining the structure of the FCS domain (C. Kim, personal communication, June 8, 2008). This conclusion was based primarily on problematic structure evaluations. Attempts to crystallize the FCS domain following NMR failed because of the extremely soluble nature of the domain.

This study reexamines the FCS domain's structure by using an alternative crystallographic approach. Two versions of the human FCS domain, hPh1 and hPh3, and one version in *Drosophila melanogaster*, sex comb on midleg (Scm) were purified and used for this study based on prior success with FCS domains (C. Kim, personal communication, June 8, 2008).

This alternative method used protein fusion to couple a binding protein to the FCS domain. Maltose binding protein (MBP) was chosen because of its success in previous structural studies (18-20). With this new approach, two versions of the FCS-MBP sequences of hPh1, hPh3 and Scm with two different linker sequences were cloned, providing six distinctly different FCS-MBP samples. The FCS-MBP crystals produced by this study, a small but crucial domain of PRC1 could provide insight into fundamental mechanisms of PcG protein repressed chromatin structures and their function in organismal regulation and development.

EXPERIMENTAL PROCEDURES

Protein Cloning and Expression.

 hPh1 (residues 796-828), hPh3 (residues 781-813), and SCM (residues 59-130) amino acids were attached to MBP amino acid sequence with a His-tag (6H) via two different linker sequences. The sequences were cloned via restriction enzymes into pETMxt and pBADMxt vectors. The pBADMxt and pETMxt vectors were transformed into ARI814 and BL21(DE3) pLysS E. coli cells respectively (21). The cells were added to a starter culture of Luria-Bertani (LB) media with Ampicillin solution. Chloramphenicol was also added to the BL21(DE3) pLysS culture to maintain the pETMxt plasmid. These starter cultures were grown overnight on an incubated shaker at 250 RPM and 37°C. The overnight starter cultures were used to inoculate several 1L volumes of LB media with proper antibiotics. The cells were grown and induced with 20% Arabinose for ARI814 cells and Isopropyl-β-D-Thiogalactopyranoside for BL21(DE3) pLysS cells. The growth culture cells were harvested and frozen at -80°C.

Protein Purification.

 A typical protein purification protocol involved re-suspending the cells from the harvested culture in a buffer consisting of 50mM Tris (pH8), 200 mM NaCl, 25 mM imidazole (pH 7.5), 10mM β-mercaptoethanol, and 1 mM PMSF. The cells were lysed by sonication, centrifuged in AvanatiJ20: JA20/15K, and separated into supernatant and cellular debris. All proteins were extracted from the supernatant by means of Ni affinity chromatography (NiSepharose). Tobacco etch virus (TEV) was used to cut the His-tag from the fusion protein. This was followed by ion exchange chromatography (HiTrap SP and HiTrap Q column). Protein solutions were concentrated using a Millipore stirred Ultrafiltration unit to approximately 30 mg/ml. Figure 1 schematically represents the protein constructs following purification with their approximate masses.

FCS domain	Protein construct product after purification from pETMxt vector	Protein construct product after purification from pBADMxt vector
hph1	MBP — SS — FCS hph1 44747.8 Da	MBP — SSAM — FCS hph1 44950.1 Da
hph3	MBP — SS — FCS hph3 44809.0 Da	MBP — SSAME — FCS SCM 45140.4 Da
SCM	MBP — SSAM — FCS SCM 49181.0 Da	MBP — SSAM — FCS hph3 49310.1 Da
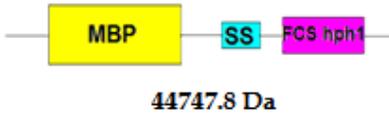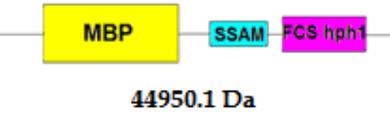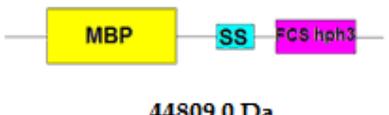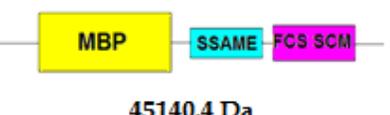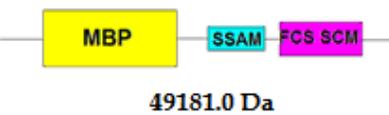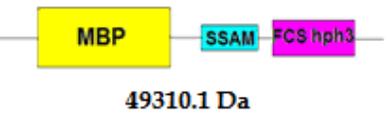
Figure 1. Schematic drawings (not to scale) of varying FCS domains expressed by their appropriate vectors. Below the illustrations are the approximate molecular weights of the protein products. Yellow box represents MBP. The blue box represents the varying linker sequences. Pink box is the FCS domain of hph1, hph3, and Scm.

