Receptor Activation Is Distinct from Hormone Binding in Intact Lutropin-Choriogonadotropin Receptors and Asp<sup>397</sup> Is Important for Receptor Activation*

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Hormone binding to receptors on the cell surface triggers a sequence of events (receptor activation and signal generation) leading to activation of effectors in the cytoplasm. Receptor activation and signal generation are difficult to study as both are intimately associated with hormone binding. The lutropin-choriogonadotropin (LH/CG) receptor offers a unique model to differentiate and examine receptor activation and signal generation from hormone binding. It belongs to a subfamily of glycoprotein hormone receptors within the G-protein-coupled receptor family. This receptor subfamily has several structural features different from the structures of other G-protein-coupled receptors. These receptors consist of a large extracellular N-terminal half and membrane-associated C-terminal half of similar size. The truncated N-terminal half alone is capable of high affinity hormone binding, whereas the truncated C-terminal half alone is capable of low affinity hormone binding and cAMP induction. However, this distinction between the high affinity hormone binding and low affinity hormone binding associated with cAMP induction has not been established in intact receptors. As a step to identify a structural element which is responsible for receptor activation and signal generation, we have identified an extracellular Asp of the C-terminal half of the LH/CG receptor which is unique and common to the glycoprotein hormone receptors. Evidence is presented for the first time that Asp<sup>397</sup> is important for induction of cAMP synthesis but not essential for hormone binding. Since extracellular Asp<sup>397</sup> cannot interact with G-protein in the cytoplasm, the inability of the mutant LH/CG receptors with an Asp<sup>397</sup> substitution to induce cAMP synthesis is likely to be caused by a defect in the intermediate steps (receptor activation and signal generation) between hormone binding and activation of G-protein. Therefore, our results not only demonstrate that receptor activation and signal generation are distinct from high affinity hormone binding in intact LH/CG receptors, but they also identify an amino acid important for the processes.

Hormone receptors on the cell surface trigger a cascade of hormonal responses in an orderly sequence, including hormone binding on the cell surface and activation of effectors in the cytoplasm. These are the two steps which can be readily assayed. Between these two events there exist poorly defined intermediate steps as manifested by the fact that hormone binding does not necessarily activate effectors (1). These intermediate steps will be tentatively called receptor activation and signal generation. To study these sequential events and their mechanisms, numerous mutant receptors have been examined in the past. Most important mutant receptors are proven to be defective either in hormone binding or in activation of effectors. For example, mutations in cytoplasmic regions of receptors successfully demonstrated that G-protein-coupled receptors engage effectors through the cytoplasmic part of the receptors, particularly the third cytoplolop (2, 3). On the other hand, enlightening studies on receptor activation and signal generation have not been easy due to difficulties in distinguishing them from hormone binding and activation of effectors. The LH/CG receptor offers a unique model for studying receptor activation and signal generation.

The LH/CG receptor belongs to a subfamily of glycoprotein hormone receptors within the G-protein-coupled receptor family (4, 5). This receptor subfamily has several structural features different from the structures of other G-protein-coupled receptors cloned to date. Their extracellular N-terminal half is considerably larger than the N-terminals of other G-protein-coupled receptors. It is as large as the rest of the receptor molecule, including the seven transmembrane domains, three exoloops, three cytoloops, and cytoplasmic C-terminal. Both the N-terminal half and the C-terminal half of the receptor have hormone contact sites according to studies with photoaffinity labeling (6, 7) and receptor peptides (8). Recently, it was demonstrated that the truncated N-terminal half alone is capable of hormone binding with an affinity comparable to that of intact receptors (9-11), however, it is not capable of inducing hormone action (11, 12). On the other hand, the truncated C-terminal half is capable of low affinity hormone binding and cAMP induction (11, 12). These results clearly distinguish receptor activation and signal generation from high affinity hormone binding in the truncated receptor halves. Whether it occurs in intact receptors is unknown. If it does occur, then receptor activation and signal generation are likely to involve low affinity hormone binding and therefore require a structural element in the extracellular part of the C-terminal half of the receptor. Such a structural element would likely play a key role in receptor activation and signal generation as well as in the transition from high affinity hormone binding to receptor activation and signal generation. The structural element may be common and unique to the glycoprotein hormone receptors, since these receptors share high sequence homologies in the C-terminal half and the identical signal pathway involving G-protein.

