Identification of Binding Domains of the Growth Hormone-Releasing Hormone Receptor by Analysis of Mutant and Chimeric Receptor Proteins

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The hypothalamic peptide GH-releasing hormone (GHRH) stimulates the release of GH from the pituitary through binding and activation of the GHRH receptor, which belongs to the family of G protein-coupled receptors. The objective of this study was to identify regions of the receptor critical for interaction with the ligand by expressing and analyzing truncated and chimeric epitope-tagged GHRH receptors. Two truncated receptors, GHRHΔN, in which part of the N-terminal domain between the putative signal sequence and the first transmembrane domain was deleted, and GHRHΔC, which was truncated downstream of the first intracellular loop, were generated. Both the receptors were deficient in ligand binding, indicating that neither the N-terminal extracellular domain (N terminus) nor the membrane-spanning domains with the associated extracellular loops (C terminus) are alone sufficient for interaction with GHRH. In subsequent studies, chimeric proteins between the receptors for GHRH and vasoactive intestinal peptide (VIP) or secretin were generated, using the predicted start of the first transmembrane domain as the junction for the exchange of the N terminus between receptors. The chimeras having the N terminus of the GHRH receptor and the C terminus of either the VIP or secretin receptor (G_NV_C and G_NS_C) did not bind GHRH or activate adenylate cyclase after GHRH treatment. The reciprocal chimeras having the N terminus of either the VIP or secretin receptors and the C terminus of the GHRH receptor (V_NG_C and S_NG_C) bound GHRH and stimulated cAMP accumulation after GHRH treatment. These results suggest that although the N-terminal extracellular domain is essential for ligand binding, the transmembrane domains and associated extracellular loop regions of the GHRH receptor provide critical information necessary for specific interaction with GHRH. (Molecular Endocrinology 12: 750–765, 1998)

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INTRODUCTION

GH-releasing hormone (GHRH) is a peptide hormone secreted from the hypothalamus that stimulates the proliferation of pituitary somatotrophs and induces the synthesis and secretion of GH by these cells. High-affinity binding of GHRH to its receptors on pituitary somatotrophs results in G protein coupling, adenylate cyclase activation and cAMP production, Ca^{2+} influx, increased expression of the GH gene, and enhanced GH secretion (1, 2). The GHRH receptors of several species have been cloned (3–6), and inactivating mutations in the receptor have been found in heritable GH deficiency diseases in both mice (7, 8) and humans (9, 10). Additionally, transcripts for alternatively spliced forms of the GHRH receptor truncated in the third intracellular loop have been found in patients with GH-producing pituitary tumors, although a role for these mutant receptors in tumorigenesis has not been established (11, 12).

The GHRH receptor is a G protein-coupled receptor (GPCR) with seven potential membrane-spanning domains and belongs to family B, group III (B-III) of the GPCR superfamily (13). It is highly homologous to the receptors for secretin (SEC) (14), vasoactive intestinal peptide (VIP) (15), pituitary adenylate cyclase-activating peptide (PACAP) (16), glucagon (17), glucagon-like peptide-1 (GLP-1) (18), and gastric inhibitory peptide (GIP) (19), which are also grouped in family B-III (13). These receptors are related to the receptors for PTH (20), PTH-related peptide (21), CRH (22), and calcitonin (23), which have a longer N-terminal extracellular domain but share many common amino acids and a similar overall structure to the receptors of family B-III (24).

The receptors of family B-III have several conserved residues in the N-terminal extracellular domain, includ-

ing six cysteines, an aspartate, a tryptophan, and a glycine at positions corresponding to amino acids 60, 65, and 100 in the GHRH receptor (24). Mutation of the conserved cysteines in the VIP receptor (25), the aspartate in the GHRH (7, 8, 26), VIP (27), and glucagon (28) receptors, and the tryptophan or glycine in the VIP receptor (27) results in a loss of hormone binding and hormone-stimulated cAMP accumulation in cells expressing these mutant receptors. The N-terminal domains of the receptors of this family are predicted to have a similar structure, presumably maintained by disulfide bonds between the conserved cysteines, and are thought to be involved in ligand binding (29). An α-helical region within this N-terminal domain is hypothesized to be involved in a coiled-coil interaction with an α-helical region in the respective hormone (30), again suggesting a role for this domain in ligand binding.

The superfamily of GPCRs bind to, and are activated by, a diverse group of ligands ranging from ions and small peptides to large glycoproteins, and different receptors exhibit considerable variation in the structural determinants of ligand recognition (31–36). Ligand binding by several receptors in family B has been studied using mutagenesis and chimeric receptor approaches. These studies reveal that the large N-terminal extracellular domains of the GLP-1 (37), VIP (38, 39), and PACAP (40) receptors are sufficient for low levels of specific ligand binding. However, other studies indicate that the presence of one or more extracellular loop regions and residues within the transmembrane domains is required for high-affinity ligand binding by the receptors for secretin, VIP, glucagon, calcitonin, PTH, GIP, and CRH (38, 39, 41–52), indicating that family B receptors are likely to exhibit multiple determinants of ligand interaction.

Other than the known importance of the conserved aspartate at position 60, which is mutated in the *little* mouse, there is no information about the role of specific domains or residues of the GHRH receptor in ligand binding and signaling. As an initial step toward understanding the molecular mechanism of agonist-dependent activation of the GHRH receptor, we designed experiments to identify domains of the GHRH receptor required for high-affinity GHRH binding. We generated two truncation mutants of the human GHRH receptor and four chimeras between the human GHRH receptor and the receptors for VIP or secretin and studied their ligand-binding and signaling properties.

RESULTS

Generation and Analysis of Epitope-Tagged GHRH, VIP, and Secretin Receptors

The analysis of mutant and chimeric receptors requires determination of the levels of receptor protein expressed and confirmation that the receptor is appropriately localized to the cell membrane. The amino acids encoding an influenza virus hemagglutinin (HA) epitope (YPYDVPDYA) were therefore introduced at the C-terminal ends of the wild-type GHRH, VIP, and secretin receptors, allowing detection of receptor protein using a monoclonal antibody against the epitope. In addition, the amino acids encoding the FLAG epitope (DYKDADDDK) were introduced in the N terminus of the wild-type GHRH receptor, downstream of the predicted signal peptide. A schematic representation of the GHRH receptor depicting the location of these epitope tags, as well as the boundaries of truncations and chimera junctions to be discussed subsequently, is shown in Fig. 1.

To ascertain whether the epitope tags affected receptor function, expression constructs for the wild-type and epitope-tagged receptors were transiently transfected into HeLa T4 cells, and the binding and signaling properties of the receptors were compared. Binding of GHRH to membranes of cells expressing the wild-type (GHRHR.*wt*), HA-tagged (GHRHR.HA), and FLAG-tagged (GHRHR.F) GHRH receptors was measured in competition assays (Fig. 2A), and it was observed that the membranes of cells expressing the wild-type and HA-tagged GHRH receptors showed a similar dose-dependent competition of GHRH binding, with ED_{50} values for competition of 5.2 and 3.2 nM, respectively. In contrast, membranes of cells expressing the FLAG-tagged GHRH receptor construct did not bind GHRH. From Scatchard analysis of saturation-binding data, the apparent dissociation constants (K_D) for GHRH binding by the wild-type and HA-tagged GHRH receptors were determined to be 36 and 31.6 pM, respectively, with maximal binding (B_{MAX}) values of 50.9 and 34.2 pmol/mg protein (data not shown). In response to GHRH stimulation, cells expressing the wild-type and HA-tagged forms of the GHRH receptor showed a similar dose-dependent accumulation of cAMP (Fig. 2B) with ED_{50} values of 5.7 and 4.4 nM, respectively. Cells expressing the FLAG-tagged GHRH receptor did not accumulate cAMP, consistent with the inability of this receptor to bind GHRH. Receptor constructs with the FLAG epitope tag were therefore used only for comparing the cellular localization of various receptor proteins in intact cells. In studies not shown, the presence of the C-terminal HA epitope tag was found not to affect the properties of the epitope-tagged VIP (VIPR.HA) and secretin (SECR.HA) receptors compared with their wild-type counterparts.

