A Constitutively Active Somatic Mutation of the Human Lutropin Receptor Found in Leydig Cell Tumors Activates the Same Families of G Proteins as Germ Line Mutations Associated with Leydig Cell Hyperplasia

TAKASHI HIRAKAWA AND MARIO ASCOLI

Department of Pharmacology, University of Iowa, Iowa City, Iowa 52242

Using a Leydig tumor cell line (MA-10) transiently transfected with the human lutropin receptor (hLHR) and mutants thereof, we examined the identity of the G proteins activated by the agonist-engaged hLHR-wild type (wt) and by three of its naturally occurring constitutively active mutants. Two of the mutants examined, L457R in transmembrane helix 3 and D578Y in transmembrane helix 6, are germ-line mutations found in boys with Leydig cell hyperplasia and precocious puberty. The third, D578H, is a somatic mutation found in Leydig cell tumors in boys with precursor puberty. We show that the hLHR-wt and the three mutants activate the G\textsubscript{s}, G\textsubscript{i/o}, and G\textsubscript{q/11}, but not the G\textsubscript{12/13} families of G proteins. The activation of these G proteins by the hLHR-wt occurs only when engaged by agonist, but their activation by the L457R, D578Y, and D578H mutants occurs independently of agonist stimulation. We conclude that the G proteins activated by constitutively active mutants of the hLHR associated with Leydig cell hyperplasia or tumors are identical and are the same as those activated by the agonist-engaged hLHR-wt. If there was preferential activation of some G protein families by the somatic D578H mutation found in Leydig cell tumors as opposed to the germ line mutations found in Leydig cell hyperplasia, then one could envision mechanisms by which the D578H mutant would be oncogenic. The data presented here suggest that such mechanisms do not need to be considered. (Endocrinology 144: 3872–3878, 2003)

A RATHER LARGE number of naturally occurring mutations of the hLHR gene associated with precocious puberty in boys has been reported in the last decade (reviewed in Refs. 1–3). The overwhelming majority of these mutations are germ line mutations found in boys with familial or sporadic precocious puberty and Leydig cell hyperplasia (1–3), but there is one mutation (D578H in transmembrane helix 6) that is somatic in nature and is found in Leydig cell tumors of boys with precocious puberty (4, 5). When expressed in COS-7 cells, the D578H mutant of the hLHR was reported to display constitutive activity when assayed for cAMP and inositol phosphate accumulation, whereas a similar germ line mutation of the hLHR associated with Leydig cell hyperplasia and male precocious puberty (D578Y, see Refs. 6–8) displayed robust constitutive activity when assayed for cAMP accumulation and little or no constitutive activity when assayed for inositol phosphate accumulation (4, 9, 10). In contrast, our recent studies using a mouse Leydig tumor cell line (MA-10) transfected with the D578H and D578Y mutants, or with another germ line mutation of the hLHR associated with Leydig cell hyperplasia and male precocious puberty (L457R in transmembrane helix 3; see Ref. 11) have shown that they all display constitutive activation of the cAMP, inositol phosphate, steroidogenic, and ERK1/2 responses (12).

Although the second messenger pathways activated by the three constitutively active mutants (CAMs) and the hLHR-wild type (wt) in transfected MA-10 cells are the same (12), it is not known if these pathway are activated by the interaction of the CAMs with the same families or different families of G proteins. For example, because different isoforms of phospholipase C can be stimulated by G\textsubscript{q/11} or by G\textsubscript{i/o} (13) and both of these have been implicated as mediators of the LHR-induced activation of phospholipase C (14–16), the agonist-independent stimulation of the inositol phosphate cascade elicited by the different CAMs could either be due to the preferential coupling of each CAM to the G\textsubscript{i/o} or G\textsubscript{q/11} families or to the identical coupling of all the CAMs to the G\textsubscript{i/o} and/or the G\textsubscript{q/11} families. Similarly, because the G\textsubscript{i/o}-dependent activation of different isoforms of adenylyl cyclase can be dramatically modulated by G\textsubscript{i/o} (17), one can envision at least two distinct pathways that could result in the agonist-independent elevated levels of cAMP detected in MA-10 cells expressing one or more of the CAMs. One pathway would involve constitutive activation of G\textsubscript{i/o} by all the CAMs. A second pathway could involve a high degree of constitutive activation of G\textsubscript{i/o} leading to the liberation of large amounts of G\textsubscript{q/11} which could in turn potentiate the activation of adenylyl cyclase by a low degree of constitutive activation of G\textsubscript{s} (17). Using the same reasoning, one can also envision pathways whereby the constitutive activation of the cAMP and inositol phosphate cascades by the different CAMs could occur by virtue of their ability to activate families of G proteins that are either identical or different from those activated by the agonist-engaged hLHR-wt.

