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GRANT NUMBER DAMD17-98-1-8228

TITLE: Analysis of Ligand Binding ErbB Receptor Tyrosine Kinases

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09/22/99
**Title:** Analysis of Ligand Binding ErbB Receptor Tyrosine Kinases

**Authors:** Kathryn M. Ferguson, Ph.D.

**Performing Organization:** University of Pennsylvania

**Funding Numbers:** DAMD17-98-1-8228

**Abstract:**
Growth factor-receptor tyrosine kinases (RTK's) play a central role in the coordination of cell growth, differentiation and other activities in multicellular organism. Molecular lesions in and/or aberrant expression of RTK's can lead to cancer. There is a particularly strong correlation between the erbB2 (neu/HER-2) receptor and breast cancer. The cell-surface location of the erbB2 receptor makes it an obvious target for novel therapies. The purpose of this research is to gain insight into the structure of the extracellular domain of this receptor and its mode of activation in order to aid in the development of new antagonists. ErbB2 is one of a family of 4 RTK's which also includes the epidermal growth factor receptor (EGFR). The first step in the stimulation of a response by a growth factor is dimerization of the receptor upon binding of the cognate ligand. We have crystals of the EGF induced homodimer of the soluble extracellular domain of EGFR that diffract to better than 2.8 Å. Determination of this structure is in progress. This structure will provide insight into the specific molecular events that drive erbB receptor dimerization. A key question is whether the dimerization is mediated only by receptor-receptor contacts, or whether the ligand is bivalent in nature, simultaneously contacting both receptor molecules in the dimer.

**Subject Terms:**
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- erbB receptor
- X-ray crystallography
- growth factor
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- ligand binding

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Introduction

Many intracellular communication signals are mediated by hormones and growth factors that bind to specific receptors on the cell surface. These receptors are responsible for transmitting the signal to the inside of the cell where a cascade of events is triggered leading to a cellular response. Many growth factor receptors share a broadly similar architecture; a large extracellular ligand binding domain, a single membrane-spanning domain, and a cytoplasmic domain consisting of a tyrosine kinase domain plus regulatory sequences. Receptors of this type, of which there are more than 50, are termed receptor tyrosine kinases (RTK's). Signal transduction through RTK's involves dimerization upon binding of the cognate growth factor resulting in stimulation of the intracellular tyrosine kinase domain of the receptor. Autophosphorylation on specific tyrosines in the RTK leads to the recruitment of downstream signaling molecules through the interaction with their Src homology 2 (SH2) domains. A great deal is known about the protein-protein interactions involved in these downstream events. There have been numerous structures of SH2 domains interacting with phosphopeptides, and the X-ray crystal structures of the tyrosine kinase domains from two receptors have been determined. In contrast there is relatively little structural information about the extracellular domains of RTK's. The molecular interactions that mediate the dimerization event are not understood. Much more is known about the mechanism of receptor dimerization of the cytokine receptor family, which have yielded more readily to biophysical studies. The striking picture from the X-ray crystal structure of a single human growth hormone (hGH) molecule simultaneously interacting with two receptor molecules has strongly influenced the thinking in the field about possible mechanisms of RTK dimerization. In particular the concept of multivalent ligands has been developed. More recent biophysical studies have now demonstrated this paradigm holds true for a number of RTK's. In this research I aim to complement these biophysical studies with the structural details for the case of the erbB family of RTK's. The erbB family of RTK's comprises 4 members, EGFR (erbB1), erbB2 (neu, HER-2) erbB3 and erbB4. The erbB ligands fall into two distinct classes: the EGF agonist that all bind to and activate EGFR [refs. 17-21], and the neuregulins (NRG) that bind to erbB3 and erbB4 [refs. 22-24]. To date erbB2 remains an orphan receptor. Each of the erbB ligands contains an EGF-like domain of 50-90 amino acids that, although in some cases only a small portion of the native ligand, is sufficient for its biological function. The interaction between EGF and EGFR is the best characterized of all erbB receptor/ligand complexes. Binding of monomeric EGF to the soluble extracellular domain of EGFR (s-EGFR) induces its dimerization to form a final (EGF)\textsubscript{2}:(s-EGFR)\textsubscript{2} complex. The dimerization step could occur by either of two mechanisms, which are not mutually exclusive. Dimerization could be receptor mediated, with an EGF-induced conformational change exposing a receptor-receptor interaction site. Alternatively, dimerization could be ligand-mediated, with each EGF molecule binding simultaneously to both receptors in the dimer. The reality may lie between the two extremes. The paradigm of bivalent ligands established in other studies makes the ligand-mediated model attractive. One of the most direct means of answering the question of whether EGF is mono- or bivalent in its interaction with EGFR is to solve the three dimensional structure of the entire complex.

