Endocrine, *vol. 26, no. 3, 241–247, April 2005* 0969–711X/05/26:241–247/$30.00

Identifying Protein Interactors in Gonadotropin Action

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Systems biology integrates a variety of diverse approaches to the study of the cellular pathways comprised of protein networks. Following an initial ligand–receptor binding event, transduction of the signal is modified in a variety of ways via downstream protein interactions. Protein interactions can occur between two proteins or, alternatively, an interaction between two proteins can be facilitated by an adapter protein. Protein interactions can affect the spatial and temporal distribution of ligand–receptor complexes in cells, attenuating or prolonging signaling. With regard to gonadotropin receptors, protein interactions have been primarily studied in terms of desensitization and termination of signal transduction, or for their role in trafficking. The purpose of this review is to describe protein interactions that mediate gonadotropin receptor functions and to highlight some emerging interactions, as well as some of the caveats inherent in the attempt to uncover these pathways.

Key Words: Gonadotropins; receptors; adapter proteins; signaling.

Introduction

Signaling in endocrine tissues originates with protein and steroid hormones, which travel in the circulatory system, and growth factors, neurotransmitters, and cytokines, which signal locally. Activation of both G protein–coupled receptors (GPCRs) and the tyrosine kinase growth-factor receptors follows agonist binding. In the case of growth-factor receptors, signaling involves dimerization of receptors subsequent to ligand binding *(1)*. The glycoprotein hormones are cystine-knot cytokines, and the family includes luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and chorionic gonadotropin (CG). The receptors for LH are GPCRs and are present in Leydig cells of the testis and thecal cells of the ovary. The receptors for FSH are in granulosa cells of the ovary and Sertoli cells of the testis.

Received April 12, 2005; Revised April 21, 2005; Accepted April 22, 2005.

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The canonical pathway of glycoprotein hormone–mediated cellular activation is through the cAMP/protein kinase A (PKA) pathway *(2)*. Additionally, protein kinase B (PKB/Akt) phosphorylation subsequent to gonadotropin treatment occurs via the PKA pathway, and potentially via guanine nucleotide exchange factors that activate downstream proteins, which, in turn, activate phosphoinositol-3-phosphate kinase (PI3K) that phosphorylates PKB *(2)*. Indeed cAMP activation of GTPases propagates the signal through downstream cascades including Erk 1/2, PI3K, and the mitogen-activated kinase p38 (MAPK) *(2)*.

In addition to these pathways of cellular activation, it is now appreciated that GPCRs *(3)* and growth-factor receptors *(4)* can interact with a number of cytoplasmic scaffold and adapter proteins, which can link the receptors to various signaling intermediates and intracellular effectors (Fig. 1). These adapter proteins illustrate that GPCR function is more complex than previously appreciated. The gonadotropin receptors have not been studied greatly in this regard. The complexity of the proteins interacting with receptors offers fine control of receptor function and compartmentalization. Identification of GPCR interacting proteins should provide additional understanding of the systems biology of gonadotropin receptor signaling.

Identification of Interacting Proteins

The search for interacting proteins that comprise the protein interaction pathways governed by and mediating gonadotropin signals is an important area of research. Previously, interactions have been anticipated by knowledge of cellular pathways. For example, in the case of the luteinizing hormone receptor (LHR), it was reasonable to expect that chaperones involved in the glycosylation pathway would interact with immature forms of the LHR *(5)*. Also, interactions between arrestin and FSH receptor (FSHR) were anticipated and could be detected by chemical crosslinking studies *(6)*.

The yeast two-hybrid screen has been the mainstay of investigators attempting to identify protein–protein interactions *(7)*. This system has been used to identify two new protein interactors with LHRs. One of these proteins, called p38JAB1, binds to immature LHRs and is involved with degradation of LHRs *(8)*. Another, called GIPC, binds to LHR and is involved in sorting and recycling of LHRs *(9)*.

