Interaction of Human Follicle Stimulating Hormone Receptor with Caveolin within the cell

membrane

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Abstract

The human FSH receptor is a g protein-coupled receptor (GPCR) expressed on the surface of granulosa cells in the ovary and Sertoli cells in the testes. FSHR has a sequence of amino acids consistent with a caveolin interaction motif ($\varphi X\varphi XXXX\varphi XX\varphi$) found between amino acids 479-489 in the primary receptor sequence in the putative 4th transmembrane domain. Caveolin is a protein found in cell membrane micro domains such as lipid rafts. These densely packed regions of the membrane are enriched for sphingolipids and cholesterol and are thought to be involved in signal transduction. Caveolin can be found in caveolae, flask-like structures which are a specific type of lipid raft, or in non-caveolae lipid rafts. Although the receptor has this motif, it was unknown if hFSHR biochemically interacted with caveolin. To test this hypothesis, hFSHR was isolated by immunoprecipitiation from HEK-293 cells stably transfected with hFSHR cDNA. Analysis by western blot of co-immunoprecipitated proteins revealed the presence of caveolin-1. Together these results suggest that caveolin-1 plays an important role in cell signaling by sequestering hFSHR into lipid rafts for normal receptor function.

Introduction

Follicle-stimulating hormone is a hormone produced by the anterior pituitary in response to gonadotropin-releasing hormone, sometimes called luteinizing-hormone-releasing hormone (LHRH). FSH targets the granulosa cells in the ovaries and the Sertoli cells in the testes to lead to the maturation of gametes. In males FSH promotes spermatogenesis in the Sertoli cells within the testes and causes the primary spermatocytes to undergo meiosis. In women FSH targets the granulosa cells within the ovaries to encourage proper development of the follicle which contains the egg to be released during ovulation [4]. It is for these reasons that FSH is a prime target for the development of new forms of birth control.

Follicle-stimulating hormone is a glycoprotein consisting of an alpha and beta subunit. The alpha unit of FSH is identical to the alpha subunits of leutinizing hormone and thyroid stimulating hormone. It is the beta subunit that varies for each hormone and interacts with the receptors of that molecule [3]. In order for a molecule to be considered a hormone it must enter into the bloodstream and travel to its target tissue. Blood reaches every cell of the body. What prevents hormones from affecting every cell is the fact that a hormone cannot illicit a response without a receptor present. Hormones can only bind to their specific receptor and receptors for each hormone are only expressed in specific cells. A hormone is only as good as its receptor and if the receptor does not function properly the hormone cannot send a signal.

Follicle stimulating hormone acts through its receptor. Human follicle stimulating hormone receptor is a G protein-coupled receptor occurring in the testes, ovaries, and the uterus. In the testes FSH binds to the Sertoli cells, causing changes that nourish the

developing sperm cells through the stages of spermatogenesis. FSH functions in the ovaries by binding to the granulosa cells and causing many changes within the developing follicle. If the follicular cell fails to respond to FSH then it is discarded.

The receptor has 7 transmembrane domains that span the cell membrane in alpha helices. Within the 4th transmembrane domain there exists a caveolin interaction motif. The caveolin interaction motif is a sequence of amino acids in the alpha helix of the transmembrane domain. This is a sequence of amino acids ordered either φxφxxxxφ or φxxxxφxxφ, where φ is one of the aromatic amino acids, tryptophan, phenylalanine, or tyrosine, and x is any amino acid [11]. When spanning the lipid bi-layer the amino acids form an alpha helix with the aromatic amino acids oriented toward the outside of the helix. Many proteins that contain this amino acid sequence have been shown to interact directly with the caveolin family of proteins. Pharmacology research has shown that caveolin's role in cell signaling has the potential to be manipulated to create new forms of treatment and therapy [8].

Many different types of receptors have been proven to bind with caveolin within the membrane. Many are signaling proteins like tyrosine kinases [2]. Several of the molecules proven to have this interaction are G protein coupled receptors, similar to FSHR, including dopamine receptor (1A), and glucagon-like peptide receptor which is involved in insulin secretion (Table 1).

Table 1. Cav-1 interacting GPCRs that contain a CIM

Receptor	CIM Sequence	Transmembrane domain
Adenosine receptor A1	228-**Y**A**F**RIQK**F**-295	No
D(1A) Dopamine receptor	313-**F**DV**F**VW**F**GW-321	Yes
Glucagon-like peptide 1 receptor	247-EGV**Y**L**Y**TLLA**F**-257	Yes
hFSHR	479-**F**A**F**AAAL**F**PI**F**-489	Yes

Caveolin has been found not only to interact with these receptors, but in some cases play an integral role in proper signaling and function [11][12][13].

