ELEXIS Based Characterization of Kek1 Interactions with DER and ErbB2

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Victoria A. Scott

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APPROVED:

Joseph B. Duffy, Ph.D.
Biology and
Biotechnology WPI
Project Advisor
Abstract

ErbB1-4 are members of the human epidermal growth factor receptor (EGFR) family of tyrosine kinases. ErbB2 has been implicated in many different types of cancer, most notably breast cancer. Structural studies reveal that ErbB2 and the Drosophila EGFR (DER) share significant similarity. DER is known to be inhibited by Kekkon 1, a transmembrane leucine-rich repeat immunoglobulin, or LIG protein. To investigate the mechanism of binding to DER and determine if Kek1 could bind ErbB2, an enzyme-linked extracellular interaction screen (ELEXIS) was utilized.
Acknowledgements

First of all, I would like to thank Professor Duffy for giving me the opportunity to join the lab and broaden my knowledge of protein purification and analysis. Receiving the offer to remain in the lab after my summer internship as an MQP student was one of the most exciting moments I have had at WPI. It was at that moment that Duff helped me to realize that the work that I do in the lab has the ability to make a difference. Throughout this project I have been motivated by Duff to continue to work hard and achieve the goals set for myself and the project. Thank you Duff for being an amazing advisor over this past year, and I know I wouldn’t have obtained the knowledge regarding protein purification and analysis that I now have if it weren’t for you.

I would also like to thank the other members of the Duffy lab, graduate student Alex Putnam and MQP student Julie Mazza, for making this project such an enjoyable experience. Thank you to Alex for mentoring me throughout this entire project, from teaching me the basics to helping me troubleshoot problems that arose. If it weren’t for you I would not have accomplished as much as I did in the past year working on this project. Thank you to Julie for being the most understanding and helpful lab partner. You made the lab environment very enjoyable and I couldn’t imagine having done the project without you.

Thank you to Katie Crowley for organizing the lab meetings and coordinating the schedules of everyone in the Duffy lab. I also thank Dave Adams, my academic advisor, for guiding me through my undergraduate career and in assisting me in the decision to complete my MQP in the Duffy lab. Lastly, I am extremely grateful to all the faculty and staff of the Biology and Biotechnology Department of WPI for all their help and support throughout my undergraduate career.
# Table of Contents

Abstract .......................................................................................................................... 1  
Acknowledgements ....................................................................................................... 2  
Introduction .................................................................................................................. 4  
Methods and Materials ................................................................................................. 8  
Results ............................................................................................................................ 15  
Discussion ..................................................................................................................... 22  
References ..................................................................................................................... 25
Introduction

According to the Centers for Disease Control and Prevention, cancer is one of the leading causes of death in the United States, second only to heart disease (CDC, 2014). It is known that extracellular domains of cell-surface receptors and ligands mediate cell-cell interaction, including adhesion, communication, and signaling initiation (Özkan et al., 2013). The epidermal growth factor receptor (EGFR/ErbB) family of tyrosine kinases (TK) is correlated to the pathogenesis of several types of cancer, including prostate, lung, glioblastoma, and 30% of breast cancers (Kovacs, Zorn, Huang, Barros, & Kuriyan, 2015). Further evaluation of this receptor in model organisms has shown that the EGFR in Drosophila melanogaster (DER) is able to be bound and inhibited by Kekkon1 (Kek1), a founding member of the Drosophila Kekkon family (Alvarado, Rice, & Duffy, 2004). The Kekkon family is a family of transmembrane proteins composed of an immunoglobulin (Ig) domain and leucine-rich repeats (Alvarado et al., 2004). The focus of this project is to investigate the binding of DER and ErbB2 to Kek1, with particular focus on the role of DER domain V’s role in the binding mechanism.

