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Gonadotropin Receptors

Role of Post-translational Modifications and Post-transcriptional Regulation

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This review focuses on the post-translational modifications of LH and FSH receptors and recent studies on the regulation of LH receptor expression mediated by an RNA binding protein. Both LH and FSH receptors undergo extensive post-translational modifications. N-linked glycosylation occurs co-translationally and plays a role in the maturation and processing of the receptor, while palmitoylation is involved in receptor endocytosis and post-endocytic trafficking. A third type of post-translational modification is phosphorylation and its function has been reviewed. Finally, the regulation of LH receptor at the mRNA level by an RNA binding protein is discussed in the context of ovarian function.

Key Words: Luteinizing hormone receptor; follicle stimulating hormone receptor; LHR mRNA binding protein; post-translational modifications; post-transcriptional regulation.

Introduction

Cloning of the rat LH receptor in 1989 by McFarland et al. *(1)* and the porcine LH receptor by Loosfelt et al. *(2)* paved the way for the cloning of the FSH receptor *(3)* and the LH receptor from other species, including the human *(4)*. The LH and FSH receptors belong to the superfamily of G protein–coupled receptors (GPCRs) *(1,5)*. GPCRs have similar overall structure including seven-transmembrane-spanning helices connected by three extracellular loops and three intracellular loops as well as an N-terminus located at the extracellular side and a carboxy terminal on the intracellular side. The GPCRs are divided into three major subfamilies based on sequence and structural homology including the Class A rhodopsin family of which the luteinizing hormone receptor (LHR) and follicle stimulating receptor (FSHR) are members. The LH receptor is often designated as the LH/hCG receptor owing to its ability to bind LH and hCG with comparable affinities. The extracellular domains of FSHR and LHR contain a number of irregular leucine-rich repeats that are implicated in the binding of their respective ligands. Although beyond the scope of this review, it should be noted that the crystal structure of the hormone-binding domain of the FSH receptor has been solved to a resolution of 2.9 Å, and based on sequence similarity, it was predicted that the structure of the hormone-binding domain is conserved among the glycoprotein hormone receptors *(6)*. Both FSHR and LHR play important roles in reproductive development and function. Mutation of these receptors can lead to pathological conditions such as infertility and arrested follicular development in females and Leydig cell hypoplasia and precocious puberty in males *(7,8)*. This review will discuss (1) the post-translational modifications of the receptors as they relate to receptor trafficking to the cell membrane and their processing following ligand–receptor interaction and (2) new aspects of the regulation of LHR expression under physiological conditions.

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Post-Translational Modification of LH and FSH Receptors

There are four types of post-translational modifications found in LH and FSH receptors including glycosylation, palmitoylation, phosphorylation, and ubiquitination. While glycosylation and palmitoylation occur during the biosynthesis of the receptor, available evidence suggests that phosphorylation occurs following ligand–receptor interaction to facilitate receptor endocytosis. The current understanding of the biological importance of glycosylation, palmitoylation, phosphorylation, and ubiquitination in the LHR and FSHR is summarized below.

N-Linked Glycosylation of the FSH Receptor

During translation of FSH and LH receptor mRNA, the growing polypeptide chain is inserted into the endoplasmic reticulum (ER) and is glycosylated on asparagine residues within the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline. As the polypeptide elongates, the transmembrane-spanning helices are woven in and out of the ER membrane leaving the large extracellular domain and loops within the ER lumen and the C-terminal tail and intracellular loops within the cytosol. N-linked glycosylation begins in the ER as the core sugars, N-acetylglucosamine (GlcNAc), mannose, and glucose (Glc3Man9GlcNAc2), are added to the polypeptide at the same time. Modifications of

the sugars begin almost immediately with the removal of two of three glucose residues by glycosidases I and II *(9)*. The monoglucosylated receptors can then interact with calnexin/calreticulum, two chaperones that facilitate receptor folding (for review see ref. *10*). In fact, both the LHR and FSHR have been shown to interact with these chaperones *(11)*. Following this interaction, the third glucose residue is removed from the receptor and if it is properly folded, the receptor is transported to the Golgi where the carbohydrates undergo further processing. If the receptor is not properly folded after chaperone interaction, the receptor is reglucosylated and may reassociate with calnexin or calreticulum *(12, 13)*. Eventually, if the receptor is unable to fold correctly, it is targeted for degradation. The role of glycosylation varies widely among proteins, but often glycosylation facilitates protein folding by increasing solubility, facilitating interactions with chaperones, or preventing aggregation *(14)*. This may also be true for both the FSH and LH receptors.