The specific aims of this research are as follows:

- Determine the X-ray crystal structure of the EGF induced s-EGFR homodimer
- Characterize the determinants of ligand specificity of domains 1 and 3 for each of the erbB receptors using biophysical and structural approaches.
- Determine the structure of an activated heterodimer between erbB2 and another erbB family member.
The major accomplishment of this funding period was to obtain well diffracting crystals of s-EGFR/EGF complex (see Task 4 below). In light of this, the emphasis of the research has shifted toward the determination of this structure and, at present, away from considerations of the ligand binding specificity of domains 1 and 3 of each erbB receptor (Task 3). This still interesting question will be revisited at a later time in the funding period. Progress on each task is described.

**Task 1 Obtain purified receptor extracellular domains (months 1 - 18)**

Recombinant baculoviruses have been generated which allow the secretion from insect cells of the full length extracellular domains of EGFR, 2, 3 and 4 (s-erbBs). C-terminal cleavable His-tags are incorporated to facilitate the first purification step using Ni-affinity chromatography. Production and purification of these proteins is routine with yields ranging from 0.3 mg/L (s-erbB2) to 1.5 mg/L (s-EGFR). Typically 5 - 10 liters of a suspension culture of Sf9 insect cells are grown, in Sf900-II medium (Gibco/BRL), to a density of 2.5x10^6 cells/ml, and infected at a multiplicity of infection (MOI) of about 5 with freshly amplified, high titer virus solution. Cultures are incubated for a further 96 hours. The clarified conditioned medium is diafiltered against 3.5 volumes of 25 mM Tris, 150 mM NaCl, pH 8.0 (buffer A), using a Millipore Prep/Scale-TFF 30 kDa cartridge and concentrated to approximately 300 ml prior to loading onto a 5 ml Ni-NTA Superflow column (Qiagen). After extensive washing with buffer A, the column is washed sequentially with 2 column volumes of buffer A containing 30, 50, 75, 100 and 300mM imidazole, pH 8.0. Typically the majority of the protein elutes in the 75 and 100 mM fractions. Fractions are concentrated in an Centriprep 30 (Amicon) and loaded on a Pharmacia Superose 6 gel filtration column. At this stage the s-erbBs are greater than 95% pure as assessed by SDS-PAGE analysis (Figure 1).

For crystallization, s-EGFR is further purified by cation exchange chromatography. Fractions from the gel filtration column are pooled, diluted 1.5 fold with 50 mM MES pH 6.0 and loaded on to a BioRad S2 sulfopropyl cation exchange column, pre-equilibrated with 25 mM MES pH 6.0. A gradient in NaCl is developed and the protein elutes at about 200 mM NaCl. The purification utilizes no harsh treatments such as those used to elute protein from antibody affinity columns, which we feel is important in generating material suitable for crystallization. Purified s-erbBs are buffer exchanged into 25 mM Hepes, 100 mM NaCl, pH 8.0, concentrated to between 20 and 100 μM, and stored at 4° C.
Concentrations are determined by absorbance at 280 nM using the calculated extinction coefficients from number of Trp and Tyr in each protein.

Recombinant baculoviruses have also been generated to express the isolated ligand binding domain 3 from each of the four receptors. C-terminal His tags are incorporated to aid in purification of this 30 kDa domain. Domain 3 from EGFR (s-EGFRdom3) has been purified, using a procedure similar to that described above for purification of the full length receptor except that a 10 kDa molecular weight cut off cartridge, and Pharmacia Superose 12 column are employed, as is required for this much smaller (30 kDa) protein. The yield of s-EGFRdom3, about 0.2 mg/L, is lower than for full length s-EGFR. The expressed protein is also less homogeneous. The expressed protein migrates as a doublet on SDS-PAGE, as visualized by Coomassie blue staining and Western blot with antibody to the C-terminal His-tag. These two species can be separated by cation exchange chromatography (Figure 2A) but the yield at this step is low. Attempts were made to improve the homogeneity of s-EGFRdom3 by enzymatic deglycosylation using endoglycosidase H. The protein could be digested to a single species (Figure 2B), which was essentially deglycosylated, some residual sugar remained as assessed using an immunoblot kit for detecting glycoproteins from BioRad. This deglycosylated s-EGFRdom3 was however unstable and precipitated during purification attempts. At this stage further work on producing large quantities of the subdomains 1 and 3 of s-erbB receptors has been postponed while effort is diverted to other more promising areas of research (Task 5).

