Mutation of Individual Serine Residues in the C-terminal Tail of the Lutropin/Choriogonadotropin Receptor Reveal Distinct Structural Requirements for Agonist-induced Uncoupling and Agonist-induced Internalization*

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We have previously mapped the agonist-induced phosphorylation of the rat lutropin/choriogonadotropin receptor (rLHR) to a locus of four serines (Ser635, Ser639, Ser649, and Ser652) located in the C-terminal tail. The removal or mutation of this locus delays the time course of agonist-induced uncoupling of the rLHR from its effector system without affecting the overall magnitude of uncoupling, and it retards the endocytosis of the agonist-receptor complex.

We have now prepared and analyzed four new rLHR mutants in which each of these serines were individually mutated to alanines. The data presented show that each mutation reduces agonist-promoted rLHR phosphorylation by 20–40%. Mutation of Ser635 or Ser639 delayed the time course of agonist-induced uncoupling to about the same extent as the simultaneous mutation of all four serines. Mutation of Ser649 or Ser652 also retarded agonist-induced internalization, but the magnitude of this decrease was less than that induced by the simultaneous mutation of all four serines. Mutation of Ser649 had no effect on agonist-induced uncoupling but retarded agonist-induced internalization to the same extent as the simultaneous mutation of all four serines. Mutation of Ser652 has little or no effect on either of these two parameters.

Co-transfection studies with dominant-negative arrestins and dominant-negative dynamin reveal that, despite differences in their rates of internalization, rLHR wild-type, rLHR-S639A, and rLHR-S649A are internalized by an arrestin- and dynamin-dependent pathway.

These data show that the structural requirements needed for the agonist-induced uncoupling and internalization of the rLHR are distinct.

Phosphorylation of G protein-coupled receptors (GPCRs) on serine and/or threonine residues is an important event in agonist-induced desensitization. GPCR phosphorylation by second messenger-dependent kinases attenuates signaling by uncoupling the receptors from their cognate G proteins, whereas phosphorylation by the G protein-coupled receptor kinases (GRKs) facilitates the interaction of the receptors with a family of inhibitory proteins called arrestins (1, 2). This phosphorylated receptor-arrestin interaction uncouples the receptors from their cognate G proteins and targets the activated receptor to clathrin-coated pits for subsequent internalization (1–4). Thus, the complex formed by the phosphorylated GPCR and arrestin serves as a common intermediate for the uncoupling of the receptor from its cognate G protein and for receptor internalization.

Using human kidney 293 cells stably transfected with the rat lutropin/choriogonadotropin receptor (rLHR) cDNA, we showed that, like many other GPCRs, the rLHR becomes phosphorylated on serine residues when the cells are stimulated with an agonist (lutropin (LH) or choriogonadotropin (CG)) (5). The identity of the kinases that mediate the agonist-induced phosphorylation of the rLHR is not known; however, neither kinase A nor kinase C can fully account for the agonist-induced phosphorylation of the rLHR (5, 6). The involvement of one of the GRKs in the agonist-induced phosphorylation of the rLHR is suggested by the finding that overexpression of GRK2, GRK4, or GRK6 enhances agonist-induced phosphorylation.2 A GRK-catalyzed phosphorylation of the rLHR is also suggested by functional studies showing that co-transfection of the rLHR with GRK2 or GRK4 diminishes the hCG-induced cAMP response (7).

By analogy with what is known about other GPCRs (see above) we proposed that the agonist-induced phosphorylation of the rLHR was responsible for the agonist-induced uncoupling of this receptor from its effector system (5). Further analysis of rLHR mutants truncated at residues 653, 631, or 628 (designated rLHR-653R, rLHR-631R, or rLHR-628R) and a full-length rLHR mutant with multiple serine substitutions (designated rLHR-5S/T→A) mapped the agonist-induced phosphorylation to a cluster of four serine residues (Ser635, Ser639, Ser649, and Ser652) present in the C-terminal tail and established some functional consequences of phosphorylation of this

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1 The abbreviations used are: GPCR, G protein-coupled receptor; LH, lutropin/choriogonadotropin receptor; rLHR, rat LH receptor; wt, wild-type; GRK, G protein-coupled receptor kinase; LH, lutropin; CG, choriogonadotropin; hCG, human CG; βAR, β-adrenergic receptor; oLH, ovine lutropin; HA, hemagglutinin.
2 M. d. F. M. Lazari and M. Ascoli, unpublished observations.
cluster (6, 8, 9). Rat LHR-t653, a truncated form of rLHR that retains Ser635, Ser639, Ser649, and Ser652, displays little or no reduction in the agonist-induced phosphorylation, as well as a normal time course and magnitude of agonist-induced uncoupling. On the other hand, rLHR-t631 and rLHR-t628, two truncated forms of the rLHR that lack Ser635, Ser639, Ser649, and Ser652, and a full-length receptor mutant in which these four residues were simultaneously mutated to alanines (i.e. rLHR-5S/T-A) display a 90–100% decrease in agonist-induced phosphorylation and a delay in the rate of agonist-induced uncoupling. The magnitude of agonist-induced uncoupling observed under prolonged agonist stimulation is unaffected, however (8, 9).

It has been known for many years that one of the consequences of agonist binding to the LHR is the endocytosis of the agonist-receptor complex (10). Although the endocytosis of the agonist-bound LHR has been shown to occur via coated pits (11), the rate of endocytosis of the agonist-receptor complex is routed to the lysosomes, where both the agonist and the receptor are degraded (11, 14). This pathway ultimately leads to an agonist-induced reduction in the density of cell surface receptors by routing the receptor to a degradative route rather than a recycling pathway (14, 15). In keeping with current views on the internalization of other GPCRs, we have shown that the activation of the rLHR is necessary for efficient endocytosis (12, 13). Moreover, the importance of the phosphorylation of the four-serine locus mentioned above in the endocytosis of the receptor-bound agonist was documented by the finding that cells expressing rLHR-55/T-A internalize the bound agonist at a slower rate than cells expressing the wild-type rLHR (9).

