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FOREWORD

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Abstract

The project is a postdoctoral training fellowship for Dr. Robert Nolte, to support X-ray crystallographic studies of cancer-related macromolecular complexes. (1) We have expressed various constructs of the human estrogen receptor DNA-binding domain, but we have failed to obtain crystallizable protein. Success of other groups in this and related efforts have led us to propose a modified statement of work, as outlined below, consistent with the central training goals of this project. (2) We have prepared crystals of a complex between a six Zn-finger segment of the transcription factor TFIID and a 31 base pair DNA site. This structure will reveal significant aspects of DNA recognition by Zn-finger proteins, which regulate many loci important for cell growth. (3) During the coming project year, Dr. Nolte will also embark on structural studies of Src-related tyrosine kinase activity, as described in the body of the progress report.

Introduction

The goal of this project is postdoctoral training for Dr. Robert Nolte in the area of structural biology and more specifically X-ray crystallography as applied to problems in cancer and to breast cancer in particular. It was proposed to crystallize the hormone binding domain (HBD) of the estrogen receptor, in order to determine its structure and the implications of structure for the mode of hormone recognition. As summarized more fully in the body of this report, a number of constructs were prepared, without success in obtaining soluble crystallizable protein. Because other groups have now achieved similar goals, (Bourquet et al., 1995; Renand et al., 1995; Wagner et al., 1995), we request a change in the statement of work (SOW) to two other, related projects in the structural biology of cancer, in order to ensure rapid progress in Dr. Nolte's training, especially in X-ray crystallographic structure determination. The proposed new SOW and its relation to the old SOW are outlined below.

The problem : Cancer involves anomalies in cellular regulation. Two key regulatory steps are the transduction of signals from the cell surface and the control of specific gene expression. The estrogen receptor, on which the original SOW focused, participates in both these steps. It receives hormonal signals and in response, activates defined genes. The recently discovered BCRA1 gene appears to encode a nuclear protein that may regulate gene expression (Miki et al., 1994). Progress in structural studies of tyrosine-kinase mediated signaling pathways and protein/DNA complexes that regulate gene expression makes the possibility of structure-based drug discovery and development a real one, but many basic principles remain to be discovered. The modified SOW outlined here focuses on different cases of gene regulation and signal transduction. The goal remains to use X-ray crystallography to uncover the basic molecular structural features governing these processes and to train Dr. Nolte in this field.

Previous work

(i) A number of structural studies of protein/DNA complexes involved in transcriptional regulation have provided the guidelines of how to think about specific recognition (see, for example, Harrison, 1991 and Steitz, 1990). An important notion to emerge both from the structural studies and from studies of transcriptional regulation at many complex promoter/enhancer sites is that large multi-protein complexes bound to DNA are critical for the sort of combinational control seen in cells of all higher eukaryotes (and in human cells in particular). Work in our laboratory on the polymerase III transcription factor known as TFIIIA has led to crystallization of a complex between a part of the factor containing six "zinc fingers" (each finger is a distinct DNA-binding domain) and a 31 base pair DNA site. This is a larger complex than any so far studied from this class of transcriptional regulatory proteins.

(ii) Protein tyrosine kinases are responsible for many key signaling events in normal and cancer cells. The kinases related to Src (so designated because of its discovery in Raus sarcoma virus) have been especially widely studied, in part because of the early importance of Src in revealing the existence of oncogenes and oncogene products. We have determined the structure of the Src-homology 2 (SH2) and Src-homology 3 (SH3) domains of lymphocyte kinase (Lck), and we are engaged in an effort to crystallize the auto-inhibited form of the full Lck protein. An important adjunct to this effort, which could spawn a number of drug-design strategies, will be to apply the expression approaches developed for Lck to another Src-like kinase such as Fyn, as well as to the protein that is "next" in the Lck-initiated pathway of T-cell signaling, zeta associated protein 70 (ZAP-70).

