

AD _____

Award Number: DAMD17-98-1-8190

TITLE: Structural Characterization of a Novel HER2/neu Binding
Ets Factor, ESX

PRINCIPAL INVESTIGATOR: C. Nunes
C. Benz, M.D.

CONTRACTING ORGANIZATION: The University of California
San Francisco, California 94143-0962

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020125 152

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 98 - 30 Jun 01)	
4. TITLE AND SUBTITLE Structural Characterization of a Novel HER2/neu Binding Ets Factor, ESX		5. FUNDING NUMBERS DAMD17-98-1-8190	
6. AUTHOR(S) C. Nunes C. Benz, M.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of California San Francisco, California 94143-0962 E-Mail: nunes@msg.ucsf.edu		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Understanding the structural basis of protein-nucleic interactions involved in breast cancer cell proliferation would prove beneficial to the rational design of therapeutic strategies aimed at reducing proliferation. We have focused on two proteins, ESX and Fmu, which serve as models for understanding the structural role of protein-nucleic acid interactions in the growth control of breast cancer. ESX, an Ets family transcription factor, binds to the Ets response element of the HER2 promoter and upregulates HER2 transcription. Fmu, a cytosine methyltransferase that modifies rRNA, is the bacterial homolog of human nucleolar p120, an enzyme overexpressed in human breast tumors and likely critical to the proliferative phenotype. The research goal is to determine these protein structures by x-ray crystallography and define the specific contacts with their binding targets to enable the design of small molecule modulators of proliferative function. As crystals of ESX have not been obtained, we have focused on Fmu structure determination. We present the structures of Fmu alone, and an Fmu-cofactor complex. Structure determination of Fmu will provide insight into RNA binding and modification, and present a novel target for breast cancer intervention			
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 11
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

TABLE OF CONTENTS:

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....8

Reportable Outcomes.....9

Conclusions.....10

References.....11

INTRODUCTION:

Esx is an Ets family transcription factor that binds to the Ets response element of the *HER2/neu* promoter (GAGGAA) where it causes up-regulation of HER2 transcription. Overexpression of the *HER2/neu* proto-oncogene in breast cancer occurs in approximately 20% of cases and is related to poor patient prognosis. In our initial proposal, we planned to express, purify, crystallize and determine the three-dimensional structure of Esx by x-ray crystallography. Elucidation of the Esx structure would be beneficial for the development of novel therapeutics aimed at blocking Esx transactivating function. Due to the difficulties in obtaining crystals of Esx, our focus has shifted to structural studies of Fmu, a novel protein that will provide insight into the role of an rRNA methyltransferase implicated in breast cancer. Fmu, a 429 amino acid RNA m⁵C methyltransferase from *E. coli*, methylates the 5-carbon of C967 of 16S rRNA. It is composed of a 270 amino acid C-terminal methyltransferase domain, and a 159 amino acid N-terminal region that is proposed to be an RNA binding domain. Fmu is the bacterial homolog of yeast Nop2 and human p120, which all share a conserved methyltransferase domain that is flanked on each end by variable regions.⁽¹⁾ Nucleolar human p120 is a proliferation associated protein which has been found to be over-expressed in tumor cells. Most human malignant tumors contain much higher levels of protein p120 than normal resting cells⁽²⁾, and expression of p120 is used as a prognostic indicator. Furthermore, increased expression has been correlated with early recurrence and shorter survival in breast cancer patients.⁽³⁾ p120 has also been shown to be a marker of aggressiveness in lung, prostate, colorectal and oral carcinoma.^(4,5,6,7) Recent work has shown that the inhibition of p120 (by ribozyme vectors, anti-p120 antibody, or anti-sense DNA) in tumor cell lines, or mice injected with tumor cells results in decreased tumor cell growth^(8,9), and these results suggest p120 is an attractive target for therapeutic intervention. As p120 is homologous to Fmu, structural information obtained for Fmu would be directly applicable to p120. Here we propose to express, purify, crystallize and determine the structure of the multi-domain RNA modifying enzyme Fmu by x-ray crystallography. The ultimate goal of this research is to use structural analysis to define inter-domain interactions of Fmu or specific contacts between Fmu and rRNA, which can later be targeted for the design of small molecule modulators of proliferative function. The following is a final summary of the entire research project, including the initial attempts to obtain crystals of Esx, and the successful structure determination of Fmu.