Advantages of the screen include amplification of signal, identification of low-affinity interactions, ease of use, and

the lack of necessity to predict prey proteins. The primary disadvantages are that membrane proteins may not fold properly or may not be soluble; several complementary assays need to be carried out to eliminate false positives; proteins that only interact after posttranslational modification may not be detected; interactions take place in the nucleus, which is not always the compartment of interest; and interactions may be detected that are bridged by an adapter protein.

A variety of new approaches are available for the toolbox of systems biologists to enable the identification of interaction networks that will aid in defining the systems biology of the gonadotropin response. It is important to remember that it is not just the interaction of proteins with receptor that is of interest, but the downstream interactions as well. In this regard, it is worth noting that the use of tagged protein libraries is employed in a now leading approach to identifying protein–protein interactions. Three strategies have been developed for detecting protein interactions. One of these is the tandem affinity purification (TAP) of tagged proteins *(10)*. The key advantages of this system are specificity and decreased background noise. A competing technology is the use of stable isotopic labeling in cell cultures, followed by mass-spectrometric analysis of cell extracts *(11)*. Any method based on mass spectrometry will be sensitive. In addition an emerging method for detecting protein interactions is the use of multidimensional separation methods prior to mass spectrometric analysis *(12)*.

Arrestin: An Adapter Protein That Interacts With Gonadotropin Receptors

Adapter proteins are defined as proteins that can associate with two or more partners. This interaction can increase the specificity or efficiency (thermodynamically) of a signaling pathway. One of the best studied examples of an adapter protein is beta arrestin, which until recently has been considered to function as an adapter by binding to clathrin *(13)*. Now it is appreciated that, in addition to binding to receptors, arrestin both self-associates, acting as a scaffold, and also binds to a number of other proteins including AP-2, Mdm2, and ubiquitin *(14,15)*. Arrestin has long been known to operate in a mechanism for GPCR desensitization and internalization. Recently, it was found *(16)* that beta-arrestin-2 (also known as arrestin 3) binds to the single transmembrane-spanning type III transforming growth-factor-beta receptor; thus, the role of arrestins as adapters continues to broaden.

When a GPCR is activated, desensitization can occur, as the GPCR becomes a substrate for GPCR kinase (GRK) binding and phosphorylation. Unlike the growth-factor receptors, which are single-pass receptors, the GPCR are structures with a seven-transmembrane-domain helix arrangement. Members of the glycoprotein hormone receptor subfamily of GPCR have one long extracellular N-terminal extension and a rhodopsin-like transmembrane domain *(17–19)*. Overexpression of either GRK or arrestin can attenuate signaling by FSH in nongonadal *(20)* or gonadal (Sertoli) cells *(21)*. That observation is not solely interpreted as attenuation of signaling by virtue of phosphorylation of receptor. Kinase inactive mutants of GRK can desensitize FSH signaling as well, indicating that the mere binding of GRK is sufficient to damp signaling *(22)*. It is also conceivable that signal attenuation is due to GRK interaction with heterotrimeric beta/gamma G-protein subunits that have dissociated from the receptor upon agonist binding *(23)*. Arrestin does bind to phosphorylated GPCRs, which results in steric hindrance, preventing G-protein reassociation with the receptor and inducing desensitization. We can further imagine that the avidity of the interaction of arrestin with the receptor will affect the temporal pattern of re-sensitization. Thus, a difference in affinity between arrestin-2 and arrestin-3 for any given GPCR may determine residency time on the receptor. This could affect the cell's decisions on whether to recycle or to internalize.

In addition, we could consider that the spatial distribution of arrestin affects the period until re-sensitization. For example, there is evidence that arrestin can recruit phosphodiesterases (the enzymes that degrade cAMP) into a receptor compartment *(24)*. In addition, arrestin associates with a host of other proteins, including ERK1/2, JNK kinase, and p38 MAP kinase *(14,25)*. The C-terminal tail of a GPCR, by determining the stability of the receptor–beta-arrestin complex, controls the extent of beta-arrestin–bound ERK activation, and influences both the subcellular localization of activated ERK and the physiologic consequences of ERK activation *(26)*. This paradigm shift considers arrestin as an initiator and director of new signals from the GPCRs that it desensitizes rather than simply as an attenuator of signaling *(27)*.

