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The Formation of a Salt Bridge Between Helices 3 and 6 Is Responsible for the Constitutive Activity and Lack of Hormone Responsiveness of the Naturally Occurring L457R Mutation of the Human Lutropin Receptor*

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The human lutropin receptor (hLHR) plays a pivotal role in reproductive endocrinology. A number of naturally occurring mutations of the hLHR have been identified that cause the receptor to become constitutively active. To gain further insights into the structural basis for the activation of the hLHR by activating mutations, we chose to examine a particularly strong constitutively activating mutation of this receptor, L457R, in which a leucine that is highly conserved among rhodopsin-like G protein-coupled receptors in helix 3 has been substituted with arginine. Using both disruptive as well as reciprocal mutagenesis strategies, our studies demonstrate that the ability of L457R to stabilize an active form of the hLHR is because of the formation of a salt bridge between the replacing amino acid and Asp-578 in helix 6. Such a lock between the transmembrane portions of helices 3 and 6 is concurrent with weakening the connections between the cytosolic ends of the same helices, including the interaction found in the wild-type receptor between Arg-464, of the (E/D)R(Y/W) motif, and Asp-564. This structural effect is properly marked by the increase in the solvent accessibility of selected amino acids at the cytosolic interfaces between helices 3 and 6. The integrity of the conserved amino acids Asn-615 and Asn-619 in helix 7 is required for the transfer of the structural change from the activating mutation site to the cytosolic interface between helices 3 and 6. The results of *in vitro* and computational experiments further suggest that the structural trigger of the constitutive activity of the L457R mutant may also be responsible for its lack of hormone responsiveness.

The lutropin receptor (LHR),[1] a member of the rhodopsin-like, Family A, G protein-coupled receptors (GPCRs), plays a pivotal role in reproductive physiology. The ligand for the LHR is either LH, produced by the pituitary in both males and females of postpubertal age, or hCG, produced by the placenta of pregnant females. LH and hCG are members of a family of related hormones termed the glycoprotein hormones, the other members of which are follitropin (FSH) and thyrotropin. The glycoprotein hormones are composed of two non-covalently associated subunits, a common α subunit and structurally related, but distinct, β subunits. The nearly identical nature of the β subunits of LH and hCG, however, permits the LHR to bind either hormone. The three glycoprotein hormone receptors, the LHR, FSHR, and TSHR, contain relatively large extracellular domains composed of multiple leucine-rich repeats that have been shown to confer high affinity binding of their respective ligands (see Refs. 1–3 for reviews). The very recently released crystal structure of hFSH in complex with the ectodomain of its receptor (4) represents the first step toward understanding the yet obscure mechanism of hormone-induced receptor activation of the glycoprotein hormone receptors.

In recent years, there have been numerous naturally occurring mutations of the gene for the human LHR (hLHR) that have been shown to result in constitutive activation of the receptor (reviewed in Refs. 5 and 6). These mutations have been found in young boys with gonadotropin-independent precocious puberty, also called testotoxicosis or familial male precocious puberty. In these boys, the heterozygous presence of a constitutively active hLHR results in the inappropriate synthesis of testosterone when pituitary LH levels are still prepubertal, thus causing an early onset of puberty. The activating mutations of the hLHR have been localized to different transmembrane helices, with many of them clustered within helix 6. Cells expressing an activating hLHR mutant exhibit elevated levels of basal cAMP as compared with cells expressing the wild-type (WT) hLHR, although not necessarily as high as those levels of cAMP observed in hLHR(WT)-expressing cells that are incubated with a saturating concentration of hCG. In particular, we had identified an activating mutation of the hLHR, L457$^{(3.43)}$R,[2] which exhibited elevated basal cAMP levels, but unresponsiveness to further hormonal stimulation despite maintaining a normal binding affinity for hCG (8). This

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[1] The abbreviations used are: LHR, lutropin receptor; hLHR, human lutropin receptor; GPCR, G protein-coupled receptor; hCG, human chorionic gonadotropin; FSH, follitropin; WT, wild-type; SAS, solvent-accessible surface; hFSHR, human follitropin receptor.

[2] The amino acid numbering in superscript is that proposed by Ballesteros and Weinstein (7). In this nomenclature, the first number indicates the helix and the numbers thereafter indicate the position of the helical residue relative to the most highly conserved residue within that helix, which is denoted as 50.

cellular phenotype is also observed in membranes isolated from L457$^{(3.43)}$R-expressing cells, where basal adenylyl cyclase activity is elevated in L457$^{(3.43)}$R membranes, but the activity is not increased further with hCG addition (9). Therefore, the L457$^{(3.43)}$R mutation appears to stabilize the hLHR in a structure that has intermediate activity (*i.e.* it is constitutively active but not as active as the agonist-occupied wt hLHR) and it is unable to transduce the binding of agonist into a fully active conformation.

The L457$^{(3.43)}$R mutation is localized to helix 3 and involves the substitution of a leucine residue that is highly conserved in Family A GPCRs (10, 11). Examination of hLHR mutants in which this leucine residue was mutated to arginine, lysine, histidine, alanine, or aspartate showed that only amino acids with positively charged side chains caused constitutive activation and concomitant hormone unresponsiveness (9). Computational models of the hLHR achieved by different approaches all converged toward the prediction that the positively charged side chains would be positioned such that they would interact with Asp-578$^{(6.44)}$ in helix 6, possibly perturbing the interactions between helices 6 and 7 (9). The aspartate at 6.44 is unique to and conserved within the glycoprotein hormone receptor family of GPCRs. The breakage of the H-bond found in the WT hLHR between Asp-578$^{(6.44)}$ and Asn-615$^{(7.45)}$ has been suggested to be the trigger of the constitutive activity induced by mutations of Asp-578$^{(6.44)}$ to Gly, Leu, His, Phe, and Tyr (12, 13). Thus, the integrity of Asn-615$^{(7.45)}$ and Asn-619$^{(7.49)}$ proved to be an essential requirement for the constitutive activity of the Asp-578$^{(6.44)}$ mutants (12).

The present studies, based upon *in vitro* and computational experiments, were undertaken to test the hypotheses inferred from the modeling of the hLHR(L457$^{(3.43)}$R) mutant regarding the basis for its constitutive activity and to explain its lack of hormone responsiveness. Our data do indeed support the predictions of computational modeling that a salt bridge between positions 3.43 and 6.44 promotes both the hormone-independent active state of the hLHR and the loss of hormone responsiveness. Furthermore, the integrity of Asn-615$^{(7.45)}$ and Asn-619$^{(7.49)}$ appears to be required for the full constitutive activity of the Leu-457 mutants.