BODY:

Technical Objective 1: Expression and purification of protein domains for structural analysis.

Esx:

In the initial proposal, four fragments of Esx were identified as potential candidates to screen for crystallization in addition to the full-length protein. These fragments were selected based on interesting homologies to other known protein domains, proteolytic data, and information obtained from genomic mapping. Full length Esx is 371 amino acids (aa) long and approximately 42 kD.

The fragments selected for crystallization studies included:

- a) DNA-binding domain (aa274-371)
- b) DNA-binding domain + two-thirds SOX domain (aa206-371)
- c) DNA-binding domain + SOX domain (aa188-371).
- d) Pointed domain + one-third SOX domain (aa1-205).

Plasmids for expression of these fragments of Esx, in addition to the full-length protein, were constructed using pET vectors containing a T7 polymerase promoter and encoding a 6-histidine residue tag. Bacterial cells, BL21(DE3)pLysS, were induced with IPTG (isopropyl- β -D-thiogalactopyranoside) to express T7 polymerase which drives high levels of RNA expression for the appropriate inserted fragment. Three to four hours following induction, the cells were harvested by centrifugation, resuspended in lysis buffer, and sonicated in order to lyse the cells. Following sonication the lysed cells and their cytosolic milieu were centrifuged to separate the soluble fraction from the insoluble fraction. In all cases the protein was resolved in the insoluble fraction, having been expressed in inclusion bodies, and hence required denaturing purification. The inclusion bodies were isolated using a sucrose separation step, in which the aggregates of protein settle through a layer of 52% sucrose upon centrifugation. Following this purification step, the protein was solubilized using 8M Urea. The denatured protein solution was purified using a Nickel column to which the N-terminal 6-histidine residue tag binds. Further purification by gel filtration was required to obtain >95% pure material suitable for crystallization trials. Following gel filtration, the protein required refolding, or removal of urea. The process of refolding proved to be quite difficult. Several techniques were tried, including refolding while the protein was bound to the column, step dialysis in which the urea concentration was slowly reduced to 0, and simple dialysis. The resultant protein, in all cases, was marginally soluble and polydisperse, as shown by Dynamic Light Scattering experiments, rendering unlikely the appearance of crystals. However, the protein was functional in DNA-binding assays.

Fmu:

The cloning of the *fmu* gene and production of the wild-type protein has been previously described.⁽¹⁰⁾ Selenomethionine-labelled Fmu used for MAD phasing experiments was produced from 4 liters of BL21(DE3)pLysS cells as per the method of van Duyne.⁽¹¹⁾ The purification of SeMet-labelled protein is as follows: 4 liters of cells were pelleted and resuspended in 50 ml Buffer A (20mM Tris HCl (pH 7.5), 50mM NaCl, 1mM MgCl₂, 0.5mM DTT, 10% glycerol), and the resuspended cells were lysed by sonication. The lysed cells were centrifuged for 20

minutes at 12,000 rpm to remove the cellular debris. The supernatant was decanted into a new tube and loaded onto a pre-equilibrated 30 ml Q-sepharose column (Buffer A). The column was washed with ~10 column volumes of Buffer A, and the protein eluted with a gradient of 0 to 0.5 M KCl in buffer A. The fractions were pooled after checking by SDS-PAGE and dialyzed overnight against Buffer B (10mM KPi (pH 6.8), 0.5mM EDTA, 0.5mM DTT 10%glycerol). The dialyzed sample was loaded onto a HAP column pre-equilibrated with buffer B and eluted with a linear gradient of 10 to 500mM KPi (pH 6.8) in buffer B. The pooled fractions were concentrated to about 6 mg/ml.

Technical Objective 2: Crystallization of protein.

Esx:

Crystal screens were initiated using full-length Esx protein and two other fragments, (a) and (b) from above. Each fragment contains the ETS DNA-binding domain, and has been tested for crystallization alone, and in complex with a 16 base pair DNA substrate. The DNA substrate, a subset of the HER2 promoter element, was purified using HPLC. Both strands were purified separately, then annealed by placing in a 95°C water bath. The annealed oligomers were then desiccated, and resolubilized in water as needed. In initial screens of crystallization conditions, the hanging drop method was used. Protein or protein-DNA complex was mixed 1:1 with well buffer in 4 microliter drops. Drops were placed on siliconized cover slips and inverted over 1 ml of well buffer. Well buffers were obtained from commercially available sparse matrices containing greater than 10 distinct precipitants combined with different salts and additives (Hampton Research). To date there have been no visible protein crystals of full-length Esx, or either of the fragments alone or in complex with DNA.