Purpose of present work

The broad goal is to enhance our understanding of specific protein-protein and protein-DNA interactions in gene regulation and in signal transduction. In particular, we are making rapid progress in the X-ray crystallographic structure determination of a complex between a larger DNA-binding segment from the polymerase III regulatory factor TFIIIA and its cognate site. We are also building upon our own recent success in structural studies of regulatory domains from Src-like protein kinases to establish a broader program. Thus, Dr. Nolte will first complete the TFIIIA/DNA structure determination (in collaboration with Dr. Ray Brown of this laboratory), a task that should take about 9 months. He will then join our effort on Lck and initiate a parallel project on either Fyn or ZAP-70, as the state of our overall project requires. The cancer relevance of these studies is described in the body of the report.

Methods.

All of the projects described here involve expression of proteins, or of specific protein domains, in order to crystallize them for investigation by X-ray diffraction. X-ray methods are the best way to determine the detailed atomic structure of a protein, a necessary pre-requisite for molecular analysis of function and drug-targeting strategies.

Body of Narrative

Estrogen Receptor: The estrogen receptor (ER) plays a significant role in the onset and treatment of breast cancer. Details of its structure could potentially lead to the design of new therapeutic agents and increase our understanding of the role this receptor plays in the onset of cancer. The goal of the first year of this training grant was to express and crystallize the hormone binding domain of the ER and use of X-ray crystallography to determine the molecular details of the binding of estrogen and anti-estrogen compounds to the ER.

Previous work in this laboratory had concentrated on constructs containing only the hormone-binding domain (HBD) beginning at either residue 297 or 342 and ending with residue 551 or 590 (Figure 1). These four constructs failed to yield soluble protein, as required for the proposed crystallographic studies. During the first year of this grant protein expression constructs were made containing larger portions of the ER in an attempt to refine the domain boundaries of the HBD under the premise that the previous domain boundaries did not encompass the complete hormone binding domain. The constructs were made using a polymerase-chain reaction (PCR) method to obtain the DNA sequences corresponding to the fragments of interest. The fragments were subcloned into a pRSETa expression vector (Invitrogen), using the Nhe1 and Bamh1 restriction sites, and then transfected into *E. coli* strain BL21. A summary of the expression constructs created during the first year of this grant period is shown in Figure 1. The constructs are labeled to represent the different modular domains that have been identified in the nuclear receptor family. Little is known about A/B,D, and F domains. The C domain is the DNA binding module, and the E domain is the hormone binding domain. Many of these plasmid constructs were engineered to contain a polyhistidine tag on the N-terminus, which was used in the purification of the proteins.

Expression tests of four of the new constructs are shown in Figure 2. This figure shows that some protein is being made by these constructs under control of the inducible promoter (evident by comparing the "Uninduced" and "Induced" lanes for each construct.) A Western blot of these gels (not shown) with a polyclonal antibody specific to the C-terminal 30Kd of the Human Estrogen Receptor was used to confirm the expression of the ER constructs, and identify the position of these expression products (shown as white arrows in the Figure). However, these proteins are insoluble and remain in the pellet fraction

after ultracentrifugation. The "DE" and "DEF" constructs showed a moderate level of protein expression, but the protein likewise remained in the insoluble fraction during purification. The "CDE" and "CDEF" constructs failed to express at a significant level when initially transformed into BL21 or BL21-pLysS lines. Western blots using an antibody to the C-terminal portion of the ER showed that a small amount of protein was produced. Two sequential arginine codons rarely used in *E.coli* were noticed in the "C" domain, which may have been responsible for the expression failure. A helper plasmid containing an arginyl-tRNA (Brinkmann et al., 1989) was co-transfected with the constructs, resulting in more efficient expression through this region. Expression of the "CDE" and "CDEF" constructs did not, however, yield significant quantities of protein. The "ABCDE" construct also expressed protein with the helper plasmid present, but it was rapidly degraded, and isolation of the unclipped product was not successful. The full-length ER construct did not express any observable protein in this system.