With these considerations in mind, the experiments presented herein were designed to identify the G proteins that are activated when the three naturally occurring CAMs of the hLHR mentioned above are expressed in MA-10 cells and to
compare them with those activated by the agonist-engaged hLHR-wt.

Materials and Methods

Plasmids, cells, and second messenger assays

The preparation of expression vectors (all in the pEF1/V5-His vector from Invitrogen, Carlsbad, CA) encoding for the hLHR-wt and mutants thereof modified with the myc-epitope at the N terminus has been described (12). The origin and handling of MA-10 cells has also been described (18). Transfections were performed in cells plated in 35-mm wells using Lipofectamine as described earlier (12), except that the posttransfection 48-h incubation at 30 C was replaced with a 37 C incubation for 24 h. Each well was transfected with 1-2 μg of plasmid DNA to equivalent levels of receptor expression as described earlier (12).

Assay of G protein activation by trypsin sensitivity

This was performed basically as described in Ref. 19. Briefly, MA-10 cells transiently expressing the hLHR-wt or mutants thereof (usually 6-35-mm wells for each blot shown) were washed twice with a cold isotonie buffer [150 mm NaCl, 20 mm HEPES (pH 7.4)], scraped into cold lysis buffer [10 mm Tris, 5 mm EDTA (pH 7.4) supplemented with 10 μg/ml benzanidine, 10 μg/ml soybean trypsin inhibitor, and 5 μg/ml leupeptin] and centrifuged at 45,000 × g for 10 min at 4 C. The pellet was then resuspended in approximately 150 μl of a solution of 1% [3-cholamidopropyl]dimethylammonio]-1-propanesulfonate in 1% H9262 H9251 NaCl, 20 mm HEPES (pH 7.4) and homogenized using five to six strokes of a motor-driven Teflon homogenizer. The protein concentration of the homogenized membranes was then adjusted to 10 mg/ml, and they were used immediately or stored at −80 C until ready to use. The homogenized membranes (50 μg of protein) were incubated in a buffer containing 25 mm MgCl2, 100 mm NaCl, 0.7% [3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate, 1 mm EDTA, 25 mm HEPES (pH 7.4), and 10 μm GDP. GTPγS (final concentration = 50 μm) and human chorionic gonadotropin (hCG) (final concentration = 25 nm) were added as indicated and the membranes were incubated for 30 min at 30 C (the total volume of the reaction was 30 μl). One microliter of a 1 mg/ml solution of N-tosyl-Phe chloromethyl ketone-treated trypsin was then added and the incubation was continued for 5 min (for Gαs, Gα12, or Gαi or activation assays) or for 15 min (for Gαs and Gαi activation assays) at room temperature. Finally, the reaction was stopped by addition of 6 μl of a 5-fold concentrated version of sodium dodecyl sulfate sample buffer and the samples were boiled for 3 min. After resolution on 12% SDS-PAGE gels and electrophoretic blotting (12) the different Ga-subunits were visualized by incubating the membranes with the appropriate primary antibodies (1:300 dilution for Gαs, Gα12, or Gαi activation assays) or for 15 min (for Gαs and Gαi activation assays) at room temperature. All immune complexes were ultimately visualized using the Super Signal West Femto Maximum Sensitivity system of detection from Pierce (Rockford, IL) and a Kodak (Rochester, NY) digital imaging system.

Hormones and supplies

Purified hCG (ICR-127, ~13,000 IU/mg) was purchased from Dr. A. Parlow of the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases and purified recombinant hCG2 was provided by Ares Serono (Randolph, MA). Cell culture medium was obtained from the Cell Production Core of the Diabetes and Endocrinology Research Center of the University of Iowa.

