Inhibition of Signal Transduction by a Splice Variant of the Growth Hormone–Releasing Hormone Receptor Expressed in Human Pituitary Adenomas

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We have previously shown that some of human growth hormone (GH)-producing pituitary adenomas preferentially express a larger transcript of GH-releasing hormone (GHRH) receptor (GHRH-R). This transcriptional variant is presumed to be produced by alternative messenger RNA splicing and contains premature stop codon in frame, predicted to yield a truncated GHRH-R. Functional expression study indicated that the variant receptor was unable to transduct GHRH signals. To determine the functional relationship between the splice-variant and the wild-type GHRH-R, the expression vector for the variant GHRH-R transcript was transfected into COS-7 cells together with or without that for the wild GHRH-R transcript. In cells transfected with both GHRH-R expression vectors, GHRH-dependent cyclic adenosine monophosphate (cAMP) induction was decreased to 39% of that in the cells transfected with the wild-type GHRH-R expression vector alone. This inhibition was found to be irrespective of the concentration (10^{-8} to 10^{-5} mol/L) of GHRH. These findings suggest that the splice variant form of GHRH-R functions as a dominant-negative modulator in GHRH-induced cellular signaling.

THE PRODUCTION AND SECRETION of growth hormone (GH) from the anterior pituitary cells are stimulated by the hypothalamic hormone.[1-3] The proliferation and differentiation of pituitary somatotrophs are also regulated by GH-releasing hormone (GHRH).[4,5] These biological actions of GHRH are mediated through its receptor (GHRH-R). Recent cloning and sequence studies have shown that GHRH-R belongs to a member of the seven-transmembrane segment receptor superfamily characterized by G-protein recognition sequence on its intracellular face.[6-8] Nearly all of the seven-transmembrane segment receptors have sequence similarity with one another, although their ligands and effector systems are distinct.[9] It has been demonstrated that mutations of the specific regions in the seven-transmembrane segment receptors made them inactive[5,10-12] or constitutively active.[13,14] In some cases of hyperfunctioning thyroid adenomas, somatic mutations in the third cytoplasmic loop of thyrotropin receptor confer constitutive activation of adenylyl cyclase, which may result in tumorigenesis and increased thyroid hormone synthesis.[13] Mutations of GHRH-R causing constitutive activation have not yet been demonstrated, whereas the mutations of Gsα protein have been shown in some GH-producing pituitary adenomas.[15]

Recently, we have shown that some of the human GH-producing pituitary adenomas preferentially express an isoform of GHRH-R transcript,[16] which is found to be the same as the transcriptional variant form b of GHRH-R recently reported by Tang et al.[17] This transcript is larger in length than the previously reported GHRH-R[7,8] and is presumed to be produced by alternative mRNA splicing.[16,17] The splice variant contains premature stop codon in frame, predicted to be truncated at the carboxyl terminal of the third intracytoplasmic loop (Fig 1A). Our previous work demonstrated that GHRH did not increase intracellular cyclic adenosine monophosphate (cAMP) levels in COS-7 cells transfected with the variant GHRH-R transcript expression vector.[16] Thus, the splice variant form is assumed to be unable to transduct GHRH signals.

Responses of GH release to GHRH stimuli have been demonstrated to be variable among GH-producing pituitary adenomas. Some of them failed to release GH in response to GHRH, and others showed the excessive response.[18] The reason for such variance remains unknown. Since almost all of the GH-producing pituitary adenomas express the splice variant, as well as the wild GHRH-R, to various extent,[16] it is worth determining whether the variant form has some effect on the signal transduction conveyed through the wild-type GHRH-R. In the present investigations, we transfected the larger GHRH-R transcript expression vector into COS-7 cells together with the wild GHRH-R expression vector. The results indicate that the splice variant GHRH-R functions as a dominant-negative inhibitor of the wild GHRH-R.