Current pharmaceutical forms of birth control function by disrupting the FSH pathway. Pills contain estrogen and progesterone that feed back negatively on the production of GnRH. Low levels of GnRH causes reduced production of FSH. Low levels of FSH will cause developing follicles to undergo atresia and be discarded. This greatly reduced the chance of ovulation and therefore pregnancy [10].

Disruption of the hormone cycle within the body and increasing levels of hormones beyond their normal levels can have some very adverse side effects. Most hormone pills contain a combination of estrogen and progesterone. By taking these contraceptives hormone levels remain high in the blood stream and are not allowed to go through the natural cycle which triggers ovulation. Research has suggested that prolonged exposure to elevated estrogen levels is linked to an increased risk of venous thromboemolism, and a higher risk of myocardial infarction and stroke [1]. By focusing on hFSHR's interaction with caveolin instead of regulation of hormone levels these risks can be bypassed. By understanding how the receptor and caveolin interact we can develop new forms of birth

control, and fertility treatments by either increasing or disrupting signaling to the granulosa cells.

FSHR and caveolin interactions could theoretically be used to develop methods of male birth control. While FSHR is present in many tissues within women it is less so in males. In men, FSHR is found primarily in the testes to promote sperm production. If this signaling could be disrupted than it may be possible to develop male contraceptives.

The opposite of this is also plausible. For women or men who are having infertility problems related to their follicle stimulating hormone levels. By understanding the interactions of the receptor it may be possible to increase these reactions to promote proper cell function and development. New fertility drugs and treatments could be developed around these interactions. We believe the key to understanding FSH signaling is linked to caveolin and caveolae.
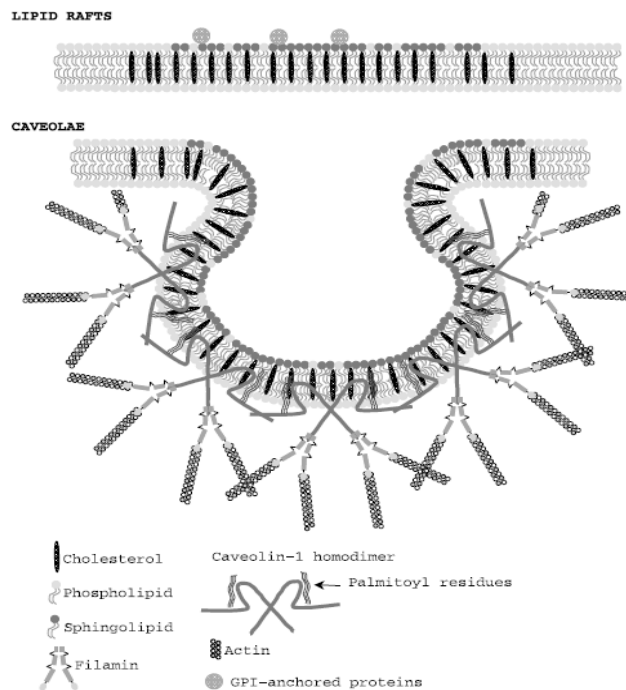

Figure 1. Diagram illustrating lipid raft composition and caveolae. Caveolin-1 scaffolds hold the flask shape of the caveolae by Taken from Quest, Leyton, and Párraga (**2004**).

Caveolin are proteins which reside in caveolae, a distinct subset of lipid rafts. Lipid rafts are domains of the cell membrane that are rich in cholesterol and sphingolipids which cause them to be densely packed and resistant to detergents. These lipid rafts act as signaling platforms regulating receptors and other signaling molecules. Caveolae, which is Latin for little caves, are flask shaped lipid rafts that are involved endocytosis and various signaling processes (Figure 1).

The caveolin family of proteins, particularly caveolin-1, has been shown to have more important roles than just holding the shape of caveolae. Caveolae are required for proper signaling of some receptors [12]. Caveolin-1 also influences the protein makeup of a cell membrane through its interaction with receptors. Cells without caveolin-1 expression are often depleted of most cell surface proteins [14]. Receptors that interact with caveolin are dependent on caveolin to signal properly and to be sequestered into the lipid raft.

In our research we hope to prove that human follicle-stimulating hormone receptor interacts with caveolin-1 within the caveolae lipid rafts. Several other studies have isolated GPCRs by using the same co-immunoprecipitation we have employed [12]. Some have even used methods to study caveolae and molecules that reside in them [7]. We will grow hFSHR-transfected HEK293 cells to use in the immunoprecipitation experiments. This cell line has already been used successfully to study the interactions of other receptors with caveolin [9]. We hope that by understanding more about hFSHR and its interactions that we will be able to use the information to develop techniques to either increase or decrease fertility.