Epidermal Growth Factor Receptor - Structure and Function

There are four known human ErbB proteins, EGFR/ErbB-1/HER1, ErbB-2/HER2, ErbB-3/HER3, and ErbB-4/HER4 and each receptor is essential for normal cellular development (Normanno et al., 2006). These receptors are composed of an extracellular ligand-binding domain, a short helical transmembrane domain, a cytoplasmic TK domain, and an approximately 230-amino acid long C-terminal non-globular tail (Ward & Leahy, 2015). This structure is illustrated in Fig. 1.

Within the extracellular region, the ErbB receptors are separated into four subdomains, commonly named domains I-IV. There are eleven known EGF-like ligands that bind to at least one of the receptors, leading to formation of particular ErbB homo- or heterodimers (Ward & Leahy, 2015). These ligands are growth factors.
specific to ErbB binding that are produced via autocrine (produced by same cell as receptor) or paracrine secretion (produced by neighboring cells) (Wilson, Gilmore, Foley, Lemmon, & Riese, 2009). The different known ligands and the ErbB receptors they bind to are illustrated in Fig. 2.

The dimerization of the receptors then leads to the activation of downstream signaling affecting cell regulatory systems. As seen in Fig. 3, the main process activated by dimerization of EGFR/ErbB1 is the RAS/Raf/MAPK pathway that controls downstream regulation of cell-cycle progression, proliferation, and differentiation (Okines, Cunningham, & Chau, 2011). The MAPK pathway is a signaling module that is highly conserved in order to relay information to the nucleus regarding cell maintenance throughout the developmental process (Montagut & Settleman, 2009). Dysregulation, excessive activation, of this pathway also plays a role in tumor development (Montagut & Settleman, 2009).

The structural components of the EGFR that are necessary for ligand binding are domains I and III, while domains II and IV are involved in the dimerization process (Normanno et al., 2006). The ErbB family have a particularly unique dimerization and activation process. The dimerization process is promoted by a dimerization loop when the receptors are bound to ligands, inducing the conformational change that exposes the
loops for binding (Normanno et al., 2006). This process can be seen in Fig. 4.

Proper regulation of the ErbB tyrosine receptor kinase family is essential for the regulation of the cell cycle, and as noted above when dysregulation occurs, cell growth rates may vary, contributing to a broad range of cancer types.

**Kekkon Transmembrane Protein Family - Structure and Function**

Prior work in the Duffy lab demonstrated that the extracellular portion of Kek1, specifically the LRRs, and domain V of DER are essential for their interaction (Alvarado et al., 2004). There are nine LIG proteins found in *Drosophila* and six of the proteins belong to the Kekkon family of transmembrane leucine-rich repeat and immunoglobulin domain proteins (Maclaren et al., 2004). A structural representation of the Kekkon family molecules is illustrated in Fig. 5. The method through which Kek1 inhibits DER is via a bipartite process. As seen in Fig. 6, the inhibition of DER requires predominantly the LRRs and transmembrane domain, but not Ig or the majority of the intracellular domain (Alvarado et al., 2004). The LRRs of Kek1 mediate the binding to DER, the transmembrane portion promotes the inhibition of the downstream signaling, and the PDZ domain bindings site within the intracellular domain is responsible for correct trafficking of Kek1 (Alvarado et al., 2004; Derheimer et al. 2004).
A drug called Herceptin is known for inhibiting the overexpression of ErbB2 in people that have HER2-positive breast cancer. Herceptin functions by blocking the dimerization of the receptors, therefore preventing ErbB2 dependent signaling. This is important for the HER2-positive breast cancer patients because due to ErbB2’s open conformation with its dimerization loop available for binding, it is able to create a homodimer without the presence of a ligand (Ward & Leahy, 2015). The process through which Herceptin blocks dimerization can be seen in Fig. 7 as therapeutic type A, which is the extracellular binding arm being blocked by the protein or antibody designed for that binding site.

