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Emerging Roles for the FSH Receptor Adapter Protein APPL1 and Overlap of a Putative 14-3-3 τ Interaction Domain with a Canonical G-protein Interaction Site

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Abstract

The interaction of cytoplasmic proteins with intracellular domains of membrane receptors can occur at several opportunities, including: during biosynthesis, while in membrane residency and during internalization and recycling following ligand binding. Since the initial discovery that it interacts with the FSH receptor (FSHR) together with additional members of a potential signaling complex, APPL1 has been shown to interact with a variety of membrane receptors. Recent subcellular localizations of APPL1 place it in dynamic and varied venues in the cell, including at the cell membrane, the nucleus and the early endosomes. Another adapter protein family the 14-3-3 proteins, are largely recognized as binding to phosphorylation sites but recent work demonstrated that in the case of FSHR, the 14-3-3 site overlaps with the canonical G-protein binding site. G-proteins appear to sample the environment and exchange between the membrane and intracellular locales and this binding could be mediated by or modulated by receptor interactions at the 14-3-3 binding site. Observations that multiple proteins can interact with cytoplasmic domains of GPCRs leads to the inescapable conclusion that either the interactions occur via orderly replacement or exchange, or that receptors are simultaneously occupied by a variety of adapters and effectors or even that oligomers of dimeric GPCRs provide for platforms that can simultaneously interact with effectors and adapters.

FSH Receptor (FSHR) Organization

Follicle stimulating hormone (FSH) is essential for ovarian folliculogenesis in females and spermatogenesis in males. The follicle stimulating hormone receptor (FSHR) protein is a member of the G-protein coupled receptor (GPCR) family and is comprised of a large extracellular domain and a transmembrane domain. The seven α -helices of the

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transmembrane domain are interconnected by three extracellular (ecL1, ecL2 and ecL3) and three intracellular loops (icL1, icL2 and icL3) (1). It is an open question as to how many of the FSHR subunits form a functional receptor. Biochemical studies demonstrated that the minimal unit of FSHR that is stable to detergent extraction appears to be a dimer (2). It is likely that higher order arrays exist (3).

A model of the FSH receptor transmembrane domain has been proposed which has proven useful in understanding the role of cysteine residues as attachment sites for lipid anchors (4). The formation of palmitoylation sites on the FSH receptor C-terminal tail potentially provides for the formation of an additional loop icL4 (4). The recent crystal structures of opsin and beta 2 adrenergic receptors have provided further opportunities to visualize the three dimensional structure of the cytoplasmic face of a G-protein coupled receptor (5;6). Whereas activation of GPCRs appears to require only the monomeric form of receptor, it is anticipated that regulation and activation will be more complex and dependent on cellular localization (7). Also, such data suggest the idea that the relationships of the monomeric receptors to each other may be more important for targeting than how they form functional units for signaling (8;9). The FSH receptor forms oligomers in the endoplasmic reticulum and clipping of the C-terminal tail is required for insertion into the plasma membrane (2). Dimerization of receptor in the endoplasmic reticulum will have consequences for genetic diseases of heterozygosity, where mutant forms of receptor which do not traffick to the cell surface may impair normal processing of receptors from the wild type allele by heterodimerization followed by retrograde proteosomal degradation of the heterodimeric complex (10). Conversely, it could be anticipated that FSHR heterozygosity may yield fertility even in the presence of inactivating mutations, by virtue of the wild type alleles giving rise to functional homodimers as well as functional heterodimers.

Juxtaposition of the receptor monomers while not necessarily positioning the intracellular loops' three dimensional binding sites for various effectors such as G-proteins, can certainly affect the extent to which one or more adapter proteins might associate with the receptor. In its simplest embodiment, G-protein interaction is thought to be facilitated by a movement of helix VI and a shift in icL3, allowing for docking of the G-protein (6). It is now suggested that G-protein interaction with receptors may involve random collisions and not stable complexes (11). In this paradigm, ligand binding could lead to amplification of the signal by the expected dissociation of the G-protein subunits, one of which activates adenylyl cyclase leading to production of cAMP, which in turn activates protein kinase A (PKA) (12). It is not known if more than one protein can bind to a receptor dimer at a time, if an adapter-occupied receptor can accommodate a G-protein or what the ratio's are of differentially occupied receptors.

