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Mutagenesis of the Caveolin Interaction Motif in Follicle Stimulating Hormone Receptor

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by

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Abstract:

Follicle Stimulating Hormone Receptor is a G-protein coupled receptor that localizes to rigid domains in the cell membrane known as caveolae. In the fourth transmembrane domain, there is a region of the receptor that contains a putative caveolin interaction motif (CIM), an alpha helix with 4 critical phenylalanine residues. These residues are critical to the localization of the receptor to caveolae. The goal of my research has been to mutate these phenylalanine residues to the amino acid leucine. Our hypothesis is that this conservative mutation will result in a mutant receptor that is no longer able to localize to the caveolae but still travels to the plasma membrane. Furthermore, if the receptors localize to other regions of the cell membrane, we hope to discover how this affects signaling. This would help demonstrate the necessity of receptor localization to caveolae for normal function. This would provide valuable information about signaling, and may help to create a novel way to inhibit FSH signaling by interrupting FSHR-caveolin interactions.

Introduction:

The phospholipid bilayer of cells has been characterized by the fluid mosaic model. This model suggests that phospholipids and other components of the membrane are free to change position with one another and diffuse freely. This allows macromolecules such as proteins and anchored molecules to move freely and interact with one another. Cholesterol, a relatively planar and nonpolar molecule, is able to fill the gaps between phospholipids and stabilizes the membrane and maintains its viscosity in various temperatures.[1]

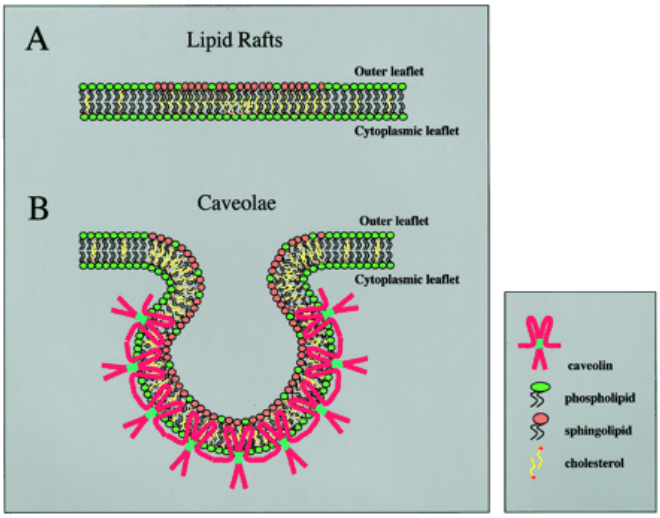
Proteins that exist in the cellular membrane are free to interact with one another as they diffuse. Among these proteins are receptors, which interact with extracellular signals to alter or activate proteins on the inside of the cell, thus transmitting the desired signal. In the body,

hormones act as chemical messengers to bring signals of various bodily needs to distant tissues via the bloodstream. Hormones must bind to a receptor in the target cells in order to produce physiological changes. The hormone-receptor interaction is specific, and most hormones act only on a single complementary receptor. Thus, receptors only exist or are expressed on cells that must react to the signal. An example of this is follicle stimulating hormone (FSH) and its receptor (FSHR). FSH is used in both men and women. In men it acts on the Sertoli cells in the testes to promote spermatogenisis. In females it acts on granulosa cells in the ovaries to promote follicular growth. FSH binds to FSHR, which is a G-protein coupled receptor (GPCR). A GPCR is a receptor with seven transmembrane domains, each of which is a nonpolar alpha helix. The ligand causes a conformational change that allows the coupled G protein to exchange GDP for GTP, while simultaneously shedding is inhibitive β and γ subunits. The active α subunit, which is anchored to the cell membrane then diffuses to activate adenylyl cyclase to produce intracellular cAMP.[3]

