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Award Number: W81XWH-04-1-0641

TITLE: Mechanisms of ErbB2 Tyrosine Kinase Regulation in Breast Cancer

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REPORT DATE: July 2005

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2005	Final	1 Jul 2004 – 30 Jun 2005

4. TITLE AND SUBTITLE
Mechanisms of ErbB2 Tyrosine Kinase Regulation in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0641

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Beth Israel Deaconess Medical Center
Boston, MA 02215

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. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The c-erbB-2 proto-oncogene (also called HER2 or neu) encodes the ErbB2 protein, a transmembrane receptor tyrosine kinase (TK) whose overexpression is an important determinant in the initiation and progression of human breast cancer. Phosphorylation of downstream targets by the ErbB2 TK is the key event leading to activation of the signaling pathways associated with development and progression of aggressive breast cancer. This project focuses on the elucidation of the three-dimensional structure of human ErbB2 TK in the apo form and in complex with non-hydrolyzable ATP analogs and EGFR/ErbB2 family-specific inhibitors, using X-ray crystallography. To crystallize the ErbB2 protein, the DNA fragments encoding ErbB2(704-990) and ErbB2(704-1255) were cloned into the eukaryotic expression vector pGAPZA for production in P. pastoris cells. Each construct was introduced into P. pastoris by electroporation and the clones, which can express recombinant ErbB2(704-990) and ErbB2(704-1255) proteins, were induced for large scale protein preparation. Unsuccessful attempts to purify the proteins lead to a new strategy for producing these recombinant proteins in baculovirus expression system. The expression vector pFastBac1 was chosen for production of ErbB2(704-990) and ErbB2(704-1255) proteins in Sf9 and Sf21 insect cells. So far, Bacmids containing the DNA fragments were made and verified by PCR. It is still in progress to transfect the insect cells with the recombinant Bacmids for the production of ErbB2 proteins.

15. SUBJECT TERMS
ErbB2, P. pastoris, baculovirus, crystallization

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	

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INTRODUCTION

The *c-erbB-2* proto-oncogene (also called *HER2* or *neu*) encodes the ErbB2 protein, a transmembrane receptor tyrosine kinase (TK) whose overexpression is an important determinant in the initiation and progression of human breast cancer (1). ErbB2 overexpression occurs in 25-30% of human breast tumors and is associated with enhanced malignancy and metastatic potential, reduced responsiveness to standard chemotherapy, and poor overall survival (2). A large series of *in vitro* and *in vivo* experiments have shown that increased ErbB2 TK activity enhances the expression of malignant phenotypes (1,3). The well-established role of ErbB2 in aggressive breast cancer pathogenesis makes this oncoprotein an ideal target for developing anticancer agents specific for ErbB2-overexpressing breast cancer cells. A novel approach to breast cancer therapy would involve the structure-based rational design of small-molecule selective inhibitors of the ErbB2 function (4). The enhancement of malignant phenotypes by the increase in ErbB2 TK activity provides the rationale to target the ErbB2 TK for antitumor therapy. Since phosphorylation of downstream targets by the ErbB2 TK is the key event leading to activation of the signaling pathways associated with development and progression of aggressive breast cancer, blocking of this enzyme would lead to growth inhibition and suppression of malignancy of the ErbB2-overexpressing breast cancer cells. However, despite the critical role of the ErbB2 TK in breast cancer its atomic structure has not been reported to date. Therefore, this proposal focuses on the structural analysis of the ErbB2 TK using X-ray crystallography. This project focuses on the elucidation of the three-dimensional structure of human ErbB2 TK in the apo form and in complex with non-hydrolyzable ATP analogs and EGFR/ErbB2 family-specific inhibitors, using X-ray crystallography.

BODY

During the one-year award period we proceeded with the experiments proposed in the Statement of Work. A description of our progress in these studies follows below.