MATERIALS AND METHODS

Hormones and Supplies—Highly purified hCG was purchased from Dr. A. Parlow and the NIDDK National Hormone and Pituitary Program and was iodinated as previously described (14). A less pure preparation of hCG (purchased from Sigma) was used only for the determination of nonspecific binding in ^{125}I-hCG binding assays. ^{125}I-cAMP and cell culture media were obtained from the Iodination Core and the Media and Cell Production Core, respectively, of the Diabetes and Endocrinology Research Center of the University of Iowa. Tissue culture reagents were purchased from Invitrogen and Corning plasticwares were obtained from Fisher Scientific.

Plasmids and Cells—The WT hLHR cDNA was kindly provided by Ares Advanced Technology (Ares-Serono Group, Randolph, MA) and was subcloned into pcDNA 3.1 (Invitrogen). Mutagenesis was performed using the PCR overlap extension method (15, 16). The entire region amplified by PCR, as well as the sites of ligation, were sequenced to ensure that there were no unintended mutations of the amplified cDNA. DNA sequencing was performed by the DNA Core of the Diabetes and Endocrinology Research Center of the University of Iowa.

Human embryonic 293 cells were obtained from the American Type Tissue Collection (CRL 1573) and were maintained at 5% CO_2 in growth media consisting of high-glucose Dulbecco's modified Eagle's medium containing 50 μg of gentamicin, 10 mM HEPES, and 10% newborn calf serum. Cells for experiments were plated onto 35-mm wells that had been precoated for 1 h with 0.1% gelatin in calcium and magnesium-free phosphate-buffered saline, pH 7.4. Cells were transiently transfected at 50–70% confluence following the protocol of Chen and Okayama (17) except that the overnight precipitation was performed in a 5% CO_2 atmosphere. Cells were then washed with Waymouth's MB752/1 media modified to contain 50 μg of gentamicin and 1 mg/ml bovine serum albumin, after which fresh growth media was added. The cells were used for experiments 24 h later.

For this study, in a given experiment cells were transfected with varying concentrations of plasmids encoding the hLHR(WT) as well as several other mutants. ^{125}I-hCG binding to intact cells and basal and hCG-stimulated cAMP were then determined within a given experiment as described below. Data were plotted using PRISM (GraphPad Software).

Binding Assays to Intact Cells Expressing the hLHR—HEK293 cells were plated onto gelatin-coated 35-mm wells and transiently transfected as described above. On the day of the experiment cells were placed on ice for 15 min and washed two times with cold Waymouth's MB752/1 containing 50 μg/ml gentamicin and 1 mg/ml bovine serum albumin but no sodium bicarbonate. To determine the maximal binding capacity, the cells were then incubated overnight at 4 °C in the same media containing a saturating concentration of ^{125}I-hCG (500 ng/ml) with or without an excess of unlabeled crude hCG (50 IU/ml). To terminate the assay, the cells were placed on ice and the contents of each well were scraped into a plastic tube on ice and combined with a 1-ml wash using cold Hanks' balanced salt solution modified to contain 50 μg/ml gentamicin and 1 mg/ml bovine serum albumin. The tubes were centrifuged at 4 °C and the pellets resuspended in 2 ml of the same wash media. After a second centrifugation, the supernatants were aspirated and the pellets counted in a γ counter.

Measurement of cAMP—HEK293 cells were plated on gelatin-coated 35-mm wells and transfected as described above. On the day of the experiment cells were washed twice with warm Waymouth's MB752/1 media containing 50 μg/ml gentamicin and 1 mg/ml bovine serum albumin and placed in 1 ml of the same medium containing 0.5 mM isobutylmethylxanthine. After 15 min at 37 °C, a saturating concentration of hCG (100 ng/ml final concentration) or buffer only was added and the incubation was continued for 60 min at 37 °C. The cells were then placed on ice, the media aspirated, and intracellular cAMP was extracted by the addition of 0.5 N perchloric acid containing 180 μg/ml theophylline and then measured by radioimmunoassay. All determinations were performed in triplicate.

Computational Modeling—The model of the hLHR employed in this work was achieved by comparative modeling by means of MODELLER (18), using the crystal structure of bovine rhodopsin as a template (19). The details of comparative modeling have been described elsewhere (13).

Among many different input arrangements tested, a unique structure was finally selected and used for building the input structures of the receptor mutants. These structures were obtained by substituting the mutated residue in the WT input structure by means of the molecular graphics package QUANTA (release 2000; www.accelrys.com). The WT and mutant structures were energy minimized and subjected to molecular dynamics simulations using CHARMM24 (20) following the computational protocol that was previously employed for simulating the *ab initio* hLHR model (21). For each single mutant, different starting conformations of the mutated side chain were probed by molecular dynamics simulations. These conformations were assigned by using different rotamer libraries and checking for the absence of bad contacts between the mutated side chain and its neighboring amino acid residues. The selected conformations of the substituting side chain in the single mutants were employed in the input structures of the multiple mutants. The receptor structures averaged over the 200 structures collected during the last 100 ps of a 150-ps molecular dynamics trajectory and minimized were finally employed for the comparative analysis.

RESULTS

As has been previously described (8, 9) and is shown herein in Fig. 1*A*, the naturally occurring mutation L457$^{(3.43)}$R in helix 3 of the hLHR results in marked constitutive activation of the receptor. This was determined in 293 cells transiently transfected with varying concentrations of plasmid encoding WT or mutant hLHR, yielding cells expressing a range of cell surface hLHR numbers as determined by ^{125}I-hCG binding to intact cells. As shown in Fig. 1*A*, at all concentrations of receptor, cells expressing hLHR(L457$^{(3.43)}$R) produced far more intracellular cAMP under basal conditions than cells expressing hLHR(WT), confirming the constitutively active nature of this mutant. Previous molecular dynamics simulations of the hLHR(L457$^{(3.43)}$R) mutant and hLHR(WT) suggested that in the WT receptor Asp-578$^{(6.44)}$ in helix 6 nor-

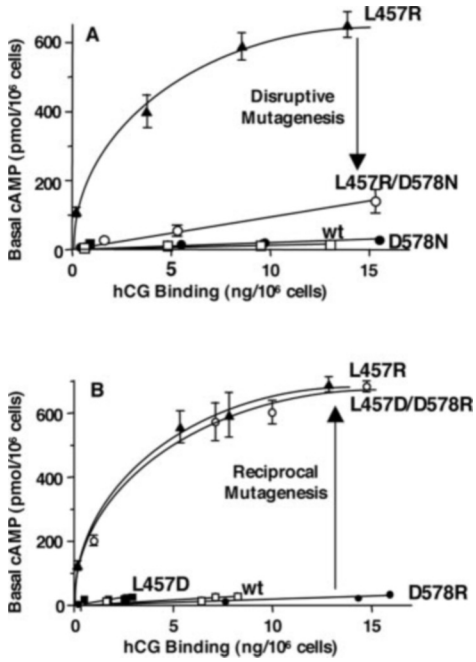

FIG. 1. **Formation of a charge-reinforced H-bond between positions 3.43 and 6.44 causes constitutive activation of the hLHR.** HEK293 cells were transiently transfected with increasing concentrations of cDNAs encoding the indicated hLHR constructs. In the same experiment, cell surface receptors (reflected by hCG binding assays to intact cells) and basal cAMP levels were determined as described under "Materials and Methods." One experiment, representative of at least three is shown. Data show the mean ± S.E. of triplicate determinations within the experiment.