FSH Receptor Signaling

In recent years the complexity of FSH signaling has become appreciated. This complexity is likely governed by cytoplasmic FSH receptor-interacting proteins which determine the residency time of signaling complexes in different cellular compartments. Biological efficacy has been typically evaluated by the kinetics of ligand receptor interaction. The binding event is clearly important from a standpoint of ensuring interaction of ligand and receptor, despite low physiological levels of hormone in the blood. However, the biological responsiveness of cells can be transient or long lived depending on the readout chosen. The best example of transient response is the production of cAMP. Rat granulosa cells in culture secrete most of the cAMP produced in response to FSH (data not shown). The low levels of cAMP detected in the cells is probably further reduced in specific compartments where phosphodiesterases are targeted (13). A recent example of sustained activation of shows that persistent phosphorylation of extracellular regulated kinase (ERK) can lead to aromatase

activation in granulosa cells (14). Less clear is the fate of internalized occupied receptors, and their interactions with signaling effectors and terminators within the cell.

FSH activates p38 mitogen activated protein kinase (MAPK) in rat granulosa cells (15) and the ERK pathway (16). FSH also stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (SGK) in rat granulosa cells (17). Stimulation of the downstream kinases such as ERK1/2 occur via activation of the small G-protein Ras or via β -arrestins (18) whereas the activation of Akt appears to involve the Src family of kinases (19). Following FSH stimulation elevation in mRNA for SGK, Activin A, P450_{scc} and CYP19 (aromatase) is observed while caveolin message is dramatically decreased (20). Interestingly, constitutively active PKA raises P450_{scc} similarly as FSH but CYP19 message is dramatically elevated by FSH compared to constitutively active PKA (20). Additionally, dominant negative PKB abolishes CYP19 expression (21). These data demonstrate that additional pathways work in concert with PKA for full FSH induction of estrogen production. One potential pathway which has been largely ignored and deserves further study is the IP₃/Ca²⁺/calmodulin/protein phosphatase pathway which may be related to the PKB pathway. This pathway controls phosphorylation status of GATA family members and in particular, the control of ovarian and testicular gene transcription by the GATA family members is of interest. The role of calcium signaling in granulosa cells has been suggested to affect variants of FSH receptor which may signal as growth factor-like receptors (22). Extracellular calcium is not required for cAMP production in response to FSH but does play a role in expression of P450_{scc} in porcine granulosa cells cultured in defined media without serum or growth factors (23). FSH also causes an increase in intracellular calcium in a cAMP-independent manner (24).

While the steroidogenesis pathways provide for monitoring of differentiation, less is known about pathways that influence apoptosis, proliferation or metabolic support to the oocyte. Treatment of granulosa cells with estradiol lessens the effect of Fas ligand-induced apoptosis (25;26). Although the field has used steroid production as an indicator of differentiated function of granulosa cells, these readouts may not be optimal for elucidation of intracellular signaling pathways that could govern metabolism and proliferation, or even recruitment of adjacent cells into a synchronized community (27). Indeed, these parameters might best be studied not by stimulation with FSH alone. Rather, it seems appropriate that future studies examine the interactions of FSH stimulation with adiponectin, EGF and epiregulin stimulation, to name a few. Moreover, educated guesses about which pathways to interrogate may not be as fruitful as interrogation of the transcriptome using mutants of the FSHR so that timing is not an issue.