The experiments presented here were designed to determine which of the four serine residues present in this locus of the rLHR become phosphorylated and to more carefully define the role of each of these residues in the agonist-induced uncoupling and internalization of the rLHR. To this end, we constructed and analyzed four new rLHR mutants in which Ser635, Ser639, Ser649, or Ser652 was individually mutated to an alanine residue in the context of the full-length rLHR. These mutants were analyzed for phosphorylation, uncoupling, and internalization.

**Materials and Methods**

**Plasmids and Cells**—The cloning of the rat luteal LH/CG receptor cDNA and the template plasmid containing the full-length coding region plus portions of the 5′- and 3′-untranslated regions of the wild-type rLHR cDNA have been described previously (16). The individual Ser to Ala mutants were constructed using polymerase chain reaction strategies to alter the nucleotides coding for these residues. The sequence of the entire region of each mutant cDNA generated by polymerase chain reaction was verified by automated DNA sequencing. The mutant and the entire region of each mutant cDNA generated by polymerase chain reaction was verified by automated DNA sequencing. The mutant and the entire region of each mutant cDNA generated by polymerase chain reaction was verified by automated DNA sequencing. The mutant and the entire region of each mutant cDNA generated by polymerase chain reaction was verified by automated DNA sequencing. The mutant and the entire region of each mutant cDNA generated by polymerase chain reaction was verified by automated DNA sequencing. The mutant and the entire region of each mutant cDNA generated by polymerase chain reaction was verified by automated DNA sequencing.

**Hormone Binding and Signal Transduction Assays**—Equilibrium binding parameters for hCG were measured during an overnight incubation (4 °C) of intact cells with a fixed concentration of [125I]hCG and increasing concentrations of hCG as described previously (6, 8). Concentration-response curves for the hCG-induced increases in cAMP accumulation were obtained by measuring total cAMP levels in cells that had been incubated with at least five different concentrations of hCG for 30 min at 37 °C in the presence of a phosphodiesterase inhibitor. The different parameters that describe the concentration response curves were calculated as described elsewhere (6, 8).

**Internalization Assays**—The endocytosis of [125I]hCG was measured in cells that had been briefly preincubated (i.e. 10 min at room temperature) with 40 ng/ml [125I]hCG. At the end of this preincubation, the free hormone was removed by washing, and the cells were re-incubated in fresh, hormone-free medium at 37 °C for up to 4 h. After the desired interval, the cells were placed on ice, and the medium was saved. The cells were briefly treated with an isotonic pH 3 buffer (10, 13). The radioactivity that was released by the acid treatment was considered to be surface-bound, whereas the radioactivity that remained cell-associated was considered to be internalized. The medium was precipitated with trichloroacetic acid, and the acid-insoluble and acid-soluble radioactivity were considered to be degraded and degraded hormone, respectively (10, 13). Because there is little or no dissociation of the receptor-bound hCG during this incubation (10), the rate of disappearance of the receptor-bound hCG can be used to measure the rate of internalization.

The rates of internalization (k)e were thus calculated from the slopes of linear regression fits to plots of the In of the surface-bound hCG versus time. The half-life of internalization (t₁/₂) is therefore defined as 0.693/kₑ.

**Immunoblots**—Expression of the transfected arrestins and dynamin was ascertained by immunoblots using the ECL system of detection. The different arrestin constructs were detected using a polyclonal rabbit anti-arrestin antibody (21) or a polyclonal rabbit antibody directed against the N-terminus of arrestin-1, which was obtained from Santa Cruz Biotechnology. The blots were developed using the ECL system of detection. The different arrestin constructs were detected using a polyclonal rabbit anti-arrestin antibody (21) or a polyclonal rabbit antibody directed against the N-terminus of arrestin-1, which was obtained from Santa Cruz Biotechnology.

3 All data were combined because no differences were noted when hLH or hCG were used.
shown that these two cell lines bind hCG with a Kd of 30,000 cpm/ng. [32P]Orthophosphate was obtained from NEN Life Sciences as described previously (24), to give a specific radioactivity of 25,000–30,000 cpm/ng. The response ratio shown in the far right was calculated by dividing the average maximal response obtained for hCG over the average maximal response obtained for cholera toxin. Each number represents the average ± S.E. of 3–6 independent experiments. The response ratio shown in the far right was calculated by dividing the average maximal response obtained for hCG over the average maximal response obtained for cholera toxin. Each number represents the average ± S.E. of three independent experiments for the cell lines expressing the mutant receptors or nine independent experiments for the cell lines expressing rLHR-wt. The results obtained with 293L(wt-12) and 293L(wt-17) were combined because they seem to be indistinguishable.

### Agonist-induced rLHR Phosphorylation—The Effect of individual Ser to Ala mutations on rLHR phosphorylation was determined by immunoprecipitation of the rLHR mutants in which each of these four serines was individually mutated to alanine (9). In more recent experiments, we switched to using oLH as the agonist, because the actions of LH and CG are indistinguishable under these conditions, but the rate of dissociation of the bound oLH is much faster than that of the bound hCG (25, 26). Thus, when using oLH as the agonist, most of the bound oLH dissociates from the rLHR prior to the immunoprecipitation, whereas a substantial amount of the hCG remains receptor-bound. This turns out to be an important consideration in immunoprecipitation experiments because some of the receptor antibodies that we have started to use (see under “Materials and Methods”) do not recognize the agonist-bound receptor. As such, when using hCG as the agonist, it is possible to misinterpret a decrease in the 32P signal as a decrease in phosphorylation, whereas in reality, the decrease is simply due to the inefficient immunoprecipitation of the hCG-receptor complex as compared with the free receptor.

### RESULTS

#### Preparation and Functional Properties of rLHR Mutants—
Phosphoamino acid analysis and phosphorylation experiments utilizing three different C-terminal truncations of the rLHR and a full-length mutant with multiple Ser to Ala mutations have identified Ser635, Ser639, Ser649, and Ser652 as the major locus of rLHR phosphorylation in transfected cells (6, 8, 9). For the experiments presented here, we prepared four new full-length rLHR mutants in which each of these four serines was individually mutated to alanine. Each mutant cDNA (designated rLHR-S635A, rLHR-S639A, rLHR-S649A, and rLHR-S652A) was transfected into human kidney 293 cells, and clonal lines stably expressing each of the mutants were obtained. All mutant receptors bound hCG with an affinity comparable to that detected in cells expressing rLHR-wt (i.e. about 200 pmol/cell, see Table I). Of the several clonal lines obtained with each mutant, we chose those with the highest cell surface receptor density for further study (Table I).