The rat FSHR is glycosylated at two of three glycosylation (N174, N182, and N276) consensus sequences present in the extracellular domain, and it appears that the presence of carbohydrates at either one of these residues is sufficient for receptor folding *(15)*. For example, mutation of either of the two sites (N174 or N276) does not affect receptor expression or binding at the cell surface *(15)*. However, mutation of both sites simultaneously prevents receptor binding at the cell surface, suggesting that at least one intact glycosylation site is required for receptor function. Additionally, treatment of cells with tunicamycin, a chemical inhibitor of N-linked glycosylation, abolishes ligand binding, while enzymatic removal of carbohydrates from mature, cell-surface FSH receptors does not affect ligand binding or affinity, suggesting that the carbohydrates are not involved in ligand interaction *(15)*, but are likely involved in receptor processing. Thus, like many other GPCRs, it appears that glycosylation facilitates folding of the rat FSH receptor. The human FSHR has four potential glycosylation sites, although which sites actually contain carbohydrates is not known and their role has not been investigated.

N-Linked Glycosylation of the LHR

Six potential N-linked glycosylation sites are found in the extracellular domain of the rat, human, and porcine LHR, although the role of these carbohydrates in receptor folding is still not clear. In 293 cells, all six of these sites in rat are glycosylated *(16)*, whereas it was reported that in insect cells the first site (N77Q) was not glycosylated *(17, 18)*. All sites except for the fifth site appear to be glycosylated in porcine LHR *(19)*. Thus, the extent of glycosylation may depend on species and/or cell type. Extensive studies have investigated the role of glycosylation of the rat LHR *(16–18,20–24)*, although there is no consensus on its function. Most studies show that glycosylation of the receptor is not required for receptor binding or signaling, as enzymatic removal of carbohydrates from the mature receptor does not alter these parameters *(20,22,23)*. Additionally, all studies agree that treatment with tunicamycin significantly decreased or abolished ligand binding *(16,17,21,23)*. However, there is no agreement on the mechanism responsible for decreased binding. For example, Davis et al. *(16)* suggest that decreased binding is a result of decreased protein synthesis, as tunicamycin has also been shown to decrease protein synthesis *(16)*. However, by monitoring ^{35}S-Met incorporation into proteins from tunicamycin-treated and control cells, Ji et al. *(21)* showed that overall protein synthesis was not affected by tunicamycin, while incorporation of ^{3}H-acetylglucosamine was significantly decreased *(21)*. This finding suggests that tunicamycin prevented glycosylation without affecting protein synthesis. Studies using site-directed mutagenesis to further examine LHR glycosylation have also led to contradictory data and interpretations *(16–18)*. However, studies thus far suggest that the last three glycosylation sites are not important for receptor function or folding *(16–18)*. Different groups have used different experimental paradigms to examine the role of LHR glycosylation and perhaps these differences have led to different conclusions *(16–18)*. A more recent study has suggested that mutations at the first three sites decrease processing of the immature receptor to the mature, cell-surface receptor, thereby reducing cell-surface expression and ligand binding *(24)*.

Of the six potential glycosylation sites in porcine LHR, carbohydrates are found at all sites except for the fifth *(19)*. Using mass spectroscopy, the first three sites were shown to contain carbohydrates, although a small percentage of isolated receptors did not contain the full complement of carbohydrates at each one of the three sites *(19)*. Because these receptors were isolated as hormone–receptor complexes, carbohydrates at these sites might not play a significant role in receptor folding or ligand binding *(19)*. Thus, carbohydrates at the fourth and sixth glycosylation sites are the only likely candidates that could play a role in receptor folding and/or function in porcine LH receptor.

Ubiquitination of LHR and FSHR

The addition of the low molecular weight protein ubiquitin can target proteins for degradation by the proteasome. More recently, ubiquitin has been shown to play a role in endocytosis *(25)*. Both the rat LHR and the human FSHR are substrates of ubiquitination *(24,26)*, although the exact role of this modification is not yet clear. Prevention of ubiquitination increased steady-state levels and cell surface expression of the FSH receptor, suggesting that ubiquitination may target the receptor for degradation *(26)*. Further studies are needed to assess the significance of ubiquitination of both the LHR and FSHR.

Palmitoylation of LHR and FSHR

Palmitoylation is a common modification among GPCRs. Typically, palmitate forms a thioester linkage with conserved cysteine residues in the C-terminal tail *(27)*. This provides

an additional site by which the receptor is anchored to the membrane and as a result creates a fourth intracellular loop *(28)*. While palmitoylation of the rat LH receptor has been studied in detail, whether the FSH receptor is palmitoylated has yet to be examined.