Expression of Truncated GHRH Receptors

For several GPCRs, it has been possible to broadly localize hormone-binding domains by expression and analysis of deletion constructs generated by the removal of large regions of the receptor protein. To determine whether either the N terminus or the C terminus of the GHRH receptor could bind ligand in the absence of the other domain, two truncation mutants were generated and transiently expressed in

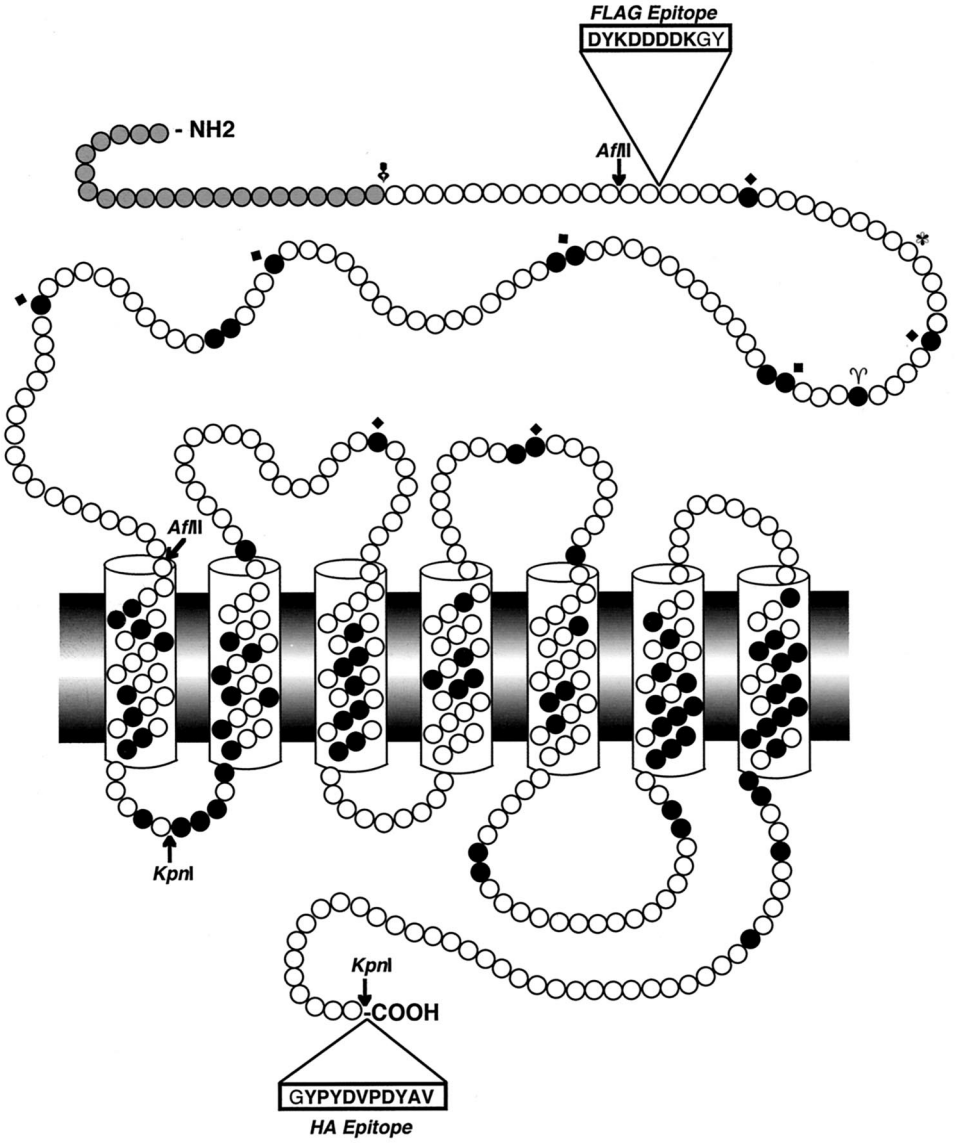

Fig. 1. Structure of the GHRH Receptor Showing the Location of Epitope Tags and Endpoints of the Truncated and Chimeric Mutant Receptors

The seven membrane-spanning domains are shown as *cylinders* crossing the lipid bilayer. The *gray shaded circles* represent the putative signal sequence, and the *black shaded circles* represent amino acids that are conserved in the related receptors for the hormones secretin, glucagon, GLP-1, GIP, VIP, and PACAP. The (✓) represents the putative signal-peptide cleavage site, the (❀) represents the consensus site for N-linked glycosylation, the (♈) represents aspartate residue 60, which is mutated in the *little* mouse, and the (♦) represents the cysteines that are conserved within family B-III receptors. The location of the *Afl*II and *Kpn*I sites used to make truncations and fusions are indicated by *arrows*. The location and sequences of the FLAG and HA epitope-tags are also shown.

HeLa T4 cells. The amino acids between the signal peptide and the first transmembrane domain were deleted in the receptor GHRHΔN by removing sequences between the two *Afl*II sites, and the amino acids downstream of the first intracellular loop were deleted in the receptor GHRHΔC by removing sequences between the two *Kpn*I sites (Fig. 1). Immunoprecipitation of the metabolically labeled HA-tagged receptor proteins (Fig. 3A) shows that the two truncated receptors, GHRHΔN and GHRHΔC, were expressed at or above levels of the full-length receptor. The apparent molecular masses were approximately 40 kDa and 20 kDa, respectively; however, the immunoprecipitated protein from cells expressing GHRHΔN showed the presence of two products, differing in size by 4 kDa. The cellular localization of the full-length and truncated epitope-tagged receptors was determined by immunofluorescence analysis using confocal microscopy with intact (for the FLAG-tagged receptors) or permeabilized (for the FLAG- and HA-tagged receptors) cells

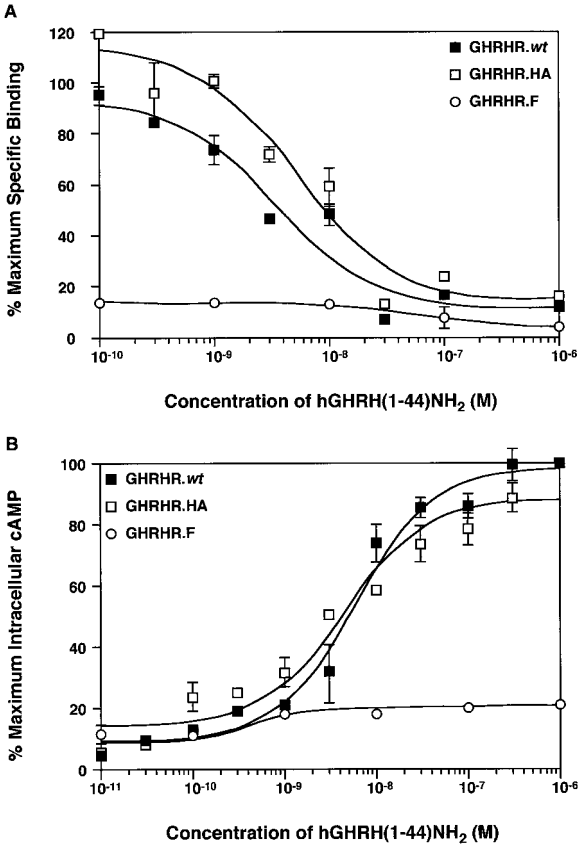

Fig. 2. Binding and Signaling Properties of Epitope-Tagged GHRH Receptors

A, Dose-dependent competition of GHRH binding to the wild-type and epitope-tagged GHRH receptors expressed in HeLa T4 cells. The relative amount of input radioligand bound in the presence of increasing concentrations of competitor is shown for each of the receptor constructs. The amount of GHRH bound in the absence of competitor corresponds to 8.2 pmol/tube. B, Dose-dependent cAMP accumulation after GHRH treatment of HeLa T4 cells transfected with wild-type and epitope-tagged GHRH receptors. The relative amount of intracellular cAMP produced in response to increasing concentrations of hormone is shown for each of the receptor constructs. The amount of intracellular cAMP accumulated in cells treated with 1 μM GHRH corresponds to 52 pmol/well. Data points represent the mean of duplicate samples with the range of values indicated by the *error bars*; each panel is representative of at least two independent experiments.

(Fig. 3B). The full-length (GHRHR) and C-terminally truncated (GHRHΔC) receptors were localized similarly; receptor protein was distributed both on the cell surface and intracellularly, with the latter presumably representing receptor protein being transported to, or internalized from, the cell surface. For these two receptors, similar localization patterns were observed using antibodies against either the N-terminal FLAG tag or the C-terminal HA tag in permeabilized cells. In contrast, cells expressing the N-terminally truncated receptor (GHRHΔN) appeared to accumulate protein preferentially in intracellular membranes, with a smaller proportion of the total receptor expressed on the cell surface. A perinuclear localization of this receptor protein was observed using both the FLAG and HA antibodies in permeabilized cells (Fig. 3B).

Analysis of Truncated GHRH Receptors

Binding of GHRH to membranes of HeLa T4 cells expressing the HA-tagged full-length or truncated forms of the GHRH receptor was measured in competition assays (Fig. 4A). The truncated receptors GHRHΔN and GHRHΔC did not bind GHRH when expressed by themselves or when coexpressed. In addition, neither truncated receptor decreased the ligand-binding ability of the full-length GHRH receptor when coexpressed with it. GHRH treatment did not stimulate cAMP accumulation in cells expressing the truncated receptor GHRHΔN, expressed either alone or together with the receptor GHRHΔC (Fig. 4B), consistent with the results of the binding studies. Coexpression of either GHRHΔN or GHRHΔC with the full-length GHRH receptor did not diminish activation of adenylate cyclase by the full-length receptor (Fig. 4B).