Task 1. To express, crystallize, and determine the crystal structure of the unphosphorylated apo ErbB2 tyrosine kinase protein. To crystallize the ErbB2 TK domain it is necessary to produce large amounts (multi-milligram quantities) of this protein in bacterial or eukaryotic cells as a soluble protein and purified it to homogeneity. Toward this goal, DNA fragments encoding the ErbB2 TK (residues 704-990) and the entire cytoplasmic domain of ErbB2 (residues 704-1255) were cloned into pGAPZA vector (Invitrogen) for expression in *Pichia pastoris*. In both constructs the ErbB2 protein contains an N-terminal hexahistidine tag for affinity purification by Ni-NTA resin followed by a TEV protease cleavage site to cleave off the hexahistidine tag after purification (Figure 1). Both constructs were verified by DNA sequencing of both strands and were introduced into *P. pastoris* host cell X-33 using electroporation. For each construct, several clones were selected to detect the expression of the ErbB2 proteins by Western Blots, using ErbB2 TK-specific antibodies (Calbiochem and Cell Signaling) (Figures 2 and 3). These studies showed that both the ErbB2 TK and the entire cytoplasmic domain can be expressed in *P. pastoris*. However, numerous attempts to produce large amounts of these ErbB2 proteins were unsuccessful due to difficulties in the purification of these proteins by Ni-NTA affinity chromatography. The yield of ErbB2 expression in *P. pastoris* is low compared to other kinases that have been reported in literature (5).

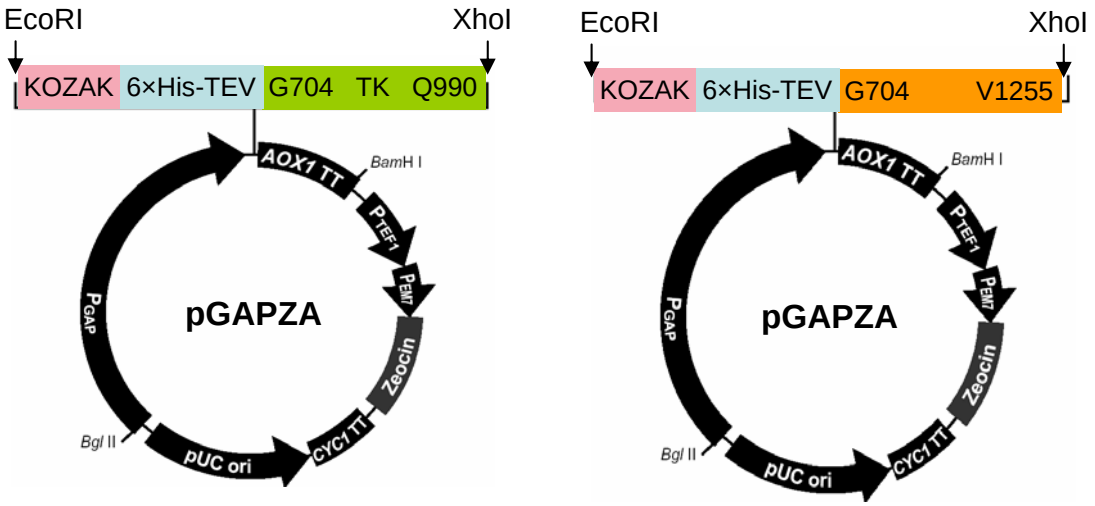

Figure 1. Maps of ErbB2 TK (residues 704-990) (left panel) and the entire ErbB2 cytoplasmic domain (704-1255) (right panel) cloned into pGAPZA vector. Zeocine is used to select the clones expressing ErbB2 proteins from these vectors.