Other cell culture supplies and reagents were obtained from Corning and Life Technologies, Inc., respectively. Polyclonal antibodies to the C-terminal regions of Ga3 (catalog no. sc-383) and Ga11 (catalog no. sc-392) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody that recognizes the C-terminal region of Ga3 and Gaα and polyclonal antibodies to the C-terminal regions of Ga12 and Ga13 (catalog nos. 371726, 371778, and 371784, respectively) were purchased from Calbiochem (San Diego, CA). Antibodies used in other Western blots included polyclonal antibodies (all from Calbiochem) that recognize Ga3 (catalog no. 371720), Ga11, Ga11 (catalog no. 371723), Gaα (catalog no. 371729), and Gaα (catalog no. 371752). Goat antirabbit antibodies coupled to horseradish peroxidase were from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma (St. Louis, MO) or other commonly used suppliers.

Results

To accomplish the experiments presented below, we had to choose a method to measure G protein activation and a suitable cell line to express the hLHR-wt and mutants thereof.

There are several methods that can be used to directly measure the activation of G proteins (15, 19-22), but we chose the trypsin sensitivity assay because of ease and because it does not involve the use of radioactive compounds (19, 20). The detection of G protein activation by this assay relies on the preferential digestion of Ga-subunits bound to GDP (i.e., the inactive state when the α-subunit is part of the heterotrimer, see Refs. 23-27) when compared with those that are bound to GTP (i.e., the active state when the α-subunit is free, see Refs. 23-27). Thus, Ga-subunits become less sensitive to trypsin digestion upon activation of the heterotrimeric G protein (19, 20).

MA-10 cells are a clonal strain of mouse Leydig tumor cells that retain many of the differentiated functions of their normal counterparts including the expression of low levels (~10,000 receptors/cell) of endogenous LHR (12, 18, 28). When engaged by hCG the low levels of endogenous LHR present in MA-10 cells mediate an increase in cAMP and progesterone accumulation as well as an increase in the phosphorylation of ERK1/2 but do not mediate an increase in inositol phosphate accumulation (12, 29). Although these findings predict that addition of hCG to untransfected MA-10 cells would protect Gαs from trypsin digestion, this was not found to be the case (Fig. 1). This apparent discrepancy is presumably because of the relative insensitivity of the trypsin degradation assay when compared with the cAMP RIA. On the other hand, because transient transfection of MA-10 cells with the hLHR-wt (to a density of ~100,000 receptors/cell) greatly enhances the hCG-induced increases in cAMP, progesterone, and phospho-ERK1/2, and it also allows the cells to respond to hCG with an increase in inositol phosphate accumulation (12), we reasoned that this manipulation would allow for the LHR-induced activation of G proteins to become detectable. The results presented in Figs. 2-4 show that this is indeed the case.

Figure 2 shows that Gα11 is readily degraded by trypsin when membranes prepared from cells expressing the hLHR-wt are incubated without any additions or with GTPγS. Incubation of these membranes with GTPγS and hCG protects Gα11 from proteolysis, however, indicating that Gα11 is activated when hCG binds to the transfected hLHR-wt. Figure 2 also show the activation of Gs by the transfected

1 Both preparations were used in this study and were found to be indistinguishable.
The detection of an hCG-induced activation of Gs\(^2\) only in MA-10 cells transfected with the hLHR-wt (compare Figs. 1 and 2) is desirable for our studies because the identification of the G proteins activated by the CAMs of the hLHR is best accomplished under conditions where G protein activation by the endogenous LHR is low or undetectable. Therefore, additional experiments designed to measure the activation of the G\(_i/o\) (Fig. 3), G\(_q/11\) (Fig. 4), and G\(_{12/13}\) (Fig. 5) families of G proteins were done using MA-10 cells transiently expressing the hLHR-wt or mutants thereof (12). Although the overall pattern of protection of the different G protein families is not identical (compare protections patterns seen in Figs. 2–4), these data show that, when engaged by hCG, the hLHR-wt can protect members of the G\(_i/o\) (Fig. 3) and the G\(_{q/11}\) (Fig. 4) families of G proteins. This degree of proteolytic protection was clearly much lower than that elicited by a combination of hCG and GTP\(_S\), however, and it is likely to be a reflection of G protein activation that is known to occur when the basal rates of GDP/GTP exchange are altered by addition of GTP\(_S\) (23–27) and/or a reflection of a small degree of constitutive activity of the hLHR-wt (12).\(^3\)

In contrast to the results obtained with G\(_i/o\), G\(_i/o\), and G\(_q/11\), we could not detect any protection of G\(_{13}\) in MA-10 cells expressing the hLHR-wt or mutants thereof (Fig. 5). G\(_{13}\) is present at very low levels in MA-10 cells, and the only available antibody that detected it also cross-reacted with several other membrane proteins that have a higher molecular weight than authentic G\(_{13}\). These cross-reacting proteins are also sensitive to proteolytic digestion as expected (Fig. 5). The possible activation of G\(_{12}\), the other member of the G\(_{12/13}\) family of proteins, could not be ascertained because G\(_{12}\) was undetectable in MA-10 cells.