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† The abbreviations used are: LH, lutropin; hCG, human choriogonadotropin.

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FIG. 1. A schematic presentation of the C-terminal half of the rat LH/CG receptor. Acidic amino acids in exoloops and transmembrane domains are indicated by bold letters.

FIG. 2. Hormone binding of mutant rat LH/CG receptors. Mutant LH/CG receptors were prepared in which acidic amino acids of the exoloops were substituted. These mutant receptors were transiently expressed in 293 cells and assayed for 125I-hCG binding to intact cells or receptors solubilized in Triton X-100 as described under “Experimental Procedures.” The data are analyzed by Scatchard plots to determine $K_d$ values and the number of binding sites. These values were analyzed by Student’s $t$ test.

Due to its extracellular location, the structural element, however, is not expected to be directly involved in contacting G-protein in the cytoplasm. Therefore, to study receptor activation and signal generation, we are interested in mutant LH/CG receptors which are capable of high affinity hormone binding but are defective in cAMP induction and in which an amino acid of extracellular regions of the C-terminal half is substituted.

In this work, we have identified an extracellular Asp at the boundary of the second transmembrane domain and the first exoloop of the LH/CG receptor, which is uniquely common to glycoprotein hormone receptors (13). Evidence is presented for the first time that this Asp is important for induction of hormone action but is not essential for hormone binding, thus distinguishing receptor activation and signal generation from high affinity hormone binding in intact LH/CG receptors. Furthermore, we have established a cell surface structural element of intact receptors that can be used to study receptor activation and signal generation.

EXPERIMENTAL PROCEDURES

Mutagenesis and Functional Expression—Mutant LH/CG receptor cDNAs were prepared in pSELECT vector using the Altered Sites Mutagenesis System (Promega) as described (14). Briefly, the multiple cloning site of the vector was cut with XhoI and BamHI and then unidirectionally ligated with full-length rat LH/CG receptor cDNA. Single strand DNA was prepared, and mutagenesis was performed according to the manufacturer’s instruction. The revertant of mutant LH/CG receptor cDNAs was also prepared in pSELECT vector.
Vectors containing mutant LH/CG receptor constructs were selected by ampicillin screening and sequencing. The resulting mutant cDNAs were subcloned into the BamHI and XbaI sites of pcDNA I/neo (Invitrogen). Mutant LH/CG receptor constructs were transfected into human embryonic kidney 293 cells by the calcium phosphate method. These cells do not express the LH/CG receptor.

**125I-hCG Binding and Intracellular cAMP Assay**—Forty-eight to 80 h after transfection, the cells were assayed for 125I-hCG binding in the presence of increasing concentrations of 125I-hCG and the Kd values were determined by Scatchard plots. hCG, batch CR 127, was supplied by the National Hormone and Pituitary Program. For intracellular cAMP assay, cells were washed twice with Dulbecco’s modified Eagle’s media and incubated in the buffer containing isobutylmethylxanthin (0.1 mg/ml) for 15 min. Increasing concentrations of hCG were then added, and the incubation was continued for 30 min at 37 °C. After removing the media, the cells were rinsed once with fresh media without isobutylmethylxanthin, lysed in 60% ethanol, freeze-thawed in liquid nitrogen, and scraped. After pelleting cell debris at 16,000 × g for 10 min at 4 °C, the supernatant was collected, dried under vacuum, and resuspended in purified water.