Electrophoresis.

SDS PAGE gels were used to determine the purity of the protein sample. They were run in an anode and cathode running buffer consisting of 0.2 M TrisHCl pH 8.9, and 0.1 M Tris, .01 M Tricine, and 0.1% SDS. 2X sample buffer was added to the sample to denature the protein. 10ul of Invitrogen Benchmark Protein Ladder were added to the last well as a reference. SDS gels ran at a constant current of 60 mA (per gel) for approximately two hours. SDS gels were run prior to purification and after the purification process.

10% Tris-glycine Native gels were used in order to determine the homogeneity of the protein sample. Proteins samples were mixed with a 6X Native sample loading buffer and run in a 1X native running buffer at a constant voltage of 120V for three hours. The native gels were run after the purification process.

Commercially purchased Coomassie Blue stain was used as a dye for SDS and native gels. Gels were incubated with Coomassie Blue stain for 15 minutes. Stained gels were destained in two solutions, 50% MeOH, 10% HAc and 5% EtOH, 7% HAc, for 60 minutes in the former solution and overnight in the latter solution.

Crystallization.

Purified samples of the protein were tested using Hampton Research's Macromolecular Crystallization Kit (crystal screens 1 and 2). The hanging drop vapor diffusion technique was used in order to screen for crystals (22). Evidence of crystals in the initial screens was followed up by repeat screens under varied conditions.

RESULTS

An adequate test expression system for DNA clones of FCS-MBP sequence constructs was determined.

The pBADMxt hph1, hph3, and SCM protein constructs that were expressed in ARI814 cells yielded more than an adequate amount of the FCS-MBP protein. As shown in Figure 2A, the SDS PAGE gel indicated large amounts of soluble at the expected protein construct's weight of about 47 kDa. Also note the over-expression of a protein of 15 kDa, which is a byproduct of the pETMxt expression system and is not the FCS-MBP protein. Similarly the pETMxt hph1, hph3, and SCM protein constructs expressed in pLysS cells produced large amounts of the FCS-MBP protein in the soluble form around 47-50 kDa as indicated in Figure 2B. This figure also shows an over-expressed protein at 10 kDa. These over-expressions are a byproduct of the pBADMxt expression system and are not relevant to this study.
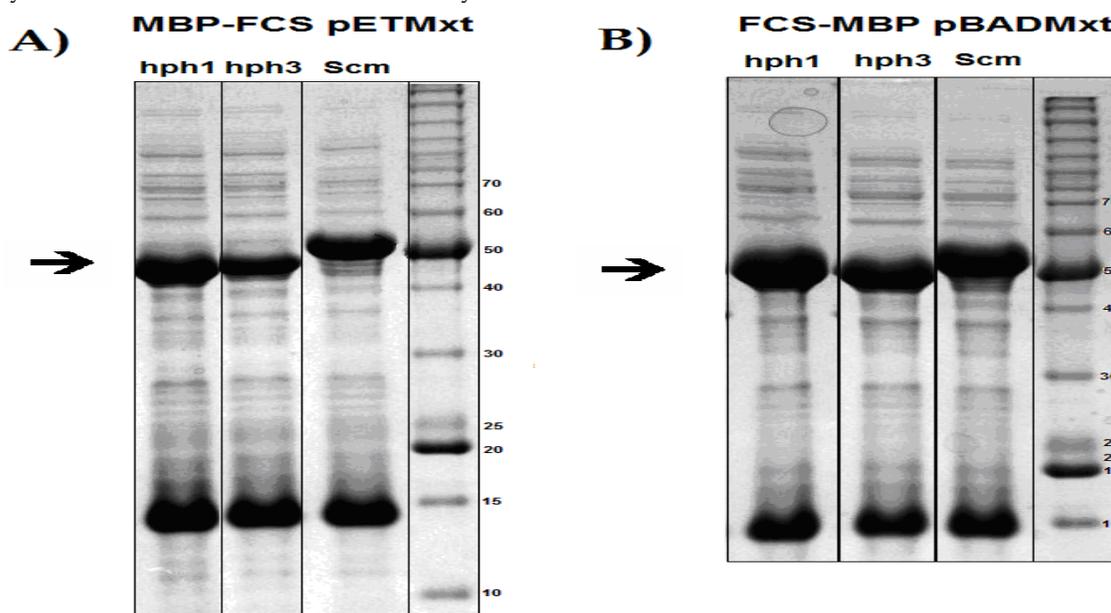
Figure 2. Test expression of soluble proteins of FCS-MBP (hPh1, hPh3, Scm) in pETMxt (A) and pBADMxt (B). FCS-MBP protein construct is located at the heavy bands (~47-50 kDa) corresponding to their appropriate molecular weight. Heavy band indicates adequate protein expression. Other bands are normal cellular proteins found in BL21(DE3) pLysS and ARI814 cells for the pETMxt and pBADMxt respectively. Molecular weight marker is in kDa.