Expanding roles of FSH Receptor Interacting Protein APPL1

Clearly, the idea that movement of FSHR within the cell is governed by interaction with cytoplasmic proteins which then influence the quality and quantity of signaling pathway output, seems a reasonable paradigm. Previous studies, using a yeast interaction trap, identified several proteins expressed by an ovarian cDNA library as interacting partners of human FSHR (hFSHR) icL1, icL2 or icL3 (28–30). One of the proteins that interacts with hFSHR icL1 was identified as an adaptor protein named APPL1 (Figure 1) which was originally identified as an Akt2 (Akt;Protein Kinase B; PKB) interacting protein (31). Initial consideration of the physiological role of FSHR-APPL1 interaction in the ovary was as an effector of FSH mediating PI3K signaling involved in the promotion and regulation of cellular metabolism, proliferation and apoptosis (30). The idea was that if Akt is bound by APPL1 then the interaction of an FSHR-FOXO1a complex with an FSHR-APPL1-Akt complex could lead to phosphorylation of FOXO1a. Indeed FSH stimulation results in phosphorylation of FOXO1a and its exclusion from the nucleus (32;33) thereby regulating

cell cycle progression by regulating p27 Kip in granulosa cells (34;35). Activation of the PI3K/Akt pathway in granulosa cells by FSH is concurrent with the induction of expression of several differentiation markers, mediated through transcriptional activation by Hif 1 (36).

Some support for the concept of a large FSHR signaling complex was engendered when it was found that in addition to APPL1, FSHR interacts with FOXO1a which is a downstream effector of the PI3K pathway (37). FOXO1a does not co-immunoprecipitate with APPL1 and at this time, the mechanism of FOXO1a association with FSH receptor is not known. However, an intriguing possibility is that Akt-APPL1-FSHR holds Akt inactive, and that FSHR-FOXO1a keeps FOXO1a tethered so that upon activation of Akt, FOXO1a can be phosphorylated. However, it is not clear if activation of Akt would rely solely on PDK1. Recently it has been shown that protein kinase CK2 can phosphorylate Akt (38). Protein kinase CK2 is also associated with the FSHR (39) providing an interesting possibility that protein kinase CK2 is the missing link for Akt phosphorylation in the absence of PDK1 activation at the FSHR signaling complex. It has recently been shown that APPL1 is selectively required for cell survival and that Akt and GSK-3 β but not TSC2 dynamically associate with APPL1 endosomes upon growth factor stimulation (40). Interestingly, the early endosomes which form with APPL1 do not contain PI(3,4,5)P₃ which the pleckstrin homology domains of Akt would bind to and so a mechanism needs to be in place for Akt to be tethered to this endosome. Thus it has been proposed that Akt may be retained at, or recruited to, the endosomal surface after PI(3,4,5)P₃ dephosphorylation by its interaction with APPL1 (41).

A homolog of APPL1 is APPL2 which lacks the C-terminal domain that interacts with FSHR (Figure 1) (37). At the time, it was reasoned based on what was known about Bin-Amphiphysin-Rvs (BAR) domain proteins, that APPL2 would co-immunoprecipitate with FSHR because it associated with APPL1 via their N-termini as would be expected for a BAR domain protein. Since APPL2 lacks the C-terminal residues that likely interacted with FSHR it would not be anticipated to interact directly with FSHR (42). Elegant cell biology studies have shown that as expected, APPL1 and APPL2 co-localize in DLD-1 cells(43). However, the subcellular co-localization of APPL1 and APPL2 may be predicted by the BAR dimerization domains. Thus the majority of APPL1 minimal BAR domains localize to large clusters of round vesicles and ring-like structures while APPL2 minimal BAR domains associate primarily with curved networks of elongated membrane structures(43). The significance of these differences is not yet appreciated.

The recent crystal structures of APPL1 dimers has provided a structural basis for the supposition of BAR domain interaction through the amine terminus (44;45). The significance of this observation is that, in theory, the dimers of APPL1 and APPL2 or heterodimers of each, can bridge interactions between two proteins, in addition to their role as a Rab5 activator. The formation of heterodimers could also have consequences. For example, Akt2 associates with APPL1 as reported but does not associate with APPL2 (37). This was the first documented difference in function between APPL1 and APPL2. Likewise, interactions between FSHR and APPL2 and between FSHR and FOXO1a evidently are distinct since FOXO1a does not associate with either APPL1 or with APPL2.