**Figure 2**

A. SDS-PAGE s-EGFRdom3. Fractions through the second of two peaks from a cation exchange purification step are shown

B. SDS-PAGE analysis showing the enzymatic deglycosylation of s-EGFRdom3 with endoglycosidase H (EndoH)
Task 2. Obtain purified ligands (months 1 - 18)

Currently we purchase all ligands from commercial sources and use them without further purification. High quality s-EGFR/EGF crystals can be grown using EGF purchased from Intergen, which is relatively inexpensive. We have available E. coli or yeast systems for several ligands which we will use if necessary. Work will proceed on this task as required.

Task 3. Characterize binding of ligands to isolated domains 1 and 3 (months 6 - 12)

In light of the difficulties discussed above in producing large quantities of the isolated domains there has been little progress in this task. Some preliminary fluorescence experiments have confirmed that the insect cell expressed s-EGFRdom3 has similar binding affinity for TGFα as does the domain3 generated by proteolysis from mammalian expressed s-EGFR. A preliminary crystallization screen of s-EGFRdom3/TGFα complex yielded no promising results.

Task 4. Obtain diffraction quality crystals of s-EGFR/EGF complex (months 7 - 18)

Purified s-EGFR was buffer exchanged into 5mM Hepes, 25 mM NaCl, pH 8.0, and concentrated to 30 mg/ml, using a Centricon (Amicon). A 1.2 fold excess of EGF was added to give final receptor concentration of 250 μM. Standard approaches were used to survey for conditions under which s-EGFR and s-EGFR/EGF complex might crystallize. These included the use of both sparse matrix and grid screening. Small needle like crystals were observed for the s-EGFR/EGF (1.2 fold molar excess of EGF) complex in several PEG/low pH conditions in an early screen. Under the same conditions no crystals formed for the protein alone. The presence of the EGF in the crystal has been confirmed by SDS-PAGE analysis of washed crystals. The EGF can clearly be seen and comparison with prepared standard samples indicates that the ligand to receptor stoichiometry in the crystal is approximately 1:1. Subsequent optimization of the crystallization conditions produced much larger crystals, suitable to test the diffraction quality. The best crystals grow from 10 - 15 % PEG 8000 at pH 6.0 to dimensions of about 0.5 x 0.2 x 0.08 mm. To test diffraction, crystals were transferred to a solution containing the same components as the reservoir plus 15% glycerol as cryoprotectant and frozen directly in liquid nitrogen in a nylon loop. Preliminary diffraction analysis was performed on MAR image plate detector using double mirror-monochromated/focused Cu Kα X-rays from a Siemens generator. The crystals diffract to better than 3.8 Å, were of space group C2, with a = 118 Å, b = 103 Å, c = 100 Å, α = 119° and one half dimer per asymmetric unit.

Crystals have been taken to synchrotron X-ray sources (see below). From test exposures taken at the National Synchrotron Light Source (NSLS)beamline X25 it is anticipated that, with an optimal crystal, it may be possible to get data to 2.5 Å. This project now represents a key component in several beam time applications at both the National Synchrotron Light Source at Brookhaven National Laboratories and the Cornell High Energy Synchrotron Source (CHESS). It is anticipated that access to synchrotron X-rays should not be a limiting factor.
Task 5. Solve the X-ray crystal structure (months 18 - 30)

(i) Collect high resolution native data (months 18 - 24)

A complete native data set to 2.8 Å has been collected on a frozen crystal at the NSLS beam line X25 operating at 1.0 Å with a Brandeis 4-module CCD-Based detector (B4). Data were processed using DENZO and SCALEPACK\textsuperscript{25}. A summary of the data collection statistics is given in Table 1.

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Table 1 Data collection statistics for Native data collected at NSLS X25.

Since these data were collected larger, better crystals have been obtained which diffract to 3.5 Å on a home source. Better high resolution native data will be collected at a future synchrotron visit.

