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Abstract:

Eph receptor tyrosine kinases (RTKs) are activated by membrane-bound ligands called ephrins. Eph RTKs are divided into two subclasses, each activated by a specific classes of the ligand ephrin. The overexpression of Eph receptors is correlated to cancer cell metastasis in several different types of cancers. Studies with the EphA2 extracellular domain (ECD) and ephrinA1 ligand have shown that upon binding of ephrin to the receptor, EphA2 undergoes increased oligomerization and activation. This indicates that oligomerization is intimately connected to kinase activity. High resolution crystal structures of the EphA2 ECD have revealed some details of these ligand bound oligomers, as well as ligand free clusters. Despite these structures, there is still no comprehensive understanding of the role that each domain plays in ligand-dependent and ligand-free EphA2 oligomerization and activation. Here we report our investigation of the role that the intracellular domain (ICD) plays on EphA2 oligomerization. We first deleted the sterile alpha motif (SAM) domain from the C-terminus of the protein and measured oligomerization with a time-resolved, fluorescence spectroscopy method with single molecule sensitivity, called fluorescence correlation spectroscopy (FCS). Deleting the SAM domain from the intracellular domain displayed reduced mobility of EphA2, and an increased brightness. From this we concluded that the SAM domain has a role in inhibiting oligomerization of the un-liganded, full-
length receptor. In future research, our aim is to investigate the role that other motifs play in receptor oligomerization. This work will have clinical applications, especially in cancers where EphA2 is mutated or overexpressed.

Introduction:

Eph receptor tyrosine kinases are the largest known subfamily of receptor tyrosine kinases. Eph RTKs are divided into two subclasses, EphA and EphB. Their ligands, ephrin are also divided into respective A and B classes. In addition, there are nine different EphA RTKs, which bind to six A-ephrin ligands, and six different EphB RTKs (as well as EphA4), which bind to three B-ephrin ligands. These Eph RTKs play a role in many aspects of cells, from morphology, to communication. What is noteworthy for this project is that RTKs conduct biochemical signals across the plasma membrane through dimerization. Eph RTK activation can be characterized by ligand binding, oligomerization followed by the phosphorylation of tyrosine residues in the juxtamembrane segment (JMS), and kinase domains of the protein. Key mutations to different domains of Eph RTKs often cause alterations in the dimerization and signaling functions of the RTKs. Post-activation of these RTKs the protein is internalized and degraded. In addition, Eph RTKs are often thought to be related to cancer metastasis. It has been shown that in some types of cancers certain Eph RTKs are overexpressed, and many Eph RTK somatic mutations are oncogenic mutations.

Mutations to Eph RTKs have different effects on the oligomerization, and therefore affect cell signaling depending on the domain the mutation is located in. Eph RTKs have many domains
including: a ligand binding domain, cysteine-rich domain, two consecutive fibronectin III domains, a transmembrane domain, juxtamembrane domain, kinase domain, SAM domain, and a PDZ binding motif.\(^1\) (Fig. 1) Our lab is highly interested in introducing key mutations, via site-directed mutation and genetic recombination, into each of the domains of Eph RTKs and observing the functional outcome on the dimerization of the receptors. Our lab elucidates changes in dimerization, and cell signaling, and how those changes influence cancer cells.

**Results:**

In order to investigate the functions of each of the domains, two serial truncations were performed on the protein. The fluorescent tag was maintained at the end of the protein despite the deletions. First a truncation was done to remove the SAM domain and everything following it, denoted as ΔSAM. A second mutation was performed to remove the Kinase domain and everything following it, denoted as ΔKS. These mutations were performed via site-directed mutagenesis, and the plasmid was expressed in a mammalian cell line termed DU145, a human prostate cancer cell line.\(^5\) DU145 cells were selected for the experiment because they express very low levels of endogenous EphA2.

Post-transfection, these cells were used in immunoblot assays with and without the ligand EA1-fc using a primary antibody (pY-EphA/B) that is against the phospho-dityrosine motif found in the juxtamembrane domain of most Eph receptors. (Fig. 3) Looking at the short exposure time in the unliganded section of the figure the
wild type and the full length receptors, denoted A2WT and A2-GFP respectively, there appears to be no phosphorylation taking place. When observing the ΔSAM construct (A2ΔS-GFP) the short incubation with the antibody shows increased phosphorylation. This shows that the receptor may be active with this truncation despite the fact that there is no ligand bound. The vector is a part of the immunoblot to show a control value for no phosphorylation. When the antibodies were applied on the ΔSAM construct given the ligand EA1-Fc phosphorylation was notably higher than either the ΔSAM construct without ligand or the full length receptor. (Fig. 4) This supports the hypothesis that the kinase domain undergoes activation upon the deletion of the SAM domain. Any small amounts of phosphorylation shown under the wild type, and full length receptors after long exposure to the antibody can be explained by endogenous protein. DU145 is an excellent candidate for this experiment specifically because it has very low levels of endogenous EphA2.
Morphological changes can also be observed with the truncation mutations (Fig. 5). Examining the morphology of the DU145 cells it can be seen that when ligand is added to the full length receptor (EphA2-GFP) the cells cluster. When no ligand is bound to the full length receptor the image resembles that of the control for no clustering (Vec/Ctl). However, the ΔSAM construct (EphA2-ΔS-GFP) shows substantial clustering without the ligand present. This clustering is similar to what is shown in the image of the full length receptor with added EA1-Fc ligand. In addition, human growth factor (HGF) normally induces cell scattering, and somewhat deters clustering. It is shown that the full unliganded receptor has no clustering in the presence of HGF. When ligand is added to the receptor, clustering occurs despite the HGF, but not quite the amount of clustering seen with the liganded receptor without HGF. When the ΔSAM construct is observed with HGF, it is shown that clustering occurs similar to that of the full length receptor with HGF. This supports the phosphorylation data shown in the previous figure. The receptor seems to undergo activation when missing the ΔSAM domain without ligand being added.
To investigate this idea further, fluorescence correlation spectroscopy (FCS) was used. FCS is able to reveal the molecular brightness of the fluorescent protein, the mobility, as well as the density of the receptor in the membrane. Molecular brightness is a measure of the photons emitted from a receptor or a complex of receptors per unit of time. The molecular brightness is directly proportional to the amount of receptors in a given complex.