Both the rat and human LHR are palmitoylated at two conserved cysteine residues in the C-terminal tail. Site directed mutagenesis was used to identify Cys 621 and 622 as the sites of palmitoylation in the rat LHR *(29,30)*, while later the analogous cysteine residues in the human (Cys 643 and 644) were identified as the sites of palmitoylation *(31)*. Mutation of the rat palmitoylation sites significantly increases the rate of receptor internalization without affecting receptor expression or cAMP production *(29,32,33)*. Further studies showed that the unpalmitoylated receptor had a greater ability to undergo PMA-stimulated phosphorylation compared to WT LHR and that the rate of WT LHR internalization increased almost to the level of the unpalmitoylated receptor by coexpression with GRK4 *(33)*. These findings suggest that unpalmitoylated receptor may be more prone to phosphorylation. Indeed, it has been reported that other unpalmitoylated GPCRs are also hyperphosphorylated *(34, 35)*, and it has been suggested that hyperphosphorylation may increase the accessibility of the endocytic machinery to the C-terminal tail of receptors *(33)*. In fact, palmitoylation may be subject to regulation, as palmitoylation is a dynamic process where one receptor may undergo multiple rounds of palmitoylation and depalmitoylation (for review see ref. *27*). Thus, depalmitoylation and as a result, hyperphosphorylation, may expedite arrestin recruitment, which could target receptors for internalization at an increased rate compared with palmitoylated receptor. However, this model does not hold true for all GPCRs. For example, mutation of the palmitoylation sites of the V2 vasopressin receptor results in decreased internalization and decreased recruitment of arrestin to the activated receptor *(36)*. Like the rat, mutation of the human palmitoylation sites leads to increased internalization *(31)*, suggesting that the role of palmitoylation is similar in both species.

Role of Palmitoylation in Post-endocytic Trafficking of LHR

Approximately 30% of internalized human LHR is recycled back to the cell surface whereas only a small fraction of the rat LHR is recycled *(37)*. Post-endocytic targeting of the human receptor is dictated partly by sequences in the C-terminal tail *(37)*. However, our studies show that palmitoylation also affects post-endocytic trafficking of the human receptor. Specifically, palmitoylated LH receptor appears to favor the recycling pathway, while unpalmitoylated receptor exhibits decreased recycling *(31)*. Prevention of LHR palmitoylation by site-directed mutagenesis reduced recycling of the human LH receptor in 293 T cells *(31)*. Additionally, recycling of a constitutively active mutant of the LHR was decreased and this mutant was shown to be palmitoylation deficient. Phosphorylation may be the mechanism by which palmitoylation influences human LHR trafficking. As mentioned earlier, unpalmitoylated receptors may exist in a hyperphosphorylated state. Because receptors are thought to undergo dephosphorylation before recycling to the cell surface, hyperphosphorylation of the unpalmitoylated receptor may make it more resistant to dephosphorylation, resulting in inefficient recycling. This idea is consistent with the report that the neurokinin 1 receptor was inefficiently recycled when stimulated with agonist at a concentration known to promote extensive phosphorylation when compared to rapid recycling seen under conditions of minimal phosphorylation *(38)*. Thus, palmitoylation deficient and hyperphosphorylated hLHR may be destined for degradation rather than recycling to the cell surface.

Post-endocytic Trafficking of the FSHR

Endocytosis and post-endocytic trafficking of the rat and human FSHR is not thought to significantly contribute to down-regulation, as most of the receptor is recycled (approx 50%) back to the cell surface rather than targeted to the lysosomes for degradation (20%) in a number of cell lines *(39)*. The significance of endocytosis in the resensitization of the FSHR is also unclear since the FSHR–FSH complex recycles to the cell surface intact *(40)*. The sequences responsible for recycling of the receptor may be located at the extreme C-terminus of the receptor, as deletion of the last eight residues redirects the rFSHR to degradation rather than recycling *(40)*. Of the last eight residues, five are conserved between rat and human FSHR suggesting that one or more of these residues may help direct the receptor–hormone complex to the recycling pathway *(40)*. The role, if any, that palmitoylation might play in post-endocytic trafficking is unclear since palmitoylation of this receptor has not been examined.

Phosphorylation of the FSH Receptor

Agonist-induced phosphorylation is an important mechanism for many GPCRs to regulate receptor responsiveness to ligand. Arrestin binds to phosphorylated receptors and uncouples the receptor from its cognate G protein thereby preventing further signaling mediated by the G protein. Arrestin also targets receptors for internalization, which can lead to down-regulation of the receptor *(41,42)*. G protein–coupled receptor kinases (GRKs) preferentially phosphorylate activated receptors and often promote arrestin association with the receptor. Arrestin has been shown to mediate internalization of both the rat and human FSH receptor, and the efficiency of association may explain the differences in the half-time of internalization between the two species (the half-time of internalization for rat approx 17 min compared to human approx 50 min) when expressed in 293T cells *(43)*. The role of phosphorylation in internalization and arrestin association has been studied more extensively for the rat FSH receptor.