(ii) Search for heavy atom derivative (months 18 - 30)

The phase information required to calculate an electron density map, from which the structure is deduced, will be obtained by the method of multiple isomorphous replacement (MIR). In this method the positions of heavy atoms in derivatives of the crystal are used to solve the phase problem. The conventional method for obtaining heavy atom derivative is to soak the crystal in solutions containing a variety of different heavy metal ions. These ions can interact with accessible functional groups in the protein crystal such as those on methionines and histidines. For large proteins a common problem with this strategy is that too many heavy atoms bind weakly to the protein. An alternative strategy that can be used in such cases is to prereact the growth factor ligand with a heavy atom that will form a stable complex with the protein.

The first stage in initiating a heavy atom derivative search is to ensure that a crystal soaked in stabilizer without any heavy atom added is isomorphous with previously collected native data. Partial data sets were collected on our home source for a number of different stabilized crystals. Crystals which were stabilized for > 6 hours were found to be nonisomorphous with those transferred from drop to cryoprotectant and frozen immediately ($R_{merge} > 20\%$). A new native data set to 3.5 Å has been collected on our home source for use in heavy atom derivative screening. This data set is of better quality than that collected at X25. The mosaicity and the $R_{sym}$ are both lower. A stronger data set will be collected at
the next synchrotron trip. The merging R factors (to 4.0 Å) for this data set against the various partial data sets collected varies from about 12% to 16%, indicating that there is some remaining nonisomorphism, perhaps arising from crystal to crystal variability. Great care will have to be taken to ensure that relatively small differences from the incorporated heavy atom can be detected and measured sufficiently accurately to permit interpretation of the heavy atom positions.

With this problem in mind a first round of heavy atom derivative screening has been initiated which focuses on large heavy atom complexes such as di-iodobis(ethylenediamine)diplatinum(II) nitrate (PIP), with the hope that the differences for such a derivative will be large enough to identify with confidence. Screening of potential derivatives of such compounds is on going.

An alternative strategy that would avoid the non-isomorphism problem is to generate seleno methionine containing s-EGFR. This has been done for other insect cell expressed protein\textsuperscript{25}. The structure could then be solved using the multiwavelegh anomalous dispersion (MAD) method, where the phasing information is obtained by measuring the anomalous differences at different wave lengths on a single crystal. Although crystal to crystal variability is not an issue, the anomalous differences are small and the diffraction intensities must be measured accurately. In light of improved quality of the data from a stabilized crystal this is a promising approach which will be explored.

A limiting factor in the progress of this portion of the research has been access to our home source, which is shared among 5 groups. Given the low symmetry and relatively weak diffraction of the s-EGFR/EGF crystals collecting a complete data set can take up to 7 days of detector time. This limitation will soon be alleviated as a new MAR detector and Seimens generator are in the final stages of installation at UPenn. In addition, a freezing system will be added to the other existing area detector opening this for use with projects that require the use of frozen crystals.

(iii) Collect heavy atom derivative data (months 18 - 30)

I have collected a data set on a crystal soaked in 0.5 mM PIP. This had as R\textsubscript{merge} of 18%. Thus far no convincing heavy atom site has been identified. Further analysis is in progress.

(iv) Compute an experimental electron density map (month 30)

No experimental phases have thus far been generated. There may however soon be the possibility to attempt to use a molecular replacement method to solve the structure. Last summer the crystal structure of the first three domains of the type-1 insulin-like growth-factor receptor (IGF-1R) was reported\textsuperscript{27}. This receptor is highly homologous to s-EGFR. Although this structure is of the protein alone, indeed this truncated IGF-1R is not able to bind ligand, the structural homology may be sufficiently high to allow the determination of a molecular replacement solution. The coordinates for the IGF-1R structure have been on a one year hold but should become available in September.

Task 6. Build the atomic model and refine the structure (months 30 - 36)

Task 6 cannot be initiated until the phase information discussed above is obtained.
Key Research Accomplishments

- Milligram quantities of pure s-EGFR, 2, 3 and 4 can be generated
- The EGF/s-EGFR receptor dimer has been crystallized
- Native data on the EGF/s-EGFR crystals to 2.8 Å resolution has been collected.
- Several potential derivatives have been characterized.

Reportable Outcomes

Application for synchrotron X-ray beam time at National Synchrotron Light Source at Brookhaven National Laboratories - 6 days awarded

Application to use Cornell High Energy Synchrotron Source (CHESS) - proposal approved; beam time can be requested as needed.

Conclusions

Major steps have been made toward solving the crystal structure of the EGF induced s-EGFR homodimer. Knowledge of this structure will be extremely valuable in understanding not only the mechanism of activation of erbB receptors but also in designing strategies for therapeutic intervention in breast cancer.
References

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