Because we could not express soluble protein in the *E. coli* expression system, we chose to solubilize the different protein products in 8M urea, followed by refolding. Purification of the solubilized material was attempted by binding the polyhistidine tagged proteins to a Ni-NTA column. The "CDEF" and "DEF" protein constructs aggregated on the column despite the presence of 7M urea, and elution with imidazole yielded only microgram amounts of protein, which were insufficient for a crystallographic study. Attempts to refold the protein on the column by slowly removing the urea prior to the elution with imidazole failed to change the tendency of the protein to aggregate. Refolding of the "DE" and "CDE" constructs was not attempted. A summary of these results is shown in Table 1.

During the tenth month of the first year we learned that several other groups had succeeded in crystallizing the HBD from homologous receptors and that the structure determinations were essentially complete. Some of these results have recently been published (Bourquet et al., 1995; Wagner et al., 1995; Renaud et al., 1995). Moreover, another group has obtained crystals of the ER-HBD itself (P.Sigler, personal communication). These structures demonstrate that the last series of constructs we made should contain the entire domains of interest. Since we failed to make soluble protein with these constructs in a *E.coli* expression system, the next logical step for this project would be to start over

using a cell culture based expression system. We therefore decided to switch the emphasis of the project in order not to compromise Dr. Nolte's training in X-ray crystallography as applied to cancer related problems since starting up a new expression system for the estrogen receptor constructs would take a significant period of time.

2.TFIIIA: The transcription factor IIIA (TFIIIA) from Xenopus laevis binds both DNA and RNA. The discovery of multiple zinc finger motifs in TFIIIA pointed to a complex mode of protein-DNA interaction. Each of these motifs is an independently folded protein domain that recognizes a specific DNA sequence of three base pairs. Nine tandem zinc fingers are present in TFIIIA, allowing the protein to bind to an extensive region of the 5S RNA gene promoter.

The first six zinc fingers of TFIIIA have been expressed in E. coli and reconstituted with a duplex of two 31 base-pair synthetic DNA strands whose sequence derives from the Xenopus oocyte 5S RNA gene promoter. Crystals of the complex were grown using "native" DNA and one of two different substituted DNAs, in which three thymidine nucleotides were modified to contain iodine at specific locations. Several diffraction data sets have been collected from native and derivative crystals with the data in some cases extending to beyond 3.2Å resolution (Table 2). The work is at the stage of interpretation of difference-Patterson functions. Crystals for additional data collection have been grown.

Many genes with key roles in cancer are regulated by zinc-finger containing proteins. Mutations within the recently identified BRCA1 gene (Miki et al., 1994) have been correlated to susceptibility of breast cancer in individuals. This gene contains a zinc finger-like domain at its amino terminus. Understanding the principles of DNA recognition by members of this family will be important for establishing a picture of their transcriptional control. In particular, the feasibility of efforts to find drugs that target gene-specific transcriptional regulation will depend on such understanding.

Src-family kinases. We have determined the structures of the regulatory domains of the lymphocyte tyrosine kinase, Lck (Eck et al., 1993; Eck et al., 1994) We are currently engaged in efforts to crystallize the auto-inhibited state of the full-length, multi-domain molecule. Src-like kinases of this sort are important in a variety of tumors, and they appear to modify proteins that have a role in

breast cancer (see, for example, Arnold et al., 1995). Upon completion of the TFIIIA project, Dr. Nolte will build upon the work on Lck kinases by carrying out parallel studies of the related Fyn structure or by attempting expression and crystallization of ZAP-70, the immediate downstream target of Lck. These studies would not commence until year 3 of the project.

Conclusion:

The revised SOW requested here for the coming year should see the completion of a crystallographic study of a complex between (TFIIIA fingers 1-6) and its cognate DNA. This structure will yield significant new information about modes of specific DNA recognition by this family of transcription factors. Other work in our laboratory on gene regulation at complex promoter/enhancers will then be poised to capitalize as the results. Dr. Nolte will himself complete this period of training and prepare to launch independent directions, by turning his attention to the cancer-related Src-like tyrosine kinases and to structural analysis of corresponding signal transduction pathways. Parallel efforts in our laboratory are proving successful at expression and crystallization of domains of the Lck kinase, and we will expand this effort to include a close homologue Fyn, as well as the downstream "effector" of Lck, ZAP-70.