Internalization of receptors is generally considered to lead to their degradation or recycling. Arrestins function to mediate internalization (downregulation) of some but not all, GPCRs. As an adapter protein, arrestin effects internalization by binding to the phosphorylated receptor in the first instance, and then to dynamin and the clathrin adapter AP2 in the next instance, thereby facilitating transport of receptor into clathrin-coated vesicles *(15)*. The three-dimensional structure of arrestin-2 provides a scaffold for the assembly of diverse molecules involved in GPCR signal transduction *(28)*. Agonist-induced activation of endogenous beta(2)-adrenergic, prostaglandin E(2), M(1)Ach, and somatostatin receptors induces arrestin-2–GFP redistribution to early endosomes *(29)*.

In the case of gonadotropin receptors, dominant negative mutants of arrestin or dynamin inhibit internalization of FSHR *(6)*. Variable rates of internalization of the gonadotropin receptors are dictated by arrestin binding to threonine residues in the third intracellular loops of these receptors *(30)*. In addition, a short stretch of amino acids in the C-terminal tail of FSHR hinders arrestin's ability to bind

to and internalize FSHR *(6)*. Differences are apparent in arrestin association, even for structurally conserved receptors. For example, unlike the LHR, the FSHR first loop must be phosphorylated for arrestin-3 to associate with the receptor *(31)*.

Another adapter role of arrestin is found in its mediation of the ubiquitination of the beta-2 adrenergic receptor. Ubiquitination is now well recognized to play an important role in GPCR turnover *(32)*. Ubiquitination of the latter receptor and of arrestin have distinct and obligatory roles in the trafficking and degradation of this prototypic GPCR *(33)*. Some specificity is apparent, as arrestin-3 but not arrestin-2 interacts with the E3 ubiquitin ligase Mdm2 *(33)*. Ubiquitination of both arrestin and the beta-2 adrenergic receptor is mediated by E3 ubiquitin ligase. Ubiquitination of beta-2 adrenergic receptor requires arrestin and internalization requires arrestin ubiquitination. The beta-2 adrenergic receptor requires ubiquitination before it can be degraded *(33)*.

Whether arrestin mediates ubiquitination of FSHR is not known. However, FSHR is ubiquitinated, and it binds ubiquitin non-covalently. Inhibition of proteosome function reduces non-equilibrium receptor internalization and increases receptor cell-surface residency *(34)*. Several isoforms of ubiquitin have been shown to interact with FSHR third intracellular loop, but the mechanism remains to be elucidated. Ubiquitination does not appear to occur on the third loop *(34)*. Therefore, the FSHR has the unique property of not only non-covalently associating with ubiquitin but also exhibiting covalent attachment of ubiquitin.

Can Adapter Proteins Participate in Receptor Oligomerization?

Recent biochemical evidence has shown that GPCRs oligomerize *(35)*. Moreover, GPCR are found in discrete microdomains of the cell membrane *(36)*. Oligomerization and movement into microdomains may be a prerequisite for the proper activation of cellular signaling pathways *(37–44)*. For example, expression of $GABA_B$ 1 receptor provides high-affinity binding without activation, and only when $GABA_B$ 2 receptor is co-expressed is signal transduction enabled, due to heterodimerization *(37–39)*. Desensitization of the LHR is associated with this receptor's movement into larger protein complexes, perhaps mediated by arrestin *(45)*. It is not known whether adapter proteins determine residency time and entry or exit into the microdomains. Nor is it clear whether oligomerization of receptors is subsequent to or required for localization of receptors into microdomains. Indeed, unlike the growth-factor receptors, the mode of GPCR oligomerization has not been well-characterized. Evidence has suggested that oligomerization involves the transmembrane domains. Thus, defective LHRs, truncated at TM domain V, can be rescued by co-expression of complete TM domain fragments or FSHR-ECD/LHR-TM chimeras *(46)*. Similarly, transactivation of unliganded gonadotropin receptors has been demonstrated *(47)*. The mechanism underlying this latter observation remains to be elucidated, but such transactivation likely requires interaction between TM domains or the ECD of the defective receptor and the rescuing partner, suggesting that oligomerization occurs. Cooperativity has been observed for agonists of GPCR. GPCR may function via oligomeric arrays *(48,49)*. In addition, LHR undergoes an increase in oligomerization during the process of activation *(45)*.