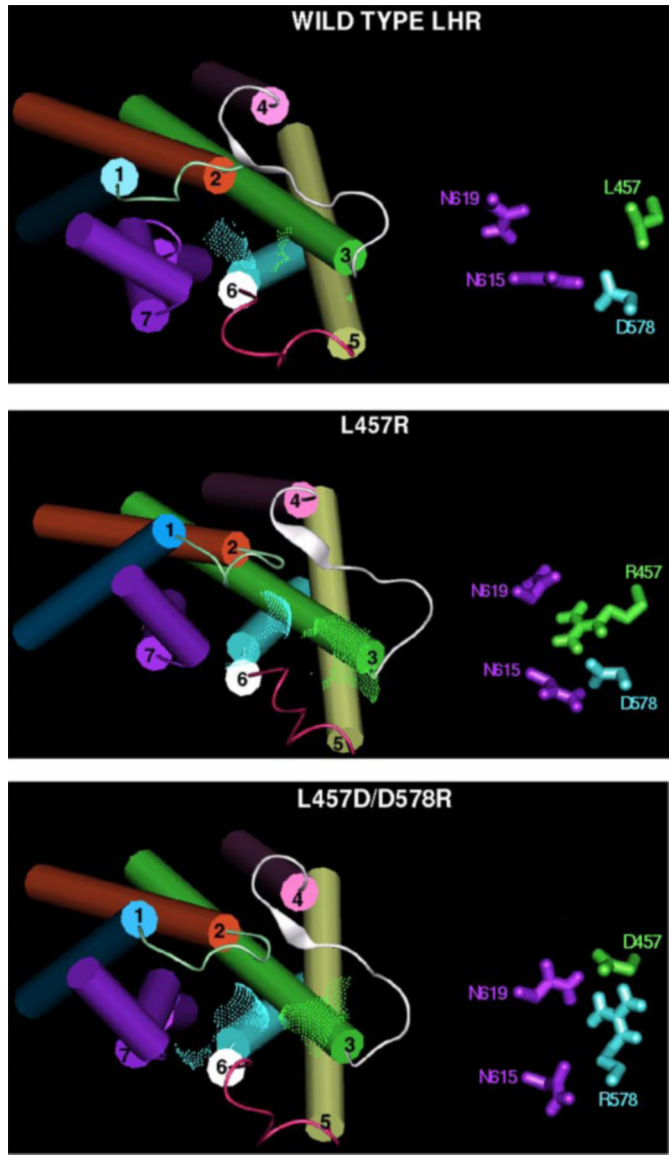

FIG. 2. **Average minimized structures of the wild-type hLHR, as well as of the L457$^{(3.43)}$R and L457$^{(3.43)}$D/D578$^{(6.44)}$R active mutants.** In the *left-hand side* of each panel, the helix bundles are shown, represented by *cylinders* and seen from the intracellular side in a direction perpendicular to the membrane surface. The extracellular domains are not shown. Helices 1, 2, 3, 4, 5, 6, and 7 are colored in *blue*, *orange*, *green*, *pink*, *yellow*, *cyan*, and *violet*, respectively, and the first, second, and third intracellular loops are colored in *light green*, *white*, and *magenta*, respectively. The solvent-accessible surface area computed over Arg-464$^{(3.50)}$, Thr-467$^{(3.53)}$, Ile-468$^{(3.54)}$, and Lys-563$^{(6.29)}$ is represented by *dots* colored according to the amino acid location. In the *right-hand side* of each panel, details of the interactions of the Leu-457$^{(3.43)}$ and Asp-578$^{(6.44)}$ mutants are shown. The amino acid side chains are colored according to their location.

mally interacts with Asn-615$^{(7.45)}$ and that replacements of Leu-457$^{(3.43)}$ in helix 3 with positively charged residues may establish a link between positions 3.43 and 6.44 via a charge-reinforced H-bond (9, 12, 13, 21). These predicted interactions in the WT and L457$^{(3.43)}$R receptors are shown in Fig. 2, *A* and *B*. To test this hypothesis, we used a strategy of both disruptive mutagenesis to first disrupt a putative interaction and then reciprocal (or complementary) mutagenesis to determine whether the interaction could be restored. If so, it would lend greater support to the effects of the disruptive mutagenesis being specific as opposed to being because of general allosteric alterations in receptor conformation.

To first examine a potential interaction of the arginine in helix 3 of the L457$^{(3.43)}$R mutant with Asp-578$^{(6.44)}$ in helix 6, we used the strategy of disruptive mutagenesis and created the double mutant L457$^{(3.43)}$R/D578$^{(6.44)}$N. If the arginine of the L457$^{(3.43)}$R constitutively active mutant is interacting with Asp-578$^{(6.44)}$ in helix 6, then we would expect this double mutant, in which Asp-578$^{(6.44)}$ was mutated, to have reduced constitutive activity. Fig. 1*A* depicts the basal activities as a function of receptor concentration of this double mutant as compared with the WT receptor, L457$^{(3.43)}$R alone and D578$^{(6.44)}$N alone. The single mutation of D578$^{(6.44)}$N behaved similarly to the WT receptor. Notably, the introduction of the D457$^{(3.43)}$N mutation into the L457$^{(3.43)}$R construct caused a marked reduction in constitutive activity as compared with L457$^{(3.43)}$R alone. These data suggest that the constitutive activity of L457$^{(3.43)}$R requires that the arginine interact with Asp-578$^{(6.44)}$. To confirm this, we performed the experiment shown in Fig. 1*B* in which this potential interaction was reestablished, but using a reciprocal amino acid pair. Thus, L457$^{(3.43)}$D/D578$^{(6.44)}$R was created to mimic an arginine-aspartate interaction between residue 457$^{(3.43)}$ with residue 578$^{(6.44)}$. Whereas in the L457$^{(3.43)}$R mutant this would entail a helix 3 arginine interacting with a helix 6 aspartate, in the L457$^{(3.43)}$D/D578$^{(6.44)}$R mutant it would now entail a reciprocal helix 3 aspartate interacting with a helix 6 arginine. As shown in Fig. 1*B*, the individual mutants L457$^{(3.43)}$D and D457$^{(3.43)}$R were not constitutively active. However, the reciprocal L457$^{(3.43)}$D/D578$^{(6.44)}$R mutant displayed as strong a constitutive activation as the L457$^{(3.43)}$R mutant. These data convincingly demonstrate that the arginine in helix 3 of the L457$^{(3.43)}$R mutant does indeed interact with Asp-578$^{(6.44)}$ in helix 6 and that this interaction stabilizes the hLHR in a constitutively active state.