Lately, studies have elucidated that the fluid mosaic model is incorrect, or at the very least, incomplete. While the majority of the membrane is fluid and dynamic, there are microdomains within the cell membrane that are rigid. These rigid microdomains are known as lipid rafts, since they are rigid structures in an otherwise “fluid” surrounding. These lipid rafts are composed of different lipids and proteins that give them their rigidity. The majority of the cell membrane is made of phospholipids known as glycerophospholipids. These glycerophospholipids are made of a glycerol backbone, an esterified saturated fatty acid chain, an esterified unsaturated fatty acid, and a phosphate group which hold one of many various head groups in place. The polar head groups make contact with the water, while the nonpolar fatty acids compress together through the hydrophobic effect. The unsaturated fatty acid causes a

bending in the fatty acid. This bending causes increased spacing and therefore decreased density. However, in lipid rafts, a phospholipid class known as sphingolipids predominate. These sphingolipids do not have a glycerol backbone, but instead have a trans-unsaturated chain that covalently bond a saturated fatty acid and a phosphate group that anchors a polar head group. The trans-unsaturated chain and the saturated chain give minimal spacing. As a result, sphingolipids allow for high density and tight packing in the cellular membrane. Lipid rafts are also packed with additional cholesterol, causing even tighter packing and higher density.[3]

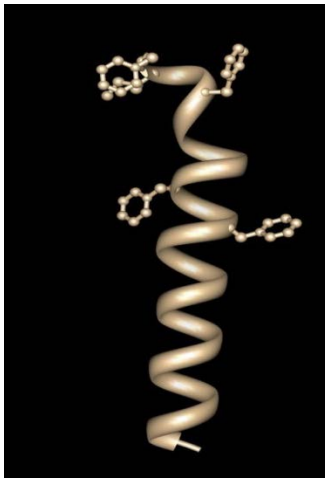
These very dense domains form around certain proteins. These proteins do not quite fit into the normal cellular membrane, leaving portions of exposed nonpolar regions. Lipid rafts, which are thicker and more dense are able to relieve this stress, allowing the protein to adopt a more stable and functional conformation. A specific protein, caveolin, is only stable in a highly curved membrane. The protein caveolin forms oligomers in the membrane to form special lipid rafts called caveolae.[1]



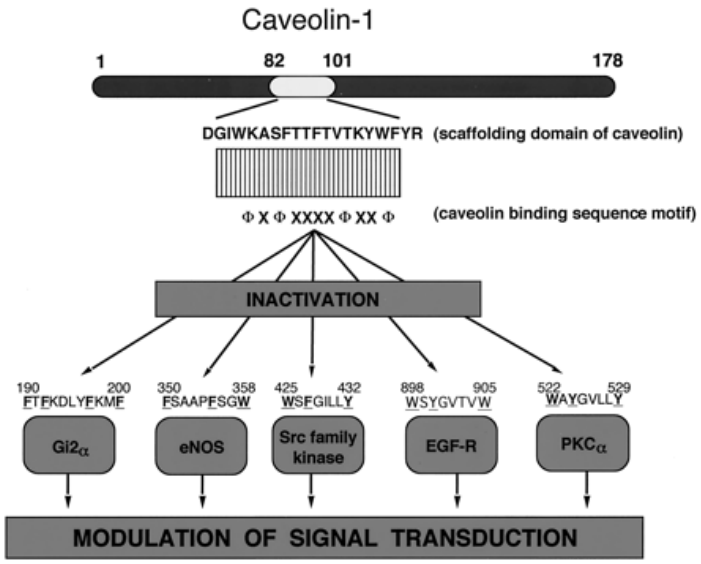
Caveolae are concave pockets of lipid rafts that contain caveolin. The pocket curvature relieves the curvature frustration of the less compatible flat membrane-caveolin interaction. It is difficult

to say whether the proteins or the lipids are accommodating to the other. For instance, it is unclear whether the caveolae are formed around caveolin to allow it to exist in a more relieved conformation, or whether the caveolin is produced so that the pockets can be formed. It is difficult to which exists to permit the structure of the other.[5]

Proteins other than caveolin can exist in caveolae; FSHR is thought to be one such protein. If this were the case, one would expect to find in the caveolae of granulosa and Sertoli cells. It is believed that by existing in caveolae, the rigid lipid raft structure could provide a signaling platform to allow rapid signal transduction. Instead of molecular messengers diffusing across the membrane for a chance encounter with its target, the target could be juxtaposed to the messenger, allowing efficient signaling. Some proteins such as FSHR have a region in their structure that encodes for association with the caveolin protein, thereby sequestering the protein to the caveolae. This interactive region is simply named the caveolin interaction motif (CIM). This region consists of four aromatic residues with a specific spacing of ΦXΦXXXXΦXXΦ (Φ being any aromatic residue and X being any residue). In FSHR, each Φ represents a phenylalanine (F). In the alpha helix where the CIM exists, the spacing of the aromatic groups allows them to all appear on the same side of a helix.