The properties of the truncated GHRH receptors indicate that both the N terminus as well as the C terminus of the receptor are required for ligand binding and signaling. We therefore pursued an alternative approach to define the ligand-binding determinants of the GHRH receptor. Chimeras between related receptors have been used with great success in defining the ligand-binding domains of several receptors, such as those for the biogenic amines, glycoprotein hormones, and small peptides, and of several receptors of family B. We generated chimeras between the VIP or secretin receptors and the GHRH receptor and studied their ligand-binding and signaling properties to determine whether the N- or C-terminal regions of the receptor were most critical for specific interaction with GHRH.

Expression of Chimeric GHRH and VIP or Secretin Receptors

As described in Table 1, the chimeras between the GHRH and VIP or secretin receptors were generated by the exchange of the N termini of the HA-tagged receptors, with the fusions made at the *Afl*II site immediately before the first transmembrane domain. The GHRH-VIP receptor chimeras were G_NV_C, which consists of the N-terminal 127 residues of the GHRH receptor and residues 144–459 of the VIP receptor, and V_NG_C, generated by replacement of the 127 residues constituting the N terminus of the GHRH receptor with the equivalent 143 residues forming the N terminus of the VIP receptor. The GHRH-secretin receptor chimeras were G_NS_C, consisting of residues 1–127 of the GHRH receptor and residues 143–449 of the secretin receptor, and S_NG_C, consisting of residues 1–142 of the secretin receptor and residues 128–423 of the GHRH receptor. The expression of the wild-type and chimeric receptor proteins was as-

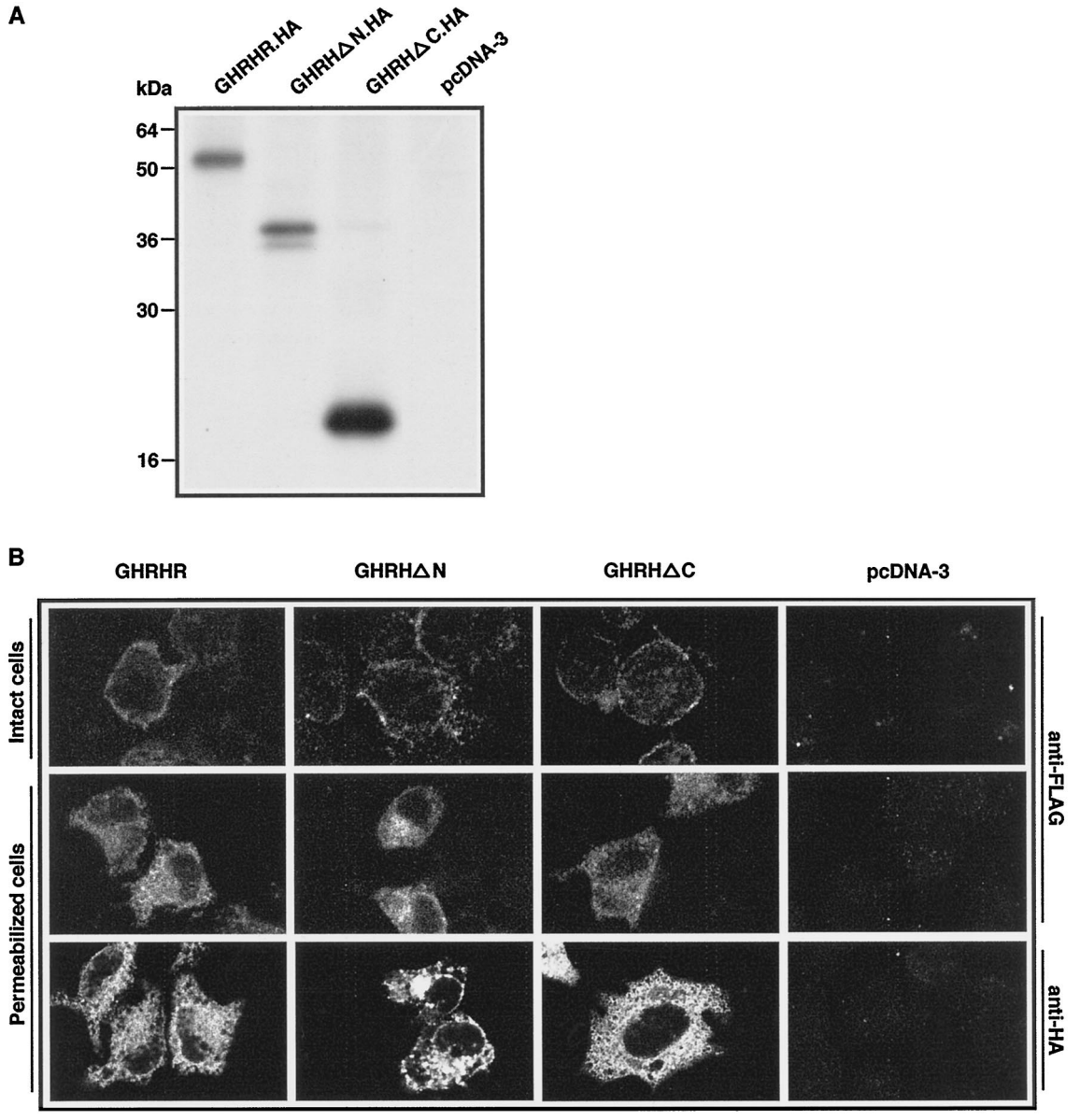

Fig. 3. Expression of Truncated GHRH Receptors

A, Immunoprecipitation of the HA-tagged wild-type and truncated human GHRH receptors. Equivalent amounts of metabolically labeled protein from HeLa T4 cells transfected with the indicated receptor constructs were immunoprecipitated using the monoclonal antibody 12CA5 against the HA-epitope and separated by SDS-PAGE on a 12% gel. The sizes of the mol wt standards included on the gel are shown on the *left*. B, Immunofluorescence localization of epitope-tagged wild-type and truncated human GHRH receptors. Indirect immunofluorescence of HeLa T4 cells transfected with pcDNA-3 or the indicated receptor constructs was performed using the anti-M2 monoclonal antibody against the FLAG-epitope (*top and middle rows*) or the 12CA5 antibody against the HA-epitope (*bottom row*). The panels in the *top row* are images of intact cells while those in the *middle and bottom* rows are images of cells permeabilized with 0.1% saponin. All slides were scanned using a confocal microscope under the same magnification and contrast settings for equivalent times, and the panels shown are representative of at least 20 fields observed in two independent experiments.

sessed by immunoprecipitation and, as shown in Fig. 5A, the proteins produced had the expected molecular sizes, and the chimeric receptors were expressed at levels comparable to, or above, those of the wild-type receptors. The chimeric receptors were glycosylated as determined by treatment with peptide-N-glycosidase F (data not shown), which reduced the observed size of the receptor proteins in accord with the number of potential glycosylation sites in the N-terminal extracellular domain of each (one in G_NV_C, three in V_NG_C, one in G_NS_C, and four in S_NG_C).

To confirm that the exchange of domains did not affect the cellular localization of the chimeric receptors, immunofluorescence analysis of permeabilized

