GIPC Binds to the Human Lutropin Receptor (hLHR) through an Unusual PDZ Domain Binding Motif, and It Regulates the Sorting of the Internalized Human Choriogonadotropin and the Density of Cell Surface hLHR*

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By using a yeast two-hybrid screen we identified GIPC (GAIP-interacting protein C terminus), a protein with a type I PDZ domain as a novel human lutropin receptor (hLHR) binding partner. Full-down and immunoprecipitation assays confirmed this interaction and showed that it is dependent on the PDZ domain of GIPC and the C-terminal tetrapeptide of the hLHR. To characterize the functional consequences of the GIPC-hLHR interaction, we used a small interfering RNA against GIPC to generate a clonal cell line that is deficient in GIPC. Studies with this cell line reveal that GIPC is partially responsible for the recycling of the hormone that is internalized by the hLHR and also for maintaining a relatively constant level of hLHR at the cell surface during hormone internalization.

The trafficking of G protein-coupled receptors (GPCRs) appears to be largely mediated by phosphorylation and/or ubiquitination of their intracellular domains (1–5) and/or by their association with trafficking proteins such as the non–visual arrestins (6, 7), N-ethylmaleimide-sensitive factor (8), ezrin-radixin-moesin-binding phosphoprotein-50/sodium-hydrogen exchange regulatory factor (EBP50/NHERF, see Ref. 4), sorting nexins (9), or GPCR-associated sorting protein (see Ref. 10). Little is known, however, about the identity of the GPCR residues that mediate trafficking, about the specificity of the proteins involved in sorting, and about common structural and/or functional features that may characterize these sorting proteins.

We have taken advantage of the high degree of amino acid sequence homology and divergent post-endocytotic fates of the rat (r) and human (h) lutropin receptor (LHR) to identify motifs present in the C-terminal tail of the LHR that are involved in the sorting of one of the internalized LHR ligands (i.e. human choriogonadotropin (hCG)) to a recycling or a degradation pathway (11–13). Based on these findings we hypothesized the following: (a) the hCG internalized by the hLHR is sorted to a recycling pathway by virtue of the ability of the hLHR to bind to intracellular proteins(s) that promote recycling; (b) that the interaction of the hLHR with these putative proteins is mediated by one or more of the recycling motifs identified previously; and (c) that the hCG internalized by the rLHR is sorted to a degradation pathway because the rLHR lacks these motifs. Because these putative sorting motifs are located in the extreme C-terminal tail of the hLHR but absent in the corresponding region of the rLHR, we sought to identify these putative proteins by using these regions of the hLHR and rLHR as “bait” in a differential yeast two-hybrid screen of a 293 cell library (the cell line where all trafficking experiments have been conducted).

The studies presented here describe the identification of a ubiquitous protein that binds to the hLHR and is involved in the post-endocytotic trafficking of the internalized hCG and in maintaining the levels of cell surface hLHR during endocytosis of the bound hormone.

MATERIALS AND METHODS

Yeast Two-hybrid Screen—The MatchmakerTM two-hybrid system 2 (Clontech Laboratories, Palo Alto, CA) was used according to the protocols provided by the manufacturer. By using PCR-based strategies, we subcloned the C-terminal 42 residues of the hLHR (i.e. residues 657–699) into the EcoR/BamHI sites of the pAS2-1 vector to generate a fusion protein with the GAL4 DNA binding domain. This plasmid was used as bait to screen a human kidney 293 cells cDNA library constructed in the pACT2 vector to generate fusion products with the GAL4 activation domain. This library was also purchased from Clontech.

Plasmids and Cells—Full-length cDNAs encoding for the hLHR and rLHR (14, 15) were subcloned into pcDNAI/Neo (rLHR) or pcDNA 3.1 (hLHR), respectively, for expression. The preparation and characterization of myc-rLHR-wt (in pcDNAI/Neo) and myc-hLHR-wt (in pcDNA 3.1)-modified forms of the LHR containing the Myc epitope at the N terminus have also been described (16, 17). The different mutants of the rLHR and hLHR used here were constructed by standard PCR strategies using the myc-rLHR-wt or myc-hLHR-wt as templates.

An expression vector (pFLAG-CMV from Sigma) coding for an N-terminal FLAG-tagged version of human GIPC (18) was kindly provided by Dr. V. Setaluri (Wake Forest University). A bacterial expression vector coding for a GST fusion protein of the full-length EBP50 (19, 20) was kindly donated by Dr. A. Bretscher (Cornell University, Ithaca, NY). A bacterial expression vector coding for a GST fusion protein of human GIPC was prepared by amplifying the full-length GIPC sequence from the FLAG-GIPC vector described above followed by subcloning into the EcoRI/BamHI sites of the pGEX-5X1 vector. The GST fusion protein of human GIPC lacking its PDZ domain (designated GIPCΔPDZ) was prepared by deletion of the nucleotides coding for amino acid residues 125–225. This was done by standard PCR strategies using the full-length GIPC GST fusion construct described above as a template. GST fusion proteins were prepared as described elsewhere (19, 20).

An oligonucleotide corresponding to inverted copies of nucleotides 240–260 (AAGGAGCTGATGGAAGATT) of the human GIPC mRNA

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† The abbreviations used are: GPCR, G protein-coupled receptor; LHR, lutropin receptor; hCG, human choriogonadotropin; r, rat; h, human; GST, glutathione S-transferase; hCG, human choriogonadotropin; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; wt, wild type.
separated by a 6-nucleotide spacer (GAGTACTG) and containing SalI and XbaI sites was synthesized commercially and subcloned into the SalI and XbaI sites of the pSupressor2 vector from Imgenex® according to their instructions. This siRNA expression vector was then used to transfect 293 cells (see below) to prepare a clonal line of GIPC-deficient 293 cells (see below).