In both of these cases, no prior purification was done to the sample, which accounts for the other bands on the SDS PAGE gels. The FCS-MBP construct has an approximate weight of 47 kDa and the bands indicated on this gel are conclusively the protein construct. An adequate expression system is essential for crystallization as it allows large amounts of proteins to be produced. Purification of the protein samples followed the proteins' growth.

FCS-MBP fusion proteins were purified to greater than 95%.

Based on the analysis and observation of the SDS PAGE and native gels (Figure 3), the observed purity of the FCS-MBP proteins was estimated to be at least 95% with some samples, FCS-MBP hph1 pETMxt, exhibiting ~99% purity. These conclusions were based on the cleanliness of the gel, as single bands generally indicate purity in a protein sample. The FCS-MBP Scm pBADMxt sample was not run in this study because of constraints and will be analyzed in the future. Minor contaminants, as indicated by the low molecular weight bands in Figure 3A and the upper bands in Figure 3B, are impurities and factored into the holistic purification estimation of the protein

constructs. With an overall percentage yielding greater than 95%, crystallization attempts followed.

A)

B)
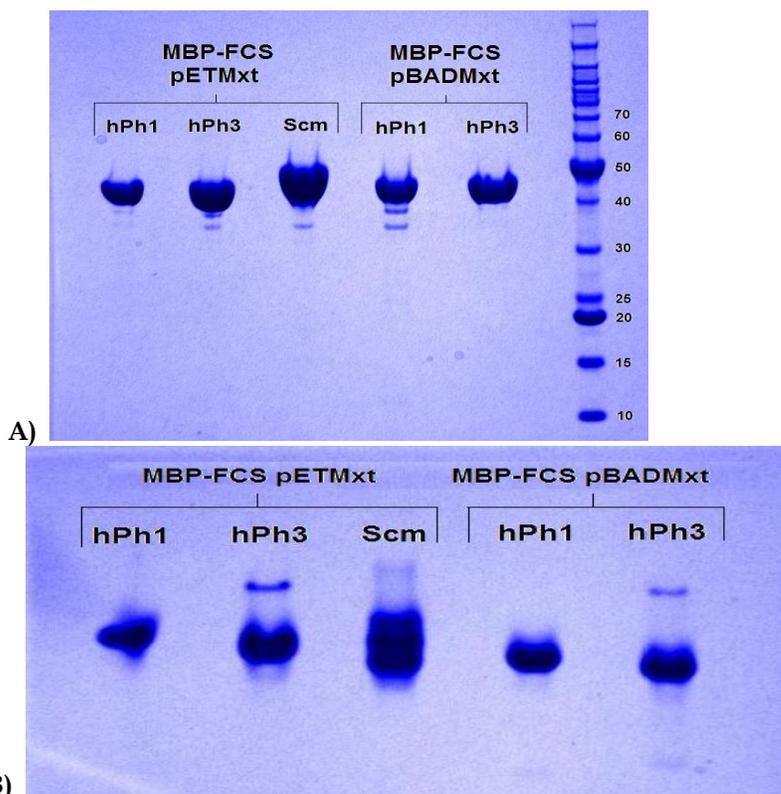
Figure 3.
A)   SDS 15% Tris-Tricine gel of purified protein samples. MBP-FCS (hph1, hph3, Scm) pETMxt and MBP-FCS (hph1, hph3) pBADMxt are ordered respectively. Low molecular weight impurities are prevalent in hph3 and Scm pETMxt as well as hph1 pBADMxt.
B)   10% Native gel of purified protein samples. MBP-FCS (hph1, hph3, Scm) pETMxt and MBP-FCS (hph1, hph3) pBADMxt are ordered respectively. Migration unity is a direct indicator of the homogeneity of the sample. hph3 and Scm pETMxt and hph pBADMxt exhibit heterogeneity indicated by multiple bands.