This shows caveolin operating as a form of scaffolding protein, interacting with other proteins to sequester them to caveolae and to modulate their function. This concept was first demonstrated *in vitro* where it was shown that a 20-amino acid peptide, part of caveolin, was a potent inhibitor of heterotrimeric G-proteins in GTP hydrolysis assays.[1] Evidence suggests that this region can selectively bind and modulate activity of a variety of proteins, including non-GPCRs, such H-ras and Src family tyrosine kinases. This specific region on the caveolin was determined as responsible for the functional effects of the protein caveolin. This region came to be termed the caveolin scaffolding domain (CSD).



Evidence suggests that a CIM is not absolutely required for binding to caveolin scaffolds, and many proteins that demonstrate a CIM do not interact with caveolin. The CIM region is more of an indication than a rule of caveolin binding. Thus the CIM existing in FSHR indicates that FSHR may be one such protein that is modulated by scaffolding to caveolin.

To determine the relevance interaction motif in FSHR, we aimed to mutagenize the sequence by changing the phenylalanines to leucines. Leucine was chosen to replace

phenylalanine because it was a conservative mutation. Leucine is approximately the same size and both are entirely nonpolar. The change would not greatly affect the overall structure (and presumably function) of the receptor, but it would interrupt the CIM because it lacks aromaticity.

The mutant receptor would divulge many pressing questions. It could reveal if the mutated CIM was still able to allow FSHR to sequester to the caveolae. If FSHR could still enter caveolae, it may or may not still function properly. If it could not be sequestered to the caveolae, it would be interesting to see if the receptor was still capable of signaling.

Methods:

The plasmid pCN42ΔB contained the wild type gene for FSHR and antibiotic resistance to ampicillin. Excision of the WT FSHR gene piece was accomplished with use of Bam HI and Not I restriction enzymes. The enzymes were added to the plasmid with buffer 3 as a buffer and diluted roughly 1:10 with DI water. Enzymatic digestion occurred at 37 ^{o}C for 4 hours in a water bath.

Ligation of mutant gene pieces and vector involved addition of purified vector and a given gene piece type. T4 ligation buffer was used with the ligase enzyme and the solution was diluted with DI water. The resulting solution with the plasmid parts was allowed to ligate overnight in a 14^{o}C water bath.

Transformation of the plasmid into *E. coli* was accomplished using two different methods. The first method involved heat shocking a solution of *E. coli* cells and plasmid at 42^{o}C for 45 seconds. Recovery (SOC) medium was then added to the cell-plasmid solution, which was then incubated at 37^{o}C with constant inversion. The transformed cells were then plated onto LB amp plates, which were then incubated at 37^{o}C overnight.

The production of the mutant gene pieces involved PCR and splicing by overlap extention (SOEing). In this process A and D primers were mixed WT pCN42ΔB (7.4 kb) along with GoTaq and diluted 1:2 with DI water to excise the WT FSHR gene piece. PCR cycled 33 times at 95^{o}C to 52^{o}C to 72^{o}C. This produced gene pieces about 1.4kb in size (of the original 2.1kb FSHR gene). The WT FSHR gene pieces were then added to a solution with A and B primers and separately to a solution with C and D primers (both containing GoTaq and DI water), and a PCR was completed for both. The B and C primers included a copy of the single phenylalanine/leucine mutation. This produced the A-B and C-D gene fragments (800b and 600b respectivey), each which contained a copy of the CIM mutation.

Gene fragments were then combined together with A and D primers and GoTaq in PCR. The existing mutation site, introduced by the B and C primers, permitted the gene fragments to anneal to one another at the mutation site. The primers then completed the final gene piece which contained one copy of the mutation. Three final mutant gene pieces were produced from this SOEing process, each with their respective single mutation to the CIM.

Results:

To determine the significance of the caveolin interaction motif in follicle stimulating hormone receptor, mutations were produced at three of the four aromatic residue sites. These mutations were all conversion of phenylalanine to leucine at the sites 481, 486, and 489 individually.