The driving force for association between any two receptor molecules may also involve the ligand. For example, the growth hormone receptor forms a dimer that is bridged by one growth hormone molecule *(50)*. The X-ray structure of the cystine-knot family member activin in complex with its receptor *(51)* shows how a dimer of activin binds two molecules of receptor extracellular domain. The reader is also referred to literature describing bone morphogenetic protein with activin II receptor (BMP7/ActIIR) *(52)* and BMP2 bound to the type 2 receptor *(53)*, as well as a crystal structure of TGFβ *(54)* in complex with its receptor *(51)*.

Biophysical studies have demonstrated that the LHRs oligomerize *(55)*, and that the temporal dynamics of receptor association can affect signaling *(45)*. However, it is not known whether the way in which the receptors oligomerize is similar for all GPCRs *(56)*.

The crystal structure of FSH in complex with FSHR's hormone-binding domain provided the first biochemical evidence that FSHRs oligomerize through the extracellular domain leucine-rich repeats 2-4, via a largely hydrophobic interaction *(57)*. The authors pointed out that those receptor's C termini in the dimeric complex are separated by a distance (74.3 Å) that, depending on linker flexibility, may be too great to accommodate contacts between associated transmembrane domains. That being the case, we must consider the possibility that adapter proteins that interact with the cytoplasmic domains of the receptors provide a mechanism to drive the association between gonadotropin receptors. The idea is certainly not farfetched, if we consider the association of rhodopsin receptors into dimers by a single arrestin molecule *(58)*. What sort of molecules could bridge the 70–80 Å gap between the transmembrane domains of associated receptor molecules? Recent evidence demonstrates that adapter-like proteins bind FSHR cytoplasmic domains, and it is reasonable that they could function either to oligomerize receptors or to organize receptor oligomers into higher-order complexes.

FSHR-Interacting Proteins

The signal-induced phosphorylation of gonadotropin receptors causes a change in receptor function. This simple modification, which can occur via either GRK or protein kinase C (PKC), has been the primary basis of signal-transduction termination in the gonadotropin systems. The idea has been put forth that simple phosphorylation is not suf-