Human kidney 293T cells are a derivative of 293 cells that express the SV40T antigen (21) and were provided to us by Dr. Marlene Hosey (Northwestern University, Chicago, IL). These cells were maintained in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 10% newborn calf serum, and 50 μg/ml gentamicin, pH 7.4, and used for all the transient transfection assays. Cells were plated in gelatin-coated 35-mm wells and transiently transfected with 0.5 μg of plasmid DNA, using the calcium phosphate methods of Chen and Okayama (22), when 70–80% confluent. After an overnight incubation with the transfection mixture, the cells were washed and used 24 h later.

FIG. 1. Identification of GIPC as a new binding partner for the hLHR. Screening of a human 293 cell library with a fragment corresponding to the last 42 residues of the C-terminal tail of the hLHR resulted in the identification of 13 positive clones. Four of these clones corresponded to the indicated regions of human GIPC. GIPC is a protein identified previously that contains a proline-rich domain, a type I PDZ domain, and an acyl carrier protein (ACP) domain (33, 34). The domain structure of GIPC and the four identified clones are shown.

FIG. 2. The association of GIPC with the hLHR depends on several of the C-terminal residues of the hLHR. Detergent lysates of 293T cells transiently transfected with the Myc-tagged versions of the hLHR-wt or mutants thereof were prepared and partially purified on a lectin column as described under “Materials and Methods.” Aliquots of the partially purified lysates containing equivalent amounts of receptors (A) were allowed to bind to GST-GIPC that had been bound previously to glutathione-agarose. The bound proteins were washed, eluted, and visualized on Western blots developed using a monoclonal antibody to the Myc epitope (9E10) and a secondary antibody coupled to horseradish peroxidase as described under “Materials and Methods” (B). A and B show only the relevant portions of a blot of a representative experiment. The 85-kDa band is the mature cell surface LHR, and the 68-kDa band is an immature precursor of the LHR found in the endoplasmic reticulum (11). The hLHR is 699 residues long, and the sequence of its C-terminal tetrapeptide is YTEC (see Fig. 4). Human LHR-t695, -t696, -t697, and -t698 are mutants of the hLHR that were C-terminally truncated at residues 695, 696, 697, and 698, respectively. C shows a Coomassie-stained gel of the GST-GIPC fusion protein (1 μg) used in the pull-down assays. Note that in addition to the full-length GST-GIPC, which migrates near the 90-kDa marker, some degradation products are detectable near the 36-kDa marker.
Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (CRL 1573). They were maintained and transfected as described above, but they were used only to obtain clonal lines of stably transfected cells expressing the GIPC siRNA vector described above or a control vector that confers G418 resistance. Clonal lines of transfected cells were obtained by selection with 700 μg/ml G418 as described elsewhere (23). The desired lines were selected based on the expression of endogenous GIPC as measured by Western blots using a rabbit polyclonal antibody to GIPC (18) kindly provided to us by Dr. V. Setaluri of Wake Forest University.

GST Pull-down Assays—The interactions between the Myc-tagged forms of the LHR and mutants thereof with GIPC, EBPF50, and derivatives were determined by measuring the ability of detergent lysates prepared from transiently transfected cells to bind to the indicated GST fusion proteins. Lysates of cells expressing the Myc-tagged LHR constructs were prepared and partially purified on a wheat germ agglutinin-agarose column as described elsewhere (16, 24), except that the lysis buffer contained 1% Nonidet P-40 and 60 mM octyl glucoside. Aliquots of lysates containing equivalent amounts of receptors were incubated with 25 μg of the appropriate GST fusion proteins bound to glutathione-agarose and washed as described previously (19, 20). The bound proteins were eluted by incubating the resin at 37 °C for 30 min followed by vigorous vortexing for 15 min at room temperature. The eluted samples were resolved on SDS gels and electrophoretically blotted as described elsewhere (24). Blots were visualized using a monoclonal antibody to the Myc epitope (9E10) followed by a secondary antibody coupled to horseradish peroxidase. The complexes were directly visualized in the blots by using a combination of the Super Signal West Femto Maximum Sensitivity system of detection (Pierce) and a Kodak digital imaging system.

Co-immunoprecipitation Assays—These assays were done using a co-transfection/cross-linking/co-immunoprecipitation approach recently developed in this laboratory (25–27). Briefly, transiently transfected cells were stabilized by cross-linking with dithiobis(succinimidylpropionate). The transfected myc-hLHR or FLAG-GIPC was immunoprecipitated from cell lysates using a monoclonal antibody to the Myc epitope (9E10) or a monoclonal antibody to the FLAG epitope (M2) and resolved on SDS gels. The gels were electrophoretically blotted, and the blots were incubated with anti-FLAG (M2) or anti-Myc (9E10) monoclonal antibodies covalently coupled to horseradish peroxidase as described elsewhere (25–27). The complexes were directly visualized using a combination of the Super Signal West Femto Maximum Sensitivity system of detection (Pierce) and a Kodak digital imaging system.

 Fate of the Internalized hCG—Transiently transfected cells were allowed to internalize 125I-hCG during a 2-h incubation at 37 °C with a saturating concentration of hormone (52 nM). After washing to remove the free hormone, the surface-bound 125I-hCG was released by a brief exposure of the cells to an isotonic pH 3 buffer (12, 28–30). This was defined as 0, and the cells (which now contain only internalized 125I-hCG) were incubated for an additional 2 h at 37 °C in medium containing an excess of non-radioactive hCG (4 μg/ml). These conditions facilitate the detection of the recycled hormone by preventing the reassociation of the recycled and released hCG with the receptor (30). At the end of this second incubation the medium was saved, and the cells were washed with cold medium. They were then briefly exposed again to the isotonic pH 3 buffer to release and measure any of the internalized hormone that had recycled back to the surface. The acid-stripped cells were solubilized with NaOH to measure residual radioactivity that remained internalized. Finally, the saved medium was precipitated with 10% trichloroacetic acid to determine the amount of degraded and undegraded 125I-hCG released (12, 28–30).