Additional structure function studies in the Duffy lab revealed that unlike the human ErbB receptors, DER contained an additional extracellular domain – domain V (Alvarado et al., 2004). Previous functional data obtained in the Duffy lab determined that domain V is the likely domain responsible for the binding to Kek1 (Alvarado et al., 2004). That lack of domain V in the human ErbB receptors suggests that Kek1 would not be able to bind and therefore inhibit the human receptors. *In order to evaluate this hypothesis and better understand the contribution of domain V to Kek1 binding the goal of this work was to use an enzyme linked extracellular interaction screen (ELEXIS) was used to assess the binding of ErbB and DER variants to Kek1.* Although ErbB1 was originally the receptor chosen to create the chimeras of DER and ErbB by prior MQP students, recent structural analysis of the ErbB family suggested ErbB2 was a better choice given its structural similarity to DER (Alvarado, Klein and Lemmon, 2009). This work revealed that DER, unlike ErbB1, which is in a closed configuration, is actually in an open conformation similar to that of ErbB2. Illustrations of the three receptors that
were evaluated for the chimera creation can be found in Fig. 8. Based on the visuals in Fig. 8, it is clear that ErbB2 was the logical choice for chimera formation.

**Methods and Materials**

**Gateway Cloning**

The Gateway® cloning system is utilized in the cloning of PCR products, analysis of gene function, and creation of expression clones for protein production. The system is based on the λ-phage site-specific pathway of recombination between att sites of bacterial as well as phage DNA. BP and LR reactions are the basis of the Gateway® cloning system and allow for gene sequences to be easily transferred between vectors without the need of restriction enzymes.

The BP reaction occurs between the gene of interest and the donor vector flanked by the attP sites. Recombination occurs between these attP sites and the result is an entry clone with the gene of interest flanked by attL sites. The LR reaction is a recombination between the attL and attR sites of the entry clone (pENTR) and a destination vector flanked by attR sites. The resulting expression clone (pEXPR) contains the desired sequence flanked by attB sites that can be expressed in cell culture. This process can be seen in an overview of the Gateway® cloning system in Fig. 9. The BP and LR reactions are made possible by the clonase enzymes that are provided with each kit. Antibiotic resistance and the presence of the ccdB gene in the unwanted clone are two selections in this cloning system that result in high efficiency. The ccdB gene causes lysis to occur in standard lab strains of *E. coli*, leading to only the proper clone being expressed in the *E. coli*.

![Figure 9: Gateway Cloning System](image-url)
In order to obtain the expression clones, pENTR clones created from the BP reactions were used in conjunction with LR reactions with either pUAST-AP (bait) or pUAST-Fc (prey) as destination vectors: 7µL of pENTR DNA, 1µL of 150ng/µL pUAST-tag, and 2 µL of LR clonase mix were incubated for 1 hour at 25°C. Next, 5 µL of each LR reaction were transformed into High Efficiency E. coli cells and plated onto LB + Ampicillin plates (50µg/ml) and incubated at 25°C overnight. Resulting colonies were inoculated and mini-prepped utilizing the Qiaprep Spin Mini-prep Kit made by Qiagen®. Each expression clone was mini-prepped for use in transfections was purified using phenol chloroform precipitation and sent to Eton Bioscience® for sequencing to verify the cloning process was complete and correct.

Cell Culture, Transfections, and Expression

The Drosophila melanogaster S3 cell line (Stephanie Mohr, DRSC at Harvard Medical School) is maintained in two different types of media, Schneider’s 12.5% FBS media and Schneider’s Heat Inactivated 10% Ultralow IgG FBS media in either 1:5 or 1:10 dilutions (dependent on growth rate) in 5 mL cultures under sterile conditions. Normal growing conditions for this cell line are 25°C without CO₂ regulation. The purpose of the 10% Ultralow IgG FBS media is to ensure minimal immunoglobulin presence when completing transfections to reduce possible interactions with the secreted proteins of interest.