The mutation process required two steps. For the first step, mutations to the wild type receptor were introduced using AB paired and CD paired primers which included the mutation. This mutation introduction also split the receptor into two fragments. The fragment produced by

the A and B primers was approximately 800 base pairs long and encoded the mutation at the end where the B primer was bound. The fragment produced by the C and D primers was about 600 base pairs long and contained the mutation where the C primer was bound. Different B and C primers were used for the different mutations, and fragment pairs were produced for each of the three mutation types.

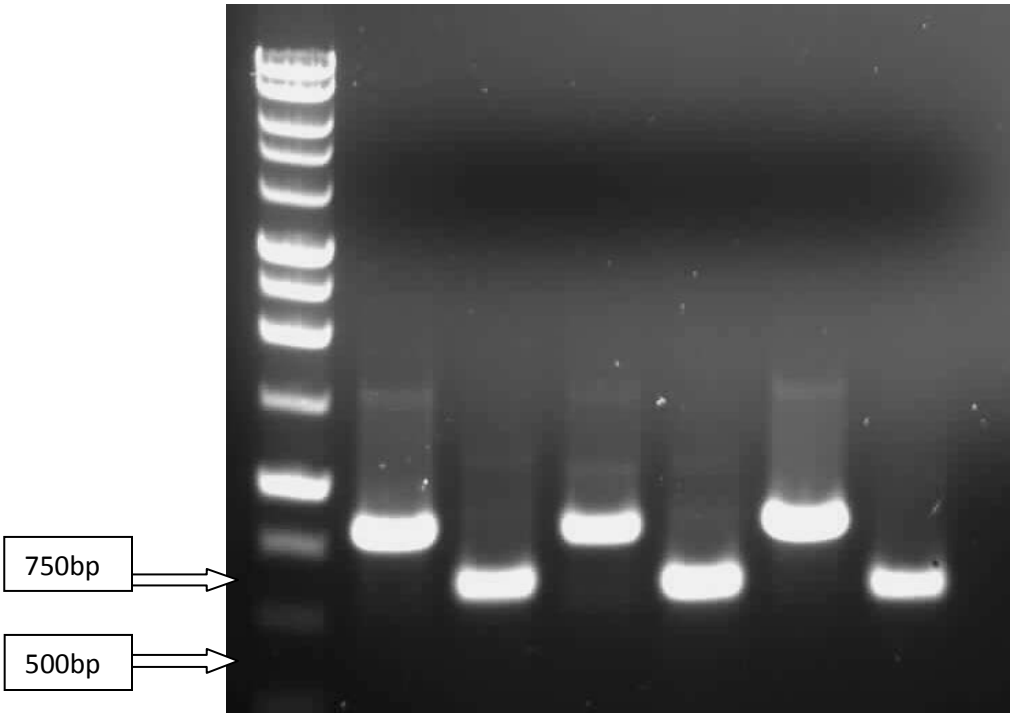


Figure 1: Mutated Fragment Pairs Produced in Step 1

The bands displayed in Figure 1 represent DNA ladder, the AB and CD fragments for the 481 mutation, AB and CD fragments for the 486 mutation, and AB and CD fragments for the 489 mutation respectively from left to right. The bottommost band of the DNA ladder represents 500 base pairs in size, and the second bottommost band represents 750 base pairs. The AB fragments are just above the 750 base pair band, as was expected from their 800 base pair size. The CD fragments were between 750 and 500 base pairs in size, as was expected from their 600 base pair size. All bands were robust, indicating large quantities of DNA produced. Lanes containing

mutation fragments contained only one band, indicating that there were no artifacts or alternative products produced. The respective band migration distance and lack of alternative products suggests that the fragments produced were the target fragments containing the introduced mutation.

With the AB and CD fragments produced, it was possible to splice them back together using SOEing. The mutation site allowed overlap between fragments, and when A and D primers were added, the PCR process produced one final gene piece of 1430 base pairs and contained the single introduced mutation site.