Fig. 3. The association of the hLHR-wt with GIPC occurs through the PDZ-domain binding motif of GIPC. Detergent lysates of 293T cells transiently transfected with the Myc-tagged versions of the hLHR-wt or hLHR-1695 were prepared and partially purified on a lectin column as described under “Materials and Methods.” Aliquots of the partially purified lysates containing equivalent amounts of receptors (A) were allowed to bind to GST, GST-GIPC, GST-GIPC(ΔPDZ), or GST-EBP50 that had been bound previously to glutathione-agarose. The bound proteins were washed, eluted, and visualized on Western blots developed in this laboratory (25–27). Briefly, transiently transfected cells were allowed to bind to GST, GST-GIPC, GST-GIPC(ΔPDZ), or GST-EBP50 that had been bound previously to glutathione-agarose. The bound proteins were washed, eluted, and visualized on Western blots developed using a monoclonal antibody to the Myc epitope (9E10) and a secondary antibody coupled to horseradish peroxidase as described elsewhere (25–27). The complexes were directly visualized in the blots by using a combination of the Super Signal West Femto Maximum Sensitivity system of detection (Pierce) and a Kodak digital imaging system.

Confocal Microscopy—Confocal microscopy experiments were accomplished as described recently (13, 25). Briefly, 293T cells were plated in eight-chamber coverslip culture vessels coated with polylysine (Biocoat from BD Biosciences). They were transfected (in a total volume of 400 μl) with 100 ng of the expression vector for the myc-hLHR-wt or co-transfected with 100 ng of the expression vector for the myc-hLHR-wt and 10 ng of the expression vector for the FLAG-GIPC. Two days after the transfection the myc-hLHR was visualized by incubating the cells for 1 h at room temperature with an anti-Myc monoclonal antibody (9E10) diluted 1/100 in phosphate-buffered saline containing 5 mg/ml bovine serum albumin, and the endogenous or transfected GIPC were visualized using the polyclonal GIPC antibody diluted 1/500 in the same

SDS gels and electrophoretically transferred to polyvinylidene difluoride membranes (31). The blots were revealed using streptavidin conjugated to horseradish peroxidase. The complexes were directly visualized and quantitated using the Super Signal West Femto Maximum Sensitivity system of detection from Pierce and a Kodak digital imaging system as described elsewhere (17). This image capture system is set up to alert us when image saturation occurs and to prevent us from measuring the intensity of such images.

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buffer. After washing three times, the cells were incubated for another hour at room temperature with a 1/100 dilution of CyTM5-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) or with a 1/2000 dilution of FITC-conjugated anti-rabbit IgG (Sigma). Finally, the cells were washed three or four times, dried, and mounted as described previously (13, 25). The CyTM5-labeled receptors and the FITC-labeled GIPC were visualized with a Bio-Rad confocal microscope at the Central Microscopy Facility of the University of Iowa. An oil 60/H11003 objective was used, and the iris opening was 2 to 2.2 for each color filter.

### Hormones and Supplies

Purified hCG (CR-127, 13,000 units/mg) was purchased from Dr. A. Parlow of the National Hormone and Pituitary Agency of the NIDDK. Recombinant human hCG was kindly provided by Ares Serono. Partially purified hCG (~3,000 units/mg) was purchased from Sigma, and it was used only to correct for nonspecific binding. 125I-hCG was prepared as described previously (32). Cell culture supplies and reagents were obtained from Corning Glass and Invitrogen, respectively. All other chemicals were obtained from commonly used suppliers.

### RESULTS

Yeast Two-hybrid Screening—Screening of 10^7 independent colonies of a human 293 cell library with a fragment corresponding to the last 42 residues of the C-terminal tail of the hLHR resulted in the identification of 13 positive clones. Four of these clones coded for regions of human GIPC (33, 34) that overlap extensively with the single PDZ domain of this protein (Fig. 1). Because clone 4 coded only for the PDZ domain of GIPC, these data suggest that the interaction of the hLHR and GIPC occurs through the PDZ domain of GIPC.

### Structural Determinants That Influence the Formation of the GIPC-hLHR Complex

The association of GIPC with the hLHR was next documented in vitro using extracts of 293T cells transiently transfected with the myc-hLHR-wt or mutants thereof and a GST fusion protein of the full-length human GIPC. As expected, lysates of 293T cells expressing the myc-hLHR-wt reveal the presence of 85- and 68-kDa bands (Fig. 2A) that represent the mature cell surface receptor and its immature intracellular precursor, respectively (11). With the exception of hLHR-E698A, which was expressed only as the 68-kDa precursor, all other mutants tested were expressed as the 85- and 68-kDa bands, and their levels of expression were similar to that of the hLHR-wt (Fig. 2A, 2B). The reasons for these differences were not investigated. A Coomassie-stained gel of GST and the different GST fusion proteins used in the pull-down assays can be found in Fig. 3C.

