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Trans-activation, Cis-activation and Signal Selection of Gonadotropin Receptors[‡]

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SUMMARY

It has been thought that when a hormone binds to a receptor, the liganded receptor activates itself and generates hormone signals, such as the cAMP signal and the inositol phosphate signal (**cis-activation**).

We describe that a liganded LH receptor or FSH receptor molecule is capable of intermolecularly activating nonliganded receptors (**trans-activation**). Particularly, intriguing is the possibility that a pair of compound heterozygous mutants, one defective in binding and the other defective in signaling, may cooperate and rescue signaling.

Furthermore, trans-activation of the binding deficient receptors examined in our studies generates either the cAMP signal or the IP signal, but not both. Trans-activation and selective signal generation have broad implications on signal generation mechanisms, and suggest new therapeutic approaches.

Keywords

LH receptor; FSH receptor; transactivation; cisactivation; signal selection

A gonadotropin receptor consists of two distinct domains, one for hormone binding (**exodomain**) and the other for signal generation (**endodomain**). The exodomain is extracellular at the N-terminus of the receptor and consists of ~250 – 300 amino acids (Hong et al., 1998, Ascoli et al., 2002) (Fan and Hendrickson, 2005). In contrast, the endodomain is associated with the membrane at the C-terminus, and comprises seven transmembrane domains (**TMs**), three extracellular loops (**exoloops**), three cytoplasmic loops (**cytoloops**) and a cytoplasmic C-terminal (Ji et al., 1998).

Exodomain and hormone binding

The exodomain comprises ~10 Leu Rich Repeats (LRR, -Leu/Ile-X-Leu/Ile). An LRR forms a loop with a short, curved parallel β strand, which interacts with the C-terminal side of the hormone (Fan and Hendrickson, 2005). The recent crystal structure of the FSH/FSHR-exodomain reveals that a number of LRRs are involved in the interaction with partially deglycosylated FSH (Fan and Hendrickson, 2005). Likewise, Ala substitutions of individual Leu and Ile residues of the LRRs of LHR impair hCG binding, suggesting their role in the hormone binding (Song et al., 2001). One of the LRRs is LRR4.

To determine the LRR4's contact points with hCG a series of the LRR4 peptide mimics, N⁹⁶L⁹⁷P⁹⁸G⁹⁹L¹⁰⁰K¹⁰¹Y¹⁰²L¹⁰³S¹⁰⁴I¹⁰⁵C¹⁰⁶N¹⁰⁷T¹⁰⁸G¹⁰⁹I¹¹⁰R¹¹¹K¹¹²F¹¹³P¹¹⁴D¹¹⁵,

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were synthesized to incorporate a Benzoylphenyl alanine (Bpa) at each of the amino acid positions in the sequence (Fig. 1). Each of the LRR4 peptides carrying a Bpa was incubated with hCG, irradiated with UV, solubilized in SDS under the reducing condition, and electrophoresed on SDS-PAGE as previously described (Jeoung et al., 2001). The photoaffinity scan of LRR4 (Fig. 1) reveals that the peptide mimics photoaffinity-labeled both of the hCG α and β subunits, preferentially hCG α . The labeling was most effective when Bpa was placed at the position of K¹⁰¹, Y¹⁰², S¹⁰⁴ or C¹⁰⁶, whereas the labeling was negligible or marginal when Bpa was positioned at L¹⁰³, I¹⁰⁵ or N¹⁰⁷. The photoaffinity-scan suggests the sequence around K¹⁰¹-C¹⁰⁶ as the preferential sites for labeling hCG, probably a contact point. Bpas at the even numbered amino acid positions in the Y¹⁰²L¹⁰³S¹⁰⁴I¹⁰⁵C¹⁰⁶N¹⁰⁷ sequence were more effective for labeling than those at the odd numbered positions, indicating their orientation toward hCG in a β strand. The labeling required UV irradiation, hCG, and the Bpa peptides, and was saturable dependent on the UV irradiation time and concentrations of hCG and the Bpa peptides. On the other hand, deglycosylated hCG, which does not bind to LHR, was not labeled at all (data not included). These results indicate the specificity of the photoaffinity labeling.