FSHR is rapidly phosphorylated on the first and third intracellular loops in response to either FSH or PMA *(44)* and possesses consensus sequences for protein kinase C (PKC) and casein kinase II *(7)*. Additionally, FSHR appears to be a substrate for GPCR kinases (GRKs) *(45)*. Both rat FSHR phosphorylation and activation are thought to be important, but not essential for internalization and arrestin interaction *(39,46)*. The importance of phosphorylation is demonstrated by the fact that expression of a dominant negative GRK2 to inhibit phosphorylation reduced arrestin-3 association and subsequent internalization of the FSHR *(47)*. Additionally, arrestin-3 association and internalization was reduced by mutation of the phosphorylation sites present in the first intracellular loop *(39)*. While phosphorylation may facilitate arrestin association and internalization, neither phosphorylation nor activation is absolutely essential for arrestin association *(46)*. For example, internalization was significantly reduced in two mutant rat FSH receptors (D389N and Y530F) that have impaired agonist-induced activation and phosphorylation *(46)*. However, overexpression of arrestin-3 is able to rescue internalization of both mutants *(46)*. Additionally, a Thr residue (Thr542) in the third intracellular loop of the rat FSH receptor was identified as an important determinant in arrestin association *(43)*. Mutagenesis studies suggest that a Thr at this position is optimal for arrestin association, as mutation to other residues (N, A, S, or D) significantly decreased arrestin association *(43)*. These results also suggested that phosphorylation of Thr542 is not required for arrestin association *(43)*. However, it should be noted that an earlier study by the same group reported that mutation of all Ser and Thr residues within the third intracellular loop of rat FSH receptor had no effect on arrestin association *(39)* suggesting that mutations might indirectly affect arrestin association.

Phosphorylation of the LH Receptor

Whereas FSHR is phosphorylated on the intracellular loops, the LHR is phosphorylated on four (rat) or five (human) serine residues present in the C-terminal tail *(48,49)*. The C-terminal tail of the LHR does not have any strong consensus sequences for PKA-catalyzed phosphorylation, although it has as many as seven potential sites for PKC-catalyzed phosphorylation *(50)* and may be a substrate for GRK phosphorylation. Neither PKA nor PKC are thought to play a significant role in LHR desensitization *(51–53)*. In fact, whether phosphorylation of the LHR plays a role in internalization and desensitization is a matter of debate. Mutation of all five phosphorylation sites in the human LHR does not affect arrestin association suggesting that arrestin recognizes and binds to structural features of the activated receptor rather than phosphorylated residues *(54)*. Additionally, coexpression of hLHR with GRK2 does not enhance arrestin association *(54)*. However, phosphorylation of the human LHR may become important in the absence of receptor activation. For example, arrestin-3 associates less efficiently with receptors containing mutations (D405N or Y546F) that impair hCG-induced activation and phosphorylation *(54)*. However, overexpression of GRK2 can rescue arrestin association with these mutants unless the phosphorylation sites are mutated *(54)*.

Phosphorylation of porcine LHR does not appear to play a role in association with arrestin. In fact, a non-phosphorylated peptide of the third intracellular loop bound to arrestin with high affinity and competed with the endogenous receptor for arrestin *(55)*. Additionally, agonist-dependent phosphorylation is not detectable in porcine LHR. It is unclear whether differences in the role of phosphorylation between human and porcine receptor is due to species diversity or differences in techniques and experimental paradigms.

Arrestin association with porcine LHR has been shown to involve the small G protein ARF6 and its nucleotide exchange factor, ARNO (ARF nucleotide binding site opener) *(56,57)*. A model by Hunzicker-Dunn and colleagues proposes that agonist binding to pLHR activates ARNO, which catalyzes the exchange of GDP for GTP on ARF6. GTP-bound ARF6 then releases membrane-bound arrestin-2 so that it can interact with the LH receptor. This model would account for the phosphorylation-independent association of the receptor with arrestin. Additionally, the model is likely to apply to other species of LHR as well as to other GPCRs. For example, Houndolo et al. *(58)* recently showed that agonist-dependent internalization of other GPCRs was inhibited in cells depleted of ARF6. Furthermore ARNO and ARF6 have been shown to be involved in the internalization of the β2-adrenergic receptor *(59)*.

Post-transcriptional Regulation of the LH Receptor

Transcription of the LH receptor gene results in multiple transcripts in gonadal tissues *(1,60)* possibly due to different transcriptional start sites in the promoter region, alternative splicing of the gene, and/or different polyadenylation sites in the 3' untranslated region of the mRNA *(1,2,61–65)*. In rat ovary, four transcripts (6.7, 4.4, 2.6, and 1.8 kb) have been identified *(1)*, with the 6.7 kb transcript being the most abundant *(1)*. The deduced size of the coding region of LH receptor mRNA is 2.1 kb and all of the transcripts except the 1.8 kb are believed to contain the open reading frame with varying lengths of the 3' untranslated region. In humans, three alternatively spliced variants (5.4, 3.6, and 2.4 kb) have been detected in the ovary *(4,66)*, although they have not yet been fully characterized.