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**A = amino acid sequences**

| Sequence | 663 | 664 | 665 | 666 | 667 | 668 | 669 | 670 | 671 | 672 | 673 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| hLHR     | GFTGSKPSQSLKLSTLHCQGTALLDKTRYTEC |       |     |     |     |     |     |     |     |     |     |
| rLHR     | GFTGSKPSQSLKLSTLHCQGTALLDKTRYTEC |       |     |     |     |     |     |     |     |     |     |
| rLHR+YTEC| GFTGSKPSQSLKLSTLHCQGTALLDKTRYTEC |       |     |     |     |     |     |     |     |     |     |
| rLHR+DSLL| GFTGSKPSQSLKLSTLHCQGTALLDKTRYTEC |       |     |     |     |     |     |     |     |     |     |

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**B = myc-rLHR added**

| kDa | 85 | 68 |
|-----|----|----|
| 1   | rLHR-wt     |     |
| 2   | rLHR+YTEC  |     |
| 3   | rLHR+DSLL  |     |

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**C = myc-rLHR pulled down with different GST fusion proteins**

![Diagram showing binding of GST fusion proteins](http://www.jbc.org/)

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*Fig. 4. When grafted into the rLHR, the C-terminal tetrapeptide of the hLHR allows the rLHR to bind to GIPC but not EBP50.* A shows the aligned amino acid sequences of the C-terminal tails of the hLHR, the rLHR and mutants thereof starting at amino acid residues 663 and 641, respectively. Detergent lysates of 293T cells transiently transfected with the Myc-tagged versions of the rLHR-wt or mutants thereof were prepared and partially purified on a lectin column as described under “Materials and Methods.” Aliquots of the partially purified lysates containing equivalent amounts of receptors (B) were allowed to bind to GST, GST-GIPC, GST-GIPC(ΔPDZ), or GST-EBP50 that had been bound previously to glutathione-agarose. The bound proteins were washed, eluted, and visualized on Western blots developed using a monoclonal antibody to the Myc epitope (9E10) and a secondary antibody coupled to horse-radish peroxidase as described under “Materials and Methods” (C). Only the relevant portions of a blot of a representative experiment are shown in B and C. Note that the relative abundance of the 85- and 68-kDa bands is reversed in the extracts of cells expressing the rLHR and hLHR (compare this figure with Figs. 2 and 3). This difference has been recognized before, and the reasons for it have been discussed (11). Also note that there is a small difference in the relative abundance of the 85-kDa band as well as in the apparent sizes of the 68- and 85-kDa bands for the rLHR-DSLL when compared with the rLHR-wt and rLHR+YTEC (B and C). The reasons for these differences were not investigated. A Coomassie-stained gel of GST and the different GST fusion proteins used in the pull-down assays can be found in Fig. 3C.

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2 The reasons for the inability of the hLHR-E698A to be processed properly were not investigated.
GIPC Binds to LHR through Unusual PDZ Domain Binding Motif

GIPC associates with the hLHR through a PDZ interacting domain in co-transfected cells. 293T cells were transiently transfected with the Myc-tagged versions of the hLHR-wt or hLHR-t695 and FLAG-GIPC as indicated. Cells were treated with or without a permeable cross-linker (see “Materials and Methods”) prior to lysis as indicated. The Myc-tagged receptors were immunoprecipitated (IP) from the lysates with a monoclonal antibody to the Myc epitope (9E10). The presence of the receptors and GIPC in the receptor immunoprecipitates were revealed by immunoblotting (IB) with the 9E10 antibody (top panels) or an anti-FLAG antibody (M2) conjugated to horseradish peroxidase (bottom panels). Only the relevant portions of a blot of a representative experiment are shown.

Collectively, these data show that the GIPC/hLHR interaction involves binding of the C-terminal tetrapeptide of the hLHR to the single (type I) PDZ domain of GIPC. Moreover, the results obtained with EBP50 suggest that the hLHR may also interact weakly with other proteins that have type I PDZ domains.

Association of the hLHR and GIPC in Transfected Cells—The association of the hLHR and GIPC was next documented in 293T cells transiently co-transfected with the myc-hLHR-wt and FLAG-GIPC. Immunoprecipitation of the myc-hLHR-wt followed by immunoblotting for GIPC readily showed the formation of an hLHR-GIPC complex (Fig. 5, left panel). The data presented in the middle panel of Fig. 5 also show that the detection of this complex can be enhanced if the complex is stabilized by cross-linking the cells prior to lysis and immunoprecipitation of the receptor.

Because the association of GST-GIPC with the hLHR was abolished by truncation of the hLHR at position 695 (see Figs. 2 and 3), we also tested for the presence of GIPC in receptor immunoprecipitates of cells co-transfected with the myc-hLHR-t695 and FLAG-GIPC. In agreement with the GST pull-down assays, these intact cell experiments revealed that the myc-hLHR-t695 does not associate with FLAG-GIPC (Fig. 5, right panel).
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Fig. 7. The transfected hLHR associates with endogenous GIPC in 293T cells. 293T cells were transiently transfected with the Myc-tagged versions of the hLHR-wt or hLHR-t695 as indicated. The cells were treated with a permeable cross-linker (see “Materials and Methods”) prior to lysis, and the transfected receptors were immunoprecipitated (IP) with a monoclonal antibody to the Myc epitope (9E10). The presence of the transfected receptors and endogenous GIPC in the immunoprecipitates were revealed by immunoblotting (IB) with the 9E10 antibody coupled to horseradish peroxidase or a polyclonal anti-GIPC antibody followed by a rabbit antibody coupled to horseradish peroxidase. Only the relevant portions of a blot of a representative experiment are shown.

In a complementary set of experiments, we co-transfected 293T cells with the myc-hLHR-wt and FLAG-GIPC and incubated them with or without a saturating concentration of agonist (hCG). The cells were then cross-linked, and the receptor or GIPC was immunoprecipitated with the appropriate antibodies, and the complementary binding partner was detected in each of the immunoprecipitates. These data (Fig. 6A) revealed that the formation of the GIPC-hLHR complex occurs independently of agonist stimulation. In four independent experiments, the ratio of GIPC to hLHR present in the immunoprecipitates was 0.78 ± 0.03 and 0.72 ± 0.13 (mean ± S.E.) in the control and hCG treated cells, respectively. The results summarized in Fig. 6B also show that GIPC binds to the mature cell surface LHR as well as its immature intracellular precursor. This latter conclusion is based on the finding that the 85- and 68-kDa species of the LHR can be detected in FLAG-GIPC immunoprecipitates.