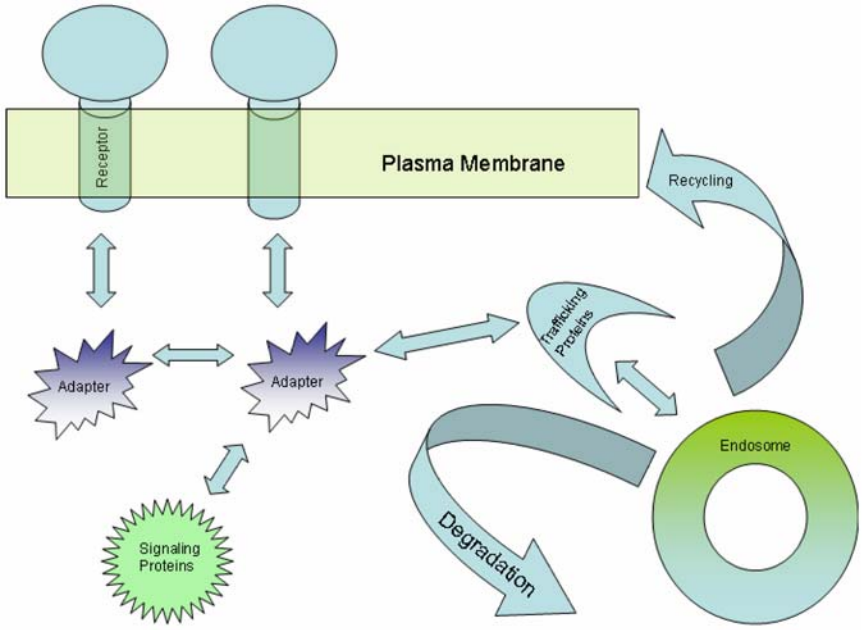

Fig. 1. Interacting proteins can coordinate the intracellular disposition of GPCRs. Adapter proteins may cause the association of two receptors, or they may coordinate the association of signaling proteins with GPCR. In addition, an adapter such as arrestin can coordinate the interaction between GPCR and trafficking protein, and can target the GPCR to the endosome or to recycling to the cell membrane.

ficient to effect the plethora of changes in protein function that occur *(59)*. In such a case, the 14-3-3 proteins can be postulated to complete the change in function. The second intracellular loop of FSHR contains structural determinants that are involved in G protein–mediated signal transduction *(60)* (Fig. 2). A 14-3-3τ isoform was identified as an interaction partner with the FSHR second intracellular loop when a human ovarian cDNA library was used as prey *(61)*. Overexpression of 14-3-3 τ dampened cAMP production *(61)*. A consensus sequence containing a serine phosphorylation site for 14-3-3 interactions is not evident in the second intracellular loop, suggesting a novel motif for binding of 14-3-3. Indeed, a potent peptide inhibitor of 14-3-3 binds specifically but lacks phosphoserine; it interacts with 14-3-3 in a manner very similar to that of phosphorylated ligands. A core motif of LDLE was found to be key to this inhibitor's activity *(62)*. Recently, it has been shown that inhibition of 14-3-3 function can lead to granulosa cell death *(63)*. GABA receptors also interact with 14-3-3 *(64)*. Thus, in addition to well-established roles that the 14-3-3 family members play in signal transduction *(59)*, they may direct organization of the glycoprotein hormone receptors. A caveat for the studies of 14-3-3 is that there are many isoforms of 14-3-3, and they bind a variety of proteins. Inhibitors of 14-3-3 may cause effects that are physiologically relevant but not specific to the particular aspect of cellular physiology being studied. Therefore, it is imperative to delineate the amino acids that are critical for interaction with 14-3-3, and to mutate these so that the only interaction that is abolished is the one between 14-3-3 and the target protein of interest.

The 14-3-3 proteins are homodimers (Fig. 3), which play a key role in signal-transduction pathways, and they bind to proteins phosphorylated on serine residues. For example, 14-3-3 proteins bind to regulators of G protein signaling proteins, keeping them inactive *(65)* and this interaction with 14-3-3 is driven by trophic factors *(66)*. Although 14-3-3 proteins can inhibit apoptosis by binding to Bad and Forkhead transcription factor, these are cytoplasmic activities of 14-3-3 and are unlikely to represent the protein's functions at the cell membrane. On the other hand, 14-3-3 proteins can interact with PDK1 an upstream activator of PKB, keeping it inactive *(67)*. In addition, 14-3-3 can interact with Rho guanine nucleotide exchange factor *(68)*. Both 14-3-3 protein and Ras interact with Raf-1, and Ras can displace 14-3-3 from Raf-1 *(69)*. Because Raf-1 is upstream of the MAPK pathway, 14-3-3 protein binding to Raf-1 can regulate kinase activity, and recruitment of 14-3-3 to the cell membrane can allow Ras (which is membrane bound) to activate this pathway *(70)*. Whether or not the interaction of FSHR with 14-3-3τ provides a scaffold for Raf-1 and a direct link to the MAP kinase pathway by bringing Raf-1 to the membrane *(71)* remains an open question.