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Table 1 : Summary of Estrogen Receptor Expression Results

| Construct | Molecular Weight | Protein Expression | Protein Soluble | Protein Refolding |
|-----------|------------------|--------------------|-----------------|-------------------|
| ABCDEF | 65 Kd | No | --- | --- |
| ABCDE | 61 Kd | Rapidly Degraded | No | --- |
| CDE | 41 Kd | Poor | No | ? |
| CDEF | 46 Kd | Poor | No | No |
| DE | 34 Kd | Moderate | No | ? |
| DEF | 38 Kd | Moderate | No | No |

Table 2 : Summary of TF3a X-Ray Data

| Crystal | Unit Cell (Å and degrees) | Resolution Range (Å) | R-sym | X-ray Source |
|--------------|-------------------------------------|-------------------------|-------|----------------|
| "Native" | 63.8x64.6x78.0 90.1, 92.1, 102.6 | 20.0 - 4.0 | 8.8 | Rotating Anode |
| Derivative 1 | 64.3x64.4x78.3 90.0, 93.0, 102.5 | 20.0 - 3.5 | 8.2 | Rotating Anode |
| Derivative 2 | 63.3x63.5x76.5 90.1, 93.5, 102.6 | 20.0 - 3.1 | 7.3 | NSLS - X12B |

R-sym: conventional crystallographic agreement factor for symmetry-related intensities

NSLS-X12B: beam line X12B at National Synchrotron Light Source, Brookhaven National Laboratory

Figure 1: Summary of Estrogen Receptor Expression Constructs

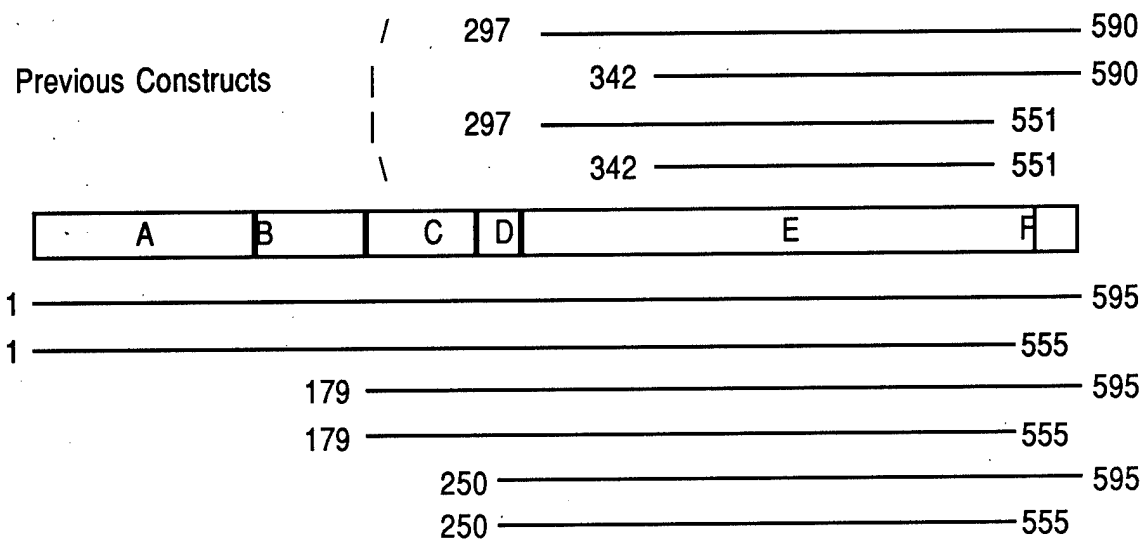


Figure 2 : Expression and Solubility Tests of Estrogen Receptor Constructs

This figure shows the expression of four of the new Estrogen receptor constructs. For each of the four constructs the following is shown. 1) Uninduced : Transfected *E. coli* without the addition of IPTG. 2) Induced : Transfected *E. coli* four hours after IPTG addition. 3) Whole Cell : Sonicated *E. coli* after four hour induction. 4) Soluble : Soluble portion of induced *E. coli* lysate (by Ultracentrifugation). 5) Insoluble : Pellet from Ultracentrifugation of induced *E. coli* Lysate.