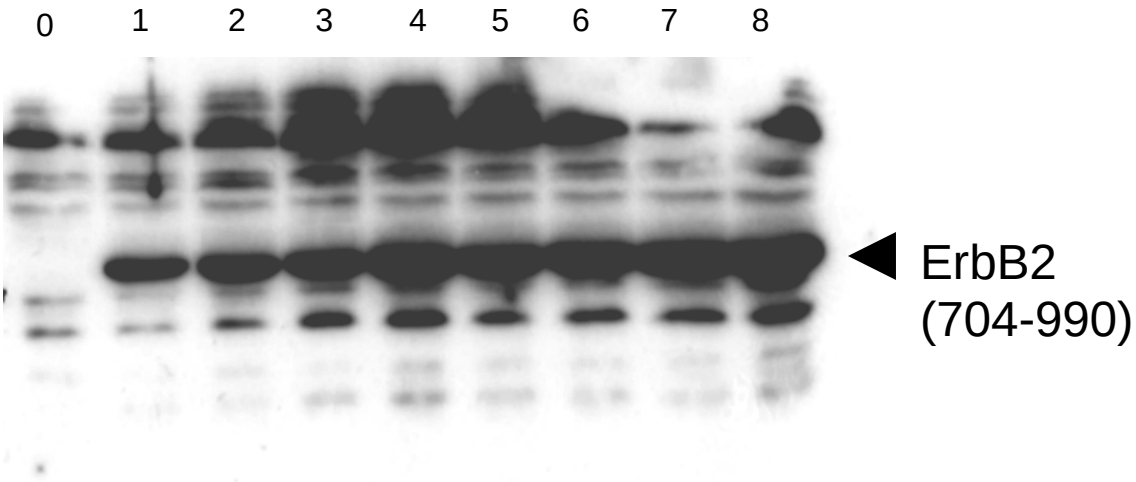

Figure 2. Western blot of selected *P. pastoris* clones expressing ErbB2 TK (residues 704-990). *Lane 0: P. pastoris* host cell X-33 electroporated with empty pGAPZA vector (control); *lanes 1-8:* recombinant strains #1-8 expressing ErbB2(704-990) protein.

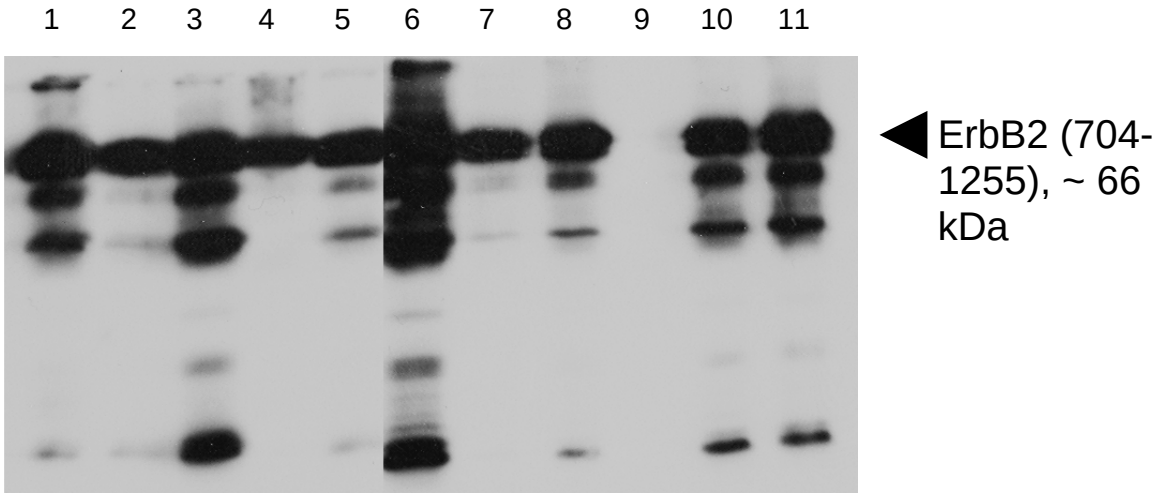

Figure 3. Western blot of selected *Pichia* clones expressing ErbB2 (704-1255) protein. Lanes 1-8: recombinant strains #1-8 expressing ErbB2 (704-1255); lane 9: *Pichia* host cell X-33 electroporated with pGAPZA empty vector; lanes 10-11: strains #9-10 expressing ErbB2 (704-1255).

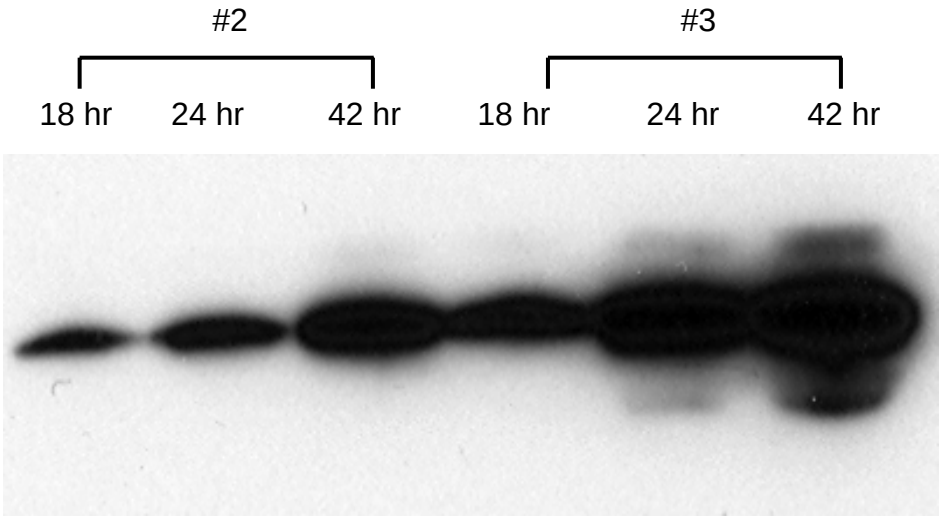

Figure 4. Induction time course of *Pichia* strains #2 and #3 expressing ErbB2 (704-1255) protein. Two *P. pastoris* strains, #2 and #3, were chosen to investigate the expression of ErbB2(704-1255). Samples were taken at different time points to run a Western Blot against an ErbB2 antibody.