The association of the transfected myc-hLHR-wt with endogenous GIPC was also documented by probing for the presence of endogenous GIPC in immunoprecipitates of 293T cells transfected with the myc-hLHR. These data show that endogenous GIPC co-immunoprecipitates with the transfected myc-hLHR-wt but not with the transfected myc-hLHR-t695 (Fig. 7).

Examination of 293T cells by confocal microscopy (Fig. 8) revealed that the endogenous or transfected GIPC shows a diffuse as well as a punctate pattern similar to that reported in HeLa cells (33). Co-localization of the endogenous (Fig. 8A) or transfected GIPC (Fig. 8B) with the transfected myc-hLHR-wt shows that they co-localize at or near the plasma membrane. In agreement with the pull-down and co-immunoprecipitation assays (Figs. 2, 3, 5, and 7), there was no apparent co-localization of the transfected myc-hLHR-t695 and the transfected GIPC (Fig. 8B).

Functional Consequences of the hLHR/GIPC Interaction—A substantial portion of the hCG that is internalized by the hLHR is recycled back to the medium as undegraded hormone, and the density of cell surface hLHR remains relatively constant during many rounds of endocytosis of hCG (12, 13, 30). Both of these phenomena are dependent on the presence of Leu683 and the C-terminal Cys699 of the hLHR (Tables I and II). Because the data presented above showed that Cys699 is also necessary for the binding of the hLHR to GIPC, we hypothesized that the formation of the hLHR/GIPC complex could be (at least partially) responsible for routing the internalized hCG to a recycling pathway and for maintaining the levels of cell surface hLHR during the endocytosis of hCG.

To test this possibility, we prepared a clonal line of 293 cells deficient in GIPC by stable transfection of the cells with a GIPC siRNA expression vector that also confers G418 resistance. Out of 21 resistant colonies tested, only two had levels of GIPC that were reduced by more than 50%. A representative Western blot of whole cell lysates of the G418-resistant cell line with the lowest levels of GIPC (designated GIPC−/G418R), the wild-type 293 cells (wt), and another stable 293 transfectant (designated G418R) expressing an unrelated vector that also confers G418 resistance are shown in Fig. 9. A quantitative assessment of GIPC levels in the GIPC+/G418R cell line performed in four independent experiments revealed that the levels of GIPC were reduced by 79 ± 0.3% compared with those of wild-type 293 cells.

The potential involvement of GIPC in the recycling of the hCG internalized by the hLHR was tested by comparing the fate of the internalized 125I-hCG in the GIPC−/G418R cells and the wild-type 293 cells transiently expressing several hLHR constructs. We chose to examine three mutants of the hLHR. The postendocytotic trafficking of the hCG internalized by the hLHR-L683A mutant is similar to that mediated by the hLHR-wt (Table I). The hLHR-t695 mutant redirects some of the internalized hCG from a recycling to a degradation pathway (Table I), and the simultaneous mutation of these two regions (i.e. the hLHR-L683A/t695 mutant) redirects more of the internalized 125I-hCG from a recycling to a degradation pathway (Table I). The routing of the hCG internalized by the hLHR-L683A/t695 mutant is in fact indistinguishable from that of the rLHR-wt (Table I), a homologous receptor that terminates in an ALHT sequence instead of a YTEC sequence (35). Because hLHR-t695 does not interact with GIPC but the L683A mutant displays a strong interaction with GIPC (Fig. 10), we hypothesized that the postendocytotic trafficking of 125I-hCG mediated by the hLHR-wt expressed in the GIPC+/G418R cells should mimic that of the hLHR-t695 expressed in the wild-type 293 cells and that the trafficking of 125I-hCG mediated by the hLHR-L683A mutant expressed in the GIPC+/G418R cells should mimic that of the hLHR-L683A/t695 mutant expressed in the wild-type 293 cells. Our hypothesis would also predict that the functional properties of the hLHR-L695 and hLHR-L683A/t695 mutants would be similar when expressed in the wild-type 293 cells and in the GIPC+/G418R cells.

Table I shows that the ratio of recycled to degraded 125I-hCG in cells expressing the hLHR-wt is lower in the GIPC+/G418R than in the wild-type 293 cells. As predicted, the ratio displayed by the hLHR-wt in the GIPC+/G418R cells is in fact comparable with that of the hLHR-L695 expressed in the wild-type 293 cells. The ratio of recycled to degraded 125I-hCG for the hLHR-L683A mutant is also lower in the GIPC+/G418R cells.
Subcellular localization of the myc-hLHR and the endogenous or transfected GIPC in 293T cells. 293T cells were transiently co-transfected with the myc-hLHR-wt only (A) or with myc-hLHR-wt and FLAG-GIPC or myc-hLHR-t695 and FLAG-GIPC as indicated (B). The cells were fixed, and the receptors (in red) were visualized using an anti-Myc monoclonal antibody (9E10) and a Cy75-conjugated anti-mouse antibody, and the endogenous (A) or endogenous + transfected GIPC (B) was visualized using a polyclonal antibody to GIPC and a FITC-conjugated anti-rabbit antibody. The cells were observed and analyzed using a Bio-Rad confocal microscope at the Central Microscopy Facility of the University of Iowa. Arrowheads highlight the co-localization of GIPC and the hLHR-wt.