Since the discovery that APPL1 interacts with Akt and FSHR, it has become clear that APPL1 is a major player in a variety of membrane protein functions (Figure 2). Interestingly full length APPL1 and APPL2 do not co-localize with subcellular compartment markers such as cis or trans Golgi, caveosomes, endoplasmic reticulum or actin, but APPL1 does recruit Rab5 to APPL1-associated cytosolic membrane structures (43) and likely also recruits others such as GTP-bound Rab21 (45). Moreover, the association of APPL proteins with phosphoinositides may impart them with regulatory properties distinct from those of

the Rab5 effector EEA1 (early endosome marker) that only binds PtdIns(3)P (43). APPL also plays a role in phosphoinositide biosynthesis. Ocr11 (oculocerebralrenal syndrome of Lowe) is an inositol polyphosphate 5-phosphatase that can be found on early endosomes. Its substrates are PI(4,5)P₂ and PI(3,4,5)P₃. This protein is recruited to endosomes and all mutations of this uncommon disease prevent its interaction with APPL1 containing endosomes (46;47). APPL1 endosomes are precursors of PI(3,4,5)P₃ positive endosomes and depletion of PI(3,4,5)P₃ causes a reversion to the precursor (41). Thus it has been proposed that the 5-dephosphorylation of PI(4,5)P₂ and PI(3,4,5)P₃ provide a switch for the maturation of APPL1 endosomes (41).

A mutation of FSHR, which prevents interaction with APPL1 does not affect the canonical FSH-mediated granulosa cell readouts of cAMP production and steroidogenesis (Thomas, R. et al, 2009, submitted). This has prompted consideration of equally important but less studied pathways that enable granulosa cell proliferation, differentiation and metabolism. In this regard, the interplay between growth factors and FSH action seems most interesting. The APPL1 endosome plays host to growth factor receptors, and this interaction suggests that APPL1 could be involved in crosstalk between FSHR and epidermal growth factor receptor (EGFR).

It has been shown that APPL1 interacts with the neurotrophin receptor (TrkA) via two modes; directly through its phosphotyrosine binding site, and indirectly through its interaction with GIPC1, a protein that is constitutively bound to TrkA and is found associated with clathrin coated pits. Reduction of either GIPC1 or APPL1 decreased signaling by the TrkA receptor to MEK, ERK and Akt. (48). Although GIPC1 interacts with the cytoplasmic tail of the LH receptor, we do not know at this time if APPL1 interacts with the LHR (49). FSH activation of Ras requires Src family kinases and epidermal growth factor (EGF) receptor tyrosine kinase activities (19). This is of considerable interest because APPL1 translocates from membranes to the nucleus in response to EGF (50) where it interacts with the nucleosome remodeling and histone deacetylase multiprotein complex. Importantly, APPL1 has been linked to the signaling activity of EGFR via the Akt and MAPK pathways (40;50) and it has been demonstrated that prolonged presence of EGFR in the APPL compartment increases not only Akt-dependent signaling but also MAPK signaling (41). Relevant questions with regard to FSH signaling are whether FSHR signal strength is affected by residency time in the APPL endosome, if EGFR and FSHR co-localize in APPL endosomes and further, whether EGFR-FSHR crosstalk is regulated by co-residency in APPL endosomes.

Another potentially fascinating connection of FSH signaling with APPL stems from the interaction of APPL with the adiponectin receptor (51). Adiponectin and its receptors are present in rat ovaries (52) and testis (53) and decreased adiponectin levels in blood are associated with polycystic ovary syndrome (PCOS) (54). Energy regulation in the cell is mediated by adenosine monophosphate kinase (AMPK) which is activated by AMP. Adiponectin can activate AMPK through APPL1-mediated cytosolic localization of the upstream kinase LKB1 or to a lesser extent by activation of PLC. This leads to release of intracellular calcium and activation of calmodulin dependent protein kinase-kinase (CaMKK) (55). Here again, it is not clear if FSHR and adiponectin receptors share the same APPL1 endosomes and if their ligands cross-regulate their actions. However, results suggest that FSH inhibits AMPK activation through an Akt-dependent pathway and promotes granulosa cell proliferation by increasing cyclin D2 mRNA expression and by reducing p27^{kip} expression (56). In particular it would be important to determine if FSH inhibits the actions of adiponectin with regards to signaling leading to lipid oxidation, glucose uptake and membrane translocation of the glucose transport 4 protein (GLUT4). Expanding roles of APPL1 have pointed to potential areas of crosstalk that may occur upon FSH stimulation. A

