Current Research on the Structure and Function of the Growth Hormone Releasing Hormone Receptor

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ABSTRACT

Growth hormone releasing hormone receptor (GHRH-R) is a family B1 G-protein coupled receptor found predominantly on pituitary somatotrophs. In the adults it is required for the normal synthesis and release of growth hormone (GH) from the pituitary. During development it is required for the normal proliferation and maturation of somatotrophs within the pituitary. Mutations of this receptor in mouse and man are associated with GH deficiency, short stature and pituitary hypoplasia. This signaling system plays important roles in growth and development, metabolism of muscle and fat, and is implicated in the regulation of cardiac and immune function, wound healing, tumor growth and the aging process. Current areas of active research discussed here include: studies of the structure of the receptor binding site and its interaction with GHRH, alternative splice variants of the GHRH-R which appear to promote tumor proliferation, truncated receptor isoforms that act as dominant negative inhibitors of wild type receptor, and the unclear physiologic role of the GHRH system in birds and fishes. (J Kor Soc Endocrinol 21:173~183, 2006)

Background

The growth hormone releasing hormone receptor (GHRH-R) is found predominantly on the somatotroph of the anterior pituitary[1,2]. In its classic physiologic pathway, GHRH produced by neurons in the arcuate nucleus of the hypothalamus is released from the median eminence and delivered to the pituitary by the pituitary portal circulation[3]. Here GHRH acts at the GHRH-R of somatotrophs to trigger both the synthesis and release of growth hormone (GH)[4]. The actions of GHRH are opposed by somatostatin and potentiated by ghrelin (a GH secretagogue), each acting at its own somatotroph receptor[5]. The integrated response to these signals is a complex pulsatile pattern of GH release to the peripheral circulation. During development GHRH is required for normal somatotroph proliferation and maturation[6]. After birth, GH acting both directly and through stimulation of hepatic IGF-I, is required for normal postnatal postural growth[3]. Genetic defects of the GHRH-R have been shown to be a cause of GH deficient dwarfism[7~14].

Besides its role in postural growth, GH acts throughout

life as a regulator of metabolism and nutrient partitioning acting anabolically (in opposition to glucocorticoids) to favor increased muscle and decreased fat, as a required factor for muscle growth in response to exercise, and to protect against muscle loss in starvation[15~17].

The GHRH/GH/IGF-1 system is also reported to have a variety of other actions throughout the body including stimulation of increased bone mineral mass[18], immune function[19], wound healing[20], heart function[21,22], skin thickness[23], sleep[24] and psychological well being [25,26]. GH levels are regulated by diet and exercise[27] and after puberty decrease steadily with age[28]. Consistent with its mitogenic effect on somatotrophs[29], GHRH is hypothesized to act as a local autocrine/paracrine factor whose local release by tumor cells may play a role in tumor growth and proliferation[30~32].

The Growth hormone releasing hormone receptor (GHRH-R) is a member of the class B1 family of G-protein coupled receptors (GPCRs), also known as class II or secretin-like hormone receptors. This is an ancient protein family with members found throughout the vertebrates, insects, worms, and possibly even plants[33].

Fig. 1. Phylogenetic distance tree derived from computer alignment of amino acid sequences of human family B1 hormone receptors. Branch points represent statistically chosen hypothetical common ancestral sequences. Branch lengths are proportional to the number of amino acid changes from that hypothetical sequence. Scale bar indicates 0.1 changes per amino acid. PAC₁-R, PACAP specific receptor; VPAC₁-R, PACAP/VIP receptor type 1; VPAC₂-R, PACAP/VIP receptor type 2.

Analysis of the human genome[34] confirms that this family encompasses a small distinct group of receptors (15 human genes) with very little sequence similarity to the large main family of rhodopsin-like (class I, family A) G protein coupled receptors which has hundreds of members (752 human genes).

Family B1 GPCRs (Fig. 1) include the known receptors for a family of peptides homologous to GHRH. These peptides include secretin, glucagon, glucagon-like peptides (GLP-1, GLP-2), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), PACAP-related peptide (PRP), peptide histidine-methionine (PHM in the human, PHI in species where the C-terminal residue is isoleucine), and glucose-dependent insulinotropic polypeptide (GIP, also called gastric inhibitory peptide)[35~37]. These homologous peptides can, to varying extents, cross-react at each other's receptors[35].

The secretin receptor was the first class II GPCR to be cloned, allowing analysis of its sequence and structure[38]. The GHRH-R was then cloned based on its sequence homology to the secretin receptor[1,2,39]. The human GHRH-R gene is made up of 13 exons separated by variably sized introns making it over 15 kb in length[40].