The potential interactions of the arginine in the L457$^{(3.43)}$R constitutively active mutant with Asn-615$^{(7.45)}$ and

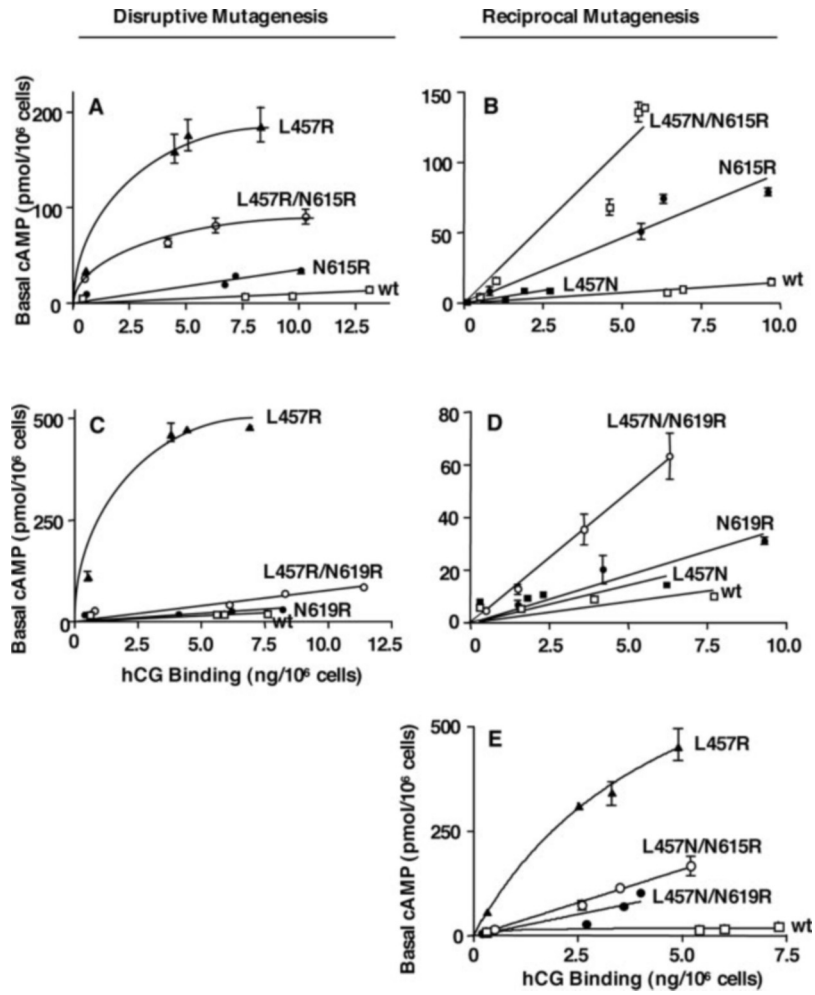

FIG. 3. **Potential interactions of the arginine in the L457$^{(3.43)}$R constitutively active mutant with Asn-615$^{(7.45)}$ and Asn-619$^{(7.49)}$ in helix 6.** HEK293 cells were transiently transfected with increasing concentrations of cDNAs encoding the indicated hLHR constructs. In the same experiment, cell surface receptors (reflected by hCG binding assays to intact cells) and basal cAMP levels were determined as described under "Materials and Methods." One experiment representative of at least three is shown. Data show the mean ± S.E. of triplicate determinations within the experiment.

Asn-619$^{(7.49)}$ in helix 7 were similarly examined. First examining Asn-615$^{(7.45)}$, a potential interaction with this residue was disrupted by the mutation N615$^{(7.45)}$R. As shown in Fig. 3*A*, the mutant N615$^{(7.45)}$R alone displayed a slight degree of constitutive activity compared with the WT hLHR examined in the same experiment. The introduction of N615$^{(7.45)}$R into the L457$^{(3.43)}$R mutant (*i.e.* the double mutant L457$^{(3.43)}$R/N615$^{(7.45)}$R) showed much greater constitutive activity than the WT hLHR, but only ~50% the constitutive activity of L457$^{(3.43)}$R. These data might implicate an interaction of Arg-457$^{(3.43)}$ with Asn-615$^{(7.45)}$. As such, a reciprocal mutant was made, L457$^{(3.43)}$N/N615$^{(7.45)}$R, which now has an asparagine as residue 457$^{(3.43)}$ and an arginine as residue 615$^{(7.45)}$. As shown in Fig. 3*B*, L457$^{(3.43)}$N alone displayed basal activity similar to the WT receptor. N615$^{(7.45)}$R alone displayed some constitutive activity. The reciprocal mutant L457$^{(3.43)}$N/N615$^{(7.45)}$R displayed even greater constitutive activity, although significantly lower compared with the L457$^{(3.43)}$R mutant (Fig. 5*A*). These data are not sufficiently supportive of a direct interaction between positions 3.43 and 7.45, in the L457$^{(3.43)}$R active mutant.

A similar pattern was observed when examining the potential interaction of the arginine in L457$^{(3.43)}$R with Asn-619$^{(7.49)}$. Thus, disruption of an interaction of the arginine in the L457$^{(3.43)}$R mutant with Asn-619$^{(7.49)}$ (as tested with the mutant L457$^{(3.43)}$R/N619$^{(7.49)}$R shown in Fig. 3*C*) resulted in a significant loss of constitutive activity as compared with L457$^{(3.43)}$R alone. The reciprocal mutant L457$^{(3.43)}$N/N619$^{(7.49)}$R was only slightly more active than L457$^{(3.43)}$R/N619$^{(7.49)}$R (Figs. 1, *D* and *E*, and 5*A*). These data are not suggestive of a direct interaction between positions 3.43 and 7.49 in the L457$^{(3.43)}$R active mutant.