Transfections were performed under sterile conditions and began with the seeding of a 6-well cell culture plate. Each well was seeded with the S3 cells in Schneider’s 10% Ultralow IgG FBS media in a concentration of 3.125 x 10⁶ cells/mL and a final volume of 1.6mL/well. After 24 hours of incubation under normal conditions, the cells were then co-transfected with the constitutive Arm-Gal4 driver and the proper pUAST responder constructs. At least 6 hours prior to the co-transfection, the transfection complex pre-mixes were created as seen in Table 1 (add the EC buffer first) and stored at -20 °C.
Once all items needed for cell culture had been sterilized and placed in the sterile cell culture hood, 6.4µL of Enhancer was added to each of the transfection complexes and the complex was incubated at room temperature (RT) for 5 minutes. After the 5 minutes, 40µL of Effectene Transfection Reagent (Qiagen®) was added and the complex was incubated at RT for 10 minutes. While the transfection complex formed, the 1.6mL of media was aspirated and each well was washed with 2mL of sterile 1X PBS. The 2mL of 1X PBS was aspirated and 1.6mL of fresh Schneider’s 10% Ultralow IgG FBS media was added to each well. After the 10 minute incubation, 0.6mL of the Ultralow media was added to each transfection complex and immediately added to the appropriate well drop-wise. The secreted proteins in the media were harvested after 7 days of incubation under normal conditions and placed into 15mL conical tubes. The harvested media was then centrifuged at 4°C to pellet any remaining cells. Post-centrifugation, the supernatant containing the protein was collected, filtered through a 0.22µm filter into a new tube, and aliquoted into 500µL samples. The first aliquot of these samples were then verified and quantified via western blot for Fc tagged proteins or alkaline phosphatase activity assay for AP tagged proteins.

**Quantification of Protein Concentration**

The quantification of the proteins expressed and harvested from the transfections is dependent upon the tag present on the protein. For AP tagged proteins, an alkaline phosphatase activity assay in a 96-well plate is utilized to quantify the concentration of protein in the aliquoted samples. This is completed utilizing a dilution series (20U/L, 50U/L, 100U/L, 150U/L, 200U/L) of human placental alkaline phosphatase (hPLAP) as the standard, diluted with harvested transfection supernatant that was not introduced to a transfection complex. The AP-tagged protein was then diluted with the same

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arm-GAL4</td>
<td>400 ng</td>
</tr>
<tr>
<td>pUAST</td>
<td>400 ng</td>
</tr>
<tr>
<td>EC Buffer</td>
<td>up to 100 µL</td>
</tr>
</tbody>
</table>
transfection supernatant as the standard utilizing a dilution series containing 10µL, 20µL, 50µL, 75µL of the sample respectively. PNPP (100µL/well) was the substrate utilized to initiate the enzymatic reaction that produced colorimetric results that were read and recorded every 20 minutes utilizing the Victor3 plate reader and program. The stop solution for the reaction is 2N NaOH and it was added to the active wells after 84 minutes of enzymatic activity. The enzymatic activity of alkaline phosphatase stimulates by the PNPP substrate provides an easily visible colorimetric response. The response can be seen in Fig. 10, which shows the hPLAP standard in row A, sErbB1-Ap dilution in row B, Dscam7 dilutions in rows C and D, and supernatant as the negative control in row E.

The concentrations of the proteins were calculated utilizing the hPLAP standard curve and the equation resulting from the linear trend line. The velocity of the sample being quantified is utilized to find the concentration in U/L by being substituted in as the y value in the equation seen in Fig. 11.
Once the concentrations in U/L were identified, the concentrations were multiplied by 13ng/U in order to determine pg/µL, which was then multiplied by the dilution fact to determine the actual amount in the sample. The volume of protein sample necessary for an ELEXIS interaction was then determined utilizing the actual amount (pg/µL) and multiplying by the 6.8ng required for each interaction.