Methods

Optiprep gradient. Before we could begin looking for a direct interaction between FSHR and caveolin we needed to establish that they both occur within the same area in the cell membrane. By isolating the lipid raft domains of the cell membrane we could begin looking for these interactions. Lipid rafts can be isolated by creating a discontinuous gradient.

HEK 293 cells stably transfected to express human follicle stimulating hormone receptor were grown to confluency in t75 flasks. The cells were then harvested and lysed by douncing. The lysed sample was then added to a 45 % solution of Optiprep medium in a centrifuged tube. This layer containing the sample was then overlaid with lower concentrations of Optiprep solution to create a discontinuous gradient as shown in figure 2. The gradient was centrifuged at 240,000 g for 18 hours. Fractions were collected starting from the top of the gradient and placed in microfuge tubes to isolate the buoyant lipid raft layer. These fractions were then separated on an SDS-PAGE gel using electrophoresis and probed using standard western blotting protocols.
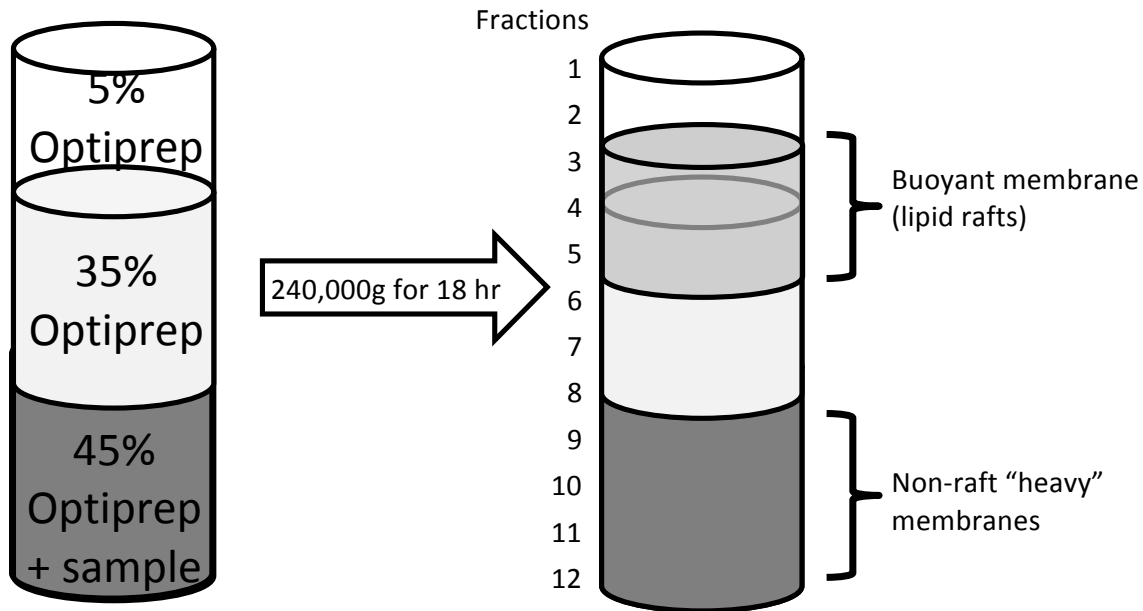
Fractions

1
2
3
4
5
6
7
8
9
10
11
12

5% Optiprep

35% Optiprep

45% Optiprep + sample

240,000g for 18 hr

Buoyant membrane (lipid rafts)

Non-raft "heavy" membranes

Figure 2. The creation of the Optiprep gradient showing the layers of medium and the separation of lipid rafts from non-raft membranes

Co-immunoprecipitation. Immunoprecipitation is a widely used method of protein detection and purification. An antibody specific to the target molecule is mounted on beaded support such as protein G sepharose. These beads are then added to a sample of whole cell lysate and the beads bind with the target protein. The antibody-protein complex is then precipitated. Any proteins not bound to an antibody are then washed away. After the wash all that is left is the target protein bound to the antibody. The target protein is then eluted from the antibody and can be analyzed by SDS-PAGE and Western blots. Co-immunoprecipitation is done in a similar fashion except the focus is to capture not just a target protein but also other macromolecules that interact with it, like FSHR (Figure 3). Traditionally co-immunoprecipitation uses a detergent to help break up the membranes within the sample. Normally this would not affect protein interactions within the cytoplasm but the interactions that are the focus of this study occur within the membrane itself and are hydrophobic. The use of a detergent would destroy these interactions and make it very

difficult for us to study these interactions. Other researchers have demonstrated that a mild detergent such as octylglucoside can be used to isolate membrane receptors and their interacting partners [9] [11].