Because the *P. pastoris* expression system did not yield sufficient amounts of the ErbB2 proteins for structural analysis, we made the decision to adopt a new strategy for producing these recombinant proteins in insect (Sf9 and Sf21) cells using the baculovirus system. In order to express the ErbB2 TK and entire cytoplasmic domain in insect cells, their encoding DNA fragments were excised from the pGAPZA vector and were subcloned into the baculovirus vector pFastBac1 (Invitrogen) (Figure 5). Following verification of the resulting constructs by DNA sequencing, these plasmids were transformed into DH10BAC competent cells containing

viral DNA. White DH10BAC colonies were selected to isolate recombinant Bacmids which contain the DNA fragment coding for the ErbB2 TK and cytoplasmic domains (Figure 6). The insertion of the ErbB2 DNA in the Bacmid was verified by the polymerase chain reaction (PCR) using the M13 forward and reverse primers.

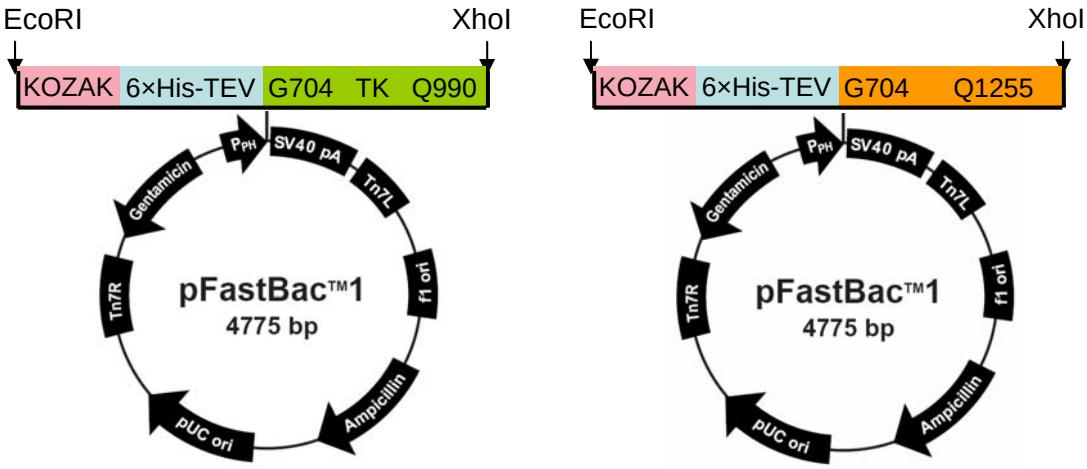

Figure 5. Maps of the ErbB2 TK (left panel) and the entire ErbB2 cytoplasmic domain (right panel) cloned into the pFastBac1 vector.