The LH receptor is primarily expressed in the theca, interstitial, granulosa, and luteal cells of the ovary and Leydig cells of the testis. However, expression of the LH receptor has also been reported in several extragonadal tissues including adrenal cortex, liver, uterus, brain, fallopian tube, and skin *(67–78)*. The physiological significance of LH receptor expression in nongonadal tissues is being pursued in several laboratories. It is well established that FSH and LH play crucial roles in follicular maturation and ovulation.

The expression of LH and FSH receptors shows considerable changes during the ovarian cycle. For example, expression of the FSH receptor increases steadily during the growth and transformation of primary follicles to antral follicles under the increasing levels of FSH and estradiol *(79)*. This leads to the induction of LH receptors, which begin to increase with follicle growth, reaching maximum levels prior to ovulation.

Using rat granulosa and luteal cells, extensive studies have been done on the regulation of LH receptor during the ovarian cycle. Expression of the receptor is undetectable in undifferentiated rat granulosa cells of small antral follicles. When the follicles undergo maturation, exposure to FSH and estrogen induces the expression of LH receptor in granulosa cells *(79)*. FSH- and estrogen-mediated induction of LH receptor in developing follicles has been reported to be mediated by transcriptional regulation. FSH or cAMP treatment of estrogen-primed rat granulosa cells was reported to cause a 10-fold increase in transcription of the endogenous LH receptor gene *(80)*. This increase in transcription of LH receptor gene was observed after 24 h of FSH or cAMP treatment. In theca cells, the LH receptor expression is detectable even at the small antral follicle stage, and the increase in receptor expression during follicular development is found to be relatively small compared to the induction in granulosa cells. During the preovulatory LH surge, the receptor expression is greatly decreased in ovarian granulosa and luteal cells and reappears during the luteinization phase *(79,81,82)*. In vitro studies using estrogen-primed rat granulosa cells have also reported a similar transient down-regulation of LH receptor by gonadotropins (FSH and hCG) or by an increase in cellular cAMP levels *(83)*. Although transient loss of LH receptor transcripts has been reported in theca cells, the fold decrease is very low when compared with the decrease in granulosa cells *(79,84)*. This section of the review will be limited to the recent understanding of the regulation of LH receptor expression during the ovarian cycle.

Down-regulation of the LH receptor has been observed in pseudopregnant rats injected with a pharmacological dose of hCG *(79,81,82,85,86)*, and this receptor down-regulation is paralleled by a specific transient loss of all LH receptor mRNA transcripts *(85–87)*. Our laboratory has observed that during ligand-induced LH receptor down-regulation, all four LH receptor mRNAs were undetectable on a Northern blot (Fig. 1) at 12 h after hCG administration and began to reappear between 24 and 48 h post-hCG injection *(85–87)*. This decline in LH receptor mRNA levels upon hCG administration was found to be specific to the LH receptor as mRNA levels of unrelated genes did not decrease under the same conditions *(86)*. The transient disappearance of LH receptor mRNA levels in the ovary could be due to a decrease in the rate of receptor gene transcription and/or an increase in the receptor mRNA degradation. Thus, the transcription rate was examined by nuclear run off assay and

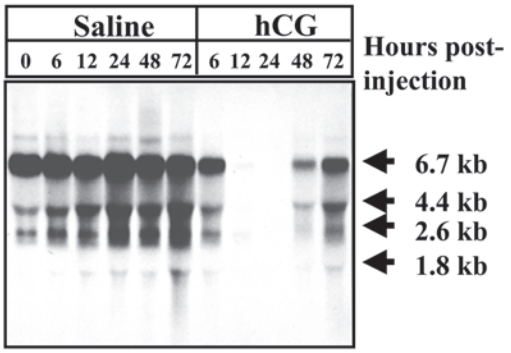

Fig. 1. Northern blot analysis of LH receptor mRNA during hCG-induced LH receptor down-regulation. Total RNA was isolated from saline-injected or hCG-injected pseudopregnant rat ovaries. Sixty micrograms of RNA was loaded onto each lane. Blots were probed using a radiolabeled cDNA corresponding to the carboxy terminus and a portion of the 3' untranslated region of rat LH receptor (nucleotides 1936–2682) [modified from Kash and Menon *(21)*, Fig. 1A; with permission from *J. Biol. Chem.*].

the mRNA decay rate by solution hybridization analysis. The results showed that the transient disappearance of LHR mRNAs did not result from decreased transcription, but occurred post-transcriptionally by rapid degradation of LH receptor mRNA *(85)*. In fact, a three-fold decrease in LH receptor mRNA half-life was observed under these conditions *(85)*.