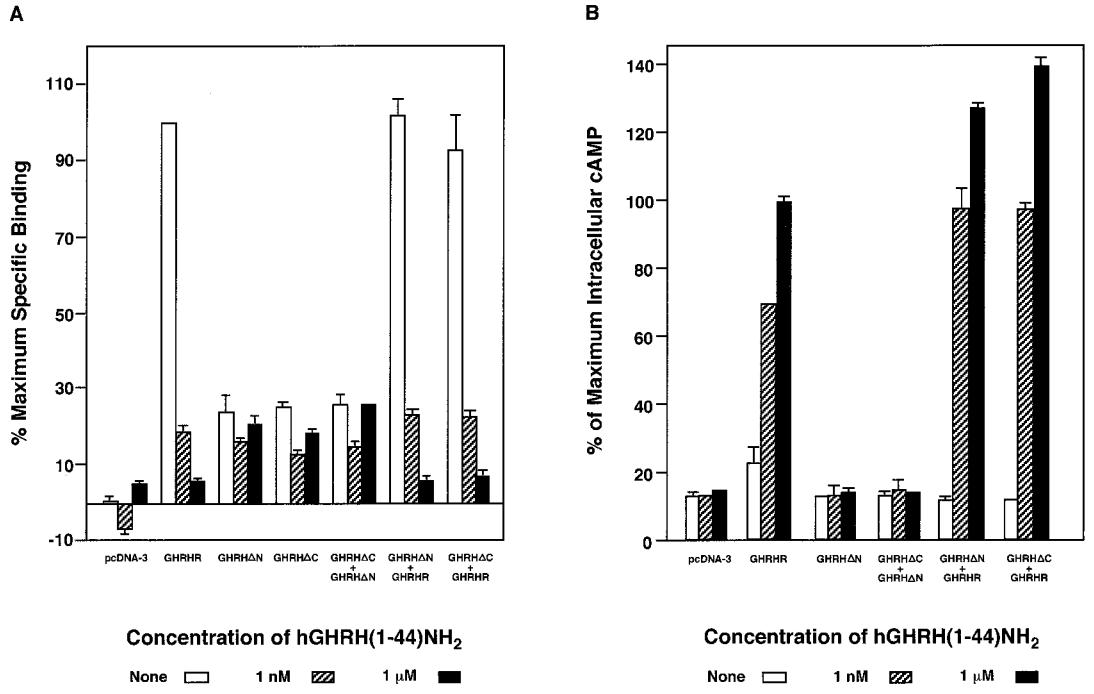

Fig. 4. Functional Analysis of Truncated GHRH Receptors

A, Binding of GHRH to membranes of cells expressing the wild-type and truncated human GHRH receptors. The relative amount of input radioligand bound in the absence and presence of two doses of unlabeled competitor is shown for each construct tested. The amount of GHRH bound in the absence of competitor corresponds to 6.7 pmol/tube. B, Stimulation of intracellular cAMP levels by GHRH in HeLa T4 cells expressing the wild-type and truncated GHRH receptors. The relative amount of intracellular cAMP accumulated in response to two doses of GHRH is shown for each construct tested. The amount of intracellular cAMP accumulated in cells treated with 1 μM GHRH corresponds to 80.2 pmol/well. Data points represent the mean of duplicate samples with the range of values indicated by the *error bars*; each panel is representative of at least three independent experiments.

cells expressing the wild-type and chimeric receptors was performed, and the cells were observed using fluorescence microscopy. As seen in Fig. 5B, the wild-type and chimeric receptors are localized in a similar manner within the cell. All receptor proteins are distributed both on the cell surface and intracellularly, with the latter presumably representing receptor protein that is being transported to, or internalized from, the cell surface.

Analysis of Chimeric GHRH and VIP Receptors

The ligand-binding properties of the chimeras between the GHRH and VIP receptors were analyzed by measurement of binding to both ligands using competition assays (Fig. 6). The chimera G_NV_C bound GHRH and VIP at extremely low levels, while the chimera V_NG_C bound GHRH at levels comparable to membranes of cells expressing the wild-type GHRH receptor (Fig. 6A) but bound VIP at much lower levels than membranes of cells expressing the wild-type VIP receptor (Fig. 6B). Scatchard analysis of saturation-binding data for binding of GHRH and VIP to the chimera V_NG_C was performed, and the K_D values were determined to be 46 pM and 156 pM, respectively. Dose-dependent accumulation of cAMP in cells transfected with the wild-type and chimeric receptors in response to treatment with GHRH and VIP is shown in Fig. 7. Cells expressing the chimera G_NV_C show an increase in intracellular cAMP in response to GHRH and VIP only at high hormone concentrations ($>10^{-7}$ M). However, cells expressing the chimera V_NG_C showed a robust accumulation of cAMP in response to treatment with both GHRH and VIP, with ED_{50} values of 1.75 and 7.95 nM, respectively, consistent with the ability of this receptor to bind GHRH, and more weakly, VIP. Interestingly, the chimera V_NG_C mediates a much stronger cAMP response to GHRH than does the wild-type GHRH receptor, although the two receptors bound GHRH at comparable levels. The binding and signaling properties of the wild-type and chimeric GHRH and VIP receptors are summarized in Table 2.

Analysis of Chimeric GHRH and Secretin Receptors

The GHRH and VIP receptors are among the most homologous in family B-III. Although advantageous from the point of minimally disrupting structure, chimeras between these receptors might not fully reveal GHRH binding determinants if these determinants are

Table 1. Construction of Chimeras between the Receptors for GHRH and VIP or Secretin

Chimera	Source of Amino Acids	Sequence at Junction
G_NV_C	GHRHR (1–127), VIPR (144–459)	◆◆ TCC ACA CTT ‖ AAG ***ACC GGC*** Ser Thr Leu ‖ Lys ***Thr Gly*** (Val)
V_NG_C	VIPR (1–143), GHRHR (128–423)	◆◆ AAT ACC CTT ‖ AAG ***ATT ATC*** Asn Thr Leu ‖ Lys ***Ile Ile*** (Val)
G_NS_C	GHRHR (1–127), SECR (143–449)	◆◆ TCC ACA CTT ‖ AAG ***GTC ATG*** Ser Thr Leu ‖ Lys ***Val Met*** (Val)
S_NG_C	SECR (1–142), GHRHR (128–423)	◆ CTG AAA CTC ‖ AAG ***ATT ATC*** Leu Lys Leu ‖ Lys ***Ile Ile*** (Leu)

◆ Denotes the nucleotides mutated by the generation of the *Afl*II site, with the original amino acids shown in *parentheses*, and ‖ denotes the chimera junction. The *italic boldface residues* are presumed to be in the first transmembrane domain of each of the chimeric receptors.

present in the analogous region of the VIP receptor. We therefore generated additional chimeras using the secretin receptor, which is less homologous than the VIP receptor to the GHRH receptor (35% *vs.* 40% identical) and which does not detectably bind GHRH. The GHRH-binding ability of the two chimeras between the GHRH and secretin receptors was determined using binding competition assays (Fig. 8). The chimera G_NS_C did not show any significant binding of GHRH while the reciprocal chimera S_NG_C bound GHRH at lower levels than the wild-type GHRH receptor, although it was expressed at higher levels than the wild-type receptor. The ED_{50} for competition of GHRH binding by the chimera S_NG_C was 1.15 nM, and the K_D value determined by Scatchard analysis of saturation-binding data was 259.3 pM. We were unable to iodinate secretin to high specific activity and therefore could not directly examine binding of secretin to these chimeras, but we studied the interaction of these chimeras with secretin by quantifying the intracellular cAMP levels in response to secretin stimulation. After treatment with GHRH, adenylate cyclase was not activated in cells expressing the chimera G_NS_C. In contrast, cells expressing the reciprocal chimera S_NG_C accumulated cAMP in a dose-dependent manner, although there was a small rightward shift in the response curve (Fig. 9A). Cells expressing either of the two chimeras G_NS_C or S_NG_C did not accumulate cAMP after treatment with secretin (Fig. 9B). The binding and signaling properties of the wild-type and chimeric GHRH and secretin receptors are summarized in Table 3.

DISCUSSION

GHRH and its receptor play an important role in the proliferation of pituitary somatotroph cells and the regulation of GH secretion (1, 2, 53), and high-affinity binding of GHRH by its receptor is a critical prerequisite for normal function of the GH axis (7–10). The molecular characterization of the association between GHRH and its receptor provides a framework for understanding their role in the regulation of GH secretion and growth, as well as their involvement in disorders of GH secretion. The approach we have taken toward this objective was to construct and analyze truncated and chimeric receptors, to begin to identify the extracellular domains of the GHRH receptor that are involved in its interaction with GHRH. Our studies of truncated receptors led us to conclude that neither the N-terminal extracellular domain nor the C-terminal domain, consisting of the transmembrane domains and the associated extracellular loops, can bind ligand in the absence of the other. The analysis of chimeras between the GHRH receptor and the receptors for VIP and secretin has revealed that although the N terminus is a critical domain for GHRH binding, the N terminus of a related receptor can be substituted, suggesting that the transmembrane domains and associated extracellular loops of the GHRH receptor form the key determinants for the specific binding of GHRH.

Studies on the GHRH receptor of the *little* mouse, in which mutation of the conserved aspartate at position 60 results in a loss of the ability of the receptor to bind ligand (26) and activate adenylate cyclase (7, 8), provided the first indication that the integrity of the N