The data presented in Table I for the different mutants expressed in the wild-type 293 cells predict that the hCG-induced decrease of the cell surface density of the hLHR-L683A mutant would be similar to that of the hLHR-wt but the hCG-induced decrease of the cell surface density of the hLHR-t695 and hLHR-L683A/t695 mutants should be greater than that of the hLHR-wt. The data presented in Table II show that these predictions are met for the hLHR-L683A mutant and the hLHR-L683A/t695 mutant. Contrary to expectations, however, the hCG-induced loss of receptors is comparable for the hLHR-wt and hLHR-t695 mutant expressed in the wild-type 293 cells (Table II). This discrepancy is likely due to the slower rate of internalization of hCG mediated by the hLHR-t695 mutant (see above).

**DISCUSSION**

GIPC was initially identified (using a yeast two-hybrid screen) as a protein that interacts with the C terminus of GaIP, a member of the regulator of protein signaling (RGS) family (33), and with the C terminus of the viral oncoprotein Tax (34). GIPC is a ubiquitously expressed small protein (333 residues, ~36 kDa) that has a proline-rich region, one type I PDZ domain (33), and with the C terminus of the viral oncoprotein Tax (34). A growing number of membrane proteins that bind to the PDZ domain of GIPC through their C termini include the glucose transporter GLUT1 (37), transmembrane Semaphorin-F (38), neurophilin-1 (39), syndecan-4 (40), tyrosinase-related protein-1 (18), the α5 and α6 subunits of integrin (41), the type III transforming growth factor-β receptor (42), the insulin-like growth factor 1 receptor (43), megalin (44), the 5T4 antigen (45), and the β1-adrenergic receptor (46). Interestingly, a region of the C-terminal tail of the TrkA (the nerve growth factor receptor) that does not include the extreme C terminus has also been shown to bind to the PDZ domain of GIPC (47).

The results presented here show that GIPC binds to the hLHR through a typical PDZ domain/C-terminal tail interaction, but the C-terminal tetrapeptide of the hLHR (YTEC) contains only one residue (the Thr in the -2 position) of the consensus sequence (X(S/T)(X)(V/L)) that appears to be recognized by type I PDZ domains (48, 49). Our analysis of the structural features of the hLHR required for the hLHR/GIPC interaction shows that the individual mutation of any of the last four residues (YTEC) of the hLHR reduce GIPC binding.
Postendocytic trafficking of the 125I-hCG internalized by selected hLHR mutants in GIPC-deficient cells

The wild-type 293 cells or the GIPC+/G418R cells were transiently transfected with the indicated constructs, and the fate of the internalized 125I-hCG was measured as described under "Materials and Methods." Results are the mean ± S.E. of 6–10 independent transfections, and they are expressed as percent of the total intracellular 125I-hCG radioactivity present at the beginning of the second phase of the experiment. The degraded and recycled hormone add up to only 60–65% of the initial radioactivity. The rest remains in intracellular compartments (data not shown). The numbers in parentheses display the mean ± S.E. of the ratios of recycled to degraded hormone.

| Receptor          | Wild-type | GIPC+/G418R |
|-------------------|-----------|-------------|
|                   | Degraded  | Recycled    | Degraded  | Recycled    |
| hLHR-wt           | 26 ± 1    | 37 ± 1 (1.39 ± 0.05) | 36 ± 1    | 29 ± 1 (0.83 ± 0.05)* |
| hLHR-L683A        | 26 ± 2    | 33 ± 1 (1.21 ± 0.08) | 40 ± 1    | 23 ± 1 (0.59 ± 0.03)* |
| hLHR-t695         | 34 ± 1    | 28 ± 1 (0.86 ± 0.05)* | 41 ± 1    | 23 ± 1 (0.57 ± 0.02)* |
| hLHR-L683A/t695   | 39 ± 2    | 19 ± 1 (0.49 ± 0.03)* | 46 ± 2    | 16 ± 1 (0.36 ± 0.03) |
| rLHR-wt           | 42 ± 1    | 18 ± 1 (0.44 ± 0.02)* | 42 ± 1    | 17 ± 1 (0.41 ± 0.02) |

* Significantly different (p < 0.01) from the same receptor expressed in wild-type cells by one-way analysis of variance.

Materials and Methods.

The wild-type 293 cells or the GIPC+/G418R cells were transiently transfected with the indicated constructs and biotinylated. The cells were then lysed immediately or incubated with hCG (52 nM) for 6 h at 37 °C prior to lysis. The amount of biotinylated receptor was measured in streptavidin blots of receptor immunoprecipitates as described under "Materials and Methods." Results are the mean ± S.E. of 8–9 independent transfections and are expressed as % of the receptor present in the non-stimulated cells.

| Receptor        | 293 cell line | 293 cell line |
|-----------------|---------------|---------------|
|                  | Degraded      | Recycled      | Degraded      | Recycled      |
|                  | (initial cpm) | (% initial)   | (initial cpm) | (% initial)   |
| hLHR-wt         | 26 ± 1        | 37 ± 1 (1.39 ± 0.05) | 36 ± 1        | 29 ± 1 (0.83 ± 0.05)* |
| hLHR-L683A      | 26 ± 2        | 33 ± 1 (1.21 ± 0.08) | 40 ± 1        | 23 ± 1 (0.59 ± 0.03)* |
| hLHR-t695       | 34 ± 1        | 28 ± 1 (0.86 ± 0.05)* | 41 ± 1        | 23 ± 1 (0.57 ± 0.02)* |
| hLHR-L683A/t695 | 39 ± 2        | 19 ± 1 (0.49 ± 0.03)* | 46 ± 2        | 16 ± 1 (0.36 ± 0.03) |
| rLHR-wt         | 42 ± 1        | 18 ± 1 (0.44 ± 0.02)* | 42 ± 1        | 17 ± 1 (0.41 ± 0.02) |

* Significantly different (p < 0.01) from the same receptor expressed in wild-type cells by one-way analysis of variance.