Taken altogether, the data examining the basal activities of the hLHR and mutants thereof suggest that the naturally occurring hLHR mutant L457$^{(3.43)}$R causes constitutive activation as a result of interactions of the introduced arginine residue in helix 3 with Asp-578$^{(6.44)}$. The conserved asparagines in helix 7 are required for the constitutive activity of the L457$^{(3.43)}$R mutant, although their direct interaction with Arg-457$^{(3.43)}$ could not be inferred based upon the results of *in vitro* experiments.

As with the basal cAMP, hCG-stimulated cAMP was examined in 293 cells expressing increasing amounts of receptor, where in any given experiment the hLHR(WT) was compared with three to four different mutants within the same experiment. For the purposes of comparing the basal and hCG-stimulated activities of all the mutants, it was necessary to design a method that would accurately standardize the data. This was made more complicated by the observations that cAMP levels (both under basal and hCG-stimulated conditions) did not always increase linearly with receptor concentration. Therefore, one could not simply divide the cAMP levels by receptor expression. Furthermore, some mutants were expressed at the cell surface at very low levels even when using maximal concentrations of plasmid for transfections. Therefore, it was imperative that all the data be compared at a single receptor concentration where all the mutants were expressed. Fig. 4 depicts a representative experiment measuring cAMP under basal (*panel A*) and hCG-stimulated (*panel B*) conditions to demonstrate how all the data were ultimately analyzed. The

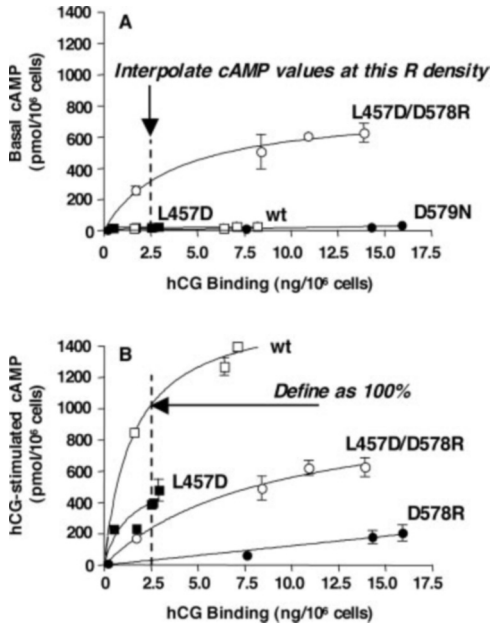

FIG. 4. **Standardization of basal and hCG-stimulated cAMP production over multiple experiments.** HEK293 cells were transiently transfected with increasing concentrations of cDNAs encoding the indicated hLHR constructs. In the same experiment, cell surface receptors (reflected by hCG binding assays to intact cells) and basal (*top panel*) or hCG-stimulated (*bottom panel*) cAMP levels were determined as described under "Materials and Methods." One experiment representative of at least three is shown. Data show the mean ± S.E. of triplicate determinations within the experiment. To normalize data over a number of experiments, the cAMP values observed at 2.5 ng of hCG binding/10^6 cells were interpolated from the curves of each experiment. Within a given experiment, the hCG-stimulated cAMP value thus obtained from cells expressing WT hLHR was defined as 100%.

mutant with the lowest level of cell surface expression was L457$^{(3.43)}$D. Therefore, we chose to analyze all the data at 2.5 ng of hCG binding/10^6 cells to ensure that cAMP levels would be detected for L457$^{(3.43)}$D and, therefore, for all other mutants. For each experiment then the cAMP levels from basal or hCG-stimulated cAMP cells were interpolated for this receptor density. When the response was linear, this determination was made mathematically using the slope of the line. When the response was non-linear, the interpolation was made graphically using expanded scales. To compare the data from different experiments, the results were then normalized to the hCG-stimulated cAMP produced by cells expressing the WT receptor in that particular experiment (Fig. 4*B*).

Fig. 5*A* depicts the basal and hCG-stimulated cAMP standardized for receptor number (all being taken from the same level of receptor expression) and expressed as a percentage of hCG-stimulated WT receptor. Fig. 5*B* expresses the hCG-stimulated cAMP levels as the -fold stimulation relative to the basal levels of cAMP for that mutant. The most notable feature is that all the mutants tested displayed a reduced hormone-stimulated cAMP response as compared with equal numbers of cell surface WT receptors. Thus, the most robust hCG-stimulated cAMP levels observed with any of the mutants were 50–60% of the maximal hCG-stimulated cAMP in cells expressing WT receptor.

Whether a mutant is defined as being responsive to hCG is, of course, also dictated by the basal levels of cAMP in the absence of any hormone. Thus, as has been shown before, L457$^{(3.43)}$R exhibits very high levels of basal cAMP, reflecting its strong constitutive activity, and hCG does not cause any further increase in cAMP levels (8, 9, 22). Although the basal levels of cAMP induced by L457R are quite high, they, nonetheless, remain only half of that observed in maximally stimulated hLHR(WT) cells. Therefore, the lack of hCG responsiveness by this mutant is not because the basal cAMP levels are already maximally stimulated. Hence, L457$^{(3.43)}$R is defined as being hormonally unresponsive. The reciprocal mutant L457$^{(3.43)}$D/D578$^{(6.44)}$R, in which the putative interaction between Arg-457$^{(3.43)}$ and Asp-578$^{(6.44)}$ in the L457$^{(3.43)}$R mutation is switched so that now the aspartate is contributed by helix 3 and the arginine by helix 6, displays as strong a constitutive activity as the L457$^{(3.43)}$R mutant. Interestingly, it too does not respond any further to hCG stimulation. Unlike the L457$^{(3.43)}$R and L457$^{(3.43)}$D/D578$^{(6.44)}$R mutants, the L457$^{(3.43)}$D and L457$^{(3.43)}$N mutants do not exhibit constitutive activity and they do respond to hCG. However, the amounts of cAMP produced in cells expressing the same numbers of L457$^{(3.43)}$R, L457$^{(3.43)}$D, and L457$^{(3.43)}$N are comparable (*i.e.* 50–60% of the hCG-stimulated cAMP in wt hLHR cells). These data suggest that any substitution of Leu-457$^{(3.43)}$ attenuates the hCG-stimulated cAMP response by 40–50% as compared with the WT receptor. Of further interest is the observation that L457$^{(3.43)}$R, which is constitutively active but unresponsive to further hormonal stimulation, when coupled with D578$^{(6.44)}$N, which is not constitutively active and is partially hormonally responsive, results in a double mutant (L457$^{(3.43)}$R/D578$^{(6.44)}$N) that is unresponsive to hormonal stimulation despite normal basal levels of cAMP. This is also observed within the context of the N615$^{(7.45)}$R and N619$^{(7.49)}$R mutants, both of which alone are partially responsive to hCG, but are devoid of hormonal responsiveness when coupled with L457$^{(3.43)}$R. However, Asn-615$^{(7.45)}$ and Asn-619$^{(7.49)}$, when coupled with L457$^{(3.43)}$N (where the same leucine is now substituted with asparagine rather than lysine), retain some hormonal responsiveness. Therefore, the substitution of Leu-457$^{(3.43)}$ with arginine either alone or in the context of other mutations, causes a major loss of hCG responsiveness. The only other mutation in this study to ablate hCG stimulation as strongly as the L457$^{(3.43)}$R mutation is D578$^{(6.44)}$R.