Endodomain and signal generation

The gonadotropin receptors are capable of generating two major signals, one to activate adenylyl cyclase (AC) to produce cAMP and the other for phospholipase C to produce diacylglycerol and inositol phosphates (IP). Among the three exoloops, exoloop 3 is the shortest with 11 amino acids and crucial for signal generation. Ala substitution of some residues impairs induction of cAMP, IP or both (Ryu et al., 1996, Gilchrist et al., 1996, Sohn et al., 2002). The L⁵⁷⁶, I⁵⁷⁷ and K⁵⁸³ are crucial for the cAMP signal, whereas the IP signal requires most of the 11 residues. The results collectively indicate the two signals are distinct, and generated by separate mechanisms at different sites. In addition to the exoloop 3 residues, D³⁹⁷ and K⁴⁰¹ in exoloop 1 are also crucial for the cAMP signal (Ji and Ji, 1995). D³⁹⁷ of TM1 and K⁵⁸³ of TM7 likely ion-pair, which probably is important for the cAMP signaling. In addition to the exoloops, Puett's group has made an important discovery that the ~10 amino acid extension upstream of TM1 is also involved in the cAMP signal generation (Alvarez et al., 1999). These signals generated at the exoloops propagate through the TMs, in particular TMs 3, 6 and 7 (Schulz et al., 2000, Angelova et al., 2000, Hirakawa and Ascoli, 2003, Munshi et al., 2003, Angelova and Puett, 2002, Shenker, 2002, Simon et al., 2002). There are a large number of natural and engineered mutations in the TMs that impact TM signal propagation (Dufau, 1998, Huhtaniemi et al., 1999, Angelova et al., 2002). The signals are ultimately transferred to G proteins at the cytoplasmic side of the receptor. For example, Gas is responsible for receiving the cAMP signal and activating adenylyl cyclase. With respect to the IP signal, there are conflicting reports whether Gai/o or Gq/11 is responsible for activating PLC and inducing IP (Kuhn and Gudermann, 1999, Salvador et al., 2001, Hirakawa and Ascoli, 2003),

Modulation of endodomain by hormone/exodomain complex

Since gonadotropins bind first to the exodomain with high affinity, the signal generation at the exoloops is likely modulated by hormone/exodomain complex (Ji et al., 1995). During the modulation, the loops 1 and 3 of the α subunit and the loop 2 of the β subunit of the hormone in the hormone/receptor complex are oriented toward the endodomain (Sohn et al., 2003) (Fan and Hendrickson, 2005). The targets include exoloop 3 (Sohn et al., 2002, Sohn et al., 2003) and exloop 2 (Nakabayashi et al., 2000, Zeng et al., 2001, Jeoung et al., 2001) (Nishi et al., 2002). The modulation likely involves the contacts among the exodomain, endodomain and hCG, in particular the sequence around S²⁵⁵ of the exodomain and exoloop 2 (Nakabayashi et al., 2000, Zeng et al., 2001, Nishi et al., 2002), as well as the contact between the hormone and exoloop 3 (Sohn et al., 2002, Sohn et al., 2003). The precise contact points, matching contact

pairs, how the contact points differentially generate the cAMP signal and IP signal, and the mechanistics of the modulation are yet to be defined.

Receptor activation: Cis-activation and Trans-activation

It is generally thought that, to activate a receptor molecule, it has to bind hormone and that only the hormone-bound receptor or receptor complex is capable of activating itself and generating hormone signals. We will call it **cis-activation** (Fig. 2). An exception is activating mutant receptors that constitutively generate hormone signals and cause diseases (Angelova et al., 2002, Shinozaki et al., 2003), which is not a subject of this proposal. In contrast to cis-activation, there is the emerging evidence that free (nonliganded) receptor molecules are activated by a liganded receptor molecule (**trans-activation**). There are only several reports on trans-activation of GPCRs, two reports on trans-activation of the thrombin receptors (Chen et al., 1994, O'Brien et al., 2000) and our reports on trans-activation of LHR (Ji et al., 2002, Lee et al., 2002) and FSHR (Ji et al., 2004). Remarkably, trans-activation is involved in signal selection. For example, FSHR generates two signals, one to activate adenylyl cyclase to produce cAMP (**cAMP signal**) and the other to activate phospholipase C to produce inositol phosphate/diacylglycerol (**IP signal**). Trans-activation of FSHR generates either the cAMP signal or the IP, but not both (Ji et al., 2004). However, the IP signal was marginal and observed in trans-activation of only one mutant, FSHR^{L27A}.