The data presented in Table I also show a comparison of the concentration response curves for hCG-induced cAMP accumulation in the clonal lines expressing the different mutants and in two clonal lines, 293L(wt-12) and 293L(wt-17), stably transfected with the rLHR-wt. We chose 293L(wt-12) and 293L(wt-17) as control cell lines because their receptor density (100,000–200,000 receptors/cell) is comparable to that of the cell lines expressing the mutant receptors, and at these levels of receptor expression, the cAMP response is basically independent of receptor density (6, 8, 19). In an attempt to correct for an inherent variability in the basal and hCG-stimulated levels of cAMP, we also measured the levels of cAMP in the different cell lines stimulated with cholera toxin and calculated a response ratio by dividing the maximal hCG response by the maximal cholera toxin response. As shown in Table I, this ratio shows that all mutants respond to hCG as well as or better than cells expressing rLHR-wt. It is noted that the concentration of hCG required to elicit the half-maximal cAMP response is 1.5–2.0-fold higher in the cells expressing the mutants than in the cells expressing rLHR-wt. This finding is consistent with previous data obtained using a mutant rLHR in which all four serine residues in question were simultaneously mutated to alanine (9).

#### Hormones and Supplies—
Purified hCG (CR-127) and oLH (AFP-5551B) were obtained from the National Hormone and Pituitary Agency of the NIDDK, National Institutes of Health. [125I]hCG was prepared as described previously (24), to give a specific radioactivity of 25,000–30,000 cpm/ng. [32P]Orthophosphate was obtained from NEN Life Sciences. Phosphate-free DMEM was purchased from ICN Biomedicals (Irvine, CA). Nonidet P-40, protease inhibitors, N,N',N'-triacetylchitotriose, protein A-agarose, and bovine serum albumin were from Sigma. Okadaic acid and cyclosporin were purchased from Alexis Biochemicals (Woburn, MA). Wheat germ agglutinin agarose was from Vector Laboratories. Cell culture supplies and reagents were obtained from Corning (Corning, NY) and Life Technologies, Inc., respectively. All other materials were obtained from commonly used suppliers.

### Table I

| Cell line               | Kd (pmol/10^6 cells) | B_max (pmol/10^6 cells) | Basal (pmol/10^6 cells) | hCG response | Cholera toxin response |
|-------------------------|----------------------|-------------------------|-------------------------|--------------|------------------------|
| 293L(wt-12) or 293L(wt-17) | ND                   | ND                      | ND                      | ND           | ND                     |
| 293L(S635A-5)           | 4.3 ± 0.7            | 39 ± 7                  | 2678 ± 489              | 1242 ± 196   | 2.16                   |
| 293L(S639A-5)           | 4.4 ± 1.2            | 70 ± 13                 | 4541 ± 220              | 1331 ± 68    | 3.41                   |
| 293L(S649A-14)          | 2.6 ± 0.5            | 62 ± 21                 | 2338 ± 775              | 830 ± 181    | 2.82                   |
| 293L(S652A-5)           | 1.8 ± 0.4            | 75 ± 26                 | 538 ± 41                | 330 ± 21     | 2.94                   |
| 293L(S639A-5)           | 1.6 ± 0.4            | 86 ± 36                 | 744 ± 187               | 360 ± 68     | 2.07                   |

* Equilibrium binding parameters were determined using intact cells during an overnight incubation at 4 °C as described under “Materials and Methods.” Each number represents the average ± S.E. of 4–6 independent experiments.

* Total (i.e., cells + medium) levels of cAMP were measured in cells incubated in the presence of a phosphodiesterase inhibitor and increasing concentrations of hCG for 30 min or in cells incubated with a single concentration (500 ng/ml) of cholera toxin for 2 h, as described under “Materials and Methods.”
of the wt and mutant receptors were immunoprecipitated, resolved on SDS gels, and visualized by autoradiography as shown in Fig. 1. The results of several experiments, such as those shown in Fig. 1, were quantitated by densitometry, and these are summarized in Table II. The results obtained with rLHR-wt are in agreement with previous data showing that this receptor is phosphorylated in unstimulated cells and that agonist-stimulation elicits a 2-fold increase in the \( ^{32} \)P signal (5, 6, 8, 9). The results summarized in Table II also show that the individual mutation of Ser635, Ser639, Ser649, or Ser652 to Ala diminishes agonist-induced phosphorylation to 57–78% of control. Because the removal or simultaneous mutation of all these four serines reduces phosphorylation to barely detectable levels (6, 8, 9), the simplest interpretation of the data shown in Table II is that all four serines are phosphorylated when cells are stimulated with agonist.

If all four serines are phosphorylated to the same extent, each individual mutation should reduce phosphorylation to 75% of the rLHR-wt. However, a reduction of this magnitude \((i.e. \ 78\% \ of \ rLHR-wt)\) was found only with the rLHR-S635A mutant. The other three mutations reduced phosphorylation to 57–65% of the rLHR-wt, suggesting that phosphorylation is hierarchical or that not all residues are phosphorylated to the same extent. Additional studies to distinguish between these possibilities were not performed because the relatively low increase in agonist-promoted phosphorylation detected in rLHR-wt \((\sim 2\)-fold; see Table II\) makes the quantitation of partial changes in rLHR-phosphorylation rather difficult.