This complex multi-intronic gene structure (a hallmark of family B GPCRs) allows for multiple alternative receptor splice forms and also can give rise to mutations caused by splicing errors.

GHRH-R mRNA is expressed predominantly in the pituitary with lesser amounts found in the hypothalamus and other areas of the brain. By Northern blot, the GHRH-R is also expressed in the placenta, kidney, testis and digestive tract where its function is not well understood[41].

Where on the GHRH-R does GHRH Bind?

For some family A GPCRs there is clear crystal structure evidence that the seven transmembrane helices form a bundle surrounding a binding pocket where small ligands can fit in and trigger activation of intracellular G proteins[42]. But for other GPCRs, including those that are activated by larger ligands, there is evidence that the extracellular domain is the hormone binding site[43]. For family B receptors the structure of the transmembrane helices is assumed to be similar to family A, but no high resolution structure has been demonstrated. Family B1 receptor ligands are helical peptides too large to fit in a transmembrane binding pocket. These receptors have relatively large extracellular domains, so it is not clear from their structure which of these receptor domains interact with the hormone ligand.

Structure/function studies of the GHRH peptide show that only the N-terminal 29 amino acids are necessary for full receptor activation[44]. Some GHRH analogs with D-arginine at position 2 have been found to act as antagonists[45]. This suggests that the N-terminal end of the peptide is crucial for receptor activation. Substitutions that stabilize the helical structure of the GHRH peptide can enhance its receptor binding affinity suggesting that the bound form of GHRH is helical[46].

The GHRH-R has a large N-terminal extracellular domain attached to the seven transmembrane bundle (Fig. 2). Studies of chimeric receptors in which the extracellular and transmembrane domains were exchanged among the closely related GHRH, VIP and secretin receptors were used to examine which of these domains determine ligand specificity[47]. The results show that neither the extracellular nor transmembrane receptor domains when expressed separately could bind hormone. The chimeric

Fig. 2. Cartoon model of GHRH-R structure showing sites of photoaffinity cross-linking. With GHRH bound to wild type receptor, cross-linkers at GHRH position 12 lie in close proximity to the stippled region, while linkers at GHRH positions 1 and 21 are close to different sites in the gray region. The single N-linked extracellular glycosylation site is shown at N. D indicates the site of a D to G point mutation (Asp60-Gly) that disrupts ligand binding in the lit mutant.

receptor constructs suggest that determinants from both the transmembranes and the extracellular domains may interact with the ligand, as no single domain predicted the function or specificity of the chimeras.

An alternative approach to determining the sites of receptor-hormone interaction is photo-affinity cross-linking. Here the radiolabeled hormone is modified with UV-activatable cross-linking groups at different sites along the hormone sequence. The hormone is then bound to its receptor and irradiated with UV light causing covalent cross-linking of the bound hormone to the receptor. The cross-linked complex is then protease digested to allow the identification of receptor sites in close contact to the bound hormone. We prepared iodinated GHRH with cross-linking groups at positions 1, 12 and 21[48]. The results suggest that the mid region of GHRH is in close proximity to the region just extracellular to transmembrane 1, while the N- and C-terminal ends of GHRH are close to domains in the C-terminal half of the receptor (Fig. 2), consistent with the N-terminus of GHRH fitting into a transmembrane pocket. Studies of other receptors in this

family have suggested similar models with the hormone's N-terminus buried in the receptor transmembrane domain and the remainder of the hormone peptide helix coordinating with extracellular domains[49]. But other models, such as a two step process with binding first to an extracellular domain and then to a transmembrane domain, can not be ruled out[50].

Loss of Function Receptor Mutants

The lit mouse is a naturally occurring GH deficient dwarf strain with reduced numbers of pituitary somatotrophs[51]. Because these mice did not respond to GHRH but their pituitary cells could release GH in response to activators of cAMP, it was proposed that the lit mouse might lack GHRH-R[52,53]. After the GHRH receptor was cloned, it was demonstrated that these lit mice had a point mutation in a conserved amino acid in the receptor's extracellular domain (Asp60-Gly) (Fig. 2) [7,8]. This defective receptor protein is produced and localized to the cell membrane, but is unable to bind GHRH[54]. Crystallographic studies of the bacterially expressed extracellular domain from the homologous CRH receptor propose that this charged aspartic acid residue is required for a salt bridge that is key to the extracellular domain structure[49].

Subsequent studies in human GH deficient patients have led to the identification of several GHRH-R inactivating mutations (Fig. 3) including one in a kindred of over 100 affected individuals[13]. It is now suggested that 10% of all human familial isolated GH deficiency type 1 is caused by GHRH-R defects[14,55]. It has also been hypothesized that activating mutations of the GHRH-R could be involved in the generation of pituitary tumors. Studies of the GHRH-R from tumors of acromegatics and other patients have failed to detect any such mutations, suggesting that activating GHRH-R mutations are at best very rare[56,57].