Controlling the stability of mRNA is a rapid and effective way of regulating gene expression in all organisms. The half-life of mRNA is influenced by its own regulatory *cis*-acting sequences located within either the 5'-leader sequence, coding region, and/or 3'-untranslated region of the mRNA *(88)*. The stability of mRNA is influenced by the interaction of various cytoplasmic/nuclear proteins (*trans*-factors) with the *cis*-acting regulatory sequences in the mRNA. In general, the formation and/or disruption of these ribonucleoprotein (RNP) complexes in response to various cellular stimuli control the stability of mRNA. *Trans*-acting factors have been identified and characterized as either mRNA stabilizing, destabilizing, or translational regulatory proteins in a number of systems *(89–94)*. Potential *trans*-acting factors that specifically regulate rat LH receptor mRNA stability during the ligand-induced receptor down-regulation in the ovary have been identified. We have identified the presence of a potential *trans*-acting factor in the homogenate of LH receptor down-regulated rat ovary, as a cytosolic protein named LH receptor mRNA binding protein (LRBP) *(87)*. LRBP binds to the coding region of LH receptor mRNA with a high degree of specificity *(87)*. We have observed a reciprocal relationship between the LHR mRNA binding protein (LRBP) and LH receptor mRNA expression during follicular maturation, ovulation, and luteinization (Fig. 2) *(87,95)*. Increased LRBP activity and decreased LH receptor mRNA levels were also observed after inducing chronic elevation of cAMP in rat corpus luteum by injection of the phosphodiesterase inhibitor rolipram *(96)*.

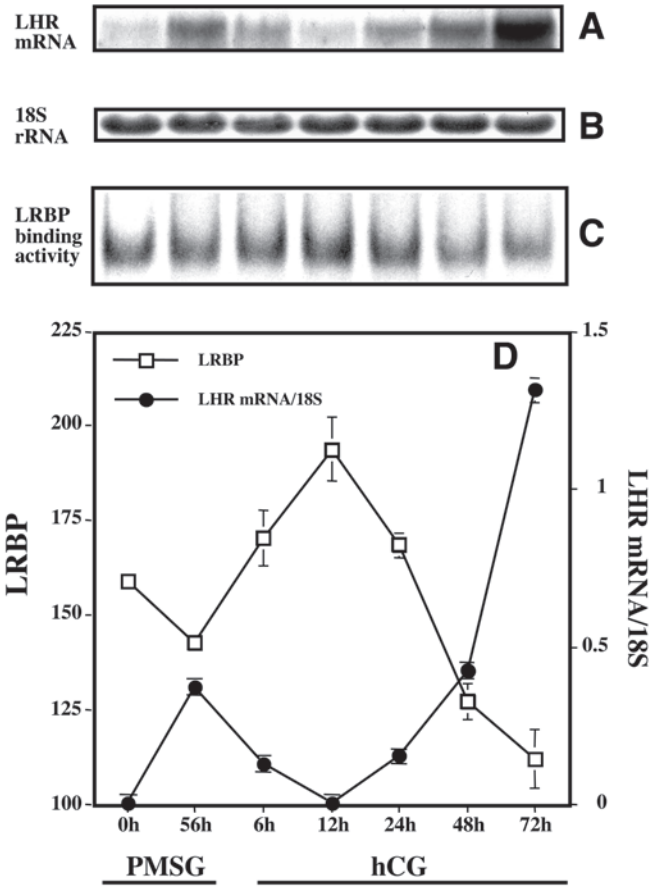

Fig. 2. LH receptor mRNA expression and RNA binding activity of LRBP during follicular maturation, ovulation, and early corpus luteum formation. Immature 21-d-old rats were injected with PMSG at 0 h and ovaries were collected immediately and 56 h post-injection. Rats were then injected with hCG 56 h post-PMSG administration and ovaries were collected at 6, 12, 24, 48, and 72 h intervals. RNA electrophoretic mobility shift analysis was performed for all time intervals using radiolabeled LH receptor mRNA with 50 µg of S100 fractions prepared from ovaries (**C**). LH receptor mRNA levels were obtained by Northern blot analysis (**A**). The Northern blot was normalized using 18S rRNA (**B**). The 6.7 kb LH receptor mRNA transcript and REMSA bands were quantitated by densitometry [modified from Nair et al. *(22)*, Fig. 5; with permission from *J. Biol. Chem.*].