Results are the mean ± S.E. of 8 independent transfections, and they are expressed as percent of the total intracellular 125I-hCG radioactivity present at the beginning of the second phase of the experiment. The degraded and recycled hormone add up to only 60–65% of the initial radioactivity. The rest remains in intracellular compartments (data not shown). The numbers in parentheses display the mean ± S.E. of the ratios of recycled to degraded hormone.

TABLE II

HCG-Induced down-regulation of selected mutants of the hLHR in GIPC-deficient cells

The wild-type 293 cells or the GIPC+/G418R cells were transiently transfected with the indicated constructs and biotinylated. The cells were then lysed immediately or incubated with hCG (52 nM) for 6 h at 37 °C prior to lysis. The amount of biotinylated receptor was measured in streptavidin blots of receptor immunoprecipitates as described under "Materials and Methods." Results are the mean ± S.E. of 8–9 independent transfections and are expressed as % of the receptor present in the non-stimulated cells.

| Receptor          | 293 cell line | 293 cell line |
|-------------------|---------------|---------------|
|                  | Degraded      | Recycled      | Degraded      | Recycled      |
|                  | (initial cpm) | (% initial)   | (initial cpm) | (% initial)   |
| hLHR-wt           | 26 ± 1        | 37 ± 1 (1.39 ± 0.05) | 36 ± 1        | 29 ± 1 (0.83 ± 0.05)* |
| hLHR-L683A        | 26 ± 2        | 33 ± 1 (1.21 ± 0.08) | 40 ± 1        | 23 ± 1 (0.59 ± 0.03)* |
| hLHR-t695         | 34 ± 1        | 28 ± 1 (0.86 ± 0.05)* | 41 ± 1        | 23 ± 1 (0.57 ± 0.02)* |
| hLHR-L683A/t695   | 39 ± 2        | 19 ± 1 (0.49 ± 0.03)* | 46 ± 2        | 16 ± 1 (0.36 ± 0.03) |
| rLHR-wt           | 42 ± 1        | 18 ± 1 (0.44 ± 0.02)* | 42 ± 1        | 17 ± 1 (0.41 ± 0.02) |

* Significantly different (p < 0.01) from the same receptor expressed in wild-type cells by one-way analysis of variance.

A Western blot of whole cell lysates of the wild-type 293 cells (designated wt), a clonal 293 cell line stably transfected with a GIPC siRNA expression vector that also confers G418 resistance (designated GIPC+/G418R), or a clonal 293 cell line stably transfected with an irrelevant vector that also confers G418 resistance (designated G418R) is shown. The blot was developed with a polyclonal antibody to GIPC and a secondary anti-rabbit antibody coupled to horseradish peroxidase. IB, immunoblot.

FIG. 9. Endogenous GIPC can be reduced by siRNA in 293 cells. A Western blot of whole cell lysates of the wild-type 293 cells (designated wt), a clonal 293 cell line stably transfected with a GIPC siRNA expression vector that also confers G418 resistance (designated GIPC+/G418R), or a clonal 293 cell line stably transfected with an irrelevant vector that also confers G418 resistance (designated G418R) is shown. The blot was developed with a polyclonal antibody to GIPC and a secondary anti-rabbit antibody coupled to horseradish peroxidase. IB, immunoblot.

(Fig. 2). The finding that addition of the YTEC motif promotes binding of a related protein (the rLHR) to GIPC whereas addition of a more traditional type I PDZ binding tetrapeptide (DSL1) does not promote binding to GIPC also suggest that there is selectivity in the hLHR/GIPC interaction (Fig. 4). Finally, the finding that the YTEC motif supports a weak interaction between the hLHR and EBP50, another protein that contains two type I PDZ domains (35), suggests that physical and functional interactions between the hLHR and other PDZ-domain containing proteins are also possible (Fig. 4).

GIPC has been reported to serve a number of roles such as the regulation of protein sorting in the biosynthetic (42, 45) or endocytic pathways (37, 44), the anchoring of proteins to the plasma membrane (45), the clustering of signaling components and transmembrane receptors (47), and the regulation of signaling by membrane receptors (43, 46). The results presented here show that GIPC regulates the fate of the hCG internalized by the hLHR and the levels of cell surface LHR during prolonged exposure to hCG.

The rat (r), mouse, and porcine lutropin receptors are among the few G protein-coupled receptors (GPCRs) that recycle poorly following internalization. These receptors are routed mostly to a lysosomal degradation pathway that has been particularly well characterized by using biochemical (12, 29, 50–52) and microscopic approaches (13, 53, 54). Thus, it is now known that the complex formed by the rat, mouse, or porcine LHR and one of its agonists (hCG) is internalized via clathrin-coated pits (53) by a pathway that requires the involvement of a non-visual arrestin and dynamin (29, 51). The rodent or porcine LHR/hCG complex is resistant to dissociation by the mild acidic pH that prevails in the endosomes (50), and a substantial proportion of the internalized complex is routed to the lysosomes where it dissociates prior to degradation (50, 52–54). By promoting the accumulation of the hCG/LHR complex in a compartment where it can be degraded, this pathway is ultimately responsible not only for the degradation of hCG (28) but also for a substantial loss of cell surface LHR that ensues following exposure of target or heterologous cells expressing the rodent or porcine LHR to agonists (13, 36, 55, 56). Surprisingly, however, the fate of the hCG internalized and the trafficking of the highly related hLHR is different from that of the porcine or rodent LHR. A substantial portion of the hCG
GIPC Binds to LHR through Unusual PDZ Domain Binding Motif

Fig. 10. GIPC associates with hLHR-L683A. Detergent lysates of 293T cells transiently transfected with the Myc-tagged versions of the hLHR-wt or mutants thereof were prepared and partially purified on a lectin column as described under “Materials and Methods.” Aliquots of the partially purified lysates containing equivalent amounts of receptors (A) were allowed to bind to GST-GIPC that had been bound previously to glutathione-agarose. The bound proteins were washed, eluted, and visualized on Western blots developed using a monoclonal antibody to the Myc epitope (9E10) and a secondary antibody coupled to horseradish peroxidase as described under “Materials and Methods” (B). A and B show only the relevant portions of a blot of a representative experiment. C shows a Coomassie-stained gel of the GST-GIPC fusion protein (1 μg) used in the pull-down assays. Note that in addition to the full-length GST-GIPC which migrates near the 90-kDa marker, some degradation products are detectable near the 36-kDa marker.