The experiments shown in Figs. 3 and 4 were done using antibodies that recognize two different members of the G\(_i/o\) family or the G\(_{q/11}\) family, respectively. Our attempts to directly identify the members of the G\(_i/o\) and G\(_q/11\) families that become activated by the hLHR-wt and mutants thereof were hindered by the availability of antibodies that are useful for the trypsin sensitivity assays. In our experience, the best antibodies for these assay are those directed against the C-terminal ends of the different Ga-subunits, but these are not available for all members of the G\(_i/o\) and G\(_q/11\) families. Therefore, the only way to more accurately address the identity of the G proteins activated

\(^2\) We did not attempt to measure the hCG-induced activation of other G proteins in untransfected MA-10 cells because the cAMP pathway is the only G protein-dependent pathway known to be stimulated by hCG in these cells (12, 18, 28).

\(^3\) The reasons why a small degree of GTP\(_S\)-induced protection is detectable with G\(_i/o\) and G\(_q/11\) but not G\(_s\) were not investigated.
by the hLHR was to use available antibodies against the different members of these families to probe Western blots of MA-10 cell lysates. The results presented in Fig. 6A for different members of the Gi/o family show that only Gαi3 and Gαo are detectable in MA-10 cells. The more intense signal detected with the Gαi3/o antibody when compared with that detected with the Gαi3 antibody could be due to a higher abundance of Gαo relative to Gαi3 or to a higher avidity of the antibody for Gαo relative to Gαi3. Because this is the antibody used in the activation assays we can readily conclude that the activation assay shown in Fig. 3 detects mostly the activation of Gαo. Likewise, the results presented in Fig. 6B show that only Gα11 is detectable in MA-10 cells and imply that the activation assay shown in Fig. 4 is most likely detecting the activation of Gα11 rather than Gαq. Functionally, this should not make any difference because Gα11 and Gαq have very similar or identical functional properties (32).

Because the activation of phospholipase C by the LHR can be mediated by Gβ/γ-subunits of the Gi/o family as well as by Gαq and Gαo (14–16) and the hLHR-wt and CAMs can couple to members of the Gq/11 (Fig. 4) and the Gi/Go families (Fig. 3), we also determined if the activation of phospholipase C is mediated by the same or by different families of G proteins. This was tested using pertussis toxin, which prevents the receptor-mediated activation of the Gi/Go family but not that of the Gq/G11 family (23–27). We found that pertussis toxin had no effect on the basal or hCG-stimulated levels of inositol phosphates in MA-10 cells expressing the hLHR-wt, or on the constitutively elevated levels of inositol phosphates found in MA-10 cells expressing the hLHR-L457R, -D578Y, or -D578H mutants (data not shown). These experiments demonstrate that, in MA-10 cells, the LHR-induced activation of inositol phosphate accumulation is mediated by Gq/G11 rather than Gi/Go. This is true not only for the agonist-engaged hLHR-wt but also for each of the three CAMs studied here.

**Discussion**

The identity of the G proteins activated by the agonist-engaged wt LHR has been previously examined by two dif-
different groups of investigators (15, 16, 21, 33). One group reported that the recombinant mouse LHR expressed in mouse L cells or the endogenous LHR present in bovine luteal membranes activated G\(_s\) and G\(_i\)-2 but not G\(_q/11\), G\(_{12}\), or G\(_{13}\) (15), whereas the other group reported that the endogenous LHR present in porcine follicular membranes activates G\(_s\), G\(_i\), G\(_{13}\), and G\(_{q/11}\) (21, 33). The data presented here show that, when engaged by hCG, the recombinant hLHR-wt expressed in MA-10 cells activates G\(_s\), G\(_{o}\), and G\(_{11}\) but not G\(_{13}\). The identify of the G proteins that mediate the LHR-induced activation of phospholipase C has also been investigated in heterologous cell types where it has been shown that this effect can be mediated by the G\(_{q/11}\) subunit of the G\(_i/o\) family as well as by G\(_{16}\) (14–16). Our data show that the agonist-provoked increased in inositol phosphate accumulation detected in MA-10 cells expressing the hLHR-wt or the agonist-independent increase in inositol phosphate accumulation detected in MA-10 cells expressing the L457R, -D578Y, and -D578H mutants is mediated by the G\(_{q/11}\) family of G proteins, instead of the G\(_{16}\) family. Clearly our results and those of others (14–16, 21, 33) are not in complete agreement regarding the identity of the G proteins that are activated by the agonist-engaged LHR-wt or the identity of the G proteins that mediate the activation of phospholipase C. These differences could be attributed to the tissue and/or cell examined as well as the species of origin of the LHR. We have used a mouse Leydig tumor cell line expressing the recombinant hLHR, whereas others have used the endogenous LHR present in porcine and bovine ovarian cells or the recombinant murine LHR expressed in mouse L cells, monkey kidney cells, or insect Sf9 cells.