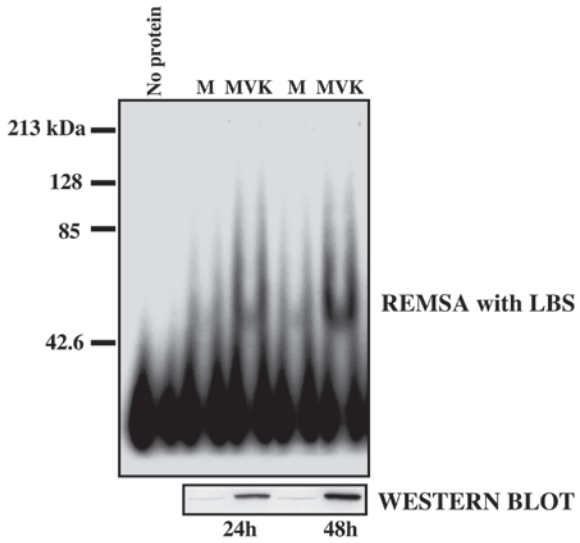

Fig. 3. Recombinant mevalonate kinase binds to LRBP binding site (LBS) of LH receptor mRNA. Human embryonic kidney cells (293) were transiently transfected with pCMV4-rMVK (MVK) and empty vector (M) and cytoplasmic proteins (S100 fraction) were isolated 24 and 48 h after transfection. REMSA was performed using radiolabeled LBS with 10 µg of S100 fractions. Western blot analysis was performed with S100 fractions (10 µg) from vector alone and pCMV4-rMVK transfected cells using MVK antibody [modified from Nair and Menon *(25)*, Fig. 7; with permission from *J. Biol. Chem.*].

To further characterize the relationship between LRBP binding activity and LH receptor mRNA down-regulation, LRBP was partially purified from S100 fraction (cytosolic protein fraction) and then used to identify the LRBP interacting region on the LHR mRNA by hydroxyl radical footprinting analysis *(97)*. The contact site was identified as a polypyrimidine-rich bipartite sequence in the coding region of LHR mRNA between nucleotides 203 and 220 (5'-UCUC-X7-UCUCCCU-3') *(97)*. Mutagenesis studies of the contact site revealed that all the cytidines in this region are important for binding *(97)*. After identifying the site of LRBP interaction and its specificity, the function of LRBP as a regulator of LH receptor mRNA half-life was examined by measuring receptor mRNA decay in vitro in the presence of the partially purified LRBP preparation. We developed an in vitro mRNA decay system using polysomes isolated from pseudopregnant rat ovary to perform this test *(95)*. The polysomes prepared from control rat ovaries were incubated with exogenously added LHR mRNA in the presence and absence of partially purified LRBP from receptor down-regulated rat ovary *(95)*. We observed an increased LH receptor mRNA decay in the presence of LRBP compared to the control without LRBP *(95)*.

LRBP was subsequently purified to homogeneity using Northwestern blots to identify the protein band possessing LH receptor mRNA binding activity. This protein band was excised and eluted from a second polyacrylamide gel *(98)* and homogeneity of the eluted protein was confirmed by one-dimensional and two-dimensional gel electrophoresis *(98)*. The purified LRBP was then subjected to N-terminal microsequencing and the resulting sequence (NH2-MLSE VLLVSA-COOH) was used for homology search and revealed a perfect match with rat mevalonate kinase (MVK) *(98)*. The identity of the protein was further confirmed by MS-MALDI.

For further characterization, rat mevalonate kinase was cloned from the LH receptor down-regulated ovary. Overexpression of MVK in human embryonic kidney cells (293 cells) showed all of the characteristics of rat LRBP with respect to specificity of LHR mRNA binding sequence (Fig. 3) *(98)*. Further support for MVK's role as a regulator of LH receptor mRNA was obtained under in vivo conditions. For example, when pseudopregnant rats were treated with hCG,