In order to verify and quantify Fc-tagged proteins, a western blot is completed (Figure 12). The standard utilized for the western is a dilution of the Fc protein which represents 10ng, 20ng, 40ng, and 80ng per well to be utilized in calculating the concentration of the samples. The protein samples are normally run in triplicate with 4µL of the sample in each well. The sample mixes are made as master mixes and then separated into separate Eppendorf tubes for the 5 minute boiling process before loading onto the gel in 1X electrophoresis buffer set up within the Bio-Rad electrophoresis apparatus. The gel runs for 45 minutes at constant 0.02 amperes. The transfer process of the protein onto the nitrocellulose membrane is completed in a sandwich orientation within the clamped transfer chamber. The gel and membrane are sandwiched between two appropriately sized pieces of filter paper, which is then placed between two pads, all of which have been calibrated in the 1X transfer buffer. The transfer process is run for 1 hour and when complete the membrane is stained with Ponceau stain, rinsed with
dH₂O, and then is blocked for 1 hour at RT with 1% Casein (Sigma) dissolved in TBS and 0.1% Tween20 (TBST) on a rocking platform. The blot is then washed for 5 minutes with TBST and then is incubated overnight (16 hours) at 4°C with the Gt-anti-Fc primary antibody (1:5000 dilution in 1% Casein). After the 16 hour incubation, the blot is washed with TBST at RT and then incubated with a 1:20000 dilution of the Ms-anti-Gt secondary antibody in 1% Casein for 1 hour at RT on a rocking platform. The blot is once again washed with TBST and then the blot is incubated in West Femto HRP Substrate solution for 5 mins, excess substrate is wicked off, and then the chemi-luminescence is immediately detected and recorded using the Bio-Rad Gel Doc apparatus and Quantity One program.

![Verification western blot to determine appropriate sized proteins.](image)

Figure 12: Verification western blot to determine appropriate sized proteins.

In order to verify that the Quantity One program had calculated the concentrations correctly, the Fc standard is plotted in order to create a baseline for the samples, which is then utilized to calculate the concentrations.

**ELEXIS: Interaction Assay**

The ELEXIS assay is a high throughput ELISA-based assay (Figure 13). The entire procedure is able to be completed in approximately 24 hours. The assay began
with seeding the 96-well plate with 50µL in each well to be utilized with a 1:166.67 dilution of the anti-AP antibody (Thermo Fischer Scientific) and then incubated for 16 hours at 4°C on a rocking platform.

After the 16 hour incubation, the wells were washed with 300µL of PBST (1X PBS + 0.05% Tween20). The wells were then blocked with 400µL of 1% Casein and incubated for an hour and 30 minutes at RT on a rocking platform. During the blocking process, the protein mixes were created utilizing 100ng of Fc-Prey, 520µU of AP-bait, 5ng of anti-Fc-HRP antibody (Jackson Labs), and supernatant up to a total volume of 50µL. After the blocking incubation was complete, the block was removed and the protein mixes were added to the appropriate wells. The plate was then covered with aluminum foil and incubated at RT on a rocking platform for 4 hours. Next, a working stock of the TMB Ultra HRP Substrate (Thermo Fischer Scientific) was obtained and the volume in the stock was 100µL per active well on the interaction plate with an additional 1mL of substrate to account for pipette error. After the 4 hour incubation, the interaction mixes were removed and the wells were washed with PBST while keeping the plate covered in foil. After the last wash, the residual PBST was left in the wells until the plate was in the gel doc room and ready for the 100µL of HRP substrate to be added to each active well. The plate was read and data recorded every 10 minutes at 590nm for 1 hour in the
Victor3 plate reader. At the 1 hour time point, 100µL of the 1M H₃PO₄ stop solution was added to the active wells and a last plate reading was completed at 450nm.

**Results**

**Receptor Variant Construction and Verification**

Given the structural similarities between DER and ErbB2, and the importance of the domain V of DER, two specific variants were constructed to initiate a more detailed structure/function analysis. The first variant created was sDER+ErbB2IV, which is the result of swapping domains IV/V of the *Drosophila* receptor with domain IV of the human ErbB2 receptor. This was created to determine if domain IV of ErbB2 compensates for the loss of domain V in DER. The second variant created was sErbB2+DERV, which is the result of adding domain V of the fly receptor onto the end of domain IV in the human receptor. This was produced in order to test if DER domain V is sufficient to confer binding to Kek1.