The most important finding presented in this paper is that a constitutively active somatic mutation of the human LHR found in Leydig cell tumors activates the same families of G proteins as germ line mutations associated with Leydig cell hyperplasia. This finding is important because it has been previously proposed (4) that the somatic mutation found in the Leydig cell tumors of boys with precocious puberty (i.e., the D578H mutant) is unique in its ability to activate two signaling pathways (i.e., cAMP and inositol phosphate accumulation) and that it differs from the germ line activating mutations found in boys with Leydig cell hyperplasia and...
precocious puberty (exemplified here by the D578Y and L457R mutants), which were reported by this group to activate only the cAMP pathway. It was further proposed that the unique constitutive activation of the cAMP and the inositol phosphate signaling pathways by the D578H contributes to the neoplastic transformation of Leydig cells (4). The data presented here clearly do not support this hypothesis. The finding that the different CAMs activate the same families of G proteins is novel and should not be considered redundant with our previous report showing that these three CAMs activate the Gs,Gi/o, and Gq/11 families of G proteins when expressed in MA-10 cells (14). Therefore, the finding that the hLHR-L457R, -D578Y, and -D578H mutants activate the same families of G proteins is important because it formally excludes the possibility that their previously described ability to activate the same second messenger pathways (12) is mediated by their preferential activation of different G protein families. This new finding is, in turn, important for the understanding of the pathophysiology of reproductive disorders and Leydig cell tumors. If there was preferential activation of some G protein families by the D578H mutant as opposed to the L457R and D578Y mutants then one could envision mechanisms by which the D578H mutant would be oncogenic whereas the L457R and D578Y mutants would not be. The data presented here suggest that such mechanisms do not need to be considered. The finding that the agonist-engaged hLHR-wt and CAMs thereof activate the same families of G proteins is also novel and not necessarily anticipated from our results on the activation of second messenger pathways for the same reasons mentioned above.

In summary, our data show that the agonist-engaged hLHR-wt and three of its naturally occurring CAMs activate the Gs,Gi/o, and Gq/11 families of G proteins when expressed in MA-10 cells. The LHR-mediated activation of Gs is presumably responsible for cAMP accumulation and the LHR-mediated activation of the Gq/11 family is responsible for inositol phosphate accumulation. The functional consequences of the LHR-mediated activation of the Gi/o family in MA-10 cells remain to be established.

Acknowledgments

Received March 25, 2003. Accepted May 12, 2003.
Address all correspondence and requests for reprints to: Dr. Mario Ascoli, Department of Pharmacology, 2-319B BSB, 51 Newton Road, University of Iowa, Iowa City, Iowa 52242-1109. E-mail: mario-ascoli@uiowa.edu.

This work was supported by NIH Grant CA-40629 (to M.A.) and a fellowship from the Lalor Foundation (to T.H). The services and facilities provided by the Diabetes and Endocrinology Research Center of the University of Iowa (supported by NIH Grant DK-25295) are also gratefully acknowledged.

References


FIG. 6. Expression of the different members of the G_i/o and G_q/11 families of G proteins in MA-10 cells. Lysates of MA-10 cells were prepared and aliquots containing the same amount of protein (~100 µg/lane) were resolved on sodium dodecyl sulfate gels and electrophoretically blotted as described in Materials and Methods. The presence of the different members of the G_i/o and G_q/11 families of G proteins was ascertained by incubating individual strips from the blots with antibodies that recognize the indicated Go-subunits. The results of a representative experiment (repeated twice more) are shown. The migration of two molecular weight markers are shown on the left. The unmarked arrows between these two markers show the position of migration of authentic recombinant Go_i (top panel) or Go_q (bottom panel).