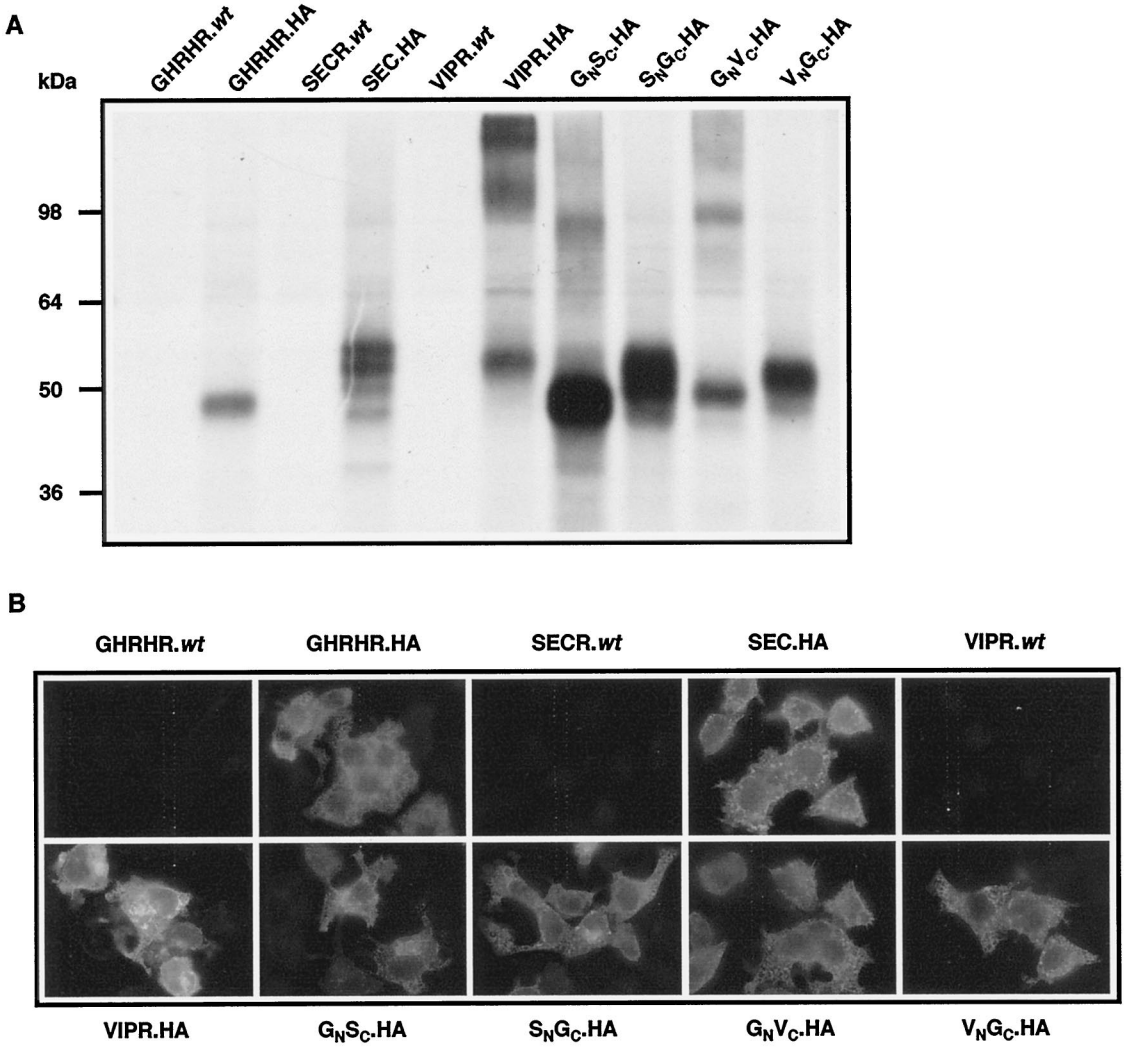

Fig. 5. Expression and Cellular Localization of Wild-Type and Chimeric GHRH Receptors

A, Immunoprecipitation of wild-type and chimeric HA-tagged GHRH, VIP, and secretin receptors. Equivalent amounts of protein from metabolically labeled HeLa T4 cells transfected with the various receptor constructs were immunoprecipitated and separated by SDS-PAGE on a 10% gel. The sizes of the mol wt standards included on the gel are shown on the *left*. B, Immunofluorescence localization of HA-tagged wild-type and chimeric receptors in cells permeabilized with 0.1% saponin was performed, and the cells were observed by fluorescence microscopy. All slides were photographed using the same exposure times, and the panels shown are representative of at least 20 fields observed in two independent experiments. The monoclonal antibody 12CA5 against the HA epitope-tag was used for both immunoprecipitation and immunofluorescence analyses.

terminus of the GHRH receptor is essential for ligand binding. In this study, we found that the insertion of a FLAG epitope tag at position 37 in the N terminus of the receptor resulted in inactivation of the receptor. Although this effect could be due in part to altered expressed or localization of the FLAG-tagged receptor, our data are consistent with this insertion directly affecting the ligand-binding properties of the receptor. From computational structural studies on the receptors of family B, it has been proposed that an α-helical region in the N terminus of these receptors interacts with an α-helical region in the C terminus of the bioactive region of the corresponding peptide hormone to form a coiled-coil motif (30). For the GHRH receptor, this α-helical region is predicted to extend from residues 26–40 and to interact with an α-helix formed by residues 13–23 of the hormone. Inactivation of the receptor by the introduction of the FLAG epitope tag at position 37 might therefore result from the interruption of this α-helical domain.

Deletion of the N terminus affected transport of the receptor GHRHΔN to the cell surface and resulted in accumulation of the receptor protein in intracellular membranes, despite inclusion of the signal peptide in this construct. This abnormal localization of GHRHΔN could be a consequence of the absence of the site for N-linked glycosylation or of defective transport due to the proximity of the signal peptide and the first transmembrane domain. The larger of the two bands seen in immunoprecipitation analysis of extracts from cells

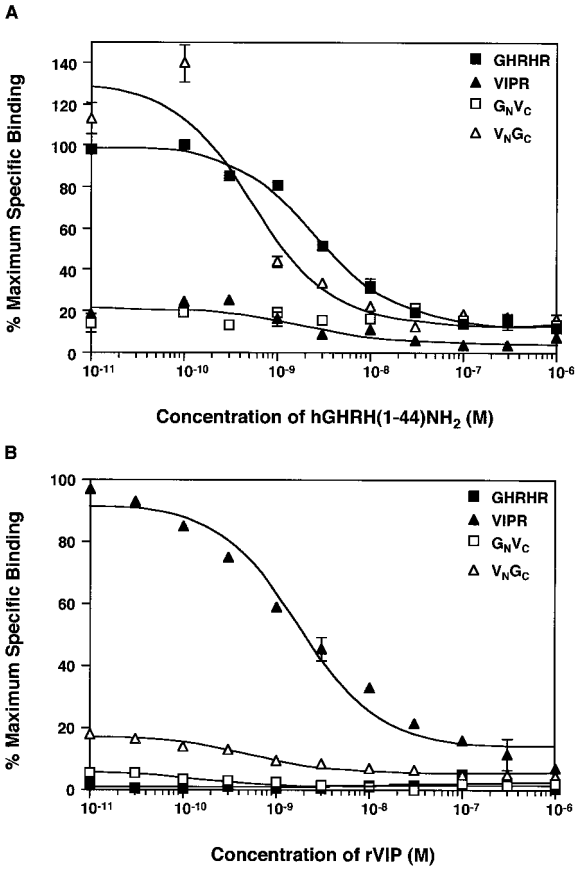

Fig. 6. Binding of GHRH and VIP to Wild-Type and Chimeric GHRH and VIP Receptors

Dose-dependent competition of GHRH (A) and VIP (B) binding to membranes of cells expressing the wild-type and chimeric GHRH and VIP receptors. The relative amount of input radioligand bound in the presence of increasing concentrations of the respective unlabeled competitor is shown for each construct tested. The amount of GHRH and VIP bound in the absence of competitor corresponds to 8.3 and 7.4 pmol/tube, respectively. Data points represent the mean of duplicate samples with the range of values indicated by the *error bars*; each panel is representative of at least three independent experiments.

expressing GHRHΔN is likely to be receptor protein in which cleavage of the signal peptide has not occurred, potentially due to structural constraints resulting from the adjacency of the first transmembrane domain. The receptor GHRHΔN did not bind GHRH in a competition assay using total cell membranes, nor did it activate adenylate cyclase upon hormone treatment, implying that the N terminus is essential for GHRH binding. The deletion of the N termini of the receptors for VIP and glucagon also resulted in proteins that did not bind to their respective ligands (27, 48), suggesting that the N terminus plays an important role in the formation of the binding site of receptors of family B-III.

The receptor truncated in the first intracellular loop, GHRHΔC, was localized within cells in a manner similar to the wild-type GHRH receptor but did not bind GHRH, indicating that although essential for ligand binding, the N terminus alone is not sufficient for interaction with GHRH. Our results with GHRHΔC are similar to those obtained with a C-terminally truncated glucagon receptor (48) but are distinct from those obtained with a similarly truncated PACAP receptor that specifically bound PACAP, although with significantly lower affinity than the wild-type PACAP receptor (40).