Two t75 flasks of transfected HEK293 cells were grown to confluency for each treatment. The cells were then washed with 5ml of ice cold 1x PBS. 5ml of ice cold PBS/EDTA was used to lift and harvest the cells from the flask. Cells were then transferred to a 15ml conical tube and centrifuged at 3000xg for five minutes at 4°C to pellet the cells. Supernate was removed and the cells were resuspended in 1ml of octylglucoside lysis buffer (10 mM Tris-Cl, pH 8, 150 mM NaCl, 60 mM octyl glucoside, 1 protease inhibitor pellet per 10ml). Tubes were incubated on ice for 10 minutes and were sonicated for 5 cycles of 10 seconds each to lyse the cells. Lysate solution was then transferred to microfuge tube and centrifuged at max speed for 15 minutes for 15 minutes at 4°C. Supernate was then transferred to a new tube and 100 microliters were save for whole lysate controls. 5 micrograms of primary antibody were added to one tube while 5 micrograms of IgG$_{2B}$ antibody were added to a second tube as a control. Samples incubated overnight at 4° C. 100 microliters of protein G slurry was added to each tube and allowed to incubate rocking end over end for 2 hours at 4° C. Beads were centrifuged for 30 seconds at max speed at 4° C. Supernate was removed and transferred to a new tube. Beads were resuspended in 0.5ml of ice cold lysis buffer and centrifuged at max speed for 30 seconds at 4° C. This wash step was repeated twice more. Beads were resuspended in 140 microliteres of 2x sample buffer and heated to 70° C for 5 minutes. Tubes were centrifuged for 30 seconds at max speed at room temperature. Supernate containing purified sample was then placed in a new tube to be analyzed.
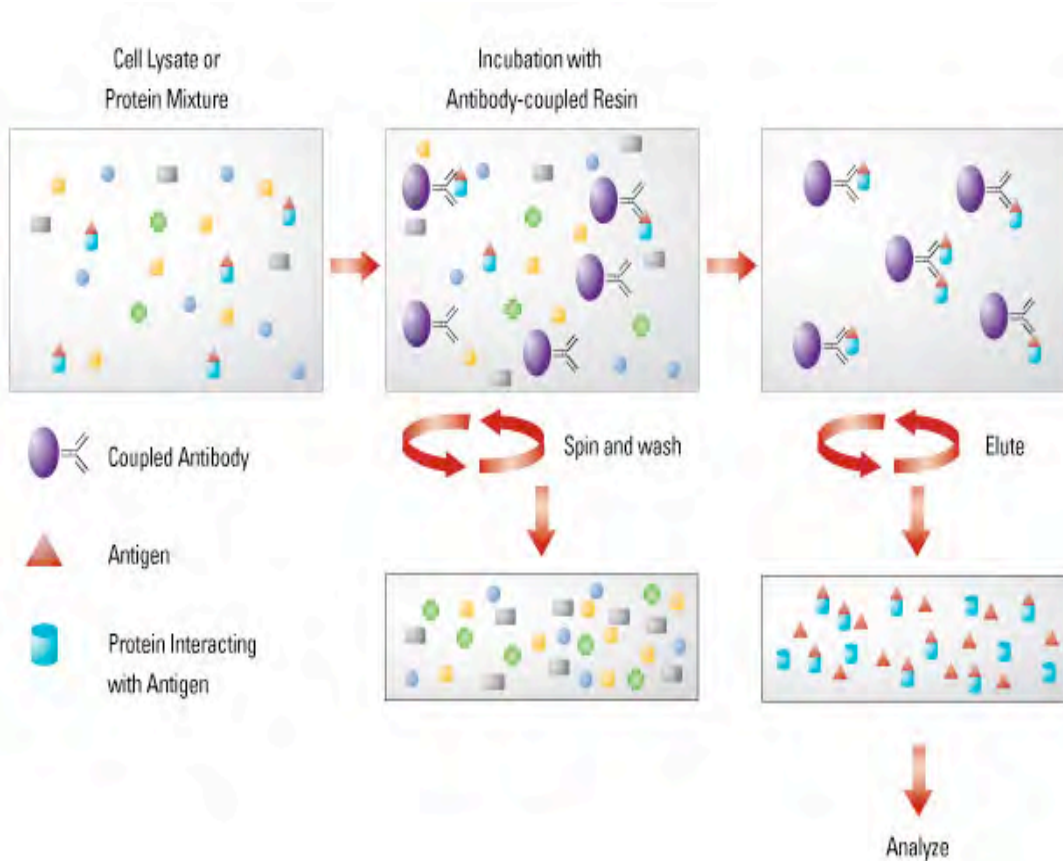
Figure 3. Process of co-immunoprecipitation by which proteins can be isolated and analyzed using an antibody against a specific target protein. Taken from piercenet.com

"Good Mojo" Protocol. As we probed our western blots for caveolin and FSHR extra bands kept appearing in our blots. These extra bands were appearing because of the coupled antibodies that were still in our samples from the actual co-immunoprecipitation procedure. The secondary antibodies bind to any antibody already present on the blot. In order to see only the bands that are a result of the proteins we are looking for we use what we nicknamed the "good mojo" protocol. This procedure more effectively blocks the membrane exposing only the desired proteins.