Since the ELEXIS assay utilizes secreted versions of the bait and prey proteins, the variants were created using either full-length variants (including transmembrane and cytoplasmic domains), previously constructed, as a template or constructed *de novo* (see materials and methods for details). Putative positive pENTR and pEXPR clones were then characterized by restriction digest and/or sequencing (Fig. 15 & 16).
Together the restriction and sequencing analyses confirmed the construction of both variant expression clones. The next important component of the project was the verification and quantification of the secreted proteins harvested from the transient transfections.

**Receptor Variant Expression - Validation and Quantification**

The quantification of the protein samples was crucial for the ELEXIS procedure because that procedure is highly dependent on the concentrations of tagged proteins.
utilized. The AP-tagged protein concentrations were calculated utilizing an alkaline phosphatase enzymatic activity assay (Materials and Methods).

Table 2: sKek1-AP Concentration Calculations Based on hPLAP Standard.

<table>
<thead>
<tr>
<th>sKek1-AP Calculations</th>
<th>sKek1-AP Volum e (µL)</th>
<th>sKek1-AP Velocit y</th>
<th>Units* 13ug/U</th>
<th>sKek1-AP Amoun t (pg/µL)</th>
<th>Dilutio n Factor</th>
<th>Actual Amoun t (pg/µl)</th>
<th>Amount for ELEXIS</th>
<th>Volum e for ELEXIS (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.0903</td>
<td>86.6</td>
<td>86.6*13</td>
<td>1125.8</td>
<td>1</td>
<td>1125.8</td>
<td>6.8 ng/ interaction</td>
<td>6.040 1</td>
</tr>
<tr>
<td>75</td>
<td>0.0119</td>
<td>65.5</td>
<td>65.5*13</td>
<td>851.5</td>
<td>1.33</td>
<td>1132.4 95</td>
<td>6.8 ng/ interaction</td>
<td>6.004 4</td>
</tr>
<tr>
<td>50</td>
<td>0.0076</td>
<td>45.3</td>
<td>45.3*13</td>
<td>588.9</td>
<td>2</td>
<td>1177.8</td>
<td>6.8 ng/ interaction</td>
<td>5.773 4</td>
</tr>
</tbody>
</table>

Table 3: sKek2-AP Concentration Calculations Based on hPLAP Standard.

<table>
<thead>
<tr>
<th>sKek2-AP Calculations</th>
<th>sKek2-AP Volum e (µL)</th>
<th>sKek2-AP Velocit y</th>
<th>Units* 13ug/U</th>
<th>sKek2-AP Amoun t (pg/µL)</th>
<th>Dilutio n Factor</th>
<th>Actual Amoun t (pg/µl)</th>
<th>Amount for ELEXIS</th>
<th>Volum e for ELEXIS (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.101</td>
<td>97.3</td>
<td>97.3*13</td>
<td>1264.9</td>
<td>1</td>
<td>1264.9</td>
<td>6.8 ng/ interaction</td>
<td>5.37 59</td>
</tr>
<tr>
<td>75</td>
<td>0.0761</td>
<td>72.4</td>
<td>72.4*13</td>
<td>941.2</td>
<td>1.33</td>
<td>1251.7 96</td>
<td>6.8 ng/ interaction</td>
<td>5.43 21</td>
</tr>
<tr>
<td>50</td>
<td>0.052</td>
<td>48.3</td>
<td>48.3*13</td>
<td>627.9</td>
<td>2</td>
<td>1255.8</td>
<td>6.8 ng/ interaction</td>
<td>5.41 48</td>
</tr>
</tbody>
</table>

Tables 2 and 3 show the process through which the concentrations of Kek1-AP and Kek2-AP were calculated. The velocity of each sample was determined by plotting the absorbance values obtained from the plate reader data versus the time at which that absorbance measurement was taken. This process is illustrated in Fig.17 and the velocities are the slope of the line within the equation for each sample dilution. The data presented suggests that both AP-tagged secreted Kek1 and Kek2 proteins were able to be expressed.
Figure 17: Plot of sKek1-AP and sKek2-AP dilution series absorbance (405nm) plotted against time (mins)

While the AP tagged proteins could be detected by their associated enzymatic activity, the Fc-tagged variants were quantified utilizing the western blot process. The samples were run in triplicate in order to determine the average trace densities of the bands on the membrane, which were then compared to an Fc standard dilution that was included on all western blots.
Figure 18: Quantification blot of the Fc tagged variants (sDER+ErbB2IV and sErbB2+DERV).