potential area of interest for future research is the effect of FSH on energy balance and proliferation in the granulosa cell, likely mediated via calcium signaling.

Overlap of the 14-3-3 interaction site on FSHR icL2 with a G-protein binding site

The 14-3-3 family of proteins has been known for some time to be involved in regulation of signaling pathways, specifically via interaction and sequestration of phosphorylated signaling molecules and recently specific interactions have been identified by transgenic mouse proteomics (57). Of interest to FSH signaling, proteins found to associate with 14-3-3 included calcium/calmodulin dependent protein kinase (CaMK), regulatory subunit of protein kinase A, protein phosphatase 2 and 3, phosphodiesterases, G-protein receptor kinase interactor 1 and inositol 1,4,5 triphosphate receptor (IP₃R) (57). Interaction of FSHR icL2 with 14-3-3 τ protein has been shown by a yeast two-hybrid screen (29). Over-expression of 14-3-3 τ in HEK293 cells stably transfected with FSHR decreased cAMP levels by two-fold at the highest dose of FSH tested, suggesting a role for 14-3-3 τ in signal transduction. In concert with this finding, FSHR icL2 has also been shown to be involved in the coupling of the receptor to the Gs protein and maintaining the receptor molecule in an inactive state (58).

To precisely determine the site of interaction between 14-3-3 τ and FSHR icL2, a series of alanine scanning mutants was constructed in icL2. As seen in Figure 3, mutation of amino acids (a.a.) ⁴⁴⁷TLE⁴⁴⁹ and ⁴⁵⁰RWH⁴⁵² to alanine abrogates the association between 14-3-3 τ and FSHR icL2 in a β -galactosidase assay in a yeast two hybrid assay. This assay was performed with the original clone of 14-3-3 τ identified in a yeast interaction trap (29) which had a deletion of the first 42 a.a. The deletion encompasses α -helices 1 and 2 (59)(60) and much of the dimerization domain of 14-3-3 τ but not the protein interaction domains (61). No interaction was observed between full-length 14-3-3 τ and wild type (Wt) FSHR icL2 or with the alanine scanning mutants (data not shown). Monomers of the 14-3-3 proteins have been shown to be active (62) and they exist in both monomeric and dimeric states, possibly using the dimerization process to control their cellular activities (61). Since the primary sequence of icL2: ⁴⁴⁷TLE RWH⁴⁵² encompasses the ERW motif important in G-protein interaction and receptor activation (63).

A set of single mutations was generated in which alanine was substituted for each amino acid, so that functionality could be tested. The mutants T447A, L448A and H452A demonstrated high levels of surface binding to radiolabeled hFSH (Figure 4A), with lower levels of binding for R450A and W451A and barely detectable binding for E449A. The ability of the mutants to bind hFSH is consistent with the levels of protein expression when the mutants are overexpressed in HEK293 cells (Figure 4B). The mutants T447A, L448A and H452A have high levels of mature fully glycosylated FSHR and also appear to bind FSH, whereas E449A, R450A and W451A have little or no mature FSHR and consequently, little FSH binding. It has recently become appreciated that 14-3-3 proteins play a role in regulation of endoplasmic reticulum localization of membrane proteins but these are largely considered to involve phosphorylation dependent interactions (64). Whether the defective trafficking of these mutants is due to abrogation of 14-3-3 interaction will require further study. The observation that these residues constrain the helices raises a concern that mutation of these residues could have induced an untoward and deleterious conformational change. The two charged residues in the E(D)RY motif are known to constrain TMVI in the helix bundle constituting the so called "ionic lock". For example in rhodopsin, Arg135 (TMIII) interacts with Glu247 (TMVI) and Glu134 (TMIII) interacts with Thr251 (TMVI). However in the active structure of opsin there is an interhelical association of Lys231 and Glu247 (TMVI) whereas Arg135 (TMIII) interacts with Tyr223 (TMVI) (6;65;65).