After transferring the sample to a membrane using standard western blot techniques the membrane was washed in 1x TBST. The membrane was then blocked with unlabeled

streptavidin in 3% BSA (100 microliters streptavidin/ 10ml 3% BSA) for one hour at room temperature. The membrane was then washed with 1x TBST 3 times, for 5 minutes. The membrane was then blocked with free biotin in 3% BSA (1 mg biotin/ 10ml 3% BSA) for one hour at room temperature. The membrane was then washed with 1x TBST 3 times, for 5 minutes. The membrane was then probed with a biotinylated antibody in 3% BSA (10 microliters antibody/ 10ml 3% BSA) overnight at 4° C. The membrane was then washed with 1x TBST 3 times, for 5 minutes. The membrane was then probed with streptavidin-HRP in 3% BSA (20 microliters streptavidin-HRP/ 10ml 3% BSA) for one hour at room temperature. The membrane was then washed for a final time with 1x TBST 3 times, for 5 minutes. The membrane was then developed according to standard western blot protocols.

Results

To determine if follicle stimulating hormone receptor occupies the same region of the cell membrane a discontinuous gradient was created using HEK 293 cells transfected to express the receptor. The gradient was created using Optiprep and centrifuged. After the centrifugation there was a clearly visible layer that was composed of buoyant lipid rafts. Fractions were then isolated and the lipid raft fraction was captured between fractions 7 and 9.

When the fractions were analyzed using western blotting techniques and probed for FSHR and caveolin-1 it was shown that they both occupy the lipid raft domains of the cell (Figure 4.)
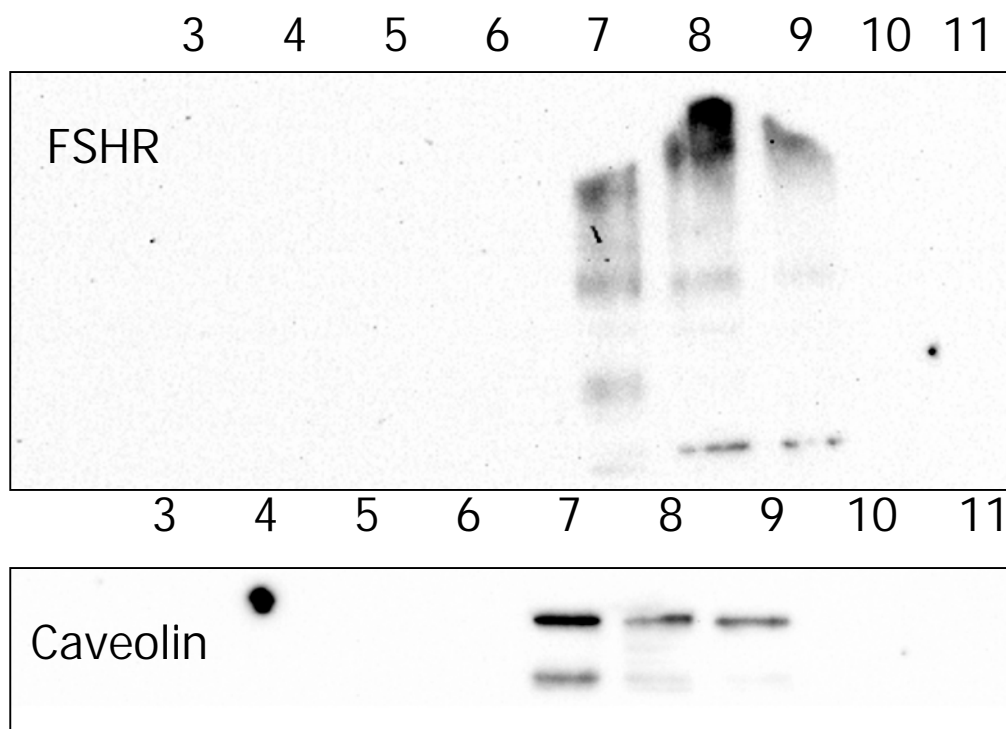

Figure 4. Western blot of the fractions from the Optiprep gradient. Faction numbers are descending from top of column toward the bottom. Blots were probed for FSHR (top) and caveolin-1 (bottom) respectively.