DISCUSSION

The revised ternary model for GPCR activation postulates that a given cell surface GPCR is in equilibrium between resting and active states and that the binding of agonist or the introduction of certain mutations stabilizes an active state of the receptor thereby shifting the equilibrium to that form of the receptor (23–25). Our knowledge of GPCR structure and function has been greatly facilitated by the determination in 2000 of the high-resolution crystal structure of the resting state of bovine rhodopsin (19), the cornerstone of the Family A subgroup of GPCRs (26). Despite there being only a few highly conserved residues and even fewer conserved motifs between the transmembrane helices of the hundreds of different Family A GPCRs, it is thought that these receptors share a structural mimicry such that the transmembrane helices likely adopt an overall structure similar to that of rhodopsin (11, 27). Collectively, the results of *in vitro* experiments aimed at structurally probing Family A GPCRs and those of sequence analyses suggest that comparative modeling of GPCRs using the rhodopsin structure as a template is likely to produce reliable results, at least for the 7-helix bundle. In this respect, almost all the LHR domains except for the N- and C-tails and the first extracellular loop could be modeled by deriving the structural restraints from the rhodopsin structure (1, 12, 13).

In the past several years, numerous constitutively activating mutations of the hLHR have been identified in boys with gonadotropin-independent precocious puberty (5, 6). All have been found to be due to substitutions of residues located with the seven-transmembrane helices of the receptor, with a large

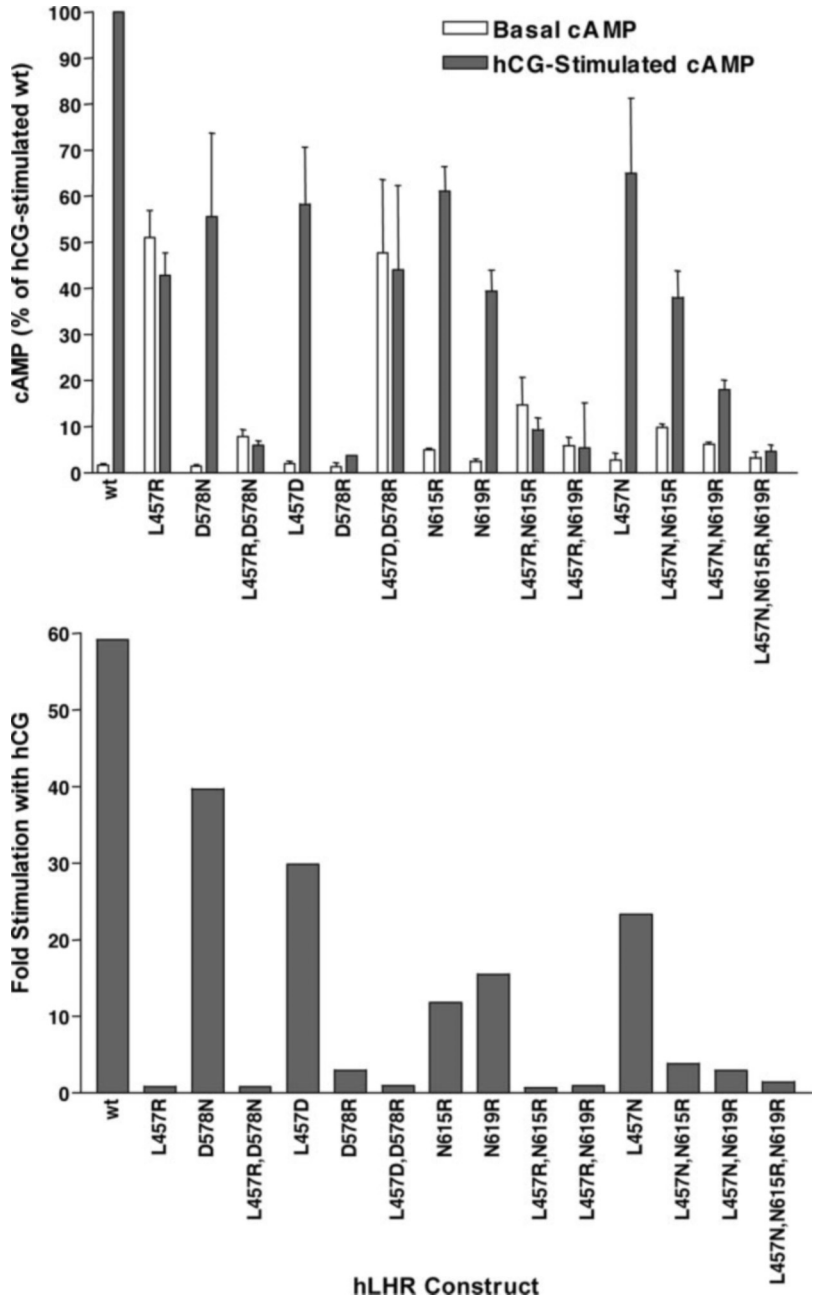

FIG. 5. **Summary of basal and hCG-stimulated cAMP levels in WT hLHR and mutants thereof expressed at the same cell surface densities.** HEK293 cells were transiently transfected with increasing concentrations of cDNAs encoding the indicated hLHR constructs. In the same experiment, cell surface receptors (reflected by hCG binding assays to intact cells) and basal hCG-stimulated cAMP levels were determined as described under "Materials and Methods." The cAMP values observed at 2.5 ng of hCG binding/10^6 cells were interpolated from the curves of each experiment. Within a given experiment, the hCG-stimulated cAMP value thus obtained from cells expressing WT hLHR was defined as 100%. The *top panel* shows the mean ± S.E. of data from at least three independent experiments for each construct and is expressed as the percent of hCG-stimulated cAMP in cells expressing the WT hLHR. In the *bottom panel*, the data from the *top panel* were used to express the hCG-stimulated cAMP as the -fold increase over basal.