Moreover the interaction between TMIII and TMVI enabled by the E(D)RY motif are less so in the β 2-adrenergic receptor (66;67). Taken together these data suggest that mutagenesis of these residues would not necessarily result in a conformational instability which would result in retrograde degradation and decreased membrane residency of GPCRs. Consistent with all structures is the orientation of the E(D) residues towards the inner helix bundle and the Y(x) residues towards solvent. Importantly, since the carboxyl terminal peptide of Gs α interacts with R135 (of the ERY motif) in the active structure of opsin (6), it is clear that orientation of these residues towards the inner helix bundle does not preclude their interactions with cytoplasmic adapter proteins.

Interestingly, although H452A FSHR mutant bound hFSH at Wt levels, very little FSH appeared to be internalized (Figure 4B). The mutants T447A, L448A and W451A internalized hFSH although at reduced levels. The mutants that internalized hFSH also produced cAMP when stimulated with hFSH. T447A, L448A and W451A, which show considerable levels of internalization, produce high levels of cAMP when exposed to hFSH (Figure 5). As expected, H452A has very low levels of internalization and cAMP production was not detectable.

These data show that internalization is linked to signaling. Thus H452A FSHR mutant exhibited little conformational perturbation, as evidence by its ability to bind FSH with high affinity but it was not internalized and could not signal. It is tempting to speculate that signaling is a concerted process requiring endosome formation, and that this is linked to activation of G-protein. It is of course entirely possible that H452A failed to load G-protein, since it has been shown using FRET measurements that heterotrimeric G-proteins precouple with GPCRs (68). In any case, this is the first demonstration that an amino acid contiguous with the canonical ERW motif has been implicated in G-protein signaling. In fact, recent structural studies of G-protein coupled receptors have indicated that the ERW motif may be more relevant to conformational stability of the transmembrane helices, than to direct interaction with G-proteins. In this regard, it is interesting to note that in the model of the FSHR intracellular domains (4), the histidine 452 residue is at the interval between helix III and the random coil of icL2 (Figure 6). Mutations of residues that progress into helix IV were of no consequence to interaction of icL2 with the 14-3-3 bait in the yeast two hybrid assay. Notably, the side chains of histidine and tryptophan (ERWH) project into the same space and are solvent accessible. The juxtaposition of these two residues could play an important role in the process of G-protein loading and activation and/or internalization. The most resolved information available so far on the receptor domains participating in the intermonomer interfaces in GPCR homo-dimers concerns a very limited number of systems, namely rhodopsin (8;69) opsin (65), squid rhodopsin(70), the β -2-adrenergic receptor (67) and the D2-dopamine receptor (71–73). Recently, a disulphide trapping approach was used to study 5HT_{2c} serotonin receptor dimerization (9). Collectively, in vitro evidence seems to highlight different portions even for the same receptor as being involved in intermonomer contacts. The most recurrent interface would involve either H1-H1 (H stands for helix) or H4-H4 or H4-H5 contacts. These arrangements of the dimeric receptor would seem to provide for molecular access to H452 and W451 residues.

It is difficult to ignore the significance of the ERWH sequence as a canonical G-protein interaction site and how mutations of this sequence abrogate interaction in the yeast two hybrid assay with the 14-3-3 bait. The (E/D)R(W/Y) motif of the majority of GPCRs belonging to the rhodopsin/ β -adrenergic receptor subfamily is present as ERW in the glycoprotein hormone receptors and has been implicated in G protein interaction and receptor activation (63). Therefore a future hypothesis to be tested is whether this motif may play a role in FSH mediated signaling and that 14-3-3 interaction with this motif may modulate such signaling.