After the results of the Optiprep gradient it was then possible to move on to co-immunoprecipitation experiments. The first set of co-immunoprecipitations was done to see if we could in fact isolate the protein we were trying to precipitate, in this case follicle stimulating hormone receptor (Figure 5.) The sample showed that we indeed isolated FSHR and that none was found in the IgG$_{2B}$ control IP. Large amounts of receptor were present in the whole cell lysate.
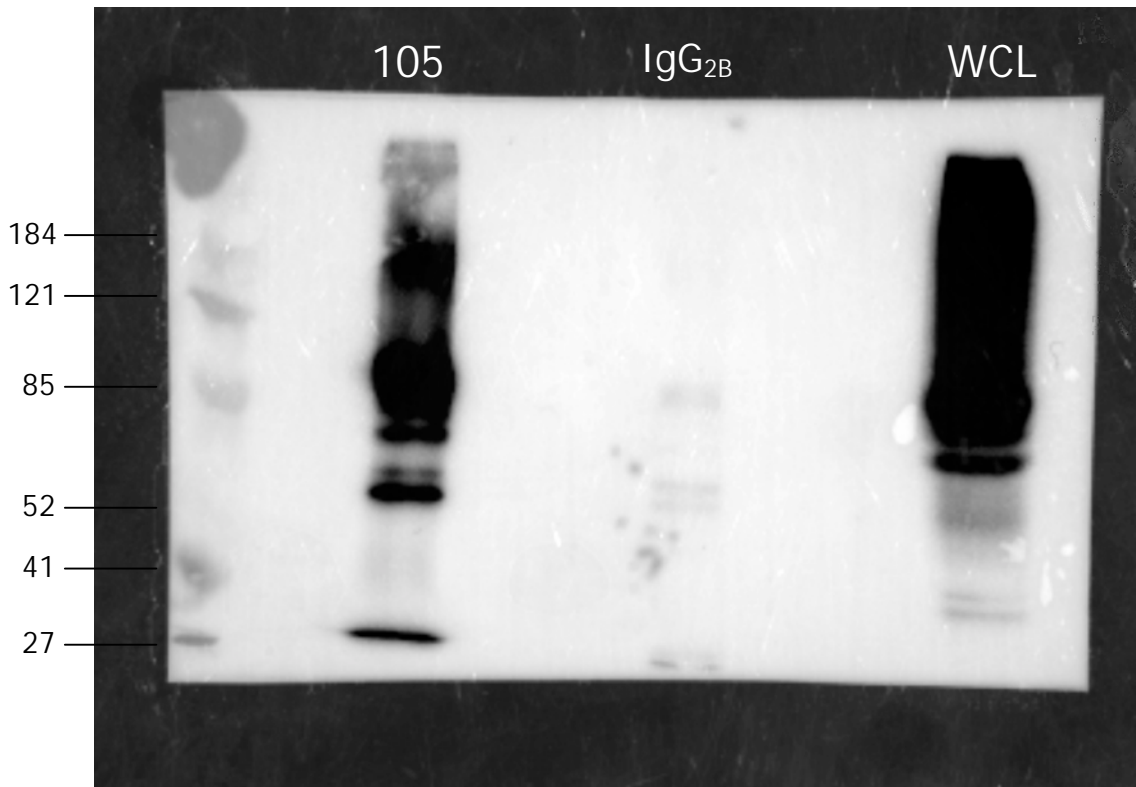

Figure 5. Early western blot showing successful immunoprecipitation of FSHR. Molecular weight markers on the left and whole cell lysate controls on the right.