Although coexpression of N- and C-terminal domains of receptors can result in partially functional receptors (32, 54, 55), suggesting reconstitution of receptors by domain-domain interaction, coexpression of the two truncated forms of the GHRH receptor, GHRHΔN and GHRHΔC, did not reconstitute ligand-

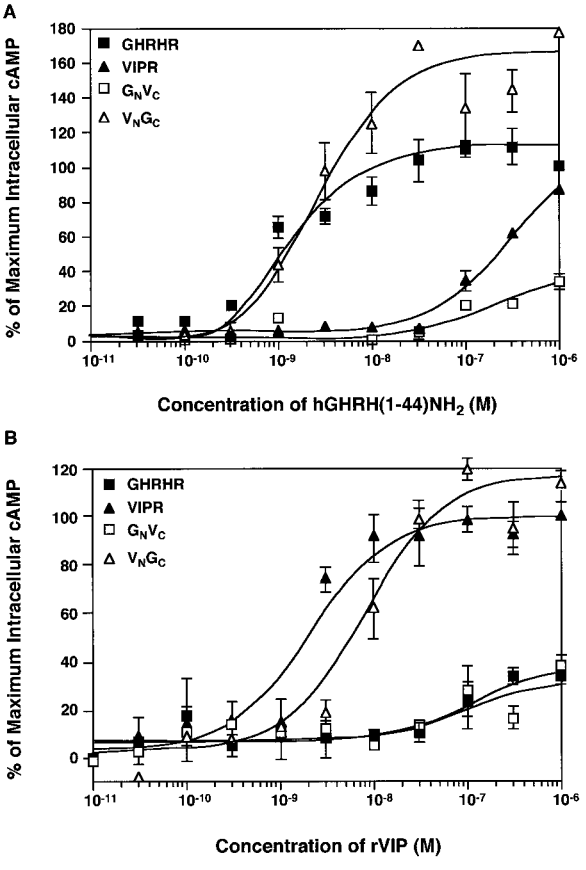

Fig. 7. Stimulation of cAMP Production by GHRH and VIP in Cells Expressing Wild-Type and Chimeric GHRH and VIP Receptors

Dose-dependent cAMP accumulation in HeLa T4 cells expressing wild-type and chimeric GHRH and VIP receptors after treatment with GHRH (A) and VIP (B). The relative amount of intracellular cAMP produced in response to increasing concentrations of hormone is shown for each construct tested. The amount of intracellular cAMP accumulated in cells treated with 1 μM GHRH and VIP corresponds to 280.4 and 229.8 pmol/well, respectively. Data points represent the mean of duplicate samples with the range of values indicated by the *error bars*; each panel is representative of at least three independent experiments.

Table 2. Binding and Signaling Properties of the GHRH-VIP Wild-Type and Chimeric Receptors

Property	GHRHR.HA	VIPR.HA	G_NV_C.HA	V_NG_C.HA
GHRH binding, ED_{50}	2.7 2.9 nM	ns	ns	0.5, 0.7 nM
GHRH binding, K_D	31.6, 35.1 pM	nd	nd	46.1, 45.9 pM
GHRH binding, B_{MAX}	34.2, 41.8	nd	nd	63.8, 56.4
VIP binding, ED_{50}	ns	1.8, 1.9 nM	ns	0.6, 0.8 nM
VIP binding, K_D	nd	143, 149 pM	nd	147, 165 pM
VIP binding, B_{MAX}	nd	190.3, 194.5	nd	47.5, 37.73
GHRH cAMP response, ED_{50}	1.2, 1.4 nM	116, 109 nM	84, 72 nM	1.6, 1.9 nM
VIP cAMP response, ED_{50}	ns	2.05, 2.14 nM	90.8, 92.3 nM	9.0, 6.9 nM

The ED_{50} values for binding competition and cAMP accumulation were not calculated when the binding or signaling was not substantially different than background (denoted as ns), and saturation binding was not performed for receptors in which hormone binding over background was not observed in competition assays (indicated by nd). The two values shown represent the means of duplicate samples from two independent experiments. In the text, the average of these two independent determinations is used. B_{MAX} values are expressed as picomoles bound per mg protein.

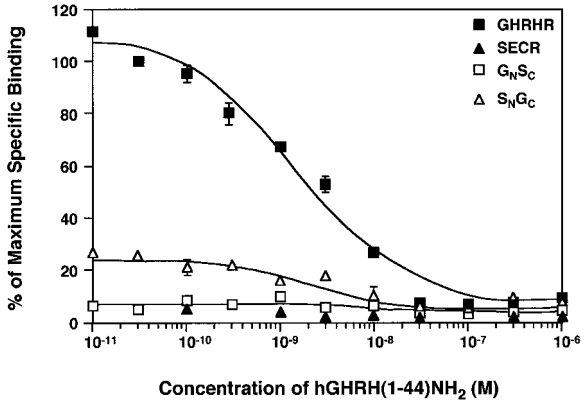

Fig. 8. GHRH Binding to Wild-Type and Chimeric GHRH and Secretin Receptors

Dose-dependent competition of GHRH binding to membranes of cells expressing the wild-type and chimeric GHRH and secretin receptors. The relative amount of input radioligand bound in the presence of increasing doses of unlabeled GHRH is shown for each construct tested. The amount of GHRH bound in the absence of competitor corresponds to 8.4 pmol/tube. Data points represent the mean of duplicate samples with the range of values indicated by the *error bars*; each panel is representative of at least three independent experiments.

binding activity. This could be due to poor colocalization of the receptor fragments or due to a lack of interaction of the two truncated proteins resulting from disruption of the complex by the presence of the first transmembrane domain in both receptor fragments. Additionally, we coexpressed the truncated and wild-type GHRH receptors and found that there was no decrease in hormone binding or signaling by the wild-type receptor, implying that the truncated receptors GHRHΔN and GHRHΔC do not act as functional dominant negative mutants.

The generation of truncation constructs of GPCRs has been useful for the identification of structural domains involved in ligand binding in several receptors (40, 54–58). However, this approach has disadvantages, in that deletion of domains or truncations can result in structural alterations and mislocalization of the receptor proteins (48, 58). The generation of chimeras between two similar but distinct receptors can be used to correlate gain or loss of function with the exchanged domain, often with minimal effects on the structure of the receptor. Chimeric receptors generated by the exchange of homologous domains have proven useful in localizing the ligand-binding domains of receptors for biogenic amines (31, 32), glycoprotein hormones (33), and small peptides (34–36), and also of several receptors in family B including the VIP, secretin, glucagon, GIP, GLP-1, CRH, and PTH receptors (38, 39, 44, 46, 47, 49, 51, 52). We therefore applied this approach to characterize the ligand-binding domains of the GHRH receptor.

The wild-type GHRH, VIP, and secretin receptors show a high degree of specificity in their interaction with their respective ligands, although some ability of GHRH to activate the VIP receptor and of VIP to activate the GHRH receptor at high ligand concentrations has been observed (3, 59). Both the VIP and secretin receptors require the N-terminal extracellular domain as well as the first extracellular loop for high-affinity ligand recognition (29, 38, 39, 41–44). Exchange of the N-terminus of the VIP or secretin receptors with the corresponding region of the GHRH receptor resulted in chimeras G_NV_C, which showed a very weak response to both GHRH and VIP, and G_NS_C, which was unresponsive to both GHRH and secretin, consistent with studies of chimeras between the VIP and secretin receptors (38, 39). However, the chimeric receptors V_NG_C and S_NG_C, having the N terminus of the VIP or secretin receptors and the C terminus of the GHRH receptor, bound GHRH and stimulated adenylate cyclase in a dose-dependent manner in response to GHRH. The chimera S_NG_C bound GHRH at lower levels but had a similar cAMP response compared with the wild-type receptor, while the chimera V_NG_C showed similar binding but a higher signaling response to GHRH than the wild-type receptor. Our observation that the chimera V_NG_C bound VIP and

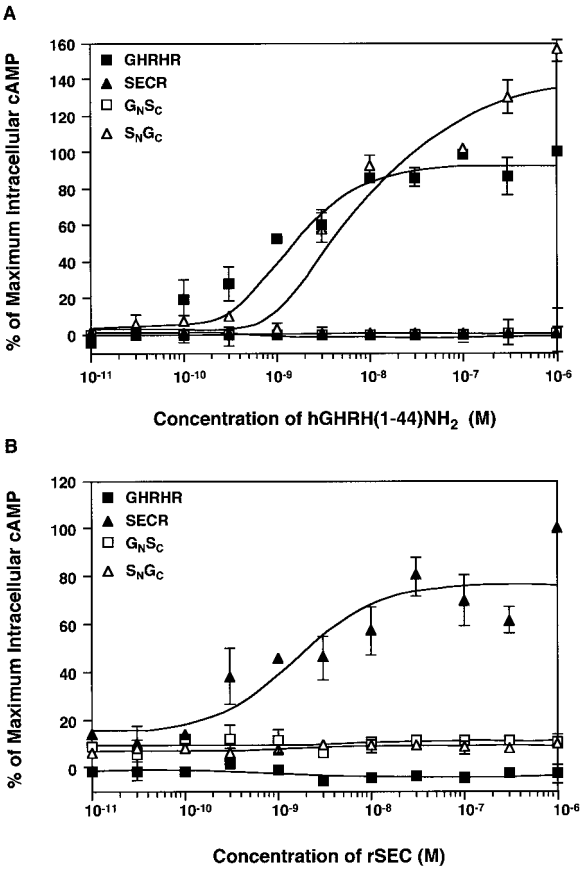

Fig. 9. Stimulation of cAMP Production by GHRH and Secretin in Cells Expressing Wild-Type and Chimeric Human GHRH and Secretin Receptors