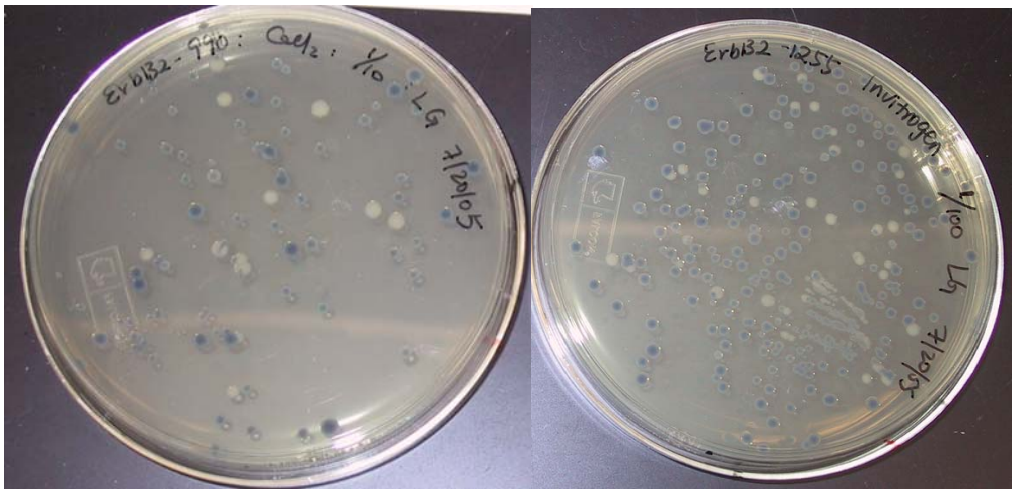

Figure 6. Selection of recombinant Bacmids containing the ErbB2 TK domain (left panel) and the entire ErbB2 cytoplasmic domain (right panel). White (ErbB2-containing) colonies were screened to verify the existence of ErbB2 DNAs by PCR using the M13 forward and reverse primers.

We are now in the process of transfecting Sf9 and Sf21 insect cells with these Bacmids in order to produce the ErbB2 proteins in large amounts required for crystallization experiments. The ErbB2(704-990) and ErbB2(704-1255) proteins will be purified using a combination of affinity, ion exchange and size exclusion chromatography.

Tasks 2 and 3. To co-crystallize and determine the structure of the ErbB2 TK complexed with a non-hydrolyzable ATP analog (Task 2) and tyrosine kinase inhibitors (Task 3). Crystallization experiments will be performed with the pure ErbB2 TK protein, ErbB2 TK complexed with the non-hydrolyzable ATP analog adenylyl diphosphonate, and ErbB2 TK complexed with the EGFR/ErbB2 TK family-specific inhibitors 2'-thioadenosine, PD-168393, CP-358774, and sporostatin, using sparse matrix screenings and vapor diffusion crystallization methods. We will take special care to purify monodisperse protein complexes for crystallization trials using size exclusion chromatography. For structure determination, crystals of selenomethionine-containing recombinant ErbB2(704-990) and ErbB2(704-1255) proteins will be used to collect multiwavelength anomalous diffraction (MAD) diffraction data using synchrotron radiation. The crystal structures will be determined with MAD methods. **We would like to emphasize that although the award period is finished, we will continue to pursue very persistently the crystallographic analysis of the ErbB2 TK domain, as outlined in the original proposal. The one-year Concept Award has provided critical support to initiate this research project and the successful completion of these studies will be reported to DOD in the future.**

KEY RESEARCH ACCOMPLISHMENTS

1. Cloning of the DNA fragments encoding ErbB2(704-990) and ErbB2(704-1255) into the eukaryotic expression vector pGAPZA for production in *P. pastoris* cells (*Task 1*).
2. Electroporation and selection of *P. Pastoris* cells expressing recombinant ErbB2(704-990) and ErbB2(704-1255) proteins (*Task 1*).
3. Induction of expression ErbB2(704-990) and ErbB2(704-1255) proteins (*Task 1*).
4. Attempts to scale-up and purify large amounts of ErbB2(704-990) and ErbB2(704-1255) proteins (*Task 1*).
5. Cloning of the DNA fragments encoding ErbB2(704-990) and ErbB2(704-1255) into the expression vector pFastBac1 for production in Sf9 and Sf21 insect cells (*Task 1*).
6. Production of Bacmids containing the DNA fragments encoding ErbB2(704-990) and ErbB2(704-1255) proteins (*Task 1*).
7. Selection and verification by PCR of the Bacmids containing the DNA fragments encoding ErbB2(704-990) and ErbB2(704-1255) proteins (*Task 1*).

REPORTABLE OUTCOMES

The experiments of the proposal are still in progress and no publications have resulted to date from these studies.

CONCLUSIONS

During the award period we made numerous attempts to find conditions that would allow the production of large amounts of the ErbB2(704-990) and ErbB2(704-1255) proteins in *P. pastoris* cells using the expression vector pGAPZA. Both these proteins could be expressed in *P.*

pastoris albeit in low amounts and there were many difficulties in their purification by Ni-NTA affinity chromatography. Because these protein fragments cannot be readily made in *P. pastoris*, we proceeded with the cloning of the DNA fragments encoding the ErbB2(704-990) and ErbB2(704-1255) proteins into pFastBac1 baculovirus vector for expression in Sf9 and Sf21 insect cells. Experiments are currently in progress to optimize the expression of these proteins in insect cells in order to proceed with the proposed crystallization experiments. The information obtained from these experiments will elucidate the mechanisms underlying the function of ErbB2 TK in breast carcinogenesis and will provide an essential structural framework for the development of new drugs against this disease.

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