Dominant Negative GHRH-Rs

An alternatively spliced mRNA encoding a GHRH-R with only the first 5 transmembrane helices is seen in normal pituitary and is more abundant in some pituitary tumors[58]. This truncated receptor (Fig. 4) was shown not to function in cAMP signaling in response to GHRH

Fig. 3. Sites of mutations reported to affect GHRH-R function. P1 to P9 are the sites of reported receptor point mutations resulting in single amino acid substitutions. T1 to T8 are sites of reported truncations. T1- Truncation found in a Brazilian kindred with over 105 effected individuals.

Fig. 4. Cartoon illustrated the GHRH-R truncations mutants found to have dominant negative activity.

and when it was co-expressed with wild-type receptor, it appeared to inhibit cAMP signaling[59]. This has been examined in more detail in a recent study using epitope tagged receptors[60] where it was found that the truncated receptor protein is expressed and associates with the wild-type receptor in some sort of multimer complex, where it blocks receptor signaling in a dominant negative fashion. We have examined a different GHRH-R truncation mutant found in a GH deficient patient from Japan[61]. While this patient was homozygous for the mutation and was severely affected, heterozygous family members also appeared of short stature, but to a lesser extent. The small population size and partial phenotype expected made it impossible to demonstrate a statistically significant heterozygote effect. This mutant receptor is truncated within transmembrane 7 (Fig. 4) and is not able to bind GHRH or signal cAMP, but like the more severe truncation just discussed, it also interferes with the

Fig. 5. cAMP signaling from the wild-type receptor is inhibited in proportion to the amount of the truncation mutant expressed.

function of the wild-type receptor in-vitro[62] (Fig. 5). These data suggest the hypothesis that the GHRH-R functional unit might be a dimer such that the presence of a bad partner could prevent the action of an associated good receptor. While heterozygote *lit/+* mice are of normal size, and no phenotype has been demonstrated in heterozygotes for human GHRH-R inactivating mutations, it is possible that some specific receptor truncation mutations could cause reduced height in heterozygotes due to this dominant negative effect.

The GHRH-R of Sheep is Truncated

To help in our studies of receptor structure/function we cloned the GHRH-R from ovine pituitary[63]. The mRNA we identified was very homologous to that of human and

Fig. 6. Sensitivity for GHRH activation of cAMP signaling is increased ten fold when the human GHRH-R is truncated by 16 amino acids.

rat, but encoded a receptor lacking the last 16 amino acids of the C-terminal intracellular tail. The identical mRNA was also found in sheep of a different breed, and no message for the full-length receptor could be identified. In contrast, we cloned the bovine receptor and found that it did not share this truncation. To establish that this was not a cloning artifact, we examined receptor protein expressed in bovine and ovine pituitaries. As predicted, the GHRH-R of sheep pituitary had the appropriately lower molecular weight on SDS gels. When the ovine receptor was transiently transfected in cell lines, we found that it's affinity for GHRH binding was the same as the human or bovine receptors, but it was ~10 fold more sensitive to GHRH in cAMP signaling assays. When the analogous truncation was put in the human receptor, it too became similarly more sensitive to GHRH in cAMP signaling assays (Fig. 6). This suggests that this C-terminal domain inhibits receptor signaling. This is consistent with results found for other receptors showing that phosphorylation of a C-terminal domain can be involved in receptor down regulation and internalization. This region of the GHRH receptor contains multiple potential phosphorylation sites and ³²P incorporation studies suggest GHRH stimulated GHRH-R phosphorylation[64].

This potentiated receptor in domestic sheep brings up an interesting hypothesis. Could the establishment of this mutated GHRH-R be the result of man's selection for animals with increased milk, meat and hair production? To answer this question we examined the GHRH-R of goats. We found that goats share the identical truncation found in sheep. Therefore, the mutation must have occurred millions of years ago in the common ancestor of goats and sheep and long before man's influence.

Mitogenic Signaling, Autocrine/Paracrine Effects and Tumor Growth

In *vivo*, decreased GHRH signaling due to inactivating GHRH-R mutation[7,65], perinatal administration of GHRH antisera[66], or knock out of the GHRH gene[14] all result in somatotroph hypoplasia. Increased GHRH signaling caused by GHRH over production by an ectopic tumor[67], activating mutations of the G protein G_s[68], or transgenic over-expression of GHRH[69] all result in somatotroph hyperplasia. In-vitro, GHRH is a mitogenic factor for somatotroph proliferation[29]. This mitogenic action would not be expected from the cAMP PKA signaling pathway known for the GHRH-R. More recent studies have demonstrated that the GHRH-R also couples to the MAP kinase stimulating the activation of ERK1 and ERK2 via a pathway that may act through the G protein $\beta\gamma$ subunits[4,70,71]. It is hypothesized that this MAP kinase pathway is the mechanism for GHRH's mitogenic effects.