For the second round of co-immunoprecipitations an anti FSHR antibody was used to co-precipitate caveolin (Figure 6). Caveolin-1 can be seen in the whole cell lysate just above the 21 KD marker. However, it is difficult to identify it in any of the lanes whether they be control or otherwise. A large unknown band occurs in all four treatment lanes.
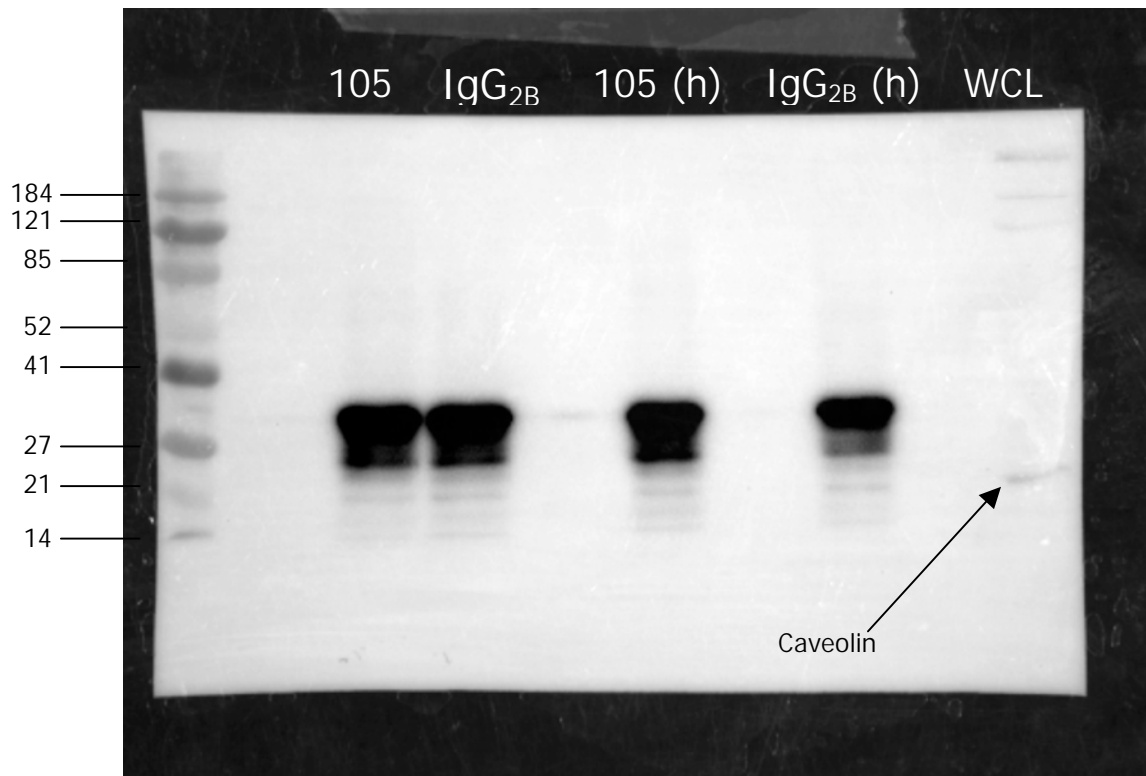
Figure 6. Western blot of samples from co-immunoprecipitation using anti-FSHR antibody. Molecular weight markers are shown on the left with whole lysate control on the right.

   Unable to identify caveolin in the samples, it was decided that a new method of probing was needed. The advantages of the good mojo protocol allow distinct bands to be seen where previously bands were indiscernible (Figure 7). Using the same samples as seen in figure 5 the bands that were previously hidden can now be seen clearly, again FSHR is not seen in the control lane.

   Another co-immunoprecipitation was done this time using an anti-caveolin-1 antibody to co-precipitate FSHR (Figure 8). In this co-immunoprecipitation there was a hormone treatment of 30 minutes and a control and the samples were then co-immunoprecipitated. A band for FSHR appears in both the control and hormone treatment Cav1 IPs between the 85 and 52 KD markers.
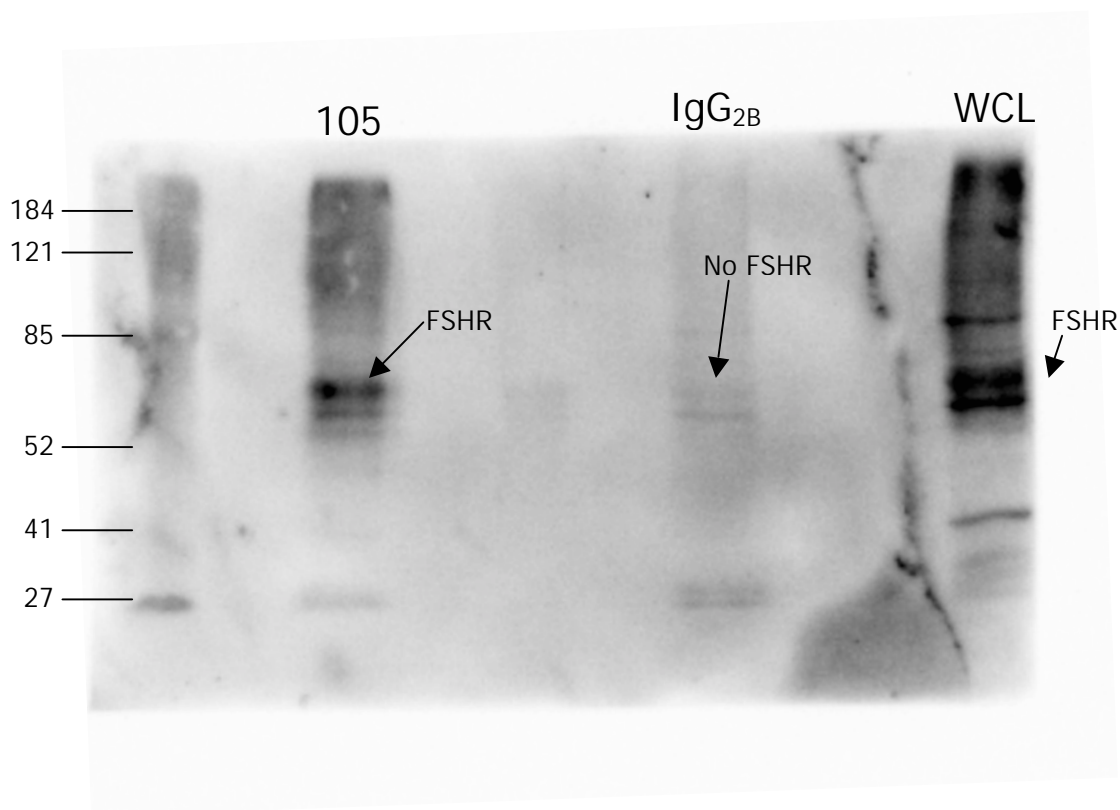
Figure 7. Western blot using the same samples as Figure 6 to emphasize the difference between a standard western blot and a blot using good mojo protocol.
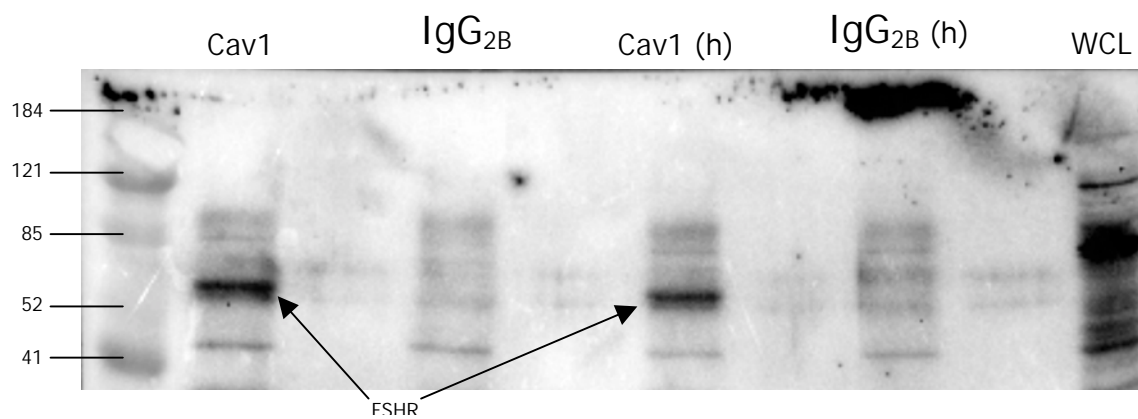