Trans-activation of LHR

As shown in Fig. 3A and 3B, signal deficient LHR^{K583R} and three binding deficient LHRs, LHR^{I55A}, LHR^{I80A} and LHR^{I105A}, were tested. To detect non-binding receptors, the Flag epitope was attached to the N-terminus of mature receptors and assayed for binding of ¹²⁵I-anti-Flag monoclonal antibody as described previously (Hong et al., 1998, Song et al., 2001). The antibody showed significant specific binding to all of the cells, indicating the surface expression of Flag-LHR^{I55A}, Flag-LHR^{I80A} and Flag-LHR^{I105A} (Ji et al., 2002). Each of the receptors cannot induce cAMP production. When cells were coexpressed with LHR^{K583R} and LHR^{I55A}, the cells bound hCG with the same high affinity as the wild type binding as shown by the similar K_d values. To avoid variations in receptor concentrations, batches of transfected cells expressing 8,000 – 15,000 of each receptor species/cell were used. The cells also produced cAMP in an hCG dose dependent manner. The maximum cAMP level of LHR^{K583R} and LHR^{I55A} was ~43 fold higher than the basal cAMP level and 33 % of the wild type maximum cAMP level. These are significant but the EC₅₀ value for cAMP was 4 fold higher than the wild type value, indicating the higher hCG concentration needed to induce the maximum cAMP level for LHR^{K583R} and LHR^{I55A} as compared to the wild type LHR. The cells co-transfected with the LHR^{K583R} and LHR^{I80A} plasmids bound hCG and responded to produce cAMP. However, the cells co-transfected with the LHR^{K583R} and LHR^{I105A} plasmids bound hCG but did not produce cAMP. These results suggest a specificity of the pairing. To test whether the rescue of cAMP induction was caused by changes in the G protein and/or adenylyl cyclase activity, the cells were treated with cholera toxin. It activates G_{αs} leading to the stimulation of adenylyl cyclase and cAMP production. All of the cells expressing the wild type LHR, LHR^{K583R}, LHR^{I55A}, LHR^{I80A}, LHR^{I103A}, LHR^{K583R/I55A}, LHR^{K583R/I80A} or LHR^{K583R/I103A} produced similar amounts of cAMP (data not shown). This result indicates no significant changes in G protein and adenylyl cyclase in the cells, regardless of co-transfection.

Next, we raised the intriguing question whether an LHR exo-domain^{+hCG} not connected to the endo-domain could activate an LHR^{-hCG}. We approached the problem using a hybrid of the LHR exo-domain attached to the transmembrane domain of CD 8 (ExoCD) that lacks the extracellular domain of CD 8 (Osuga et al., 1997). ExoCD was expressed on the cell surface,

bound hCG, but was incapable of inducing cAMP as shown in Fig. 3C and 3D. Co-expressed ExoCD with LHR^{I55A} or LHR^{I80A} bound hCG and induced cAMP production, but co-expressed ExoCD with LHR^{I105A}, LHR^{K583R-I55A} or LHR^{K583-I80A} did not. All of these cells produced similar levels of cAMP in response to cholera toxin, indicating the integrity of adenylyl cyclase and the G proteins. It is clear that the exo-domain does not have to be attached to the endo-domain for hCG binding and activation of the endo-domain. In addition, the interaction of the 7 TM domains is not necessary for rescue.

Since trans-activation requires the interaction of two receptor molecules, it is logical to question whether gonadotropin receptors dimerize. The recent crystal structure of FSH complexed with the FSHR exodomain (Fan and Hendrickson, 2005) shows a low affinity dimer with K_d value of 0.4 mM at the monomer-dimer equilibrium (Fan and Hendrickson, 2005). Chemical crosslinking data in the report suggests that FSH/FSHR complexes exist primarily as monomer and form unstable dimer, which is consistent with our observation of trans-activation. In addition, there is the evidence that other GPCRs exist as monomers and oligomers, (Roess et al., 2000) (Hunzicker-Dunn et al., 2003) (Tao et al., 2004) (Bai, 2004) (Milligan, 2004) but it is in debate whether oligomerization is necessary for receptor activation.