Fmu:

Crystal screens were initiated using Fmu purified as described above. Drops were placed on siliconized cover slips and inverted over 1 ml of well buffer. Well buffers were obtained as described for Esx. No crystals were seen in the initial trays, however a shiny precipitate was observed in drops with PEG as the precipitant. Following this result, a set of PEG conditions were made (5-30% PEG 400-PEG 8000) over a pH range of 5.5 to 8.5 (0.1M buffer) and more crystallization trials were preformed. Small crystals were observed in drops with 15% PEG 4000, pH 8.5. A finer grid screen of PEG solutions was made (12-18% PEG 4000, 0.1M Tris pH 8.0-8.6) and further crystallization trials produced crystals suitable for diffraction. In additional crystallization trials, Fmu was mixed 1:1 with its cofactor S-adenosyl-methionine (AdoMet). Crystals were visible under similar conditions as unliganded Fmu.

Technical Objective 3: Single crystal diffraction of protein

Esx:

As no crystals of Esx have been available to date, diffraction data collection has not been possible.

Fmu:

The crystals obtained for Fmu, as described above, were tested for diffraction on a home source, a Raxis IV rotating anode X-ray generator equipped with a high-speed area detector. Since cryoprotection can significantly increase the useful diffraction lifetime of protein crystals,

potential cryosolvents were screened for their ability to allow flash freezing of Fmu crystals in liquid nitrogen. Mineral Oil was determined to be the best cryoprotectant for these crystals. The crystals diffracted to approximately 3.0 Å in space group $P2_1$ ($a=59.48$, $b=48.64$, $c=86.65$, $\alpha=90.0$, $\beta=108.59$, $\gamma=90.0$). Following initial screening on the home source, crystals were taken to a synchrotron at Stanford Synchrotron Radiation Laboratory. A dataset was collected on a native crystal that diffracted to 1.65 Å, and a complex crystal (Fmu-AdoMet) that diffracted to 2.1 Å.

Technical Objective 4: Structural solution and interpretation

In addition to high quality diffraction, structural solution requires accurate phasing information.

Esx:

Structural solution of Esx has not been possible since crystals were never obtained.

Fmu:

In order to obtain phasing information, selenomethionine Fmu protein was purified as described above. SeMet Fmu crystallized in conditions identical to those for the native crystals. A three wavelength MAD (Multi-wavelength Anomalous Dispersion) experiment was collected at the Advanced Light Source at Lawrence Berkeley Laboratories. The selenomethionine Fmu crystal diffracted to 2.25 Å in space group $P2_1$ ($a=87.53$, $b=48.56$, $c=114.83$, $\alpha=90.0$, $\beta=107.79$, $\gamma=90.0$), with 2 molecules per asymmetric unit. Using this data and the crystallographic software program CNS, 14 selenomethionine sites (there are 7 per protein molecule) were found. Starting phases were calculated using these 14 sites and the software SHARP. Non-crystallographic symmetry averaging was performed since there were 2 molecules in the asymmetric unit, and initial maps were calculated. Using the graphics program QUANTA, ~80% of the model was built into the density; subsequently, this model was used for molecular replacement into the 1.65 Å native data in the program AMORE. A density modification procedure was applied in CNS, and additional model building was performed in QUANTA. The Fmu model refined to give a final R-factor of 21.8% ($R_{\text{free}} = 25.6\%$) and comprises residues Arg 5-Lys 428 and 390 water molecules. In addition, the structure of AdoMet-bound Fmu was determined to a resolution of 2.1 Å and refined to an R-factor of 23.7% ($R_{\text{free}} = 27.7\%$), with observed density for residues Arg 5-Lys 429 and 130 water molecules.

Details of the structures are described in a manuscript submitted for publication to Nature Structural Biology.

KEY RESEARCH ACCOMPLISHMENTS:

ESX:

- Expressed and purified full-length ESX and 4 protein fragments
 - Amino acids 274-371
 - Amino acids 206-371
 - Amino acids 188-371
 - Amino acids 1-205.
- Purified a 16-base pair oligomer of DNA, a subset of the HER2 promoter element.
- Screened crystallization conditions for full-length ESX and 2 protein fragments.