FCS-MBP hph1 protein pETMxt crystals were observed and refined.
The hph1 FCS-MBP protein pETMxt crystals were initially observed during the crystal screening process under the reagent containing 0.01 M Zinc Sulfate, 0.1 M MES pH 6.5, and 25% Polyethylene glycol (PEG) after 3 days of growth (Figure 4 left).
        Of all the different protein complexes examined in this study, hph1 FCS-MBP pETMxt was the only one to crystallize. Further refinement of the hph1 protein followed and resulted in more defined and larger crystals (Figure 4 right). At least a ten fold increase was observed from the initial to the refined crystals.
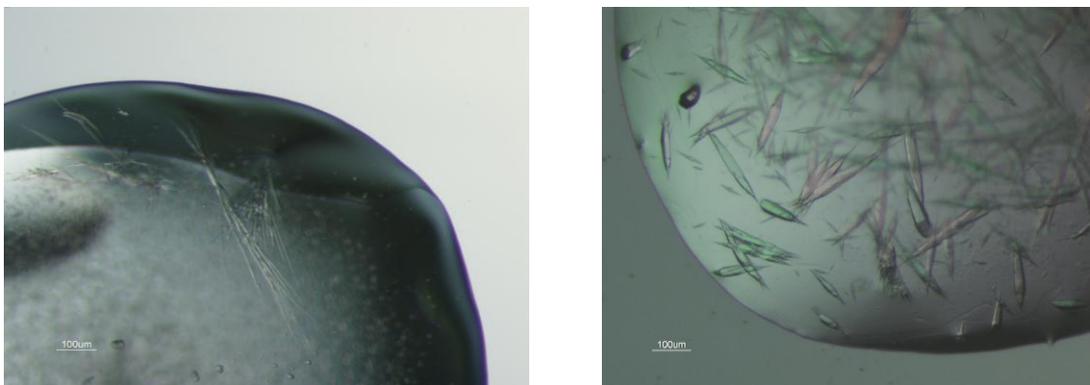
Figure 4. Crystal growth of FCS-MBP hph1 pETMxt Initial and Refined.
The crystals pictured on the left were the initial evidence of success in the screening process. Optimization of the reagents and environmental conditions led to significant lateral and transverse growth in the FCS-MBP crystals as shown in the photo to the right.

Although the crystals are assumed to be the protein MBP-FCS protein construct, it is possible that salt or other impurities precipitated out of solution given the changing environment. SDS PAGE and native gels were run on the unknown crystals to verify their identity (Figure 5).
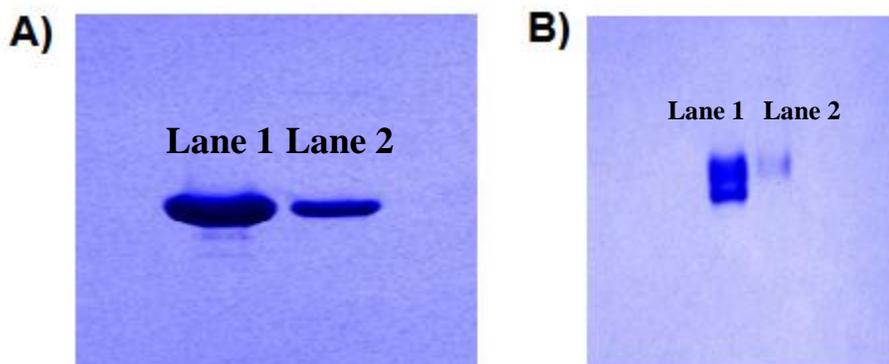
Figure 5. Identity of the observed crystals.
  A) SDS PAGE gel of FCS-MBP hph1 pETMxt. Control hph1(30mg/ml) located in lane 1 and solubilized crystals (~10 mg/ml) indicated in lane 2. Identical gel migration of the control and test sample observed. Lighter band can be accounted for by a less concentrated test sample.
  B) Native gel of FCS-MBP hph1 pETMxt. Control hph1 (30mg/ml) located in lane 1 and solubilized crystals (~10 mg/ml) indicated in lane 2. Identical gel migration of the control and test sample observed. Lighter band in lane 2 (test sample) can be accounted for by a less concentrated sample.