Trans-activation and signal selection of FSHR

Next, we tested trans-activation of FSHR. C¹⁵A, P²⁴A, D²⁶A, L²⁷A and F³⁶A mutations in the exodomain impair FSH binding without impacting their surface expression (Ji et al., 2004). For FSHR^{+FSH/-cAMP}, a hybrid of the FSHR exodomain attached to the GPI anchor sequence was constructed (Ji et al., 2004). When it was stably expressed in cells, the cells bound ¹²⁵I-FSH but did not induce cAMP (Fig. 4). The cells stably expressing ExoGPI were transiently transfected with each of the nonbinding mutants, C¹⁵A, P²⁴A, D²⁶A, L²⁷A and F³⁶A. All of the cells bound FSH, and the K_d values were similar to the ExoGPI value. The concentrations of FSH binding sites on intact cells were in the range of 22,500 -46,300/cell, which are in the range of the wild type receptor concentration. The cells coexpressing ExoGPI/FSHR^{P24A}, ExoGPI/FSHR^{D26A} or ExoGPI/FSHR^{F36A} produced cAMP in an FSH dose dependent manner (Fig. 4C). The maximum cAMP levels were substantial, ~40% of the wild type for ExoGPI/FSHR^{P24A}, ~53% for ExoGPI/FSHR^{D26A} and ~35% for ExoGPI/FSHR^{F36A}. The successful cAMP induction was particularly impressive, because the hormone binding sites were fewer than the binding site on the cells expressing ExoGPI, which did not respond to FSH and produce cAMP. These results clearly show the distinction of the hormone binding from the cAMP signal generation. This is more obvious in the case of the ExoGPI/FSHR^{C15A} pair. This pair did not respond to FSH binding to produce cAMP despite high affinity FSH binding at a comparable number of sites. No cAMP induction demonstrates specificity in the rescue of cAMP induction and cooperation of ExoGPI with nonbinding mutant FSHRs. The data in Fig. 4D show that IP was not induced by the four pairs of ExoGPI/nonbinding FSHR, ExoGPI/FSHR^{C15A}, ExoGPI/FSHR^{P24A}, ExoGPI/FSHR^{D26A} and ExoGPI/FSHR^{F36A}. This is particularly interesting, because some of the pairs successfully produced cAMP. In contrast, ExoGPI/FSHR^{L27A} induced IP production in an FSH dose dependent manner, but surprisingly, it was incapable of producing cAMP. Although the IP induction was 1.22 fold over the basal level, the maximum IP level was 55% of the wild type value and reproducible.

The results show that the pair of ExGPI and the nonbinding FSHRs collaborates to rescue the induction of either cAMP or IP, but not both. It is remarkable that trans-activation of the pairs is capable of inducing only one of the two hormone signals (Ji et al., 2004).

Significance of Signal Selection: Multiple signals from a hormone receptor are a source of undesirable side effects of hormone drugs

Hormone receptors generate multiple signals. Undesirable side effects are one of the major problems in the drug development and therapeutics on hormone receptors, as for most pharmaceuticals. For instance, type I diabetic patients take insulin to stimulate the uptake of blood glucose, but insulin can cause other side effects including those on cell growth, heart beat and blood pressure. An ideal hormone drug is to induce a specific hormone action without invoking the unintended side effects. To control a specific signal, it is necessary to determine whether multiple signals are regulated by a master-switch or each signal is controlled by a separate micro-switch.

If a receptor molecule can generate only one signal and each signal is controlled by a separate micro-switch, there is a room to choose a specific signal and manipulate its generation. In contrast, multiple signals are controlled by a master-switch, it will be difficult to generate a specific signal without invoking others. We have shown separate switches for the cAMP signal and the IP signal and defined the physical location of the micro-switched (Ryu et al., 1996, Gilchrist et al., 1996) (Sohn et al., 2002). Remarkably, trans-activation of the nonbinding FSHR mutants generates only one of the two signals but not both, which could provide a means to generate a specific hormone signal without invoking other signals ovarian cancers (Wang et al., 2003, Choi et al., 2002, Parrott et al., 2001). Our study on trans-activation of nonbinding LHRs also shows the same trend (unpublished observations). However, it is necessary to exhaustively screen nonbinding receptors for trans-activation and signal selection.

Mechanisms of Trans-activation

Our data indicate trans-activation as an intermolecular event, which requires a pair of a signal deficient receptor and a binding deficient receptor. In addition, they implicate that the hormone/exodomain complex of a signal deficient receptor modulates the endodomain of a binding deficient receptor. However, the mechanistics of the modulation and trans-activation are little known. Specific questions include whether the hormone/exodomain complex interacts and modulates the exodomain or endodomain of the nonbinding receptor, where the contact site(s) is, where the modulation site(s) is, which part(s) of the hormone/exodomain complex is essential, and which part of the endodomain might be necessary.