Fmu:

- Screened crystallization conditions for Fmu.
- Obtained crystals of Fmu.
- Expressed and purified selenomethionine-labelled Fmu.
- Identified a cryoprotectant condition and collected diffraction data for Fmu.
- Obtained phase information for Fmu data and calculated initial maps.
- Built partial model of Fmu that was used in molecular replacement to fit the 1.65 Å data.
- Completed building and refinement of the Fmu model.

REPORTABLE OUTCOMES:

Paper submitted to *Nature Structural Biology*.

Ph.D. conferred.

CONCLUSIONS:

As mentioned in the introduction, the focus of the proposal has shifted since crystals were not obtained for Esx. The progress made on Fmu, however, meets the proposed timeline in the original grant. Fmu has been expressed, purified, and crystallized, with diffraction data collected to a maximum resolution of 1.65 Å. Additionally, selenomethionine-labelled Fmu has been purified, crystallized and used for structural determination by MAD phasing methods. The resulting electron density maps are of excellent quality, and a complete model of the molecule has been built into the density. In addition, the structure of an Fmu-cofactor (AdoMet) complex has been solved to 2.1 Å. The refined protein structures define the structural basis for cofactor and RNA binding, and provide insights into the m⁵C methyltransferase mechanism of Fmu. Such information will provide a starting point for identifying regions that may be suitable for inhibitor design.

REFERENCES:

1. Reid R, Greene P, Santi D. Exposition of a family of RNA m⁵C methyltransferases from searching genomic and proteomic sequences. *Nucleic Acids Research* 27: p. 3138-3145, 1999.
2. Ren Y, Busch RK, Perlaky L, Busch H. The 58-kDa microspherule protein (MSP58), a nucleolar protein, interacts with nucleolar protein p120. *European Journal of Biochemistry* 253: p.734-742, 1998.
3. Freeman J, McGrath P, Bondada V, Selliah N, Ownby H, Maloney T, Busch R, Busch H. Prognostic significance of proliferation associated nucleolar antigen p120 in human breast carcinoma. *Cancer Research* 51: p.1973-1978, 1991.
4. Uchiyama B, Saijo Y, Kumano N, Abe T, Fujimura S, Ohkuda K, Handa M, Satoh K, Nukiwa T. Expression of nucleolar protein p120 in human lung cancer: difference in histological types as a marker for proliferation. *Clinical Cancer Research* 3: p. 1873-1877, 1997.
5. Kallakury BV, Sheehan CE, Rhee SJ, Fisher HA, Kaufman RP Jr, Rifkin MD, Ross JS. The prognostic significance of proliferation-associated nucleolar protein p120 expression in prostate adenocarcinoma: a comparison with cyclins A and B1, Ki-67, proliferating cell nuclear antigen, and p34cdc2. *Cancer* 85: p. 1569-1576, 1999.
6. Ueki T, Nakayama Y, Sugao Y, Kohno K, Matsuo K, Sugimoto Y, Yamada Y, Kuwano M, Tsuneyoshi M. Significance of the expression of proliferation-associated nucleolar antigen p120 in human colorectal tumors. *Human Pathology* 28: p. 74-79, 1997.
7. Ventura L, Migaldi M, Criscuolo M, Castelli M, Barbolini G, Ranieri A, Bifaretti G, Uboldi P, Leocata P. Nucleolar protein p120 expression in oral carcinoma. *Anticancer Research* 19: p. 1423-1426, 1999.
8. Sato K, Nishi T, Takeshima H, Kochi M, Kuratsu J, Masuko N, Sugimoto Y, ymada Y, Ushio Y. Expression of p120 nucleolar proliferating antigen in human gliomas and growth suppression of glioma cells by p120 ribozyme vector. *International Journal of Oncology* 14: p. 417-424, 1999.
9. Perlaky L, Busch RK, Busch H. Combinatorial effects of monoclonal anti-p120 antibody (Mabp120), liposomes and hyperthermia on MCF-7 and LOX tumor cell lines. *Oncology Research* 8: p. 363-369, 1996.
10. Gu XR, Gustafsson C, Ku J, Yu M, Santi D. Identification of the 16S rRna m⁵C967 Methyltransferase from *Escherichia coli*. *Biochemistry* 38: p. 4053-4057, 1999.
11. Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J. Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *Journal of Molecular Biology* 229: p. 105-124, 1993.