**Agonist-induced Uncoupling**—The time course of agonist-induced uncoupling of the LH/CG-sensitive adenylyl cyclase in 293 cells expressing rLHR-wt suggests the existence of two phases: a fast phase, which occurs within 15 min of agonist addition and leads to a 40–60% reduction in agonist-stimulated cAMP synthesis, and a slower phase, which leads to a further 20–40% reduction in the agonist-stimulated cAMP synthesis (Fig. 2 and Refs. 8 and 9). Because the removal or mutation of Ser635, Ser639, Ser649, and Ser652 affects the time course, rather than the magnitude of agonist-induced uncoupling (cf. Fig. 2 and Refs. 8 and 9), the effect of the individual Ser to Ala mutations on this process were initially tested by challenging cells with agonist after a short \((i.e. \ 15\)-min\) pre-incubation with or without agonist. Work also

| Cell line | Agonist-promoted rLHR phosphorylationa | Residual agonist-induced cAMP response \( \% \) of control |
|-----------|----------------------------------------|----------------------------------|
| 293L(wt-12) or 293L(wt-17) | 2.3 ± 0.4 (100%) | 43 ± 6 |
| 293L(S635A-5) | 1.8 ± 0.1 (78%) | 40 ± 8 |
| 293L(S639A-5) | 1.4 ± 0.2 (61%) | 69 ± 4 |
| 293L(S649A-14) | 1.3 ± 0.1 (57%) | 37 ± 7 |
| 293L(S652A-5) | 1.5 ± 0.2 (65%) | 35 ± 5 |
| 293L(S5/T → A-2) | ND | 74 ± 1 |

* Individual phosphorylation experiments were done with one of the cell lines stably transfected with a mutant receptor and either 293L(wt-12) or 293L(wt-17) cells as shown in Fig. 1. The autoradiograms were scanned and quantitated using a BioRad Molecular Imaging System, and the magnitude of the signal measured in agonist-treated cells was expressed as fold over basal. Basal levels of receptor phosphorylation varied by at most 30% in all cell lines, except for cells expressing rLHR-S639A, in which basal phosphorylation was enhanced 2–3-fold when compared to cells expressing rLHR-wt (cf. Fig. 1). The results presented for cells expressing the rLHR-wt are the average ± S.E. of six independent experiments. The results presented for the other cell lines are the mean ± range of two independent experiments. The numbers in parentheses represent percentages relative to the cells expressing rLHR-wt.

† Individual uncoupling experiments were done using two of the cell lines expressing mutant receptors and either 293L(wt-12) or 293L(wt-17) as a control. Individual cell lines were initially divided into two groups and preincubated without (group A) or with (group B) a saturating concentration of agonist for 15 min at 37 °C to induce receptor uncoupling. At the end of this preincubation, all cells were washed with neutral and acidic buffers (see under “Materials and Methods”), and each group of cells was divided into two subgroups. These were further incubated without (groups A1 and B1) or with (groups A2 and B2) a saturating concentration of agonist for 15 min at 37 °C. Intra cellular levels of cAMP were measured at the end of this incubation (in duplicate samples) and agonist-induced uncoupling was calculated as follows: [(B2 – B1)/(A2 – A1)] × 100. For example, in one experiment, the levels of cAMP (in pmol/10⁶ cells) for 293L(wt-12) cells were 245 for group A2, 17 for group A1, 150 for group B2, and 50 for group B1. Thus, in this case, agonist-induced uncoupling was calculated to be \[[(150 – 50)/(245 – 17)] × 100 = 44\%\]. In the same experiment, the levels of cAMP for 293L(S649A-15) were 140 for A2, 14 for A1, 99 for B2, and 43 for B1. Thus, in this case, agonist-induced uncoupling was calculated to be \[[(99 – 43)/(140 – 14)] × 100 = 44\%\]. In the same experiment, the levels of cAMP for 293L(S652A-5) were 116 for A2, 14 for A1, 83 for B2, and 35 for B1. Thus, in this case, agonist-induced uncoupling was calculated to be \[[(83 – 35)/(116 – 14)] × 100 = 47\%\]. The results presented are the average ± S.E. of 3–4 (mutant cell lines) or 12 (wt cell lines) independent experiments. The results obtained with 293L(wt-12) and 293L(wt-17) were combined because they seem to be indistinguishable.

‡ Not determined during the course of these experiments. However, previous results from this laboratory have shown that the basal and agonist-induced phosphorylation of rLHR-S5/T → A are barely detectable (9).
Uncoupling and Internalization of the LH/CG Receptor

One of two cell lines stably expressing rLHR-wt (either 293L(wt-12) or 293L(wt-17)) ( ), 293L(S639A-5) (○), or 293L(S5/T→A-2) (△) cells were preincubated with agonist for the indicated times at 37 °C. The free and bound agonist were then removed (see under “Materials and Methods”), and the cells were divided into two groups and incubated without or with 100 ng/ml hCG for 15 min at 37 °C. At the end of this incubation, the medium was aspirated, and the cells were used to determine the intracellular cAMP content. The amount of cAMP accumulated in the group of cells incubated without agonist was then subtracted from that present in the cells incubated with agonist, and the results were expressed as a percentage of the cAMP response determined under identical conditions but using cells that were preincubated without agonist (see under “Materials and Methods” and the legend to Table II). Each data point represents the average ± S.E. of four independent experiments. The results obtained using 293L(wt-12) and 293L(wt-17) cells were combined because they were indistinguishable.

In agreement with previous results (8, 9), the time course of uncoupling demonstrated by the clone 293L(wt-17) was intermediate between those of the clones expressing the wild-type receptor (293L(wt-12)) and rLHR-S639A cells. These results show that the early time course of uncoupling is retarded to the same extent in cells expressing rLHR-5S/T→A or rLHR-S639A and that the magnitude of uncoupling detected at later time points (i.e. 60 min or later) is basically the same in cells expressing rLHR-wt or either of these two mutants.

Agonist-induced Internalization—The effect of individual serine mutations of the rLHR on the endocytosis of hCG was measured using a protocol that allows us to follow one round of endocytosis of the bound hormone (10, 13). The half-life of internalization of hCG in cells expressing the rLHR-wt is slow (1–2 h, depending on the cell type; see Refs. 9, 10, 12, and 13) when compared with the half-life of internalization of many other ligand-receptor complexes (27). In agreement with previous data, the results summarized in Fig. 3 and Table III show that 293 cells expressing rLHR-wt (either 293L(wt-12) or 293L(wt-17)) internalize the bound hCG with a half-life of 139 min. These data also show that mutation of Ser652 had little or no effect on internalization, mutation of Ser635 or Ser639 increased the half-life of internalization 1.4–1.6-fold, and the mutation of Ser649 increased the half-life of internalization 2.8-fold. In fact, the half-life of internalization of hCG in cells expressing rLHR-S649A is similar to that measured in parallel experiments using cells expressing rLHR-5S/T→A, a mutant in which all four serines were simultaneously mutated to alanines (Table III).
TABLE III  
Rates of internalization of hCG in clonal cell lines expressing the wild-type and mutant receptors  

| Cell line                          | Half-life of internalization (min) |
|-----------------------------------|-----------------------------------|
| 293L(wt-12) or 293L(wt-17)        | 139 ± 10                          |
| 293L(S635A-5)                     | 225 ± 32                          |
| 293L(S639A-5)                     | 196 ± 25                          |
| 293L(S649A-14)                    | 392 ± 31                          |
| 293L(S652A-5)                     | 171 ± 15                          |
| 293L(S2T → A-2)                   | 551 ± 24                          |

Based on these results we conclude that Ser<sup>635</sup>, Ser<sup>639</sup>, and Ser<sup>649</sup> are needed for internalization and that the role of Ser<sup>649</sup> is particularly important.

**Roles of Arrestin and Dynamin on Agonist-induced Internalization and Uncoupling**—In order to learn more about the structural requirements for the agonist-induced internalization of the rLHR, we examined the effects of nonvisual arrestins, dynamin, and mutants thereof on the internalization of rLHR-wt, rLHR-S639A (one of the two mutants that slows down internalization and uncoupling; cf. Figs. 2 and 3, and Tables II and III), and rLHR-S649A (a mutant that blocks internalization but does not affect uncoupling; cf. Fig. 3 and Tables II and III). As already mentioned above, nonvisual arrestins are clathrin-binding proteins that act as adapters, linking GPCRs to clathrin-coated pits (3). Two mutant forms of β-arrestin, β-arrestin-V53D and β-arrestin(319–418), act as dominant negative mutants of arrestin-mediated GPCR internalization because they have reduced binding affinities for the phosphorylated GPCRs (4). Dynamin is a GTPase that participates in the fission of endocytic vesicles from the plasma membrane (28). Dynamin mutants that are deficient in GTP binding (such as dynamin-K44A) block coated pit-mediated internalization of several receptors (17, 29, 30).

The effects of arrestin-3, β-arrestin(319–418), or dynamin-K44A on the internalization of hCG mediated by rLHR-wt, rLHR-S639A, or rLHR-S649A were examined using transient co-transfection assays. The expression of each of these constructs has been documented before (4, 31), and it was verified in the present experiments using Western blots, as described under “Materials and Methods” (data not shown). In agreement with the results obtained with the stably transfected cell lines (cf. Fig. 3), the results presented in Fig. 4 show that the internalization of hCG is greater in cells transiently transfected with rLHR-wt (42% of the bound hormone was internalized and/or degraded during a 2-h internalization assay) than in cells transfected with rLHR-S639A (55% of the bound hormone was internalized and/or degraded) and much higher than in cells transfected with rLHR-S649A, in which only 25% of the bound hormone was internalized and or degraded. Co-transfection with arrestin-3 increased the internalization of hCG mediated by all three receptors by about 2-fold, but the internalization of hCG detected in cells co-transfected with rLHR-S649A and arrestin-3 (56%) was lower than that detected in cells co-transfected with rLHR-wt and arrestin-3 (75%) or rLHR-S639A and arrestin-3 (73%).

An involvement of nonvisual arrestin in the endocytosis of hCG is best documented by the finding that the two dominant-negative mutants of β-arrestin inhibit hCG internalization (Fig. 4). In agreement with previous data obtained with the β<sub>2</sub>-adrenergic receptor (4), we found that β-arrestin(319–418) is somewhat more effective than β-arrestin-V53D in inhibiting the internalization of hCG. The effects of these two dominant-negative arrestins seem to be similar for rLHR-wt, rLHR-S639A, and rLHR-S649A, however. The inhibitory effects of dynamin-K44A on the endocytosis of hCG (40–60% reduction) were somewhat more pronounced than those of the dominant-negative arrestins but were similar for rLHR-wt, rLHR-S639A and rLHR-S649A. Lastly, treatment of 293 cells expressing rLHR-wt with hypertonic sucrose, another manipulation that disrupts the assembly of clathrin-coated pits (32) also inhibited the endocytosis of hCG by about 50% (data not shown).

We also attempted to use transient co-transfection assays to test for the putative involvement of arrestin on the agonist-induced uncoupling of the rLHR. Unfortunately, the methods used to measure uncoupling in stably transfected cell lines (see legend to Table II) could not be used in transiently transfected cells because of their relatively weak cAMP response. As such, we were forced to measure uncoupling simply by measuring the magnitude of the cAMP response elicited by hCG in 293 cells transiently transfected with the rLHR-wt alone or together with different arrestin constructs. This paradigm has been used before by Premont et al. (7) to show that co-transfection of 293 cells with the rLHR-wt and GRK2 or GRK4 reduces the hCG-induced cAMP response of cells transfected with rLHR-wt only. The results of these experiments are presented in Table IV; they show that under these experimental conditions, arrestin-3 or β-arrestin(319–418) has a slight stimulatory effect on the hCG-induced cAMP response. Thus, under the same experimental conditions where we can readily measure opposite effects of arrestin and dominant-negative arrestins on the agonist-induced internalization of the rLHR (cf. Fig. 4), we cannot measure opposite effects on uncoupling.

Taken together, these results show that the agonist-induced internalization of the rLHR is arrestin-dependent. They also suggest that the agonist-induced uncoupling of the rLHR is arrestin-independent.

**Discussion**

Together with previous data from this laboratory (5, 6, 8, 9), the experiments presented here are consistent with a model in which four serines present in the C-terminal tail of the rLHR (Ser<sup>635</sup>, Ser<sup>639</sup>, Ser<sup>649</sup>, and Ser<sup>652</sup>) are phosphorylated in response to agonist stimulation. This accommodates the findings that the removal or mutation of Ser<sup>635</sup>, Ser<sup>639</sup>, Ser<sup>649</sup>, and Ser<sup>652</sup> decreases basal and agonist-induced phosphorylation by at least 90% (6, 8, 9), whereas the individual serine mutations reduce agonist-induced phosphorylation only partially (Table II). However, the reduction in the magnitude of phosphorylation detected with each mutation suggests that all four serines are not phosphorylated to the same extent or that there is a

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5 In agreement with other studies (31), preliminary experiments (not presented) performed with β-arrestin or arrestin-3 showed that these two enhanced the internalization of hCG to the same extent. Most of the experiments presented here were done using arrestin-3.

6 Complete dose-response curves for the effects of hCG on cAMP accumulation in the transiently transfected cells were not obtained. It should be noted, however, that in the case of the rLHR, agonist-induced uncoupling is due mostly (or entirely) to a decrease in the maximal cAMP response, rather than to changes in the sensitivity of this response (9). As such, the use of a single, maximally effective concentration of hCG is a reliable indicator of uncoupling (7, 9).
Ser649, and Ser652 (9) can be fully reproduced by the mutation of either Ser635 or Ser639 (Table II and Fig. 2), whereas the independent transfections. After washing (to remove the free hormone), the cells were incubated for 2 h at 37 °C to allow for processing of the surface-bound hormone, and the amounts of surface-bound, internalized, and degraded radioactivity were measured as described under “Materials and Methods” and in the legend to Fig. 2. The internalized and degraded radioactivity levels were added manually and were expressed as a percentage of the total hormone present (i.e. surface bound, internalized, degraded, and dissociated). Each column represents the average ± S.E. of three independent experiments. The numbers within each column represent percentages relative to the control cells (i.e. those transfected with the receptor plus empty vector plasmids).

### Table IV

| Plasmids co-transfected with rLHR-wt | Additions to medium | cAMP accumulation pmol/10⁶ cells |
|-------------------------------------|---------------------|---------------------------------|
|                                     | Buffer             | 100 ng/ml hCG                   |
| Empty vector                        | 5 ± 3              | 78 ± 24                         |
| Arrestin-3                          | 4 ± 2              | 118 ± 28                        |
| β-Arrestin(319–418)                 | 4 ± 1              | 126 ± 54                        |

Effects of arrestin and dominant-negative arrestin on the hCG-induced cAMP response of cells transiently expressing rLHR-wt

293 cells plated in 100-mm dishes were transfected with 10 ng of expression vectors encoding for rLHR-wt together with 10 ng of empty vector, arrestin-3, β-arrestin-V53D, β-arrestin(319–418), or dominant-K44A, as indicated. After an overnight transfection, the cells were washed, trypsinized, distributed into six-well plates, and used for internalization assays 24 h later (see under “Materials and Methods” for details). A single round of internalization assays was performed by first incubating the cells with 40 ng/ml [125I]hCG for 10 min at room temperature. After washing (to remove the free hormone), the cells were incubated for 2 h at 37 °C to allow for processing of the surface-bound hormone, and the amounts of surface-bound, internalized, and degraded radioactivity were measured as described under “Materials and Methods” and in the legend to Fig. 2. The internalized and degraded radioactivity levels were added manually and were expressed as a percentage of the total hormone present (i.e. surface bound, internalized, degraded, and dissociated). Each column represents the average ± S.E. of three independent experiments. The numbers within each column represent percentages relative to the control cells (i.e. those transfected with the receptor plus empty vector plasmids).

The data presented here clearly show that the attenuation of the agonist-induced uncoupling that results as a consequence of the removal (8) or mutation of Ser635, Ser639, Ser649, and Ser652 (9) can be fully reproduced by the mutation of either Ser635 or Ser639 (Table II and Fig. 2), whereas the slower rate of agonist-induced internalization detected in the multiple substitution mutant (9) can be fully reproduced by mutation of Ser649 (Table III and Fig. 3). The mutation of Ser652 had little or no effect on agonist-induced uncoupling or internalization. Taken together, these data show that the structural requirements for agonist-induced uncoupling and agonist-induced internalization of the rLHR are different. Some overlap between these structural motifs is suggested by the finding that mutation of Ser635 or Ser639, which induced maximal attenuation of uncoupling, also induced partial attenuation of internalization (Figs. 2 and 3 and Tables II and III).

One of the most interesting recent advances in our understanding of the biology of GPCRs is the realization that the complex formed by the phosphorylated β₂-adrenergic receptor (β₂AR) and arrestin plays a pleiotropic role in the regulation of β₂AR function (3, 4, 33, 34). Thus, it is now generally accepted that the ORK-catalyzed phosphorylation of the β₂AR increases the affinity of the β₂AR for β-arrestin and that the formation of the phosphorylated β₂AR-arrestin complex sterically hinders the β₂AR-G protein association. Because β-arrestin can also bind clathrin with high affinity, the β₂AR-arrestin complex is targeted for internalization through clathrin-coated pits. Some variations on this theme have also begun to emerge, as illustrated by the finding that agonist stimulation of the AT₁A angiotensin (29) and the m2 muscarinic receptors (31) leads to receptor phosphorylation, but the phosphorylated receptors are internalized by a pathway that does not require the participation of arrestin or clathrin-coated pits. However, overexpression of nonvisual arrestins forces the agonist-stimulated AT₁A angiotensin and m2 muscarinic receptors to be internalized via clathrin-coated pits by a pathway that requires nonvisual arrestins (29, 31).

The data presented here for the rLHR-wt expressed in 293 cells shows that the agonist-induced internalization of the rLHR, like that of the β₂AR expressed in 293 cells, requires the participation of arrestin and clathrin-coated pits (Fig. 4). Thus, the overexpression of two dominant-negative forms of β-arrestin and a dominant-negative form of dynamin, as well as a treatment
ment with hypertonic sucrose inhibit the agonist-induced internalization of rLHR-wt. In agreement with data obtained with the β2AR expressed in 293 cells (4), we found that β-arrestin-V53D is less effective than β-arrestin(319–418) in inhibiting the internalization of the rLHR. However, the magnitude of the inhibition of rLHR-wt internalization induced by overexpression of the two dominant-negative β-arrestins and the dominant negative dynamin in 293 cells is generally lower than those reported for the β2AR in the same cell line (4, 29, 34, 35). A more notable difference is with the effect of arrestin-3, as the overexpression of this protein in 293 cells has little or no effect on the internalization of the β2AR (4, 29, 34, 35), but it enhances the internalization of the rLHR-wt about 2-fold (Fig. 4). When considered together, these data suggest that the rLHR-wt has a low affinity for arrestins and that the slow rate of internalization of the rLHR-wt (t1/2 ~ 140 min; see Table III) compared with that of the β2AR (t1/2 < 30 min; see Ref. 29) may be a reflection, at least in part, of the weak rLHR-arrestin interaction.

The effects of the two dominant-negative β-arrestins and dominant-negative dynamin on rLHR-S639A or rLHR-S649A were virtually indistinguishable from those detected with rLHR-wt (Fig. 4) and support the idea that the agonist-induced internalization of these two mutants also occurs by an arrestin- and coated pit-dependent pathway. The experiments presented here are the first to address the involvement of nonvisual arrestins in this phenomenon. The involvement of clathrin-coated pits in the internalization of the LHR-wt has been previously documented using ultrastructural approaches, however (11).

Although transient cotransfection assays have been used by others (7) to show that GRK overexpression reduces the hCG-induced cAMP response mediated by rLHR-wt, the same paradigm failed to reveal opposite effects of arrestin-3 or a dominant-negative β-arrestin on the hCG-stimulated cAMP response. The overexpression of both arrestin-3 and a dominant-negative β-arrestin had a slight stimulatory effect on hCG-stimulated cAMP accumulation (Table IV). Although these results suggest the existence of an arrestin-independent pathway for the agonist-induced uncoupling of the rLHR, they may need to be interpreted with caution because transient co-transfection assays of 293 cells have also failed to detect an effect of β-arrestin or β-arrestin-V53D on the agonist-induced cAMP response mediated by the β2AR (34). Likewise, transient overexpression of β-arrestin on JEG-3 cells has no effect on the agonist-induced desensitization of the m2 muscarinic receptor unless one of the GRKs is also co-transfected (36). In fact, β-arrestin overexpression has been shown to enhance the agonist-induced desensitization of the β2AR only in stably transfected Chinese hamster ovary cells expressing large amounts of β2AR (37). On the other hand, the possibility of an agonist-promoted but arrestin-independent pathway for rLHR-uncoupling cannot be completely dismissed, as there are studies that demonstrate that the GRK-catalyzed phosphorylation of rhodopsin can partially inhibit G protein coupling in the absence of arrestin (38–40), and there is at least one study documenting that the GRK-catalyzed phosphorylation of the β2AR can also directly inhibit G protein coupling in the absence of arrestin (41).

When taken together, the effects of arrestin overexpression on the agonist-induced uncoupling (Table IV) and internalization (Fig. 4) of the rLHR-wt, as well as the effects of individual Ser mutations on agonist-induced uncoupling (Fig. 2 and Table II) and internalization of the rLHR (Fig. 2 and Table III) are consistent with the following two models.

Model 1 assumes that Ser635, Ser639, and Ser649 are involved in the interaction of the rLHR with arrestin, but the role of Ser649 in this interaction is more important than that of Ser635 and Ser639. This model also assumes that arrestin is involved in the agonist-induced internalization of the rLHR but not in the agonist-induced uncoupling of the rLHR. Model 1 accommodates all the findings presented here. Thus, the mutation of Ser635, Ser639, or Ser649 slows down internalization, but the effect of S649A mutation is more pronounced than those of the S635A or S639A mutation (Fig. 3 and Table III). Furthermore, arrestin-3 overexpression does not enhance the internalization of rLHR-S649A to the same level as that detected with rLHR-wt or rLHR-S639A (Fig. 4) because the S649A mutation reduces the binding affinity for arrestin. Because Ser635, Ser639, and Ser649 are all involved in the receptor-arrestin interaction, Model 1 is also consistent with the finding that the dominant-negative β-arrestins inhibit the internalization of rLHR-wt, rLHR-S639A, and rLHR-S649A (Fig. 4). The lack of effect of the S649A mutation and the effect of the S635A and S639A mutations on agonist-induced uncoupling (Table II) are accommodated by the assumption that uncoupling is arrestin-independent. This assumption is, in turn, supported by our inability to demonstrate an effect of arrestin-3 or dominant-negative β-arrestins on the agonist-induced cAMP accumulation (Table IV) under conditions similar to those used to detect effects on agonist-induced internalization (Fig. 4).

Model 2 assumes that Ser635 and Ser639 are involved in the interaction of the rLHR with arrestin, and Ser649 is involved in the interaction of the rLHR with another protein (protein X). This model also assumes that the agonist-induced uncoupling of the rLHR is mediated by arrestin, and the agonist-induced internalization of the rLHR is mediated by arrestin and protein X. The attenuation of agonist-induced uncoupling detected in rLHR-S635A and rLHR-S639A and the lack of effect of rLHR-S649A (Table II) can thus be explained by a reduced interaction of rLHR-S635A and rLHR-S639A with arrestin and a normal interaction of rLHR-S649A with arrestin. Conversely, the strong inhibition of internalization detected in rLHR-S649A and the partial effect of rLHR-S635A and rLHR-S639A (Fig. 3 and Table III) can be explained by a reduced interaction with protein X (rLHR-S649A) and a reduced interaction with arrestin (rLHR-S635A and rLHR-S639A). In this model, arrestin-3 overexpression does not enhance the internalization of rLHR-S649A to the same level as that detected with rLHR-wt or rLHR-S639A (Fig. 4), because the S649A mutation is predicted to reduce the binding of rLHR to protein X, and the interaction of the rLHR with both protein X and with arrestin is needed for internalization. The inhibitory effect of dominant-negative β-arrestins on the internalization of rLHR-wt, rLHR-S639A, and rLHR-S649A (Fig. 4) can also be accommodated by the assumption that the rLHR-arrestin interaction is partially responsible for internalization. What Model 2 cannot explain, however, is the lack of effect of arrestin-3 and a dominant-negative β-arrestin on agonist-induced cAMP accumulation (Table IV). If this paradigm measures uncoupling (see above), then Model 2 predicts an inhibitory effect of arrestin-3 and a stimulatory effect of the dominant-negative β-arrestin on hCG-induced cAMP accumulation.

Experiments are now being planned to directly measure the interaction of the rLHR (and mutants thereof) with arrestin and to search for other proteins that may interact with the C-terminal tail of the rLHR and affect its internalization. We will not be able to differentiate between the two models described above until one or both of these strategies are successful. Regardless of which of the two models proposed above is correct, it is clear that the agonist-induced uncoupling and internalization of the rLHR are affected by mutations of differ-
ent serine residues. This finding mirrors the results presented in a recent publication on the m2 muscarinic receptor, in which it was concluded that the agonist-induced uncoupling and internalization of this GPCR are mediated by the distinct Ser/Thr clusters present in the third intracellular loop (42). Although the study on the m2 receptor did not define the importance of individual Ser/Thr residues, it was shown that the phosphorylation of only one of the two clusters promotes uncoupling, whereas the phosphorylation of both clusters is needed to promote agonist-induced internalization. Thus, the data from these two studies, conducted with different GPCRs, lead to basically the same conclusion, that the agonist-induced uncoupling and internalization are mediated by distinct Ser/Thr residues.

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REFERENCES

1. Premont, R. T., Inglese, J., and Lefkowitz, R. J. (1995) FASEB J. 9, 175–182
2. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1996) FASEB J. 4, 281–289
3. Goodman, J. O. B., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
4. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) J. Biol. Chem. 272, 32507–32512
5. Hipkin, R. W., Sánchez-Yague, J., and Ascoli, M. (1993) Mol. Endocrinol. 7, 823–832
6. Hipkin, R. W., Wang, Z., and Ascoli, M. (1995) Mol. Endocrinol. 9, 151–158
7. Premont, R. T., Macrae, A. D., Steffel, R. H., Chung, N., Pitcher, J. A., Ambrose, C., Inglese, J., MacDonald, M. E., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 6403–6410
8. Wang, Z., Hipkin, R. W., and Ascoli, M. (1996) Mol. Endocrinol. 10, 745–759
9. Wang, Z., Liu, X., and Ascoli, M. (1997) Mol. Endocrinol. 11, 183–192
10. Ascoli, M. (1982) J. Biol. Chem. 257, 13306–13311
11. Ghinea, N., Vuhai, M. T., Groyer-Picard, M.-T., Houllier, A., Schoevaert, D., and Milgrom, E. (1992) J. Cell Biol. 118, 1347–1358
12. Hoeleber, S. R., Sairam, M. R., and Ascoli, M. (1991) Endocrinology 128, 2837–2843
13. Dhanwada, K. R., Vijapurkar, U., and Ascoli, M. (1996) Mol. Endocrinol. 10, 544–554
14. Ascoli, M. (1984) J. Cell Biol. 99, 1242–1250
15. Wang, H., Segaloff, D. L., and Ascoli, M. (1991) J. Biol. Chem. 266, 780–785
16. McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosenblit, N., Nikolos, K., Segaloff, D. L., and Seeburg, P. H. (1989) Science 245, 494–499
17. Damke, H., Bahn, T., Warnock, D. E., and Schmid, S. L. (1994) J. Biol. Chem. 269, 915–934
18. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
19. Fabritz, J., Ryan, S., and Ascoli, M. (1998) Biochemistry 37, 664–672
20. Rosemblit, N., Ascoli, M., and Segaloff, D. L. (1988) Endocrinology 123, 2284–2290
21. Rodriguez, M. C., and Segaloff, D. L. (1990) Endocrinology 127, 674–681
22. Sterne-Marr, R., Gurevich, V. V., Goldsmith, P., Bodine, R. C., Sanders, C., Donoso, L. A., and Benovic, J. L. (1993) J. Biol. Chem. 268, 15640–15648
23. Donoso, L. A., Gregerson, D. S., Smith, L., Robertson, S., Knope, V., Vrabec, T., and Ralsow, C. M. (1990) Curr. Eye Res. 9, 343–355
24. Ascoli, M., and Puetten, D. (1979) Proc. Natl. Acad. Sci. U. S. A. 75, 99–102
25. Strickland, T. W., and Puetten, D. (1981) Endocrinology 109, 1933–1942
26. Ascoli, M., and Segaloff, D. L. (1987) Endocrinology 120, 1161–1172
27. Mukherjee, S., Ghosh, R. N., and Maxfield, F. R. (1997) Physiol. Rev. 77, 789–803
28. Warnock, D., and Schmid, S. (1996) BioEssays 18, 885–893
29. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1996) J. Biol. Chem. 271, 18302–18305
30. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) Science 274, 2086–2089
31. Park-Sylaarsd, R., Gurevich, V. V.; Lee, K. B., Ptasienek, J. A., Benovic, J. L., and Hasey, M. (1997) J. Biol. Chem. 272, 23682–23688
32. Heuser, J. E., and Anderson, R. G. W. (1988) J. Cell Biol. 108, 389–400
33. Ferguson, S. S. G., Downey, W. E., Calep, A. B., Barak, L. S., Menard, L., and Caron, M. G. (1996) Science 271, 363–365
34. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. G. (1997) J. Biol. Chem. 272, 27005–27014
35. Menard, L., Ferguson, S. S. G., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1996) Mol. Pharmacol. 51, 800–808
36. Schloder, M. L., and Nathanson, N. M. (1997) J. Biol. Chem. 272, 18882–18890
37. Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M. G., Lefkowitz, R. J., and Lohse, M. J. (1993) J. Biol. Chem. 268, 3201–3208
38. Wilden, U., Hall, S. W., and Kuhn, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1174–1178
39. Krupnick, J. G., Gurevich, V. V., and Benovic, J. L. (1997) J. Biol. Chem. 272, 18811–18813
40. Xu, J., Dodd, R. L., Makino, C. L., Simon, M. I., Baylor, D. A., and Chen, J. (1997) Nature 389, 505–509
41. Pitcher, J. A., Lohse, M. I., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1992) Biochemistry 31, 3193–3197
42. Park-Sylaarsd, R., and Hasey, M. M. (1997) J. Biol. Chem. 272, 14152–14158