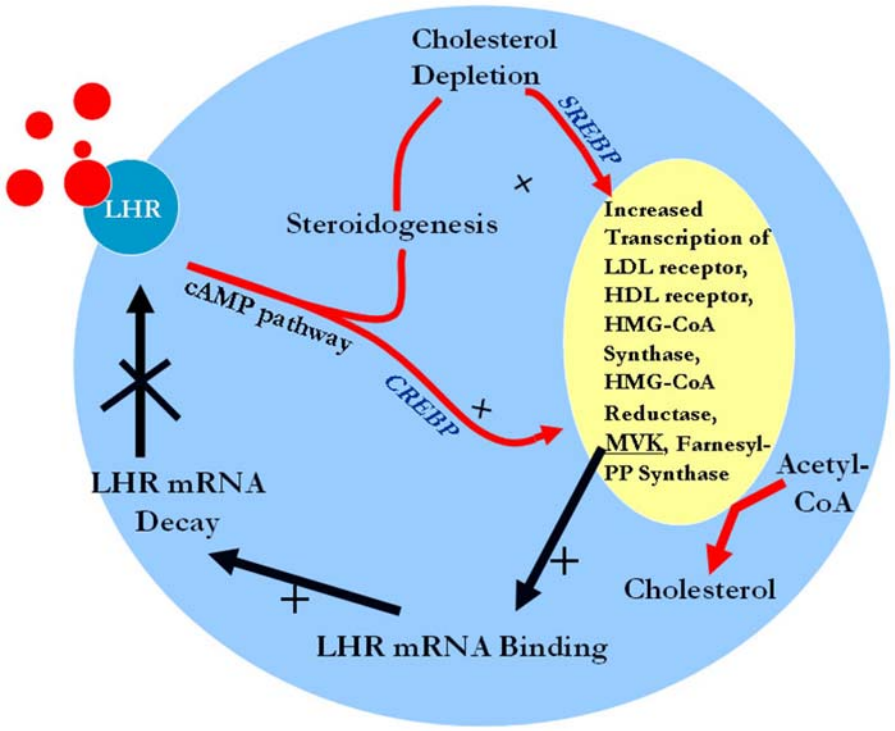

Fig. 4. A model for post-transcriptional regulation of LH receptor in the ovary: LH or hCG binding to receptor activates cAMP signal cascade. Increase in intracellular cAMP level leads to activation of steroid hormone synthesis and depletion of ovarian cholesterol. This is followed by an increased transcription of genes associated with synthesis and transport of plasma cholesterol. During this process, mevalonate kinase (MVK), an enzyme involved in cholesterol biosynthesis, binds to LH receptor mRNA and accelerates its degradation, resulting in down-regulation of receptor expression in the ovary.

an increase in MVK expression was detected by Western blot analysis and real-time PCR analysis *(99)*. The increase in MVK protein levels was preceded by an elevation in MVK mRNA levels *(100)* and followed by down-regulation of LHR mRNA. The LRBP binding activity in the cytosolic fractions prepared from these LH receptor down-regulated rat ovaries showed an increase in RNA binding activity of LRBP, which agreed with the real-time PCR data *(99)*. The comparable expression pattern of MVK and LRBP binding activity in the cytosolic fractions during receptor mRNA down-regulation is consistent with the identification of MVK as the LH receptor mRNA binding protein.

MVK, an enzyme involved in cholesterol biosynthesis, is a member of the galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (GHMP) superfamily of enzymes that are known to have a left-handed β-α-β fold, termed the ribosomal protein S5 domain 2-like fold *(100,101)*. A similar β-α-β fold exists in elongation factor G, ribonuclease P and other RNA/DNA binding proteins *(102–104)*. Therefore this fold could be the primary target of MVK interaction with LH receptor mRNA. However, this has not yet been examined. MVK binds to both ATP and mevalonate to phosphorylate mevalonate to mevalonate-5-phosphate in the *de novo* synthesis of cholesterol. We therefore examined if the binding of ATP and mevalonate had any effect on its binding to LH receptor mRNA. Binding of recombinant rat MVK to LH receptor mRNA was decreased in the presence of ATP and mevalonate *(98)*. Increased binding of MVK to LH receptor mRNA was observed when cellular mevalonate synthesis was blocked by mevastatin, an inhibitor of hydroxymethylglutaryl-CoA reductase *(98)*. The decreased binding of MVK to LH receptor mRNA in the presence of ATP/mevalonate indicates that either the ATP/mevalonate binding region of the protein might be required for the binding to LH/hCG receptor mRNA or binding of the substrates might induce a change in MVK protein conformation, thus making it unable to bind to LH receptor mRNA. This might help to maintain the conformational state of the MVK that favors catalytic function of MVK in cholesterol biosynthesis.

Based on these results we present a model of LH receptor down-regulation in the ovary (Fig. 4). According to this model, binding of LH or hCG to the receptor increases cAMP production and steroidogenesis, thereby depleting endogenous cholesterol. Cholesterol depletion releases the precursor of the sterol response element binding protein (SREBP) from the endoplasmic reticulum, which then enters the Golgi where it undergoes proteolytic processing, leading to the translocation of the transcription factor, SREBP, to the nucleus *(105,106)*. The transcription factor then induces the

expression of sterol response element (SRE) containing genes that are involved in cholesterol metabolism, including MVK. Cyclic AMP might also induce the expression of one or more of these genes independently. MVK acts as an LHR mRNA binding protein resulting in increased mRNA degradation and subsequent down-regulation of LH receptor expression. Thus, LH receptor expression is linked to cholesterol metabolism through MVK. MVK serves an additional role as an endogenous regulator of LH receptor expression in the ovary. This regulatory mechanism provides an efficient means to respond to the short-term needs of the cell in response to the constantly changing endocrine and paracrine hormonal milieu. MVK functioning as a LH receptor *trans*-acting regulator might help to balance the levels of LH receptor expression and the intracellular levels of cholesterol needed for steroid hormone biosynthesis in the ovary.

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