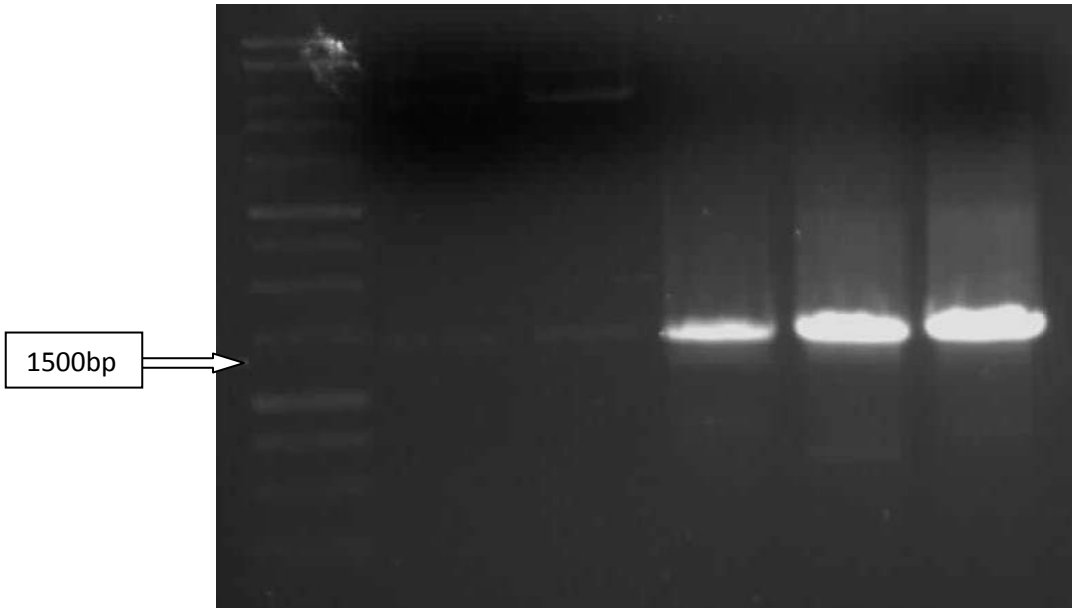


Figure 2: Recombination of Mutant Fragments in Step Two

The bands in Figure 2 represent DNA ladder, cut wild type vector, cut wild type vector, F481L, F486L, and F489L respectively from left to right. The gel displayed is slightly rotated clockwise in Figure 2, but bands all migrated equal distances, including wild type bands. The migration distance was roughly 1500 base pairs, which was consistent with the expected 1430 base pair product. Again, product bands were robust and consisted of only one band, indicating that the

SOEing effectively combined mutant fragments without production of artifacts or alternative products. The brightness of the band of the mutants indicates that a large amount of product was formed. Wild type bands were used as comparison, since mutant and wild type gene pieces should be identical in size.

To create new plasmids that contained the mutated genes instead of the wild type, wild type vector plasmid (pCN42ΔB) was digested using restriction enzymes for the Bam H1 and Not 1 sites. This expelled the wild type gene piece from the plasmid, creating a vector which could integrate the mutant gene piece cassettes.

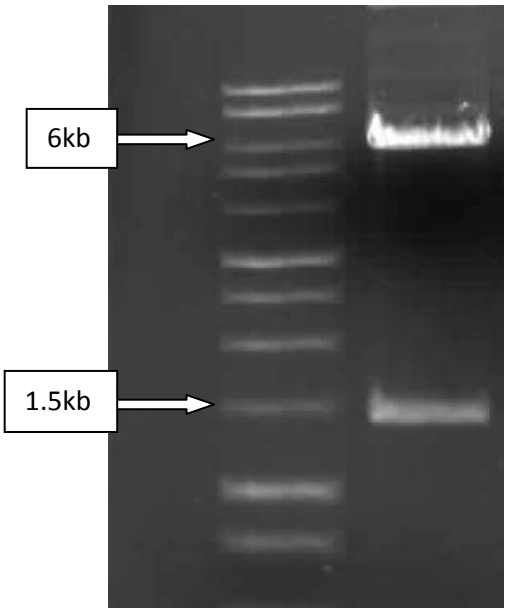


Figure 3: Digestion of WT Plasmid with Bam HI and Not I Restriction Enzymes

The digestion of the WT plasmid produced two fragments, the WT gene fragment for hFSHR and remaining vector that included ampicillin resistance. The digested products were separated by gel electrophoresis, as shown in Figure 3. The entire pCN42ΔB plasmid was about 7.4kb in size, and digestion expelled the hFSHR gene piece (about 1,430 bases) leaving roughly a 6kb plasmid piece. Figure shows that digestion was successful, and the 1.4kb gene piece was