Dose-dependent cAMP accumulation in HeLa T4 cells expressing the wild-type and chimeric GHRH and secretin receptors after treatment with GHRH (A) and secretin (B). The relative amount of intracellular cAMP produced in response to increasing amounts of the hormones is shown for each construct tested. The amount of intracellular cAMP accumulated in cells treated with 1 μM GHRH and secretin corresponds to 348.3 and 187 pmol/well, respectively. Data points represent the mean of duplicate samples with the range of values indicated by the *error bars*; each panel is representative of at least three independent experiments.

activated adenylate cyclase in response to VIP can be explained by the fact that the aspartate residue at position 196 in the first extracellular loop of the VIP receptor, which is predicted to be important in interaction with VIP (43), is also present in the corresponding region of the GHRH receptor. The VIP receptor and the chimeric receptor G_NV_C mediate cAMP accumulation in response to very high concentrations of GHRH, consistent with a low-affinity interaction of the VIP receptor with GHRH. The differences between the binding and signaling responses in the chimeric receptors S_NG_C and V_NG_C suggest that some of the determinants required for GHRH binding are present in the N terminus of the VIP receptor but not the secretin receptor. Alignment of the N-terminal regions of the three receptors (3, 24) indicates that among the residues predicted to form the α-helix suggested to be involved in the coiled-coil interaction with the ligand (30), there is a higher homology between the GHRH and VIP receptors than between the GHRH and secretin receptors. Additionally, comparison of the amino acids at positions 13–23 in the hormones GHRH, VIP, and secretin, which are predicted to form the α-helices that interact with the receptors (30), shows that this region of GHRH is more homologous to VIP than to secretin. These differences between VIP and secretin, and between their receptors, are likely to account for the differences in the GHRH-binding and signaling responses of the chimeras V_NG_C and S_NG_C.

Our data from the truncated receptors demonstrate that neither the N terminus nor the C terminus of the GHRH receptor can bind ligand in the absence of the other domain. Our studies of the chimeric receptors suggest that one or more of the extracellular loops, in conjunction with residues in the transmembrane domains, function as the determinants of binding specificity and activation for the GHRH receptor. Although the N terminus is essential for ligand binding, substitution of this domain with the corresponding domain of a related family B-III receptor reconstitutes functional GHRH binding. It has been hypothesized that all the receptors in this family have a similar structure in the N-terminal domain as a result of the conserved pattern of cysteine residues forming conserved disulfide linkages (29), and an α-helix in this region is predicted to be involved in the primary interaction with the ligand (30). From our results and those obtained from studies on other receptors in this family (38–47, 49–52, 60), it is conceivable that the N terminus of these receptors plays a primary structural role in the initial interaction with the ligand, while residues in the extracellular loops and the transmembrane domains are involved in secondary interactions with the ligand that determine the specificity of ligand binding. Our results provide a basis for future studies to identify specific receptor domains and residues involved in recognition and binding of GHRH. Chimeras in which individual extracellular loops of the receptor are substituted will enable the localization of binding determinants in a more precise manner, allowing site-directed mutagenesis to be used to define the key residues involved in specific ligand interaction. Identification of domains that are structurally important for the interaction of GHRH with its receptor will facilitate the design of specific receptor agonists and antagonists that might have therapeutic use in the diagnosis or treatment of diseases of the GH axis.

MATERIALS AND METHODS

Plasmids, Receptor Constructs, and Hormones

All receptor constructs were made in the vector pcDNA-3 (Invitrogen, Carlsbad, CA) downstream of the T7 polymerase

Table 3. Binding and Signaling Properties of the GHRH-Secretin Wild-Type and Chimeric Receptors

Property	GHRHR.HA	SECR.HA	G_NS_C.HA	S_NG_C.HA
GHRH binding, ED_{50}	1.8, 2.1 nM	ns	ns	1.1, 1.2 nM
GHRH binding, K_D	31.6, 35.1 pM	nd	nd	234.8, 283.9 pM
GHRH binding, B_{MAX}	34.2, 41.8	nd	nd	23.9, 29.2
GHRH cAMP response, ED_{50}	1.5, 1.4 nM	ns	ns	5.5 nM
Secretin cAMP response, ED_{50}	ns	1.55 nM	ns	ns

The ED_{50} values for binding-competition and cAMP accumulation were not calculated when the binding or signaling was not substantially different than background (denoted as ns), and saturation binding was not performed for receptors in which hormone binding over background was not observed in competition assays (indicated by nd). The two values shown represent the means of duplicate samples from two independent experiments. In the text, the average of these two independent determinations is used. B_{MAX} values are expressed as picomoles bound per mg protein.

promoter and were flanked by *Hin*dIII and *Xba*I sites at the 5′- and 3′-ends, respectively. Their sequences were confirmed using the dideoxy nucleotide chain termination method (Amersham Life Sciences, Arlington Heights, IL). In the numbering system used for all clones in subsequent sections, the first nucleotide of the initiation codon is defined as position 1. The cDNA clones for the rat VIP (15) and secretin receptors (14) were provided by Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan), and the cDNA for the human GHRH receptor was previously cloned in the laboratory (3). Peptide hormones were obtained from Peptides International (Louisville, KY) or Peninsula Laboratories (Belmont, CA).

Generation of Epitope-Tagged Receptor Constructs

Oligonucleotides having 20–22 nucleotides complementary to the C-terminal end of the GHRH, VIP, or secretin receptors and encoding the influenza virus HA epitope (61) were synthesized. Recognition sites for the enzymes *Kpn*I and *Xba*I were engineered on either side of the HA epitope with the stop codon within the *Xba*I site. These primers, together with upstream primers within each of the receptor cDNAs, were used to amplify the C-terminal fragment of each receptor. The PCR products were cloned in context with the respective receptor into the expression vector pcDNA-3 to generate full-length clones for the epitope-tagged receptors (GHRHR.HA, VIPR.HA, and SECR.HA).

The amino acids for the FLAG epitope tag (62) were inserted at the N-terminal domain of the GHRH receptor downstream of the putative signal peptide at residue 37 using gene splicing by overlap extension (63). An oligonucleotide primer encoding the FLAG epitope tag with a site for the enzyme *Eco*RV, 3′ of the tag and having 22 nucleotides complementary to the GHRH receptor was synthesized. This primer and a downstream primer were used to amplify a fragment corresponding to amino acids 130–580 of the receptor. A primer complementary to the sense primer for the FLAG epitope and the T7 promoter primer were used to amplify a fragment corresponding to amino acids 1–140 of the receptor. The two fragments were annealed and amplified using the two external primers, and the product was cloned in context with the C-terminal region of the receptor to generate the FLAG-tagged GHRH receptor GHRHR.F.

Generation of Truncation and Chimeric Constructs of the GHRH Receptor

Using site-directed mutagenesis (64), the recognition site for the enzyme *Afl*II (CTTAAG) was engineered in the tagged GHRH receptors as shown in Fig. 1. The site was introduced at nucleotides 100–106 and 385–390 (corresponding to amino acids 34–35 and 129–130), respectively, in the HA-tagged receptor and at nucleotides 385–390 in the FLAG-tagged receptor. This resulted in changes in amino acids 35 and 129 (Arg to Lys and Val to Leu, respectively).

Removal of the 285-bp *Afl*II-*Afl*II fragment resulted in the deletion of the extracellular domain between amino acids 35 and 129 to give the N-terminally truncated HA-tagged construct (GHRHΔN.HA). Deletion of the 278-bp *Eco*RV-*Afl*II fragment of the FLAG-tagged GHRH receptor, followed by filling-in with Klenow polymerase and ligation, generated the N-terminally truncated FLAG-tagged construct (GHRHΔN.F).

An oligonucleotide complementary to the first intracellular loop of the GHRH receptor and with nucleotides 467–472 altered to form a recognition site for the enzyme *Kpn*I, together with the T7 promoter primer, was used to amplify a 480-bp N-terminal fragment of the GHRH receptor. The PCR product was digested with *Hin*dIII and *Kpn*I and ligated with the large fragment obtained by digestion of the full-length HA-tagged GHRH receptor with the same enzymes, to generate the construct GHRHΔC.HA, truncated within the first intracellular loop. The same primers were used to amplify a 516-bp fragment using the FLAG-tagged GHRH receptor as a template, and the product was ligated into pcDNA-3 to generate the clone GHRHΔC.F.

The chimeras were constructed by the exchange of N-terminal extracellular domains between the HA-tagged receptors with the predicted start of the first transmembrane domain of the receptors as the junction (Table 1). The recognition site for *Afl*II was engineered at nucleotide positions 385–390, 427–432, and 424–439 (amino acids 129–130, 143–144, and 142–143) in the cDNAs for the GHRH, VIP, and secretin receptors, respectively, and the wild-type receptors having the epitope tag and *Afl*II site were used for all subsequent studies. The N-terminal fragments that were exchanged between the receptors were generated by digestion of the cDNAs for the wild-type receptors with *Hin*dIII and *Afl*II.