The purified FCS-MBP hph1 pETMxt protein (~ 30mg/ml), Lane 1, was used as a control to compare it to the observed crystals, Lane 2, that were harvested and re-suspended in a 1 M Tris-HCl buffer to approximately 10mg/ml. The similar migration patterns of both the SDS and native gel identify the observed crystals as the FCS-MBP protein.

DISCUSSION

In this study the strategy of fusing Maltose Binding Protein to the target FCS domain was effective in determining the structure of the FCS domain. The MBP-FCS fusion construct was

produced, purified, and crystallized. These results are more promising than those of earlier FCS domain structural studies.

To determine the structure of a protein, crystallization requires large expression rates, homogeneity, and purity. In this study, the FCS-MBP constructs exhibited all of these characteristics and produced promising observed results. Although these are essential for crystallization, that does not mean all proteins exhibiting such characteristics will crystallize. There are a multitude of other variables depending on the protein that determine the success of crystallization. For example, the FCS domain alone was incapable of crystallizing regardless of its seemingly ideal characteristics. Similarly, of the six different samples studied, only one construct was successful. Each protein has different properties defined by its structure. Indeed, because of this variation, proteins are expected to behave differently, and this accounts for the low success rate. In looking at the amino acid structures of hph1 pETMxt and hph1 pBADMxt, the only difference between the two proteins is two amino acids (AM) in the linker sequence. However, the small differences in sequence, as shown in Figure 3, exemplify the importance of the primary structure of the protein and how it affects the molecule as a whole. In this case, hph1 pBADMxt exhibited low molecular weight impurities which may have adversely affected its ability to crystallize. Of the six proteins, only one, FCS-MBP hph1 pETMxt, successfully crystallized, which is an incredible feat and a success in itself given the time.

The fusion protein construct of FCS-MBP hph1 pETMxt crystallized and, as indicated in Figure 3, exhibited exceptional purity of about 99%. This emphasizes a correlation between purity and crystallization. As expected, no crystals were evident in any of the other protein samples. More importantly, as this study has shown, the FCS domain is capable of crystallizing. As the differences between the initial crystallization and the refined crystallization indicate (Figure 4), this protein is capable of optimization. Unfortunately the refined crystals observed in Figure 4 were not quite large enough to be analyzed by X-ray crystallography. They needed to be at least triple the size in order to collect accurate data from diffracted X-rays.

Because we know this domain will purify, crystallize, and optimize, further studies will use this technique to determine its function. Indeed, employing MBP as a protein crystallization facilitator for the FCS domain is essential for FCS structural determination. Currently, further refinement of the crystal's growth is in progress. X-ray crystallography will follow pending full optimization. The data will then be analyzed and a proposed structure for the FCS domain established. With these promising results, it is only a matter of time before the structure is determined.

Structure gives insight into the mechanisms and function of proteins at the cellular level. Based on this structure, the hypothesis that the FCS domain plays an integral part in binding nucleic acids creating a higher ordered structure can be asserted, challenged, or redefined. Whatever the case may be, the insight obtained from such analysis provides a foundation for future studies.

The ability of organisms to function depends on the interaction of sub-cellular molecules and atoms, with proteins an integral part of such molecules. Polycomb group proteins are essential to the development and regulation of all organisms. The FCS domain, an ~30 amino acid sequence, as part of an important Polycomb protein repression complex, PRC1, was examined in this study. The FCS domain has been shown to take an active role in the regulatory mechanisms of the repression complexes; however, little is known about the actual function of the domain. By stepping back and examining the FCS domain structure, one could possibly establish its role in PRC1, creating a better understanding of PRC1.

The major Polycomb complexes are important because deviations from their normal functions can be detrimental to organisms. For example, PcG repression complexes such as PRC1 and PRC2 are directly associated with prostate, breast, and bladder cancer (23, 24). In Chinnaiyan's study, over-expression of EZH2 has been directly linked to cancers of various types (23). Since bladder, breast, and prostate cancer are among the most common types afflicting individuals today, it is crucial to learn how to prevent and treat them. By analyzing the structures

of functional domains, such as FCS, researchers can study protein complexes and hypothesize how they interact at the transcriptional and translation levels. In this case, by learning exactly how or why PcG proteins regulate cellular activity we could hasten the creation of anticancer drugs or establish a foundation for a better understanding of protein complexes' repression role in gene expression. Despite scientific advances, the regulatory mechanisms of genetic expression are still largely unknown. Although this study deals with a minute aspect of the regulation, success at this level is essential to piecing together the complex and highly regulated process of gene expression in all organisms.

ACKNOWLEDGEMENTS

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