That adapter proteins can drive association of the receptors is a reasonable thesis. In Fig. 3, the structure of 14-3-3 zeta dimer is shown *(72)*. A bar is drawn to indicate a distance of approx 70–80 Å. Clearly, the 14-3-3 dimer could bridge the distance proposed on the basis of the FSHR structure. However, because the association of gonadotropin receptors is partly constitutive, and because the association of 14-3-3 with FSHR is transient, it seems unlikely that

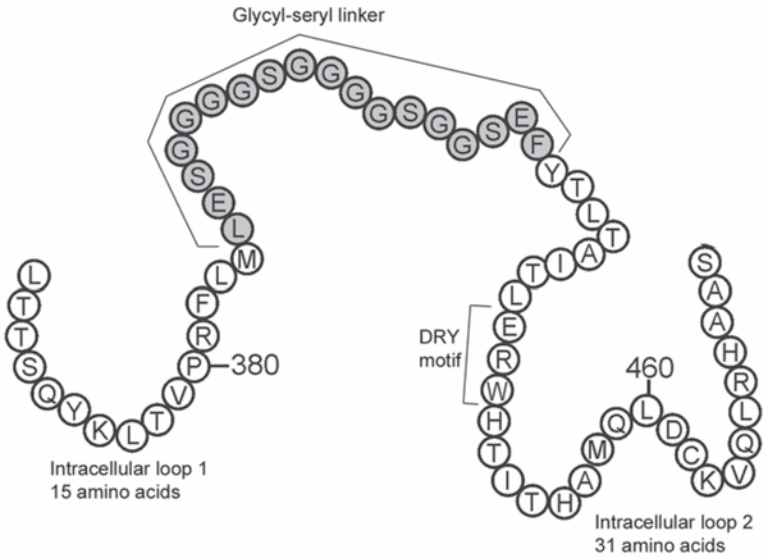

Fig. 2. Intracellular loops 1 and 2 of the human FSHR interact with adapter proteins. Note that the consensus DRY sequence is ERW in the FSHR. The DRY region and NPXXY region are believed to be involved in the activation and transformation of photoactivated rhodopsin. In an interaction trap screen, loop 1 was observed to interact with the adapter protein APPL and loop 2 was observed to interact with 14-3-3τ. A glycyl-seryl linker was inserted between loops 1 and 2 for use in an interaction screen.

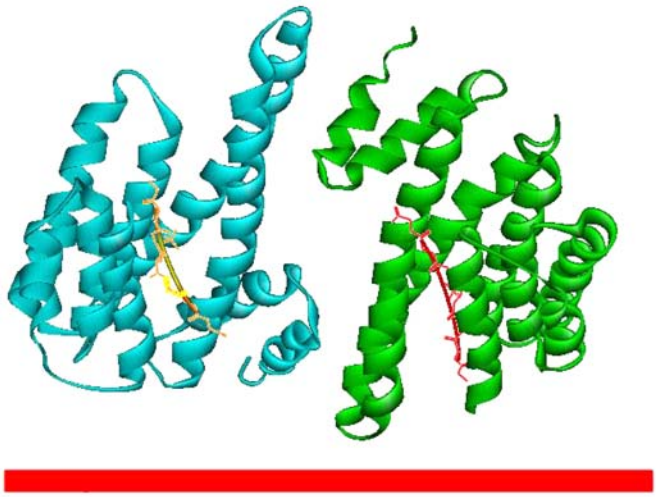

Fig. 3. Solid ribbon tracing of the 14-3-3 zeta dimer with a phospho-peptide substrate bound to each subunit. The PDB file used is 1A37. The bar illustrates a distance of 70–80 Å.

14-3-3 is involved in constitutively oligomerized receptors. However, it is intriguing to consider the possibility that 14-3-3 plays a role in the transient increase in FRET observed following activation of LHR *(45)*.