Several studies have found that synthetic GHRH antagonists can inhibit the growth and proliferation of a wide variety of human tumors and tumor cell lines. This inhibition can be demonstrated both in in-vitro models of tumor growth and also in-vivo when tumors are xenografted into nude mice[72-76]. Human small cell carcinoma of the lung, breast cancer lines, osteosarcomas, and prostate cancer cell lines were all inhibited. This antiproliferative effect of GHRH antagonists is consistent with the hypothesis that GHRH can act as a local autocrine/paracrine factor in the stimulation of cell growth[31] and with the mitogenic actions of GHRH at the somatotroph[29]. The mechanism for this action has been unclear because studies using PCR could not detect

Fig. 7. Illustration of GHRH-R splice variants SV-1, SV-2, SV-3 and SV-4. Regions encoded by intronic sequence not part of the pituitary receptor are shown striped, black regions are shared with the pituitary receptor; grey shows the signal peptide of the pituitary receptor.

Fig. 8. cAMP activation by human or chicken GHRH at HEK293 cells with and without transfection with chicken GHRH receptor.

full length GHRH-R in these cell lines responsive to GHRH antagonist[74]. Instead it was discovered that these tumor cells, and also some normal tissues such as human prostate, express alternatively spliced forms of GHRH-R messages not found in pituitary[74,77]. These alternatively spliced mRNAs encode shortened receptor splice variant proteins that were named SV1, SV2 and SV3. The major predicted splice variant, SV1 initiates at a putative alternative start codon within intron 3. In SV1 the first 87 amino acids of the normal 128 amino acid receptor N-terminal extracellular domain are replaced by a new 26 amino acid sequence with the remainder of the receptor unchanged (Fig. 7). In-vitro studies find that this new receptor with a smaller alternative extracellular

domain is still able to bind GHRH analogs[78]. The presence of SV1 message in tumor lines correlates with their response to antagonists[74]. Thus GHRH antagonist, by blocking the actions of this alternatively spliced receptor, may prove to be useful agents against a wide variety of cancers.

GHRH in Birds?

GHRH and PACAP-like peptides highly similar to those in mammals are found in a protocordate invertebrate where their function is unknown as no GH has been identified in these species[79]. GHRH-like peptides are found in fish, but their function as the regulator of GH

secretion is unclear as PACAP seems a better candidate for that role[80]. In birds, GnRH, TRH and dopamine are potent stimulators of GH release and PACAP has only low potency[81]. A GHRH-like peptide has been identified in chicken[82], but it does not cause GH release in live animals or stimulate in-vitro preparations of chicken pituitary[83]. But, in the same studies, mammalian GHRH is a very potent GH releaser in chickens, both in-vivo and in-vitro.

To try to understand this contradiction, we cloned the chicken GHRH-R[84]. This receptor is intermediate in sequence between the known GHRH-Rs of mammals and fish, and distinct from related receptors for other ligands. When the recombinant chicken receptor was expressed in cell lines, it bound human GHRH with high affinity and signaled cAMP accumulation (Fig. 8). But this receptor did not respond to the known chicken GHRH and only very weakly responded to related peptides like VIP and PACAP. When we examined membranes from chicken pituitary, we found that the endogenous chicken receptor bound human GHRH but not the chicken GHRH-like peptide. Thus our cloned receptor behaved exactly like the endogenous chicken GHRH-R and we hypothesized that there was some problem with the chicken GHRH we had tested. Mass spectrometry showed that our peptide stock was not cleaved or degraded and was present at the expected concentration, so we re-cloned the GHRH-like peptide from chicken hypothalamus and found that it did not agree with the published sequence. A lysine (Lys21) that is conserved in GHRH-like peptides from all other known species was not conserved in the published chicken GHRH sequence. In our hands, this conserved lysine was also conserved in chickens. When we tested Lys21 chicken GHRH on the recombinant chicken receptor, on chicken pituitaries, and in live chickens, we were disappointed. This new peptide was more active than before, but only weakly active with no more potency than PACAP and dramatically less potency than human GHRH. Thus the puzzle of the GHRH system in birds is not yet solved but we hypothesize that the problem is with the chicken GHRH-like peptide and we are currently testing potential solutions.

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