removed to leave 6kb vector. The absence of a band at 7.4kb indicates that essentially all plasmid copies were digested. The two bands present indicate that there was complete digestion of the plasmid, and there were no alternative products. Each plasmid expelled one gene pieces, so the number of gene pieces was equivalent to the number of vector pieces created. This explains why the vector band is brighter than the gene piece band, since each vector piece contained just more than four times as much DNA as the gene pieces.

The vector was removed from the gel and purified. The mutant gene piece cassettes were then added to the vector and ligated. The ligated products were then transformed into *E. coli* and plated on LB-Amp plates. However, no colonies formed on the plates, indicating that the transformation was unsuccessful.

Discussion:

The purpose of generating FSHR receptors with mutant CIM is to study the importance of the CIM and moreover of the receptors' location within caveolae for signaling. With SOEing, it was possible to produce the three single mutant gene pieces. These gene pieces represent a successful mutation of the CIM. In the investigation of the CIM's importance, it must be mutated and then the mutants must be observed for differences. The first step, the mutation of the gene, appeared to be successful. However, to ensure that a mutation took place one would have to either clone the mutant genes or have the gene pieces analyzed and bases characterized. Now the mutants must be expressed and observed in mammalian cells. This will require future research. The mutants must be ligated back into the plasmid to create the full, and mutated, FSHR gene. The mutant plasmid must then be amplified by transformation into *E. coli* and then harvested by lysis. The plasmid must then be transfected into human embryonic kidney cells (HEK), which

will then express the mutant receptors. With expression in HEK cells, many questions pertaining to FSHR and its CIM may be answered.

Further research may divulge how the CIM is important to the function of FSHR. With only a single mutation, it is possible that no change will be observed, and the CIM is not sufficiently mutated. If this were the case, multiple mutation FSHRs could be produced through similar SOEing methods. It will be interesting to see how the mutated FSHRs interact with caveolin. If they do not interact and are not sequestered to lipid rafts, it may or may not affect downstream signaling. It is possible that the receptors are then sequestered to flat (non-caveolae) lipid rafts. The receptor functions properly in thick caveolae, so it is reasonable to conclude that the receptor may take a different conformation outside of the caveolae, where nonpolar regions of the helix become exposed and must contort to find a more stable conformation. However, it is also possible that the receptor operates entirely normally outside of caveolae. Such speculations can only be settled through further research.

It was not possible to insert the mutant gene pieces into the pCN42ΔB vector. To troubleshoot the failed ligation prodecure, WT gene pieces were ligated with vector in varying proportions: excess vector with limited gene pieces, equimolar, and excess gene pieces with limited vector. However, none of the ligation products could be transformed into *E. coli*, nor could they be viewed on a gel due to small concentrations. This implies that there must have been some issue with the ligation process. Perhaps the ligase enzyme had been somehow inactivated, or that the ligase buffer was poorly mixed. It is also possible that the vector and gene pieces were not of sufficient quantity to produce useful amounts of mutant plasmid. The in order the HEK cells to express the plasmid, it is paramount that the ligation difficulty is resolved.

Despite the ligation problems, we were successful in creating large amount of pure mutant gene pieces with the desired introduced mutations. This was a big step toward allowing mutant expression in HEK cells. Although further work is required to complete the research, this process represented great progress in the direction of solving the FSHR-CIM questions. This would have great implications for FSHR signaling and function. With better understanding of this signaling pathway, we would have more control over it. It may be possible to alter the pathway in such a way as to adjust fertility effects in men and women, perhaps for birth control or fertility drugs. These fertility pathway alterations would stem from changes to the CIM and therefore to FSHR's signaling ability and function, its ability to integrate into the caveolae. Novel approaches to problems can become paramount when existing tacts are not sufficient or capable of solving a giving problem. By understanding the purpose of FSHR in cavealoe, we can approach fertility in a brand new way.

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