As an example, having a dimer with a fluorescent tag would show twice the molecular brightness of a monomer. The average molecular brightness of the full length receptor is 466 cpsm, and the average molecular brightness of the ΔSAM construct is 633 cpsm. (Fig. 6) This shows that the molecular brightness of the ΔSAM construct without ligand, is significantly higher than the molecular brightness of the full length receptor without ligand. Src-GFP and Myr-GCN4-GFP are monomer and dimer control values respectively. Src is a known monomer, and the GCN4 portion of Myr-GCN4 forces a dimer conformation. The molecular brightness of the ΔSAM construct is not significantly different from the dimer control, however, is significantly different from the monomer control. Again it is indicated that the deletion of the SAM domain allows oligomers, and activation of the receptor. The ΔKinase construct (EphA2ΔKS-GFP) showed an even higher molecular brightness than the ΔSAM construct. In fact, the ΔKinase construct seems to be elevated just above the dimer control value. The difference in molecular brightness between the ΔKinase construct and the ΔSAM construct can likely be
accredited to differing equilibrium distribution of oligomer states.\textsuperscript{5} Overall it can be concluded based on molecular brightness that the truncated receptors seem to have higher oligomer states.

The diffusion coefficients tell a similar story. The diffusion coefficient is a measure of mobility of the receptor within the membrane. The full length receptor has a diffusion coefficient value of 0.30 (\(\mu m^2/s\)). This is about twice the value of the \(\Delta\text{SAM}\) construct and the \(\Delta\text{Kinase}\) construct. Mobility in the membrane is measured via the diffusion coefficient utilizing fluorescence recovery after photo-bleaching (FRAP). It is concluded that the larger the membrane bound protein is, the slower the rate of diffusion.\textsuperscript{8,9} From the diffusion coefficients of the different constructs, it can be concluded, that the mobility of the truncated constructs is greatly reduced due to larger cluster formation. This confirms the previously mentioned idea that deletion of the SAM domain causes activation of the receptor regardless of ligand presence. This also shows that the kinase domain is in no way inhibiting activation of the receptor.

Overall, from the results it can be concluded that the SAM domain of EphA2 plays a major role in the inhibition of receptor activation, and signaling.
Methods:

This project used plasmids that contain human EphA2, and were labeled with eGFP fluorescent proteins at the C-terminus. The starting plasmids with not truncation mutates were created by our collaborators at Case Western Reserve University. The nucleic acid sequences of these plasmids are known, and have been dissected and annotated using a free program called Genome Compiler. Genome Compiler along with an online tool made by New England Biolabs (NEB) were used to design the truncation mutations performed. E. coli strains used consisted of DH5α, NEB5α, and NEB10β.

Site-directed mutagenesis was conducted using reagents purchased from NEB. Primers were designed for mutation as previously described. There are three options for mutation using site-directed mutagenesis which are substitution, deletion, and insertion. (Fig. 8) The goal here is to have primers with a high enough annealing affinity that they bind to the template DNA, but not so high that they are not able to be easily denatured for replication in Polymerase Chain Reaction (PCR).

Western blotting was used to measure the phosphorylation of tyrosine within the receptor. Western blotting is an experiment that allows a specific protein to be located. The antibody pY-EphA/B was introduced to bind to our protein, and a secondary antibody was introduced as a reporter of the magnitude of primary antibody binding. Another method of data collection involved a custom built fluorescence microscope. In order to detect changes in dimerization, fluorescence cross-correlation spectroscopy (FCCS) was used. (Fig. 9) FCCS can monitor molecular interactions, enzymatic reactions, and dynamic colocalization. With use of two different colored lasers, and two corresponding photon detectors, the emission of the two
fluorescent proteins can be detected. In this project these fluorophores would be eGFP as mentioned previously, and mCherry. This method allows the cross-correlation of the diffusion of proteins to be observed.

Another method to detect oligomerization is fluorescence resonance energy transfer (FRET). FRET is similar to FCCS, except FRET is used to indicate distance on a molecular level. Rather than measured correlated diffusion of fluorophores, FRET measures the energy transferred from a fluorophore in an excited electronic state to a chromophore. The amount of energy transferred between the fluorophores has a direct relation to distance. Note that when any spectroscopy data is taken for membrane bound receptors, the laser is always focused on the lamellipodial region of the cell. (Fig. 9) This is to ensure that what is being detected is the result of membrane function only. Luckily any data taken in which an organelle or anything non-membrane appeared under the laser is noticeable and thrown out as outliers. Similar outliers
appear when data is taken too close to the edge of the cell. All FCS for this project is done in vivo, and the cells often move out of the area that the laser is focused on.

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Presentation:

This project along with additional research was published with colleagues in the journal Scientific Reports, titled, “A role of the SAM domain in EphA2 receptor activation.” Published in March of 2017, this article can be freely accessed online, and is the 5th work cited in this project.

This work was also presented in poster format at the annual American Society of Biochemistry and Molecular Biology (ASBMB) meeting on the 26th of March at McCormick place Chicago IL.
Bibliography:


