Evidence for the Direct Involvement of Transmembrane Region 6 of the Lutropin/Choriogonadotropin Receptor in Activating \( G_s^* \)

(Received for publication, October 25, 1996, and in revised form, March 5, 1997)

Amy N. Abell and Deborah L. Segaloff‡

From the Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

The luteinizing hormone/chorionic gonadotropin receptor (LHR) is a heptahelical receptor that interacts primarily with \( G_s \). Previous studies by others have shown that some forms of familial male precocious puberty are associated with mutations of the human LHR in the sixth transmembrane region that result in constitutive activation of the receptor. This study demonstrates that a peptide corresponding to the lower portion of the sixth transmembrane region of the LHR can significantly activate adenylyl cyclase activity. Experiments with membranes derived from wild-type versus cyc- \( S_{49} \) cells demonstrate that the stimulation of cyclase by this peptide is due to an activation of \( G_s \). As such, our data demonstrate a direct role for transmembrane region 6 of the rat LHR in activating \( G_s \) and therefore raise the possibility that mutations in transmembrane region 6 of the LHR may directly affect the coupling of the receptor to \( G_s \). Significantly, these data are the first to demonstrate the ability of a transmembrane portion of a G protein-coupled receptor, in the absence of any contributions from an intracellular loop region, to activate a G protein.

The LHR1 is a large cell-surface glycoprotein with the characteristic structure of a member of the superfamily of G protein-coupled receptors (1). This structure includes the presence of seven putative membrane-spanning regions connected by alternating intracellular and extracellular loops, an extracellular N-terminal extension, and an intracellular cytoplasmic tail (1, 2). The rLHR is capable of binding either luteinizing hormone or chorionic gonadotropin with high affinity, resulting in the activation of \( G_s \) and adenylyl cyclase and the production of cAMP (1, 3). Although cAMP is responsible for eliciting most of the significant effects of the rLHR, at high receptor densities and in the presence of high concentrations of hormone, the rLHR also activates phospholipase C (4, 5).

Many studies of different members of the G protein-coupled receptor superfamily have focused on the determination of the sites of these receptors that physically interact with G proteins. Utilizing multiple approaches such as chimeric receptors, synthetic peptides, and deletion/substitution mutagenesis, several investigators have determined that the N- and C-terminal portions of the third intracellular loop are essential for the interaction of the adrenergic receptors with their respective G proteins (6–8). Additionally, similar experiments with the muscarinic acetylcholine receptor subfamily of G protein-coupled receptors have revealed that the third intracellular loop determines the subtype-specific coupling of the muscarinic acetylcholine receptors to distinct G proteins, with the N- and C-terminal portions of the third intracellular loop being absolutely required for activation of their respective G proteins (9–11). More important, the third intracellular loop is not the site of G protein interaction for all G protein-coupled receptors. For example, experiments utilizing synthetic peptides and fusion proteins corresponding to regions of the human neutrophil N-formylpeptide receptor demonstrated the importance of the second intracellular loop and the N-terminal portion of the cytoplasmic tail for the coupling of this receptor to \( G_s \) (12).

The sites of interaction of the LHR with \( G_s \) are not well defined. However, several of the activating mutations found to be associated with familial male precocious puberty have been found to occur in the lower portion of transmembrane 6 and in the C-terminal portion of the third intracellular loop of the hLHR (13–17). These mutations are thought to activate the receptor by altering the packing of the transmembrane helices and/or by exposing intracellular regions, which can then interact with \( G_s \). The identification of these activating mutations has led investigators to hypothesize that the third intracellular loop of the LHR may be important for the coupling of this receptor to \( G_s \) (13).

This study was undertaken to identify regions of the LHR that interact with \( G_s \). Because mutation of the LHR frequently results in intracellular retention of mutant receptors (3, 18–25), we chose to utilize an alternate approach. Hence, the following studies were performed by examining the ability of synthetic peptides corresponding to regions of the LHR to stimulate adenylyl cyclase activity. Based upon the studies of the adrenergic receptors and upon mutations of the LHR causing familial male precocious puberty, we chose to focus initially on the third intracellular loop and sixth transmembrane region of the rLHR. As shown herein, our results clearly demonstrate a role for transmembrane 6 of the LHR in the activation of \( G_s \).

MATERIALS AND METHODS

Supplies—Highly purified hCG (CR-127) was provided by the National Hormone and Pituitary Agency of NIDDK, National Institutes of Health. GDP\(\beta S\), alumina WN-3, and reagents for adenylyl cyclase assays were obtained from Sigma. AG-50W-X4 resin was purchased from Bio-Rad, and \([\gamma^{32P}]ATP\) was from DuPont NEN. Tissue culture reagents and plasticware were obtained from Life Technologies, Inc. and Corning Inc. (Corning, NY), respectively.

Peptides—rLHR peptides were synthesized as C-terminal amides and were purified by reverse-phase high pressure liquid chromatography to >95% purity. Peptides were synthesized either by Multiple Peptide Systems (San Diego, CA) or by the Mayo Protein Core Facility.
Fig. 1. Sequences and locations of the rLHR I3/TM6 and E1/TM2 peptides. The proposed topology of the transmembrane and loop regions of the rLHR is shown with the locations of the regions corresponding to the I3/TM6 and E1/TM2 peptides. These, and additional peptides derived thereof, are listed in Table I. Not shown are the extracellular domain and cytoplasmic tail of the receptor.

Mayo Foundation (Rochester, MN). As a control for the different peptide sources, some peptides were synthesized by both vendors and were found to have identical properties.

Cells—Human embryonic kidney 293 cells (ATCC CRL 1573, American Type Culture Collection, Rockville, MD), which do not express the rLHR, were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 50 μg/ml gentamicin, 10 mM Hepes, and 10% newborn calf serum and incubated at 37 °C in 5% CO2. The clonal stable 293 cell line rLHR-wt12, expressing the extracellular domain and cytoplasmic tail of the receptor, was generously donated by Dr. Mario Ascoli (University of Iowa). Human embryonic kidney 293 cells (ATCC CRL 1573, American Type Culture Collection, Rockville, MD), which do not express the rLHR, were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 50 μg/ml gentamicin, 10 mM Hepes, and 10% heat-inactivated horse serum and incubated at 37 °C in 5% CO2. S49 wild-type and cyc– mouse lymphoma cell lines were obtained from the Cell Culture Facility, University of California, San Francisco. S49 wild-type cells were grown in confluent 100-mm dishes were set on ice for 15 min and washed twice with 5 ml of ice-cold buffer A (250 mM sucrose, 25 mM Tris-Cl, and 1 mM EDTA, pH 7.4). Cells were scraped from the dishes; the dishes were aspirated; and the cell pellet was resuspended in 1 mM Tris-Cl and 5 mM EDTA, pH 7.4; the supernatant; and the cell pellet was resuspended by vortexing in buffer B. Crude preparations of S49 wild-type cells were performed essentially as described above for 293 membranes with the following modifications. 10 μg of S49 membranes in 10 μl of 20 mM Hepes, 2 mM MgCl2, and 1 mM EDTA, pH 8.0, were preincubated with rLHR peptides for 15 min at 30 °C with shaking, followed by 15 min at 4 °C. Preincubation, the following components were added: 10 μl of 230 mM Hepes and 4 mM EDTA, pH 8.0; 10 μl of 250 μM GTP; and 10 μl of the reaction mixture described above, except that MgCl2 was included at 50 mM instead of 20 mM. The reaction mixture was incubated for 20 min at 30 °C with shaking. Reactions were terminated; radiolabeled nucleotides were separated; and samples were counted as described above for 293 membrane cyclase assays.

Inhibition by GDPβS—For assays measuring the GDPβS-induced inhibition of rLHR peptide-stimulated adenyl cyclase activity, GTP (final concentration of 20 μM) was substituted with GDPβS (final concentration of 6 μM). Membranes were incubated with the indicated rLHR peptide (final concentration of 30 μM), with a saturating concentration of hCG (final concentration of 15 μg/ml), or with 100 μM forskolin. Adenylyl cyclase activity was measured essentially as described above.

RESULTS

To determine the role of the third intracellular loop and transmembrane 6 regions of the rLHR in the activation of Gs, peptide I3/TM6, corresponding to the C-terminal portion of the third intracellular loop and the lower portion of transmembrane region 6, was synthesized (Fig. 1 and Table I). As a control for I3/TM6, peptide E1/TM2 was also synthesized. As shown in Fig. 1, peptide E1/TM2 represents the N-terminal portion of the first extracellular loop and the upper portion of transmembrane 2, a region that should not be directly involved in the coupling of the rLHR to Gs. These peptides were then tested for their ability to either activate Gs or competitively inhibit the interaction of the rLHR with Gs. Membranes prepared from 293 cells expressing the wild-type rLHR were incubated with increasing concentrations of either peptide E1/TM2 or I3/TM6 in the absence or presence of a maximally stimulatory concentration of hCG, and adenylyl cyclase activity was measured. As shown in Fig. 2, E1/TM2 had no effect on either basal or hormone-stimulated cyclase activity, whereas I3/TM6 markedly altered adenylyl cyclase activity. Low concentrations of I3/TM6 (30 μM) stimulated basal cyclase activity —3-fold (Fig. 2B). The magnitude of this response is similar to that elicited by a maximal concentration of hCG (Fig. 2A).

When membranes were incubated in the presence of both a maximal concentration of hCG and 30 μM I3/TM6, this peptide
produced a further 3-fold increase in cyclase activity above the 3-fold stimulation by hCG alone (Fig. 2C). In addition to the stimulatory effects of low concentrations of I3/TM6, high concentrations (300 μM) of I3/TM6 produced a significant inhibition of both basal and hormone-stimulated cyclase activity (Fig. 2, B and C). Both the activating and inhibitory actions of the I3/TM6 peptide on basal adenyl cyclase activity were independent of the presence of the full-length rLHR as similar activities were observed in membranes prepared from untransfected 293 cells that do not express the rLHR (Fig. 3).

Experiments with other G protein-coupled receptors such as the β2-adrenergic receptor would suggest that it is the I3 portion of I3/TM6 that is responsible for the activity of this peptide (6, 7). To address this possibility, peptide I3 (Table I), corresponding to the third intracellular loop of the rLHR, was tested for activity. Over a range of 1–300 μM, however, I3 was found to have no effect on either basal or hCG-stimulated cyclase activity (data not shown). Since it is possible that the lack of activity of the I3 peptide was due to the absence of a hydrophobic sequence that would enhance its association with membranes, an I3/TM2 peptide (Table I) was also examined. This peptide, in which the C-terminal portion of the I3 sequence is now followed by a hydrophobic sequence corresponding to the upper portion of the second transmembrane helix, however, was also devoid of any stimulatory or inhibitory activity (data not shown).

The lack of activity of the I3 and I3/TM2 peptides and the identification of several activating mutations in the lower portion of transmembrane 6 of the hLHR (13, 15) raised the possibility that the TM6 portion itself may be responsible for at least some of the activity of the I3/TM6 peptide (13, 15). To address this question, peptide TM6, corresponding to the lower portion of transmembrane 6, was synthesized (Fig. 1 and Table I). When incubated with membranes expressing the rLHR, 10–56 μM TM6 peptide stimulated both basal and hormone-stimulated cyclase activity ~2-fold (Fig. 2, B and C). Much higher concentrations of peptide TM6 inhibited both basal and hormone-stimulated cyclase activity, but to a lesser extent than the I3/TM6 peptide (Fig. 2, B and C). Similar to the I3/TM6 peptide, the activity of the TM6 peptide was independent of the presence of the full-length rLHR as activity was present in 293 membranes (Fig. 3). These data show that the residues corresponding to the lower portion of transmembrane 6 are responsible for a significant part of the activity of the I3/TM6 peptide.

Control peptides were synthesized to demonstrate the specificity of the TM6 amino acid sequence for the activation of Gs. For these purposes, peptide TM6(L552P), corresponding to peptide TM6 but with the substitution of a leucine with a disruptive proline, was synthesized (Table I). In addition, peptide TM2up, corresponding to amino acids 391–398 (LLIASVDS), representing the upper portion of transmembrane 2, was synthesized (Table I). More important, unlike the TM6 peptide, the
TM6(L552P) and TM2up peptides had no stimulatory or inhibitory effects on either basal (Fig. 4A) or hormone-stimulated (data not shown) cyclase activity, suggesting a structural basis for the activity of TM6. In addition to these two control peptides, randomly scrambled versions of the TM6 peptide, designated TM6-scr1 and TM6-scr2 (Table I), were prepared. Interestingly, both scrambled peptides were capable of stimulating basal (Fig. 4B) and hormone-stimulated (data not shown) cyclase activity, identical to the parent TM6 peptide; however, both peptides inhibited cyclase activity at much lower concentrations of peptide.

To determine whether the I3/TM6 and TM6 peptides were interacting directly with Gα to stimulate cyclase activity, as opposed to nonspecifically affecting adenyl cyclase, the GTP dependence of the stimulation by these peptides was examined. Membranes prepared from 293 cells expressing the rLHR were incubated either in the presence of GTP or in the absence of GTP and the presence of the inhibitor GDPβS. Results are presented as % of adenyl cyclase activity in the absence of GTP and the presence of GDPβS as compared with the presence of GTP and the absence of GDPβS. Data shown are the means ± S.E. of four independent experiments. b, basal.

The ability of GDPβS to inhibit the activation of cyclase activity by a stimulatory dose (30 μM) of peptide was then examined. As controls, the effects of GDPβS on forskolin- and hCG-stimulated cyclase activity were also examined. As would be expected, GDPβS significantly decreased hCG-stimulated cyclase activity, whereas it had little effect on forskolin-stimulated cyclase activity (Fig. 5). The results in Fig. 5 show that GDPβS markedly decreased the stimulatory activities of both the I3/TM6 and TM6 peptides, suggesting that low concentrations of these peptides act directly on Gα to stimulate cyclase activity.

To more definitively demonstrate the interaction of rLHR peptides with Gα, the effects of the I3/TM6, TM6, and TM6(L552P) peptides were examined in membranes prepared from wild-type versus cyc− S49 cells. As shown in Fig. 6A, S49 wild-type membranes demonstrated both sodium fluoride- and forskolin-stimulated adenyl cyclase activity. As expected, S49 cyc− membranes, which lack Gα (26), showed no detectable basal cyclase activity or stimulation of cyclase by NaF, but did exhibit forskolin-stimulated cyclase activity similar to that measured in S49 wild-type membranes (Fig. 6A). Examination of I3/TM6 peptide activity showed that low concentrations of I3/TM6 (10–56 μM) produced a 3-fold stimulation of adenyl cyclase activity in S49 wild-type membranes similar to that observed in 293 membranes (Fig. 6B). Interestingly, this 3-fold stimulation was maintained in S49 wild-type membranes even at very high concentrations of peptide I3/TM6, concentrations that inhibited 293 membrane cyclase activity (cf. Figs. 3 and 6B). Similar to 293 membranes, low concentrations of peptide TM6 (10–56 μM) produced an ~2-fold increase in cyclase activity in S49 wild-type membranes (Fig. 6B). However, unlike the moderate inhibition of cyclase activity in 293 membranes by higher concentrations of peptide TM6, higher concentrations of peptide TM6 produced a further increase in S49 wild-type cyclase activity to ~4–5-fold of basal cyclase activity (cf. Figs. 3 and 6B). The control peptide TM6(L552P) had no effect on S49 wild-type cyclase activity (Fig. 6B). The ability of the TM6 and I3/TM6 peptides to stimulate adenyl cyclase activity was then examined in S49 cyc− cell membranes. As shown in Fig. 6B, the cyclase activity observed over all concentrations of both peptides tested was undetectable. The ability of the TM6 and I3/TM6 peptides to stimulate adenyl cyclase activity in S49 wild-type cell membranes, but not in S49 cyc− cell membranes, clearly demonstrates the requirement for Gα in the stimulatory

To more definitively demonstrate the interaction of rLHR peptides with Gα, the effects of the I3/TM6, TM6, and TM6(L552P) peptides were examined in membranes prepared from wild-type versus cyc− S49 cells. As shown in Fig. 6A, S49 wild-type membranes demonstrated both sodium fluoride- and forskolin-stimulated adenyl cyclase activity. As expected, S49 cyc− membranes, which lack Gα (26), showed no detectable basal cyclase activity or stimulation of cyclase by NaF, but did exhibit forskolin-stimulated cyclase activity similar to that measured in S49 wild-type membranes (Fig. 6A). Examination of I3/TM6 peptide activity showed that low concentrations of I3/TM6 (10–56 μM) produced a 3-fold stimulation of adenyl cyclase activity in S49 wild-type membranes similar to that observed in 293 membranes (Fig. 6B). Interestingly, this 3-fold stimulation was maintained in S49 wild-type membranes even at very high concentrations of peptide I3/TM6, concentrations that inhibited 293 membrane cyclase activity (cf. Figs. 3 and 6B). Similar to 293 membranes, low concentrations of peptide TM6 (10–56 μM) produced an ~2-fold increase in cyclase activity in S49 wild-type membranes (Fig. 6B). However, unlike the moderate inhibition of cyclase activity in 293 membranes by higher concentrations of peptide TM6, higher concentrations of peptide TM6 produced a further increase in S49 wild-type cyclase activity to ~4–5-fold of basal cyclase activity (cf. Figs. 3 and 6B). The control peptide TM6(L552P) had no effect on S49 wild-type cyclase activity (Fig. 6B). The ability of the TM6 and I3/TM6 peptides to stimulate adenyl cyclase activity was then examined in S49 cyc− cell membranes. As shown in Fig. 6B, the cyclase activity observed over all concentrations of both peptides tested was undetectable. The ability of the TM6 and I3/TM6 peptides to stimulate adenyl cyclase activity in S49 wild-type cell membranes, but not in S49 cyc− cell membranes, clearly demonstrates the requirement for Gα in the stimulatory
are the means, which is representative of three such experiments.

The data presented provide evidence of a direct interaction of transmembrane region 6 of the LHR with $G_\alpha$. When present at concentrations of 10–100 $\mu$M, the TM6 peptide (corresponding to the lower portion of TM6) was able to stimulate both basal and hCG-stimulated adenylyl cyclase activity. This stimulatory activity is mediated by $G_\alpha$ as peptide activity is present in S49 wild-type membranes, but is absent in S49 cyc$^-$ membranes, which lack $G_\alpha$. More important, experiments with control peptides for the TM6 peptide suggest that the activity of the TM6 peptide requires some specific, disruptable sequence. For example, the equally hydrophobic peptide TM2up failed to alter cyclase activity, demonstrating that a string of predominantly hydrophobic amino acids is not sufficient to activate $G_\alpha$. In addition, a leucine to proline substitution in the TM6 peptide resulted in a peptide with no activity, indicating that the TM6 peptide has a specific structure that is disruptable. However, clearly the amino acid sequence requirements of the TM6 peptide are not absolute as scrambled TM6 peptides had similar activating properties as the parent TM6 peptide. Results from experiments with the scrambled TM6 peptides were not entirely unexpected since the scrambled TM6 peptides consist almost entirely of homologous hydrophobic substitutions, and many researchers have shown that homologous substitutions are often well tolerated (30–32). Taken all together, the results presented suggest that the TM6 peptide possesses some specific amino acid sequence and structure necessary for its activation of $G_\alpha$. More important, these results are the first to demonstrate an interaction of a transmembrane region of a G protein-coupled receptor with a G protein.

In addition to the stimulation of adenylyl cyclase activity by TM6, a longer peptide, I3/TM6 (corresponding to the C-terminal portion of the third intracellular loop and the lower portion of transmembrane 6), stimulated cyclase activity. The effects of the I3/TM6 peptide appear to be specific since a control peptide, E1/TM2 (corresponding to a portion of the first extracellular loop and the upper portion of transmembrane 2), had no effect on cyclase activity. Also, the effects of the I3/TM6 peptide were independent of the presence of the full-length rLHR. In addition, the I3/TM6 peptide acted directly on $G_\alpha$ as activation was present in S49 wild-type membranes, but was absent in S49 cyc$^-$ membranes. However, the I3/TM6 peptide stimulated cyclase activity more effectively than the TM6 peptide, suggesting that the I3 portion of the peptide may be responsible for some of the activity of the I3/TM6 peptide and that the I3 portion may interact with $G_\alpha$. Surprisingly, examination of other peptides revealed that neither the I3 peptide (corresponding to the full third intracellular loop of the rLHR) nor the I3/TM2 peptide (corresponding to the C-terminal portion of the third intracellular loop followed by a hydrophobic anchor derived from the upper portion of transmembrane region 2) had any effects on basal or hormone-stimulated cyclase activity. Based upon the $\beta_2$-adrenergic receptor paradigm, it is generally assumed that the C-terminal portion of the third intracellular loop of the LHR is involved in the activation of $G_\alpha$. However, the only data thus far on the LHR to support this hypothesis come from the observations that some constitutively activating mutations of the hLHR result from single amino acid substitutions within the C-terminal portion of the third intracellular loop (14, 17). On the other hand, it has been shown that substitution of the three lysine residues in the C-terminal portion of the third intracellular loop of the rLHR (present also in the hLHR) to alanines had no adverse effects on either basal or hormone-stimulated cAMP accumulation in cells expressing terminal portion of the third intracellular loop. Consequently, peptide TM6, which corresponds only to the lower portion of transmembrane 6, is the same for the rLHR and hLHR. The data presented provide evidence of a direct interaction of transmembrane region 6 of the LHR with $G_\alpha$. When present at concentrations of 10–100 $\mu$M, the TM6 peptide (corresponding to the lower portion of TM6) was able to stimulate both basal and hCG-stimulated adenylyl cyclase activity. This stimulatory activity is mediated by $G_\alpha$ as peptide activity is present in S49 wild-type membranes, but is absent in S49 cyc$^-$ membranes, which lack $G_\alpha$. More important, experiments with control peptides for the TM6 peptide suggest that the activity of the TM6 peptide requires some specific, disruptable sequence. For example, the equally hydrophobic peptide TM2up failed to alter cyclase activity, demonstrating that a string of predominantly hydrophobic amino acids is not sufficient to activate $G_\alpha$. In addition, a leucine to proline substitution in the TM6 peptide resulted in a peptide with no activity, indicating that the TM6 peptide has a specific structure that is disruptable. However, clearly the amino acid sequence requirements of the TM6 peptide are not absolute as scrambled TM6 peptides had similar activating properties as the parent TM6 peptide. Results from experiments with the scrambled TM6 peptides were not entirely unexpected since the scrambled TM6 peptides consist almost entirely of homologous hydrophobic substitutions, and many researchers have shown that homologous substitutions are often well tolerated (30–32). Taken all together, the results presented suggest that the TM6 peptide possesses some specific amino acid sequence and structure necessary for its activation of $G_\alpha$. More important, these results are the first to demonstrate an interaction of a transmembrane region of a G protein-coupled receptor with a G protein.

In addition to the stimulation of adenylyl cyclase activity by TM6, a longer peptide, I3/TM6 (corresponding to the C-terminal portion of the third intracellular loop and the lower portion of transmembrane 6), stimulated cyclase activity. The effects of the I3/TM6 peptide appear to be specific since a control peptide, E1/TM2 (corresponding to a portion of the first extracellular loop and the upper portion of transmembrane 2), had no effect on cyclase activity. Also, the effects of the I3/TM6 peptide were independent of the presence of the full-length rLHR. In addition, the I3/TM6 peptide acted directly on $G_\alpha$ as activation was present in S49 wild-type membranes, but was absent in S49 cyc$^-$ membranes. However, the I3/TM6 peptide stimulated cyclase activity more effectively than the TM6 peptide, suggesting that the I3 portion of the peptide may be responsible for some of the activity of the I3/TM6 peptide and that the I3 portion may interact with $G_\alpha$. Surprisingly, examination of other peptides revealed that neither the I3 peptide (corresponding to the full third intracellular loop of the LHR) nor the I3/TM2 peptide (corresponding to the C-terminal portion of the third intracellular loop followed by a hydrophobic anchor derived from the upper portion of transmembrane region 2) had any effects on basal or hormone-stimulated cyclase activity. Based upon the $\beta_2$-adrenergic receptor paradigm, it is generally assumed that the C-terminal portion of the third intracellular loop of the LHR is involved in the activation of $G_\alpha$. However, the only data thus far on the LHR to support this hypothesis come from the observations that some constitutively activating mutations of the hLHR result from single amino acid substitutions within the C-terminal portion of the third intracellular loop (14, 17). On the other hand, it has been shown that substitution of the three lysine residues in the C-terminal portion of the third intracellular loop of the rLHR (present also in the hLHR) to alanines had no adverse effects on either basal or hormone-stimulated cAMP accumulation in cells expressing terminal portion of the third intracellular loop. Consequently, peptide TM6, which corresponds only to the lower portion of transmembrane 6, is the same for the rLHR and hLHR. The data presented provide evidence of a direct interaction of transmembrane region 6 of the LHR with $G_\alpha$. When present at concentrations of 10–100 $\mu$M, the TM6 peptide (corresponding to the lower portion of TM6) was able to stimulate both basal and hCG-stimulated adenylyl cyclase activity. This stimulatory activity is mediated by $G_\alpha$ as peptide activity is present in S49 wild-type membranes, but is absent in S49 cyc$^-$ membranes, which lack $G_\alpha$. More important, experiments with control peptides for the TM6 peptide suggest that the activity of the TM6 peptide requires some specific, disruptable sequence. For example, the equally hydrophobic peptide TM2up failed to alter cyclase activity, demonstrating that a string of predominantly hydrophobic amino acids is not sufficient to activate $G_\alpha$. In addition, a leucine to proline substitution in the TM6 peptide resulted in a peptide with no activity, indicating that the TM6 peptide has a specific structure that is disruptable. However, clearly the amino acid sequence requirements of the TM6 peptide are not absolute as scrambled TM6 peptides had similar activating properties as the parent TM6 peptide. Results from experiments with the scrambled TM6 peptides were not entirely unexpected since the scrambled TM6 peptides consist almost entirely of homologous hydrophobic substitutions, and many researchers have shown that homologous substitutions are often well tolerated (30–32). Taken all together, the results presented suggest that the TM6 peptide possesses some specific amino acid sequence and structure necessary for its activation of $G_\alpha$. More important, these results are the first to demonstrate an interaction of a transmembrane region of a G protein-coupled receptor with a G protein.

In addition to the stimulation of adenylyl cyclase activity by TM6, a longer peptide, I3/TM6 (corresponding to the C-terminal portion of the third intracellular loop and the lower portion of transmembrane 6), stimulated cyclase activity. The effects of the I3/TM6 peptide appear to be specific since a control peptide, E1/TM2 (corresponding to a portion of the first extracellular loop and the upper portion of transmembrane 2), had no effect on cyclase activity. Also, the effects of the I3/TM6 peptide were independent of the presence of the full-length rLHR. In addition, the I3/TM6 peptide acted directly on $G_\alpha$ as activation was present in S49 wild-type membranes, but was absent in S49 cyc$^-$ membranes. However, the I3/TM6 peptide stimulated cyclase activity more effectively than the TM6 peptide, suggesting that the I3 portion of the peptide may be responsible for some of the activity of the I3/TM6 peptide and that the I3 portion may interact with $G_\alpha$. Surprisingly, examination of other peptides revealed that neither the I3 peptide (corresponding to the full third intracellular loop of the LHR) nor the I3/TM2 peptide (corresponding to the C-terminal portion of the third intracellular loop followed by a hydrophobic anchor derived from the upper portion of transmembrane region 2) had any effects on basal or hormone-stimulated cyclase activity. Based upon the $\beta_2$-adrenergic receptor paradigm, it is generally assumed that the C-terminal portion of the third intracellular loop of the LHR is involved in the activation of $G_\alpha$. However, the only data thus far on the LHR to support this hypothesis come from the observations that some constitutively activating mutations of the hLHR result from single amino acid substitutions within the C-terminal portion of the third intracellular loop (14, 17). On the other hand, it has been shown that substitution of the three lysine residues in the C-terminal portion of the third intracellular loop of the rLHR (present also in the hLHR) to alanines had no adverse effects on either basal or hormone-stimulated cAMP accumulation in cells expressing
Activation of $G_s$ by Transmembrane 6 of the LHR

14591

the mutant receptor (20). While these results do not necessarily rule out a role for this region of the LHR in activating $G_s$, they do demonstrate that an amphipathic helical structure of the C-terminal region of the third intracellular loop is certainly not required for activation of $G_s$ by the rLHR. Clearly, more studies need to be performed to address the role of the third intracellular loop of the LHR in $G_s$ activation. There are therefore several possible reasons why the I3 and I3/TM2 peptides did not exhibit any stimulatory activity in this study. These include the possibility that the C-terminal portion of the third intracellular loop of the LHR does not directly interact with $G_s$ (and the constitutively activating mutations may be causing allosteric changes in transmembrane 6). Alternatively, although the C-terminal portion of the third intracellular loop of the LHR may interact with $G_s$, stimulation of $G_s$ may occur only by the coordinated interaction of this region with one or more other regions of the receptor. It may also be that these peptides are interacting with $G_s$, but in a nonproductive manner distinct from the interaction of the LHR with $G_s$. Finally, one cannot exclude the possibility that these peptides are simply not assuming the same conformation as the C-terminal portion of the third intracellular loop of the full-length LHR.

In addition to the stimulation of cyclase by low concentrations of peptides I3/TM6 and TM6, very high concentrations of both peptides I3/TM6 and TM6 were able to inhibit 293 membrane adenyl cyclase activity. However, this inhibition was severely reduced or completely absent in S49 wild-type membranes. Whether the inhibitory activity of these peptides in 293 cells is due to interactions of the peptides with $G_s$ or due to nonspecific inhibition of $G$ protein activity remains to be determined. In either case, the results would not be relevant to the overall conclusion that lower (i.e. more physiologically relevant) concentrations of these peptides exert stimulatory effects on $G_s$ that are independent of cell type.

Many investigators have identified regions of G protein-coupled receptors that possess the ability to activate G proteins. For example, peptides corresponding to the C-terminal region of the third intracellular loop of both the $\beta_2$- and $\beta_3$-adrenergic receptors were each capable of stimulating $G_s$ (7, 8). In addition, a peptide corresponding to the C-terminal portion of the third intracellular loop of the m2-aceetylcholine receptor was capable of directly stimulating both $G_s$ and $G_i$ (11). Also, a peptide corresponding to a cytoplasmic region and a portion of the transmembrane segment of the single transmembrane insulin-like growth factor/mannose 6-phosphate receptor was capable of activating $G_i$, but a peptide corresponding to the transmembrane portion alone had no effect on $G_i$ activity (33). More important, this report is the first to our knowledge to identify a peptide corresponding to a transmembrane region of a G protein-coupled receptor that has the ability to directly activate a G protein. Recent studies have suggested that transmembrane 6 of the LHR is involved in interhelical interactions (34). If this suggestion turns out to be correct, our results suggest that in addition to contributing to interhelical interactions, the lower portion of transmembrane 6 of the LHR is also capable of interacting with and activating $G_s$. Interestingly, many mutations associated with familial male precocious puberty occur in this lower portion of transmembrane 6 of the hLHR (13–17). Our data would further suggest that constitutive activation of LHRs observed with transmembrane 6 mutations that cause familial male precocious puberty may be due to changes in the interaction of the lower portion of transmembrane 6 with $G_s$, as opposed to or in addition to allosteric changes in the conformation of the third intracellular loop. Recently, Liu et al. (10) demonstrated that the insertion of one to four alanines into the lower portion of transmembrane 6 of the m2-aceetylcholine receptor resulted in constitutively activated receptors. They proposed that the activation of this receptor involved the movement of transmembrane 6 toward the cytoplasm (10). This model is consistent with our identification of the lower portion of transmembrane 6 of the LHR directly activating $G_s$. Whether this region of the rLHR activates $G_s$ while in the membrane or whether it is exposed to the cytoplasm when hormone binds remains to be determined.

Acknowledgments—We thank Drs. Mario Ascoli, Daniel McCormick, Nikolai Artemyev, and Paul Sternweis for helpful discussions and Mario Ascoli for critically reading the manuscript.

REFERENCES
1. McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosenblit, N., Nikolics, K., Segaloff, D. L., and Seeberg, P. H. (1989) Science 245, 494–499
2. Rodriguez, M. C., and Segaloff, D. L. (1990) Mol. Endocrinol. 4, 1328–1335
3. Segaloff, D. L., and Ascoli, M. (1993) Endocr. Rev. 14, 324–347
4. Gudermann, T., Birnbaumer, M., and Birnbaumer, L. (1992) J. Biol. Chem. 267, 4479–4488
5. Wang, Z., Hipkin, R. W., and Ascoli, M. (1996) Mol. Endocrinol. 10, 748–758
6. Liggett, S. B., Caron, M. G., Lefkowitz, R. J., and Hnatowich, M. (1991) J. Biol. Chem. 266, 4816–4821
7. Cheung, A. H., Huang, R. R., Crozier, S. R., Graziolo, M. P., and Strader, C. D. (1999) FEBS Lett. 277, 270–274
8. Okamoto, T., Yohitake, M., Hayashi, Y., Inagaki, M., Ogata, E., and Nishimoto, I. (1993) Cell 75, 729–735
9. Liu, J., Conklin, B. R., Lin, N., Yun, J., and Wess, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11642–11646
10. Liu, J., Lin, N., Conklin, B. R., and Wess, J. (1996) J. Biol. Chem. 271, 6172–6176
11. Okamoto, T., and Nishimoto, I. (1992) J. Biol. Chem. 267, 8342–8346
12. Schreiber, R. R., Prassasite, E. R., Ye, R. D., Cochrane, G. C., and Bokoch, G. M. (1994) J. Biol. Chem. 269, 329–331
13. Shenker, A., Laue, L., Kosugi, S., Merendino, J. D., Jr., Minegishi, T., and Cutler, G. B., Jr. (1993) Nature 365, 652–654
14. Latronico, A. C., Anasti, J., Arnhold, I. J. P., Mendonca, B. B., Domenice, S., Albano, M. C., Zachman, K., Wajchenberg, B. L., and Teigos, C. (1995) J. Clin. Endocrinol. & Metab. 80, 2490–2494
15. Kosugi, S., Van Dop, C., Gelfert, M. E., Rakl, W., Carell, J. C., Chausain, J. L., Mori, T., Merendino, J. D., Jr., and Shenker, A. (1995) Hum. Mol. Genet. 4, 183–188
16. Kremer, H., Mariman, E., Otten, B. J., Moll, G. W., Jr., Stoolna, G. B., Witt, J. M., Jansen, M., Drexler, H. P., Reiners, H. P., and Brunner, H. G. (1993) Hum. Mol. Genet. 2, 1779–1783
17. Laue, L., Chan, W. Y., Huseh, A., Kudo, M., Hau, S. Y., Wu, S., Blomberg, L., and Cutler, G. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1906–1910
18. Thomas, D., Rozell, T. G., Liu, X., and Segaloff, D. L. (1996) Mol. Endocrinol. 10, 760–768
19. Thomas, D. M., and Segaloff, D. L. (1994) Endoerinology 135, 1902–1912
20. Wang, Z., Jiaquette, J., Collison, K., and Segaloff, D. L. (1993) Mol. Endocrinol. 7, 1437–1444
21. Wang, Z., Wang, H., and Ascoli, M. (1995) Mol. Endocrinol. 9, 141–150
22. Liu, X., Davis, D., and Segaloff, D. L. (1993) J. Biol. Chem. 268, 1513–1516
23. Abell, A., Liu, X., and Segaloff, D. L. (1996) J. Biol. Chem. 271, 4518–4527
24. Rozell, T. G., Wang, H., Liu, X., and Segaloff, D. L. (1995) Mol. Endocrinol. 9, 1727–1736
25. Miller, R. T., Masters, S., Sullivan, K., Beiderman, K., and Bourne, H. (1988) Nature 334, 712–715
26. Salomon, K., and Segaloff, D. L. (1996) FEBS Lett. 272, 770–774
Evidence for the Direct Involvement of Transmembrane Region 6 of the Lutropin/Choriogonadotropin Receptor in Activating $G_s$

Amy N. Abell and Deborah L. Segaloff

*J. Biol. Chem.* 1997, 272:14586-14591.
doi: 10.1074/jbc.272.23.14586

Access the most updated version of this article at [http://www.jbc.org/content/272/23/14586](http://www.jbc.org/content/272/23/14586)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 16 of which can be accessed free at [http://www.jbc.org/content/272/23/14586.full.html#ref-list-1](http://www.jbc.org/content/272/23/14586.full.html#ref-list-1)