The Western blot in Fig. 18 shows the process through which the bands are detected utilizing the Quantity One program. The lanes are identified and the lines are positioned in the middle of the bands. The brackets are then placed around the bands upon selecting the “detect bands” option. The brackets are adjusted to fit the entire band within their parameters, and then the standard and samples are selected for densitometry analysis. Lane 10 appears to be lower concentration that the other two bands in its triplicate, while the rest of the bands appear to have the appropriate concentrations for each lane.
Figure 19: The Fc standard is plotted to obtain the equation utilized to calculate protein sample concentration.

The equation from the Fc standard plot (Fig. 19) is utilized to calculate the concentrations of the protein samples by substituting the x value with each sample's average trace density value. Based on the Westerns, it appears as though the Fc-tagged variants are of the correct size and able to be expressed.

ELEXIS Based Analysis of Receptor Variants

With all the tagged control and variant proteins in hand, their binding abilities were tested using ELEXIS. Prior work done in the lab indicates that the ability of Kek1 to bind the DER is approximately 8-10 fold higher than that of Kek2. As noted above, the absence of domain V in the human receptors suggests that they would be incapable of binding to Kek1. To investigate this and the relevance of domain V of the fly receptor to its binding a preliminary experiment with the variants and wild type receptors was carried out.
The data obtained from the interaction assay showed an 8.9 fold higher interaction reading of sKek1-AP/sDER-Fc over sKek2-AP/sDER-Fc, confirming the functionality of the ELEXIS assay. In contrast to its binding to DER, Kek1 showed essentially no interaction with ErbB2 with signals being similar to supernatant alone. This is consistent with the putative model for domain V being the critical feature driving the binding of Kek1 to DER. Further support for this was the 6.4 fold higher interaction of sKek1-AP/sErbB2+DERV-Fc over sKek2-AP/sErbB2+DERV-Fc. Swapping of domain V from DER with domain IV of ErbB2 (sDER+ErbB2IV) seemed to result in a loss of specificity and general “stickiness” as the level of background signal with this variant with both Kek2 and supernatant only was also high.

The results from the variant receptor interaction assay show promising results regarding binding within the sKek1-AP/sDER-Fc and the sKek1-AP/sErbB2+DERV-Fc interactions. The data collected from this assay can be utilized to draw simple conclusions, but in order to create a detailed description of the binding of the receptors, the assay would need to be completed in triplicate utilizing the appropriate concentrations of anti-Fc-HRP antibody. The current interaction set included several changes to the protocol, the most important change was the use of 100-fold higher concentration of the anti-Fc-HRP antibody. The interaction mix incubation time was then

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**Figure 20: sDER Variants ELEXIS Interaction Data from Time Point 10 Minutes (100-fold increased anti-Fc-HRP)**

*Fold difference = (bait/prey absorbances)/(bait/supt absorbances)*
reduced from 4 hours to 1 hour. The post-interaction washes were adjusted from being originally only 4 washes of PBST for 1 minute each, to an additional 8 washes with 1X PBS for 2 minutes each after the original 4 washes with PBST. The remainder of the protocol was followed with no changes being implemented.

**Discussion**

The goal of this project was to develop greater insight to the binding of Kek1 and the *Drosophila* EGFR using the ELEXIS approach. To accomplish this, variant constructs with both tags (AP and Fc) were generated, the constructs were transfected and expressed, the proteins were then verified and quantified utilizing western blotting or AP activity assays, and lastly, preliminary data was able to be gathered regarding the binding of the variants and wild type receptors (DER and ErbB2) to Kek1 and Kek2.