Expression of Receptor Constructs in HeLa T4 cells Using the Vaccinia-T7 RNA Polymerase Hybrid Expression System

All experiments were performed using cells transfected with various receptor constructs using the Vaccinia Virus-T7 polymerase hybrid expression system (65). Subconfluent monolayers of HeLa T4 cells cultured in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 5% FBS (GIBCO BRL, Grand Island, NY) were infected with Vaccinia virus vTF7.3 expressing the bacteriophage T7 RNA polymerase (obtained under license from Dr. Bernard Moss, NIH, Bethesda, MD), at a multiplicity of infection of 10, for 30 min in PBS/0.1% BSA. The various plasmid DNAs were incubated with liposomes (66) at the ratio of 4–5 μg of lipid per μg DNA, in Opti-MEM I medium (GIBCO BRL), for 20–30 min at room temperature. Virus was aspirated from the cells, and the DNA/liposome mixture was added to the cells and incubated at 37 C in 5% CO_2 for 15–16 h. The amount of DNA used for transfection varied with the size of the plates.

Metabolic Labeling of Transfected Cells and Immunoprecipitation of Epitope-Tagged Receptors

Cells grown in 35-mm dishes and transfected with 5 μg DNA per plate were starved in cysteine/methionine-deficient DMEM (ICN Biomedical Inc., Irvine, CA) for 30 min and labeled with 50 μCi/plate ProMix (Amersham) in the same medium for 2 h at 37 C in 5% CO_2. The cells were washed with PBS, harvested, and subjected to one cycle of freeze-thaw. The cell pellets were resuspended in 400 μl RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS] containing 0.1 mM phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin, and the membranes were solubilized by incubation on ice for 1 h with vortexing. The lysate was clarified by centrifugation and incubated with 0.5 μg of the HA-specific 12CA5 ascites fluid (a gift from Dr. Robert A. Lamb, Northwestern University) for 3–4 h at 4 C on a hematology mixer. At the end of this period, 30 μl of a 50% suspension of protein A-Sepharose beads (Pharmacia, Piscataway, NJ) in PBS were added to the tubes, and the incubation was continued for 30 min. The beads were washed four times with 500 μl cold RIPA buffer and once with cold wash buffer (50 mM Tris-HCl/pH 7.5, 150 mM NaCl, 5 mM EDTA) and resuspended in 30 μl of 2× SDS-PAGE sample buffer (50 mM Tris-HCl/pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue). The samples were boiled for 5 min before separation by SDS-PAGE using a Tris-glycine buffer with See-Blue Pre-Stained Standard (NOVEX, San Diego, CA) as size markers. The gels were fixed in 20% methanol/7% acetic acid, saturated with glacial acetic acid, impregnated with 22% wt/vol of 2,5-diphenyl-oxazole in acetic acid, dried, and exposed to Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY). For glycosylation analysis, immunoprecipitated proteins were digested for 4 h at 37 C with 0.2 U of peptide-*N*-glycosidase F (Boehringer Mannheim Corp., Indianapolis, IN) in a buffer containing 20 mM Na_2HPO_4 (pH 8.0), 20 mM EDTA, 1% NP40, 1 μg/ml leupeptin, 0.1 μg/ml pepstatin A, and 1 μg/ml aprotinin.

Immunofluorescence Localization of Epitope-Tagged Receptors

HeLa T4 cells cultured on glass coverslips in 35-mm plates were transfected as described. For permeabilized cells expressing the FLAG-tagged constructs, the coverslips were washed twice in PBS and incubated for 2 h at 4 C with 3 μg/ml of the anti-FLAG M2 monoclonal antibody (Kodak IBI, New Haven, CT) in PBS containing 0.1% saponin. After extensive washing, the coverslips were incubated at 4 C for 30 min with 2 μg/ml of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS containing 0.1% saponin and 0.2% whole goat serum, and after extensive washing with PBS, the coverslips were mounted in FITC-Guard (Testog Inc., Chicago, IL). For nonpermeabilized cells, incubation was carried out under similar conditions using primary and secondary antibodies diluted in PBS (without saponin). For cells expressing the HA-tagged receptor constructs, the coverslips were washed twice in PBS, fixed with 1% (wt/vol) paraformaldehyde in PBS for 10 min, and immunofluorescense analysis was performed as described, using 1 μg/ml of the HA-specific 12CA5 ascites fluid. The images of cells expressing the FLAG-tagged and HA-tagged receptors in Fig. 3B were optical sections obtained using confocal laser scanning microscopy with a Bio-Rad MRC 600 (Bio-Rad Laboratories, Richmond, CA) connected to a Nikon microscope using a 40× objective. All samples were scanned under the same contrast settings for equivalent times, and optical sections were taken through the central plane of the cell. Photomicroscopy of cells expressing the wild-type and chimeric receptors in Fig. 5B was performed using a 63× objective on a Zeiss Axiophot microscope (Carl Zeiss Inc., Oberkochen, Germany) with equivalent exposure times.

Measurement of Ligand Binding

For binding assays, cells grown in 10-cm dishes were transfected with 10 μg DNA per plate. Binding assays were performed using approximately 50 μg of membrane protein per reaction, prepared from transfected cells as described (3). The reactions were carried out at 25 C for 1 h in a 300-μl reaction volume and were terminated by centrifugation at 4 C for 10 min. The membrane pellets were washed once with binding buffer, and the amount of bound radioligand was measured using a γ-counter. The assays were performed with duplicate samples, and the means were used for all further calculations. For competition studies, the membrane proteins were incubated with either (3-[^{125}I]iodotyrosyl^{10})GHRH(1–44)-amide or (3-[^{125}I]iodotyrosyl^{10})VIP present at a concentration of 70 pM (Amersham), in the absence or presence of increasing concentrations of the unlabeled hormone. The nonspecific binding, determined as the percent of input counts bound in the presence of 1 μM unlabeled hormone, was approximately 13% and 7% for GHRH and VIP, respectively, and was subtracted from all raw data to give the specific bound counts. The percentage of the maximum specific bound counts for each data point was calculated using the specific counts bound by the wild-type receptor in the absence of competitor as 100%. Saturation-binding assays are representative of at least two independent experiments. The concentration of the radioligand ranging from 1.37 pM to 700 pM and the corresponding unlabeled hormone was present at 1 μM. The binding-competition data were fit to a one-site competition equation to determine ED_{50}, and Scatchard analysis of saturation-binding data was used to determine K_D and B_{MAX} values using the program GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Measurement of Intracellular cAMP Levels

For the measurement of cAMP responses, cells were transfected in 12-well plates using 2.5 μg plasmid DNA per well. The cells were treated with hormones as described (3) for 20 min at 37 C, lysed in 150 μl of cold 0.1 M HCl, and harvested, and the lysates were neutralized with an equal volume of 150 mM Tris-HCl (pH 8) containing 4 mM EDTA. The protein was removed by centrifugation for 10 min at 4 C, and the supernatants were used to assay cAMP by a competitive protein-binding assay (67) using 4.5 nM [8-^{3}H]cAMP (Amersham), as a tracer. The protein-bound [8-^{3}H]cAMP in the supernatant was measured by liquid scintillation counting using CytoScint (ICN), and a linear standard curve was performed in each assay. The assays were performed with duplicate samples, and the means were used for all further calculations. The percentage of the maximum cAMP accumulated for each data point was calculated using the cAMP accumulated in cells expressing the wild-type receptor, and treated with 1 μM hormone, as 100%. The dose-response curves were fit to a sigmoidal dose-response equation, and ED_{50} values were determined using the program GraphPad Prism (GraphPad Software Inc.).

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Hormones and the Heart

Naples, Italy, September 24–26, 1998

Conveners: Gaetano Lombardi (Naples, I) and Luigi Saccà (Naples, I)

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Topics: Insulin regulation of myocardial metabolism and performance; Biochemical basis of thyroid hormone action; Thyroid hormone and cardiovascular physiology; Overt and subclinical thyrotoxicosis and hypothyroid diseases: clinical studies and management; Effects of thyroid hormone on cardiovascular performance during non-thyroidal illness; GH/IGF-1 axis in heart failure and in cachexia: clinical implications, GH/IGF-1 in experimental heart failure; GH and heart failure; GH/IGF-1 and endothelial function; GH and metabolic risk factors; GH and premature atherosclerosis; Aldosterone in heart failure; ACE inhibitors in heart failure; Angiotensin II receptor blockade in heart failure; Beta-blockers in heart failure.

This Meeting will give a unique opportunity to gain insights into the physiological relevance of a number of hormones in relation to the cardiovascular system and to learn about their potential role in the cure of heart failure. It will be of prime interest to all those conducting basic and clinical research in cardiology, endocrinology, angiology and metabolic diseases.

Deadline for abstract submission: 15 June 1998
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