percentage clustered within helix 6. Computational modeling of the hLHR suggested that a characteristic of the activating mutation sites is that they are close to highly conserved polar amino acids, including Arg-464$^{(3.50)}$ of the (E/D)R(Y/W) motif, and Asn-619$^{(7.49)}$ of the NP*X*X*Y* motif (reviewed in Ref. 1). Indeed, this latter site lies close to both Leu-457$^{(3.43)}$ and Asp-578$^{(6.44)}$, the targets of our mutational experiments. Whereas Leu-457$^{(3.43)}$ is almost conserved in Family A GPCRs (11), the aspartate at position 6.44 is unique to the glycoprotein hormone receptors. It was initially postulated that Asp-578$^{(6.44)}$ interacts via hydrogen bonds with both Asn-615$^{(7.45)}$ and Asn-619$^{(7.49)}$ and that disruption of these interactions causes constitutive activation (28). Subsequent *in vitro* and computational experiments targeting the constitutively active hLHR mutants of Asp-578$^{(6.44)}$ in combination with mutants of the two asparagines in helix 7 support the hypothesis that in the resting state Asp-578$^{(6.44)}$ is involved in a H-bond with Asn-615$^{(7.45)}$ and that weakening of such inter-helical interactions is the trigger of the constitutive activity of the different Asp-578$^{(6.44)}$ mutants only in the presence of intact Asn-615$^{(7.45)}$ and Asn-619$^{(7.49)}$ (12). Indeed, computational modeling highlighted the essential role of the two highly conserved asparagines in helix 7 in allowing the structural information transfer from the activating mutation site, *i.e.* Asp-578$^{(6.44)}$, to the cytosolic domains (12).

Of the many constitutively active mutants of the hLHR that have been described, the L457$^{(3.43)}$R mutant is of particular interest for several reasons. First, unlike most other constitutively activating mutations of the hLHR, L457$^{(3.43)}$R results in particularly strong constitutive activity. It should be noted, that despite its greater constitutive activity, L457$^{(3.43)}$R is nonetheless not as active as the agonist-occupied WT hLHR, suggesting that, as with other constitutively activating mutants, L457$^{(3.43)}$R likely stabilizes an intermediate state of activation of the receptor. Second, the L457$^{(3.43)}$R mutant is unresponsive to further hormonal stimulation despite its ability to bind hormone with high affinity (9, 29). Because both its constitutive activity as well as its unresponsiveness to hormone

TABLE I

hLHR construct	Location of mutations	SAS	Constitutive activity[a]
		$Å^2$	
WT		28	−
L457R	L457$^{(3.43)}$R	88	+++
D578N	D578$^{(6.44)}$N	29	−
L457R/D578N	L457$^{(3.43)}$R/D578$^{(6.44)}$N	35	+
L457D	L457$^{(3.43)}$D	24	−
D578R	D578$^{(6.44)}$R	44	−
L457D/D578R	L457D$^{(3.43)}$D/D578$^{(6.44)}$R	76	+++
N615R	N615$^{(7.45)}$R	43	+
N619R	N619$^{(7.49)}$R	23	−
L457R/N615R	L457$^{(3.43)}$R/ N615$^{(7.45)}$R	57	+
L457R/N619R	L457$^{(3.43)}$R/ N619$^{(7.49)}$R	30	+
L457N	L457$^{(3.43)}$N	35	−
L457N/N615R	L457$^{(3.43)}$N/ N615$^{(7.45)}$R	55	+
L457N/N619R	L457$^{(3.43)}$N/ N619$^{(7.49)}$R	37	+
L457N/N615R/N619R	L457$^{(3.43)}$N/ N615$^{(7.45)}$R/ N619$^{(7.49)}$R	50	−

[a] Those mutants that are not constitutively active are denoted by "−" and possess basal activities similar to the WT hLHR. Weakly constitutively active mutants are denoted by "+," and strongly constitutively active mutants by "+++."

can be observed in membranes isolated from L457$^{(3.43)}$R cells, we presume that the inability of L457$^{(3.43)}$R to be stimulated by agonist is because of a structural property of the mutant and not to downstream cellular effects mediated by the mutant (9). In other studies, we have shown that in cells expressing L457$^{(3.43)}$R or other activating mutants of the hLHR there is an induction of cAMP-dependent phosphodiesterase activity because of the constitutively elevated levels of intracellular cAMP (22). As such, when the WT hLHR or another G_s-coupled receptor such as the hβ_2-adrenergic receptor are co-expressed with L457$^{(3.43)}$R, there is an attenuation of agonist-stimulated cAMP production through the WT hLHR or other GPCR that is not observed if inhibitors of phosphodiesterase are present (22). This activation of phosphodiesterase activity by L457$^{(3.43)}$R cannot, however, account for the lack of hormonal responsiveness of this mutant because hormonal unresponsiveness is observed in isolated membranes and because the cAMP measurements done in intact cells are done in the presence of a phosphodiesterase inhibitor. We cannot, however, rule out the possibility of chemical modifications to the hLHR or protein associations with the hLHR that are stable to cell lysis. The third notable feature of the L457$^{(3.43)}$R mutant is that this mutation is the result of the substitution of a very highly conserved transmembrane residue. A recent analysis of the Family A GPCR transmembrane amino acid distributions found that leucine at position 3.43 was present in 74% of this class of GPCRs (11). Fourth, computational experiments on the L457$^{(3.43)}$R mutant had predicted that formation of a charge-reinforced H-bond between positions 3.43 and 6.44 is the main trigger of the hLHR activation induced by this mutant (9, 21).

The *in vitro* studies presented herein confirm that the activating nature of the L457$^{(3.43)}$R mutation of the hLHR is essentially because of the interaction of the introduced arginine at position 3.43 with Asp-578$^{(6.44)}$. Thus, the constitutive activity of the L457$^{(3.43)}$R mutant was diminished when the potential interaction of the introduced Arg-457$^{(3.43)}$ with Asp-578$^{(6.44)}$ was disrupted by mutagenesis. Importantly, the constitutive activity could then be restored when reciprocal mutations were reinstated, which could allow the salt bridge between positions 3.43 and 6.44.