Figure 8. Western blot showing the presence of FSHR from a co-immunoprecipitation experiment using an anti caveolin-1 antibody and probed using the good mojo protocol.

Discussion

Understanding a protein's structure and its interactions is necessary when trying to develop new drugs and treatments. Every piece of evidence lends itself to the larger picture that is molecular biology. By understanding the interactions of follicle stimulating hormone receptor we can create new treatments that exploit how the receptor normally functions.

Originally we used co-immunoprecipitation with anti FSHR antibodies to search for caveolin, but this proved difficult and ultimately unsuccessful. The western blots were not clear enough to determine if caveolin was present. Our solution to this was the use of our good mojo protocol which uses biotinylated antibodies to produce clear western blots. Unfortunately we did not posses a biotinylated antibody for caveolin and were forced to do another round of co-immunoprecipitations this time using anti-caveolin-1 antibodies to isolate follicle stimulating hormone receptor. We were able to use the good mojo protocol to produce a western blot that clearly shows that FSHR and caveolin interact directly.

We have shown with our Optiprep gradient that the receptor exists in lipid rafts as we suspected and this can allow it to signal to and interact with other proteins within and around the lipid raft. We have also shown that follicle stimulating hormone receptor interacts directly with caveolin. This means that FSHR may exist in caveolae. This interaction may also mean that caveolin is not only interacting with the receptor but is crucial for sequestering it into caveolae lipid rafts so that it can function properly.

Ever since caveolin interaction motifs were noticed their importance in cell signaling has been in question. The next step is to treat with different time trials of hormones. For our experiments we only used two hormone treatments. Cells were treated either not at all or for 30 minutes with follicle stimulating hormone. It is possible that that

there is an increase or decrease in the interaction but that our 30 minute hormone treatment was too long for us to clearly see the effects. Future experiments will involve timed treatments with FSH at increasing intervals most likely, 0 minutes, 1 minute, 5 minutes, 10 minutes, 15 minutes, and 30 minutes. With these treatments we will be able to see if band intensity increases or decreases in the western blot analysis of the co-immunoprecipitation experiments which would correlate to an increase or decrease of interaction between the proteins.

Future work will also include using cells transfected with mutant receptors that have an altered or are completely lacking a caveolin interaction motif. Using these transfected cells it can be determined whether or not the caveolin interaction motif is responsible for the interaction we have observed. By understanding follicle stimulating hormone receptor and its interactions could be the keystone in development of future forms of birth control and fertility treatments.

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