**RESULTS AND DISCUSSION**

The truncated C-terminal half of the LH/CG receptor alone is capable of low affinity hormone binding and cAMP induction, whereas the truncated N-terminal half is capable of high affinity hormone binding with no cAMP induction (11, 12). In an attempt to identify a structural element in the extracellular portion of the C-terminal half of the LH/CG receptor, which is essential for cAMP induction but not for hormone binding, the amino acid sequences of glycoprotein hormone receptors were compared with those of other G-protein-coupled receptors (13). The result revealed that Asp937 of the LH/CG receptor is unique and common to glycoprotein hormone receptors but is absent in other G-protein-coupled receptors (13). It is located on the cell surface at the boundary between the second transmembrane domain and the first extracellular loop of glycoprotein hormone receptors (Fig. 1). Therefore, it is not expected to interact with G-protein. To examine its role in the hormone-receptor interaction and receptor activation to induce hormone action, a series of mutant LH/CG receptors were prepared in which Asp or Glu in the extracellular loops of the C-terminal half of the receptor was substituted.

Asp97 of the LH/CG receptor was substituted with Glu, Asn, Ala, Lys, or Arg (LH/CG-Asp97-Glu, LH/CG-Asp97-Asn, LH/CG-Asp97-Ala, LH/CG-Asp97-Lys, and LH/CG-Asp97-Arg). These mutant LH/CG receptor constructs were transfected into human embryonic kidney 293 cells which do not intrinsically express the LH/CG receptor. As shown in Fig. 2, all transfected cells were capable of 125I-hCG binding with affinities similar to that of the natural receptor (p < 0.5), whereas cells which were not transfected or were transfected with the plasmid lacking the LH/CG receptor cDNA did not bind 125I-hCG (data not included). The number of binding sites/cell was also similar (p < 0.2) except that LH/CG-Ras97-Asn, LH/CG-Ras97-Ala, and LH/CG-Ras97-Lys bound somewhat less (0.05 < p < 0.2). This reduced number of surface receptors does not appear to be due to reduced synthesis of the receptors, as the receptor number was similar (p < 0.2) when the cells were solubilized in Triton X-100 and assayed for hormone binding (Fig. 2). These results demonstrate that the mutant receptors are expressed on the cell surface and that they retain their ability to bind 125I-hCG. Furthermore, the results indicate that the substitution of Asp97 did not significantly affect the receptor’s ability to bind the hormone. This is in contrast to the dramatic effect of this substitution on the induction of cAMP synthesis, as cAMP synthesis was significantly reduced by 10,000 times or was undetected at all (Fig. 3). Not only did the affinity of the cAMP inducibility decrease but the maximum level of cAMP production diminished as well (p < 0.01). The basal levels of intracellular cAMP of transfected cells were similar. Prior to this report, the magnitude of such a negative effect

**FIG. 3. Induction of cAMP synthesis.** Cells expressing the mutant LH/CG receptors in Fig. 2 were treated with increasing concentrations of cold hCG and assayed for intracellular CAMP. The basal level of AMP was 10–20 fmol/1000 cells. NS indicates not significant as cAMP levels were slightly higher than the basal level. ND indicates not detected.
has never been observed in any mutant receptors. These results suggest that Asp$^{397}$ is important for cAMP induction but not for hormone binding and that the mutant receptors are not effectively activated to induce cAMP synthesis even at significantly higher hormone concentrations. This conclusion is consistent with the observation that the C-terminal half of the LH/CG receptor is capable of low affinity hormone binding and cAMP induction (11, 12). It is also in agreement with the conclusion that the site for the receptor activation to induce cAMP is in the C-terminal half (12).

Extracellular Asp$^{397}$ is not expected to interact with G-protein because G-protein is located on the cytoplasmic side of the membrane (15) and is coupled to cytoplasmic portion of receptors (2, 3). This is consistent with the fact that defective coupling of receptors to G-proteins is caused by mutations in the cytoplasmic domains of receptors (2, 3). Whatever the substitutes at the amino acid position 397 did in mature receptors, they resulted in unsuccessful coupling of the receptors to G-proteins without contacting G-protein. Therefore, the inability of the mutant LH/CG receptors to induce cAMP synthesis is likely to be caused by a defect in steps prior to activation of G-protein (receptor activation and signal generation).