Recently it has been demonstrated that G proteins do not only localize at the plasma membrane, but rather shuttle between the plasma membrane and intracellular membranes such as the endoplasmic reticulum and the Golgi (74;75). Indeed assembly of signaling complexes prior to insertion at the plasma membrane is a current paradigm(76;77). Since it seems that receptors interact freely with G-proteins (11) it seems reasonable to propose that premature loading of G-protein coupled receptors may be sterically blocked by the association of adapter proteins with the G-protein interaction site. Further research on this topic with regard to the FSH receptor will require imaging of fluorescently labeled FSHR interacting proteins and similarly labeled FSHR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FSH	follicle stimulating hormone
FSHR	FSH receptor

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Domain Structure of APPL Proteins

Figure 1.

Schematic of the domain architecture of APPL proteins. The BAR domain is the dimerization domain (42). The pleckstrin homology domain (PH) is the site of Rab5 interaction. The FSH receptor binding site is in the C-terminal domain, which also contains the phosphotyrosine binding domain (PTB).

Figure 2.

APPL1 interactome MAP. FSHR interacts with APPL1 potentially leading to additional associations. APPL1 forms heterodimers with APPL2. APPL1 associates with the adiponectin receptor (ADPORA1) as well as the neurotrophin receptor TrkA (48). It interacts with protein kinase B (Akt) and phosphoinositide-3-kinase (PI3K) (31) holding them in an inactive complex. Rab5 an effector of APPL1 is bound at the Cterminus (45). APPL1 is present in early endosomes associated with the marker EEA1(81). Following EGF stimulation it is found associated with Histone Deacetylase (HDZC) (50). It has been shown to associate with OCRL (47) and netrin receptor protein referred to as Deleted in Colorectal Carcinoma (DCC) (82).

Figure 3.

Scanning alanine mutant of the FSHR icL2 bait protein tested for interaction with 14-3-3 τ as previously described (29). Each underlined set of residues was changed to alanine (YTL/AAA). Region 447–452 (OD3 and OD4) of FSHR-icL2 appeared to be involved in the interaction with 14-3-3 τ and so single mutations were made in full-length FSHR and tested for function.

Figure 4.

Figure 4A. Binding of ^{125}I -hFSH to HEK293 cells transiently transfected with plasmids encoding single amino acid substitution mutants of hFSHR as previously described (2). Decreased or absent binding was consistent with the level of protein expression (Figure 4B) and indicated that the mutations did not adversely affect conformation of the receptor. Arrows indicate the H452A mutant which has good binding but did not internalize ^{125}I -hFSH.

Figure 4B. Immunoblot of cellular lysates following transfection with single amino acid substitution mutants of hFSHR was done as previously described (2). The region of hFSHR icL2 represented as single mutations is underlined: $^{441}\text{YTLTAITLERWHTITHAMQLDCKVQLRHAAS}^{471}$. Loading controls were immunostained with actin antibody.

Figure 5.

Induction of cAMP production following binding of hFSH to HEK293 cells transiently transfected with plasmids encoding single amino acid substitution mutants of hFSHR as previously described (2). Decreased or absent cAMP production was consistent with the level of protein expression (Figure 4B) and binding of radiolabeled hFSH (Figure 4A) with the exception of the H452A mutation (arrow). This mutant of hFSHR exhibited good binding, but failed to internalize and did not induce cAMP production.

Figure 6.

Orientation of a G-protein interaction motif (ERW)H in a model of FSHR cytoplasmic domain structure (4). Substitution of alanine at position His 452 affects neither trafficking to the membrane nor FSHR binding. This mutation results in a block to cAMP production and failure of the hormone receptor to internalize. The Glu 449 and Arg 450 are oriented into the center of the transmembrane domain helices whereas side chains of both His 452 and Trp 451 are oriented towards solvent.