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Fig. 1.

Photoaffinity scan of Leu Rich Repeat 4.

(A) A series of the LRR4 peptide mimics, N⁹⁶LPGLK¹⁰¹Y¹⁰²L¹⁰³S¹⁰⁴I¹⁰⁵C¹⁰⁶N¹⁰⁷T¹⁰⁸G¹⁰⁹I¹¹⁰R¹¹¹K¹¹²FPD¹¹⁵, were synthesized, in which a Benzoylphenyl alanine (Bpa) replaced one of the amino acids (as indicated above gel lanes) and a Tyr residue was attached at the C-terminus for radio-iodination. Each of the Bpa peptides was radioiodinated, incubated with cold hCG, irradiated with UV, solubilized in SDS under the reducing condition, and electrophoresed on SDS-PAGE. The gels were dried and scanned on phosphorimager for autoradiography. The radioactive bands of labeled hCG α and β are marked.

(B) The percentage of labeled hCG and the K_d value of each Bpa peptide mimics were calculated from labeling of hCG in the presence of increasing concentrations of individual ¹²⁵I-Bpa-peptides. In addition, the labeling by a constant amount of ¹²⁵I-peptide was inhibited in the presence of increasing concentrations of cold peptides, and the results were used to calculate the IC₅₀ values (the concentration of cold peptides that is required to inhibit labeling by 50% of the maximum labeling).

Fig. 2. Hypothetical models of trans-activation and cis-activation. (A) The exodomain complexed with hormone intramolecularly modulates its own endodomain (cis-activation), and (B) intermolecularly modulates unoccupied receptor (trans-activation).

Fig. 3.

Rescue of cAMP production by coexpression of LHR^{-hCG} and LHR^{+hCG/-cAMP}. HEK 293 cells were transfected with LHR^{-hCG} plasmids, LHR^{+hCG/-cAMP} plasmid, or both. Cells were assayed for ¹²⁵I-hCG binding in the presence of increasing concentrations of nonradioactive hCG (A). The results were analyzed by Scatchard plot to determine the K_d. In addition, intact cells were treated with increasing concentrations of unlabeled hCG and intracellular cAMP was measured (B). Experiments were repeated 4–6 times in duplicate. NS stands for not significant. (C) Rescue of cAMP induction by the ExoCD chimeras. The LHR exo-domain was linked to the transmembrane and cytoplasmic domain of CD 8. The resulting chimera (ExoCD) was coexpressed with LHR^{-hCG} mutants, and assayed for hCG binding and cAMP induction as described in A and B.

FSH Binding			cAMP		IP	
K _d (pM)	Receptors/cell		EC ₅₀ (nM)	Max (fmol)	EC ₅₀ (nM)	Fold Increase
1,781 ± 315	36,300 ± 2400	■ FSHR ^{WT}	0.40 ± 0.06	59.9 ± 3.7	1.7 ± 0.03	1.40 ± 0.03
316 ± 11	55,000 ± 2200	□ FSHR-ExoGPI	ND	ND	ND	ND
346 ± 16	35,300 ± 900	● FSHR-ExoGPI/FSHR ^{C15A}	ND	ND	ND	ND
513 ± 30	44,000 ± 70	○ FSHR-ExoGPI/FSHR ^{P24A}	1.05 ± 0.15	23.7 ± 2.2	ND	ND
594 ± 10	46,300 ± 7000	◆ FSHR-ExoGPI/FSHR ^{D26A}	0.85 ± 0.15	32.0 ± 2.7	ND	ND
198 ± 12	22,500 ± 4200	◇ FSHR-ExoGPI/FSHR ^{L27A}	ND	ND	1.87 ± 0.24	1.22 ± 0.05
295 ± 47	28,200 ± 2200	▲ FSHR-ExoGPI/FSHR ^{F36A}	3.06 ± 0.55	21.0 ± 1.0	ND	ND

Fig. 4.

Trans-activation of nonbinding FSHRs by ExoGPI. HEK 293 cells stably expressing ExoGPI were transfected again with FSHR^{C15A}, FSHR^{P24A}, FSHR^{D26A}, FSHR^{L27A} or FSHR^{F36A} and assayed for hormone binding and induction of cAMP and IP as described in the legend to Fig. 3.