Recently, APPL proteins (adapters with leucine rich repeats and pleckstrin homology domains phosphotyrosine binding site and leucine zipper) have also been found to associate with the FSHR *(73)*. The sequence of APPL proteins places them in the BAR domain protein family *(74)*. Thus, APPL may act as a membrane sensor that upon endocytosis of the FSHR undergoes a conformational change and binds Rab5 *(4)*. APPL proteins may also link FSHRs to other downstream pathways given that they interact with both PI3K and PKB *(75)*. The function of APPL proteins in gonadotropin receptor function is an active area of study in their laboratory.

Protein kinase CK2 (PKCK2) has hundreds of substrates, a characteristic that makes it an ideal candidate for a general oligomerization factor. There is a PKCK2 consensus site on FSHR's third cytoplasmic loop, and it is reasonable that PKCK2 acts as a scaffold by binding to FSHR. Because PKCK2 is a heterotetramer, it can in turn bind another protein with a PKCK2 consensus site, thereby recruiting that protein to the cell membrane. Two proteins that interact with PKCK2 and FSHR are arrestin and ubiquitin. It was noted above that ubiquitin associates with FSHR's third loop in a non-covalent manner *(34)*. Conceivably this association occurs via PKCK2. The question of whether ubiquitination of FSHR is dependent on the protein kinase CK2 consensus site needs to be explored. Along these lines, it is known that PKCK2 phosphorylates arrestin-3 without changing its ability to bind the endocytotic component but does regulate the formation of large arrestin-3 containing protein complexes *(76)*. Thus, it is timely to determine whether PKCK2 acts as a scaffold protein for FSHR. Recently, the interaction of PKCK2 with FSHR has been demonstrated (Fig. 4). This is the first time that this kinase has been shown to directly interact with FSHR or any GPCR for that matter. Further studies are ongoing.

In all cases, final confirmation of physiological significance of the interaction is necessary. The best way to assess this is to create mutants of the ligand pair that prevent interaction and to then test for functionality. Mutations made in

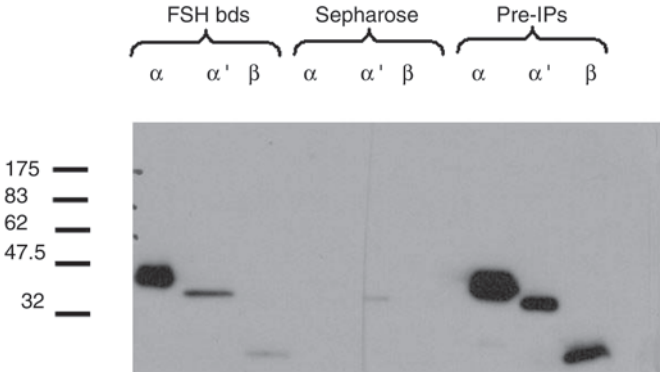

Fig. 4. PKCK2 interaction with FSHR. HEK 293 cells stably expressing FSHR were transfected with 1 μg of either HA-tagged PKCK2 α, α', or β subunit. Extracts of cells were incubated with either single-chain FSH conjugated to Sepharose 4B or with Sepharose 4B alone to determine if complexes of FSHR and the PKCK2 subunits were specifically captured. After extensive washing in minicolumns, eluates from the resins were analyzed by SDS-PAGE and transferred to Immobilon-P membranes. Blots were then probed with 5 μg rabbit anti-HA tag antibody (Upstate Cell Signaling Solutions, Charlottesville, VA) to detect HA-tagged PKCK2 subunits.

the receptor are less fraught with difficulties than mutations made in the interacting protein. This is because the interacting protein likely interacts with a host of other proteins, especially if it is an adapter protein. Therefore, mutations in FSHR's intracellular loops that do not compromise binding and trafficking but that do prevent the co-immunoprecipitation of interacting proteins with FSHR are good candidates for study, in the determination of the effects of the interacting protein on FSH function.

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