The *in vitro* experiments done in this study were also analyzed *in silico* using the computational approach previously described (12, 13, 21). This approach consisted of comparing the structural features of the WT hLHR with those of constitutively active and functionally inactive hLHR mutants known thus far to define computational indices capable of differentiating between the non-active forms of the hLHR from the constitutively active ones (13, 21). According to the latest computational model of the hLHR, which is based upon the crystal structure of rhodopsin (19), the most significant feature that makes the constitutively active mutants different from the non-active ones was the increase in the solvent accessibility of the cytosolic extensions of helices 3 and 6 of the constitutively active mutants as compared with the WT hLHR (1, 12, 13). This effect was properly described by the solvent-accessible surface area (SAS) computed over amino acids Arg-464$^{(3.50)}$, Thr-467$^{(3.53)}$, Ile-468$^{(3.54)}$, and Lys-563$^{(6.29)}$, where Arg-464$^{(3.50)}$ is the arginine of the (E/D)R(Y/W) motif (1, 12, 13). Indeed, the SAS index was below 50 $Å^2$ in the WT hLHR and non-active hLHR mutants, whereas it was above that threshold in the constitutively active hLHR mutants. This computational index, defined based upon the structural comparison between spontaneous activating and inactivating mutations, was successfully challenged in its ability to predict the functionality of a number of engineered mutants of the hLHR (12, 13).

The predictive ability of the SAS index was challenged in this study as well. The simulated mutants together with the corresponding SAS index are listed in Table I. It is worth noting that the SAS is significantly above the threshold, a feature of the active forms, only in two cases, *i.e.* L457$^{(3.43)}$R and L457$^{(3.43)}$D/D578$^{(6.44)}$R, in which position 3.43 is linked by a charge-reinforced H-bond to positions 6.44 and, less frequently, to 7.49 (Fig. 2, *B* and *C*). These data strongly suggest that the salt bridge between the transmembrane portions of helices 3 and 6 would stabilize the hormone-independent active states of the hLHR. Along the same line, computational experiments showed that the L457$^{(3.43)}$D and the D578$^{(6.44)}$R single mutants, in which the salt bridge between positions 3.43 and 6.44 cannot be formed, share the features of the non-active hLHR forms (*i.e.* SAS below 50 $Å^2$), consistent with the results of *in vitro* experiments.

Molecular dynamics simulations also predict that L457$^{(3.43)}$R and L457$^{(3.43)}$D/D578$^{(6.44)}$R, the only two significantly active hLHR mutants as determined by *in vitro* experiments, share the release of the charge-reinforced H bonding interactions found in the WT hLHR between Arg-464$^{(3.50)}$ of the (E/D)R(Y/W) motif, at the cytoplasmic extension of helix 3, and both Glu-463$^{(3.49)}$ and Asp-564$^{(6.30)}$, the latter lying at the cytoplasmic end of helix 6. At least one of these two interactions is, in fact, present in all the remaining weakly active and non-constitutively active mutants (*i.e.* "+" and "−," respectively, in Table I). Thus, consistent with the results of *in vitro* experiments, computational modeling predicts only two mutants, *i.e.* L457$^{(3.43)}$R and L457$^{(3.43)}$D/D578$^{(6.44)}$R, as significantly constitutively active (*i.e.* "+++" in Table I). In this respect, either the N615$^{(7.45)}$R or N619$^{(7.49)}$R mutation exerts a

sort of antagonistic effect on the "active state" features of the L457$^{(3.43)}$R mutant. In other words, as already observed for the active mutants of Asp-578$^{(6.44)}$ (12), also for the L457$^{(3.43)}$R mutant, the integrity of Asn-615$^{(7.45)}$ and Asn-619$^{(7.49)}$ is required for an effective structure information transfer from the mutation site to the cytosolic interface between helices 3 and 6. This does not necessarily imply direct interactions between Arg-457$^{(3.43)}$ and the two conserved asparagines.

Computational modeling also suggests that a salt bridge between positions 3.43 and 6.44 triggers the constitutive activity of the L457$^{(3.43)}$R mutant rather than a mutation-induced perturbation in the H-bond between Asp$^{(6.44)}$ and Asn$^{(7.45)}$, an interaction suggested to stabilize the inactive form of the LHR (12, 13, 28). Indeed, Asp-578$^{(6.44)}$ may interact with both Arg-457$^{(4.43)}$ and Asn-615$^{(7.45)}$. These results are in line with mutagenesis studies from the homologous human follitropin receptor (hFSHR). In fact, as shown previously, the introduction of the L$^{(3.43)}$R mutation in the hFSHR results in marked constitutive activation of this receptor (30). However, whereas the D$^{(6.44)}$G mutation in the hLHR causes constitutive activation (12, 28, 31), the analogous D$^{(6.44)}$G mutation in the hFSHR is without effect (32). These studies suggest that, in contrast to the hLHR, the breakage of the H-bond between Asp$^{(6.44)}$ and Asn$^{(7.45)}$ in the hFSHR does not cause constitutive activation. Therefore, if the L$^{(3.43)}$R mutation in the hFSHR were inducing constitutive activation solely by disrupting the interaction between Asp$^{(6.44)}$ and Asn$^{(7.45)}$, constitutive activity would not be observed.

Collectively, the results of *in vitro* and computational experiments suggest that the constitutive activity and the loss of hormone responsiveness of the naturally occurring L457$^{(3.43)}$R mutant have a common structural determinant, *i.e.* the formation of a charge-reinforced H-bond between positions 3.43 and 6.44. This is suggested by the observation that the highest constitutively active mutants of Leu-457$^{(3.43)}$ share a charge-reinforced H-bond between positions 3.43 and 6.44 and are not responsive to hCG, whereas the non-constitutively active mutants of Leu-457$^{(3.43)}$, such as L457$^{(3.43)}$N and L457$^{(3.43)}$D, do not share such a structural link between positions 3.43 and 6.44 and these mutants respond to hCG. Thus, there is a correlation between hormonal unresponsiveness and the presence of a salt bridge between residues 3.43 and 6.44. Of course, a definitive elucidation of the structural causes of the lack of hCG responsiveness of the L457$^{(3.43)}$R mutant would require an atomistic knowledge of its hormone-bound form.

The results of our *in vitro* and computational experiments challenge the commonly accepted paradigm that associates constitutive activity essentially with the release of intramolecular interactions. Our data, in fact, suggest that the primary events in the hormone-independent activation of the hLHR also include the formation of strong structural links between the transmembrane domains, although these structural links may cause the release of interactions in the distal cytosolic domains. Indeed, in the case of the active mutants of Leu-457$^{(3.43)}$, the transmembrane portions and the cytosolic ends of helices 3 and 6 undergo, respectively, formation and breakage of an interhelical linkage. The latter involves the arginine of the (E/D)R(W/Y) motif, which is predicted to become more exposed to the cytosol in the active hLHR forms. Finally, the high performance of the SAS index in predicting the functionality of single and multiple mutants of Leu-457$^{(3.43)}$ strengthens the already probed hypothesis that computational modeling can be used for *in silico* functional screening of LHR mutants (12, 13).

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