To determine the role of the side chain of Asp$^{397}$, it was substituted with another acidic amino acid, Glu. The impact of this substitution was less dramatic as the resulting mutant receptor was capable of inducing cAMP synthesis with only a slightly lower affinity at a lower maximum level. The different effects of Asp$^{397}$ and Glu$^{397}$ probably are attributable to the side chain. Although both have a negative charge at physiological pH, Asp is shorter than Glu by a CH$_2$ group. Due to this shorter side chain, the carboxylate ion of Asp is more rigid. This rigidity may explain why the carboxylate or carboxyl groups of Asp are used preferentially in active centers of proteins. It is also possible that the length of the side chain is critical for a correct interaction in tight areas. Our results indicate the importance in cAMP induction of Asp$^{397}$ due to its side chain and the negative charge. It is of interest to note that the positive charge of hCG C-terminal Lys$^{381}$ is essential for receptor activation (16, 17) and is involved in low affinity receptor binding (17), in contrast to the essential role of the negative charge of Asp$^{397}$ in receptor activation. Another interesting correlation between hCG and the LH/CG receptor is that the hCG C-terminal region is capable of low affinity receptor binding and receptor activation while the C-terminal half of the receptor is capable of low affinity hormone binding and receptor activation. Whether Asp$^{397}$ of the receptor interacts with Lys$^{381}$ of hCG is yet to be determined.

It is possible that unintended changes in other positions of the LH/CG receptor cDNA constructs might have affected the cAMP inducibility. To test this possibility the mutant receptor constructs were reverted to wild type by converting Asp$^{397}$, Ala$^{397}$, Lys$^{397}$, Arg$^{397}$, and Glu$^{397}$ in the mutant constructs to Asp$^{397}$. The cells transfected with the revertant constructs produced cAMP (Fig. 2). Furthermore, the affinity and maximum level of cAMP production were similar to those values of the wild type receptor, demonstrating that the cAMP inducibility was fully restored by the reversion. These results prove that the Asp$^{397}$ substitution itself was responsible for the loss of the cAMP inducibility.

Next, we decided to determine whether the loss of the cAMP inducibility is specifically due to the substitution of Asp$^{397}$ or whether it is a general aspect of the substitution of other acidic amino acids in extracellular loops of the C-terminal half. For this purpose, Asp$^{410}$ of the first exoloop and Asp$^{496}$ and Glu$^{496}$ of the second exoloop were individually converted to Asn or Gln. These substitutions did not have an impact on hormone binding or cAMP-induction ($p < 0.7$) as shown in Figs. 2 and 3. We have previously shown that hormone binding and cAMP induction were not noticeably affected by a similar substitution of Glu$^{496}$ of the third transmembrane domain or Asp$^{496}$ of the sixth transmembrane domain (14). On the other hand, the substitution of Asn for Asp$^{383}$ of the second transmembrane domain reduced the affinities for both hormone binding and cAMP induction (14). This negative effect of the Asp$^{383}$ substitution is strikingly different from the selective abolition of the cAMP inducibility but not hormone binding by the Asp$^{397}$ substitution. Furthermore, no Asp$^{382}$ substitution completely abolished the cAMP inducibility, as did some Asp$^{397}$ substitutions (data not included). Therefore, the loss of the cAMP inducibility upon the Asp$^{397}$ substitution appears to be specific. Taken together, these results underscore the specificity of the role of Asp$^{397}$ in cAMP induction.

In conclusion, our results not only demonstrate that receptor activation and signal generation are distinct from high affinity hormone binding in intact LH/CG receptors, but identify an amino acid important for the processes as well.

REFERENCES