Fig. 11. Proposed models for the formation of GIPChLHR complexes. A, the C-terminal tails of two molecules of the hLHR are shown as gray lines, and the two important regions, Leu683 and the C-terminal tetrapeptide (YTEC), are indicated. Two molecules of GIPC are shown in green bound through their PDZ domains to the YTEC motifs of the two hLHR molecules. An oligomeric complex is proposed to be formed by self-association of GIPC and stabilized by a direct intermolecular interaction of Leu683 of the hLHR. B, the C-terminal tail of one molecule of the hLHR is shown as a gray line, and the two important regions, Leu683 and the C-terminal tetrapeptide (YTEC), are indicated. One molecule of GIPC is shown in green bound, through its PDZ domain to the YTEC motif of the hLHR molecules. This molecule of GIPC is associated with another molecule of GIPC, which is bound through its PDZ domain to an additional as yet unidentified protein (shown in red) which is also directly bound to Leu683 of the hLHR.

internalized by the hLHR is recycled back to the medium in an undegraded form (13, 26, 30), the internalized hLHR localizes mostly to endosomes (13), and the loss of cell surface hLHR during the endocytosis of hCG in cells expressing the recombinant hLHR is minimal (13).

As shown herein (Tables I and II), the recycling of the hCG internalized by the hLHR and the maintenance of a relatively constant level of cell surface hLHR during prolonged exposure to hCG are highly dependent on the C-terminal tetrapeptide (696YTEC699) and an upstream Leu683. One or both of these motifs are missing in the rodent or porcine LHR (Fig. 4 and Ref. 12) which as mentioned above route most of the internalized hCG to a degradation pathway and display a substantial reduction in cells surface LHR during prolonged exposure to CG.

Based on the results presented here, we conclude that the recycling of the hCG internalized by the hLHR and the levels of cell surface hLHR are mediated by the constitutive association of the C-terminal region of the mature cell surface hLHR with the PDZ domain of GIPC. Because GIPC can interact with itself, apparently through its N-terminal region (37), it is possible that this interaction promotes the formation of a complex containing at least two molecules of the hLHR that are indirectly associated with each other through their bound GIPC and that this complex is stabilized by direct interaction of the two molecules of the hLHR through Leu683 (Fig. 11A). Alternatively, it is possible that another protein (as yet unidentified) binds simultaneously to Leu683 of the hLHR and to the PDZ domain of a second molecule of GIPC, which is self-associated with the molecule of GIPC that is constitutively bound to the C terminus of the cell surface hLHR (Fig. 11B). Although the model presented in Fig. 11A is the simplest, only the model presented in Fig. 11B accommodates all the data presented here as discussed below.

The involvement of the C-terminal region of the hLHR and the lack of involvement of Leu683 on the binding of the hLHR to GIPC are documented here by the pull-down and immunoprecipitation experiments (Figs. 2, 3, 5, 7, and 10). The involvement of GIPC in promoting the recycling of the hCG internalized by the hLHR and in maintaining the density of cell surface hLHR is documented by functional studies done in the wild-type and GIPC-deficient cells (Tables I and II). These functional studies show that the ratio of recycled to degraded hCG and the hCG-induced loss of cell surface receptors observed when the hLHR-wt is expressed in the wild-type 293 cells are only slightly affected by mutation of Leu683, a manipulation that would cause minimal disruption of the two putative complexes proposed in Fig. 11. The functional properties of the hLHR are affected more by removal of the YTEC motif, a manipulation that disrupts the formation of either of the proposed complexes. When the hLHR-wt is expressed in GIPC-
deficient cells, the ratio of recycled to degraded hCG and the hCG-induced loss of cell surface receptors are again slightly affected because the reduced levels of GIPC are not optimal for formation of either of the proposed complexes. Moreover, the effects of the L683A mutation, which induces only partial disruption of the proposed complexes in the wild-type cells, are amplified in the GIPC-deficient cells because the disruptive effects of the receptor mutations on the formation of the complex are synergistically amplified by the reduction in GIPC levels. Because the L683A/t695 mutation is already maximally effective in disrupting the formation of either of the proposed complexes. Moreover, the functional properties of the hLHR-L683A/t695 mutant at the cell surface is lost of the hLHR-L683A/t695 mutant at the cell surface is dominant when the hLHR-t695 is expressed in the wild-type 293 cells, the two models shown in Fig. 11 are also consistent with the finding that a substantial portion of the hCG internalized by the hLHR-L683A/t695 mutant is routed to a degradation pathway, and the hCG-induced loss of the hLHR-L683A/t695 mutant at the cell surface is not yet known, however, if GIPC is the only hLHR binding partner that regulates these processes.

In summary, the data presented here identify GIPC as a novel binding partner for the hLHR and show that a physical interaction between these two proteins is an important determinant of the sorting of the hCG internalized by the hLHR and of maintaining the density of cell surface hLHR during the endocytosis of hCG. It is not yet known, however, if GIPC is the only hLHR binding partner that regulates these processes.

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GIPC Binds to the Human Lutropin Receptor (hLHR) through an Unusual PDZ Domain Binding Motif, and It Regulates the Sorting of the Internalized Human Choriogonadotropin and the Density of Cell Surface hLHR
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