The beginning of the project involved creating the necessary constructs in order to test the hypothesis of whether DER domain V is necessary to confer binding to Kek1. The constructs were created as chimeras of DER and ErbB2 in order to conserve the structure of the proteins instead of deleting domains. The first construct created was the sDER+ErbB2IV construct, in both the AP and Fc tagged versions. The purpose of this variant was to assess whether domain IV, in addition to domain V, of DER is necessary for binding and if ErbB2 domain IV is capable of initiating binding to Kek1. The second construct created was sErbB2+DERV, also in both tagged versions, to determine if DER domain V is sufficient for binding Kek1. These expression clones were verified via sequencing and restriction digests. The sequencing was an efficient method of verifying that the appropriate construct had been made, especially with the utilization of primers that encoded the junction regions. The sequencing results were compared to the predicted coding sequence to check for any point mutations. All of these variants have been created in construct form, but only the Fc-tagged versions of the variants have been expressed and harvested as secreted proteins. This is due to the particular orientation of the ELEXIS assay as well as previous data showing that when sDER is presented as the bait, the interaction with Kek1 decreases exponentially. This may be
due to sDER-AP conferring dimerization, in turn reducing the availability of the receptor to bind to Kek1.

The western blots that were completed for the variants showed that each of the secreted proteins were the expected size (Fig. 18).

The interaction assay, although a 100-fold higher concentration of the anti-Fc-HRP antibody was utilized, showed very promising results for the ability of DER domain V to confer binding of Kek1 to ErbB2. Wild type ErbB2 exhibited no binding to Kek1 (same as background levels), while the binding of ErbB2+ domain V exhibited ∼6 fold increase in signal relative to wild type ErbB2 and approximately a quarter of the signal for DER. The interaction will need to be completed again utilizing proper concentrations to determine if the data obtained in this assay can be considered significant.

Figure 21: ELEXIS assay 96-well plate post-stop solution addition.

It is clearly visible on the plate in Fig. 21 that the interaction between Kek1-AP and sErbB2+DERV-Fc is much higher than its interaction with sKek2-AP and supernatant. The background for the interaction readings were minimally increased in
comparison to the normal procedure results from previously completed assays, which allows for generalized conclusion to be made regarding the interaction results. An important conclusion to discuss is the 6 fold difference between the interaction of sErbB2+DERV-Fc and Kek1-AP versus Kek2-AP. This difference appears to be indicative that domain V of DER may be sufficient to confer binding to Kek1, but in comparison to the positive control of sKek1-AP/sDER-Fc and the fold over background, there is a large difference between the two (Fig. 20). Thus, while the presence of domain V on ErbB2 may initiate some binding, it is clear that DER confers a much higher rate of binding to Kek1. The basis for this will require further swap and mutant receptor variants to be constructed and analyzed. Another interesting result on the interaction plate was that of sDER+ErbB2IV-Fc binding to the AP-tagged proteins and to supernatant. This suggests that this variant protein is “sticky”, which can be a result of the protein binding to the plate leading to a response.

The next steps for this project are to complete the interaction assay in triplicate while following the optimized protocol. Another future experiment could be to swap the orientation of the proteins in the interaction by utilizing the both the AP and FC tagged versions of all the proteins. Lastly, the potential for developing a cancer therapeutic for ErbB2 is likely going to result in the creation of varying versions of Kek1 that bind and inhibit ErbB2 in the same process though which Kek1 inhibits DER. In order to get to this stage of therapeutic development, greater understanding of the details of Kek1’s binding to DER would need to be gained.
References


CDC. (2014). Health, United States, 2014: With Special Feature on Adults Aged 55–64., from National Center for Health Statistics


Pharmacology & therapeutics, 122(1), 1-8.
doi:10.1016/j.pharmthera.2008.11.008

doi:10.1016/j.cell.2013.06.006
Appendix A  
ELEXIS interaction assay raw data and calculations
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<th>Time</th>
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<th>sKek2-AP</th>
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