MATERIALS AND METHODS

Cell Transfection and cAMP Determination

Human GHRH-R cDNA expression vector HPR3C, kindly provided by Dr K.E. Mayo, consisted of human GHRH-R cDNA clone HPR3 and eukaryotic expression vector pcDNA-1 (Invitrogen, San Diego, CA).[7] The *Pma*CI-*Bgl*II fragment of the larger GHRH-R transcript was subcloned into *Pma*CI and *Bgl*II sites of HPR3C so as to generate variant GHRH-R cDNA expression vector (named HPR3C-L).[16] COS-7 cells (5×10^5 cells per 100-mm dish) were transfected with various amounts of the expression vectors using lipofectin reagent (GIBCO-BRL, Gaithersburg, MD). The total amounts of transfected DNA were kept constant (4 μg) by adjustment with control vector, pUC19. The cells were harvested with trypsin-ethylenediamine tetraacetate 48 hours posttransfection and replated onto 12-well plates at a density of 2×10^5 cells per well.

The transfected cells were incubated with 1 mmol/L isobutylmethylxanthine (Sigma, St Louis, MO) at 37°C for 1 hour without serum, and thereafter the plates were chilled at 4°C for 15 minutes. The cells were treated for 15 minutes with or without various concentrations of GHRH (Peptide Institute, Osaka, Japan) at 37°C. After the medium was aspirated, the plates were quickly frozen on dry ice. The cells were lysed with 0.5 mL ice-cold 100 mmol/L HCl, processed for determination of

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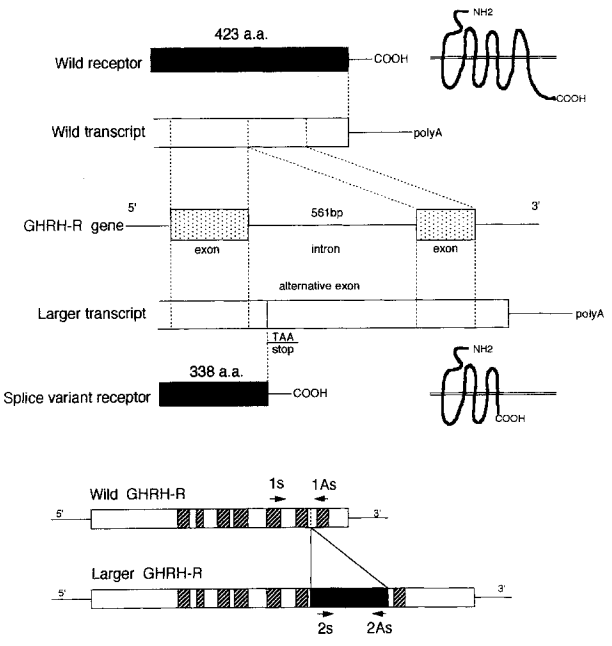

Fig 1. (A) Schematic representation of the structure of 2 isoforms of GHRH-R. The larger GHRH-R transcript is generated via alternative mRNA splicing, yielding a truncated form at the carboxyl terminal of the third intracytoplasmic loop. (B) The positions of the specific primer pairs used in RT-PCR for human GHRH-R. PCR performed with the primer pair 1s-1As was able to amplify both the wild and the larger GHRH-R transcripts, while PCR with the primer pair 2s-2As was to amplify the larger transcript alone. Hatched boxes denote the nucleotide sequence corresponding to the transmembrane domains. Black box shows 561-bp insertion in the larger GHRH-R transcript.

cAMP with an enzyme immunoassay system (Amersham International, Tokyo, Japan).

Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was isolated from the transfected cells by acid guanidinium isothiocyanate-phenol-chloroform extraction method.[19] Aliquots (5 μg) of total RNA were reverse-transcribed into single-strand cDNA using oligo(deoxythymidine)$_{12\text{-}18}$ (0.075 U; Pharmacia, Uppsala, Sweden) and Moloney murine leukemia reverse transcriptase ([RT] 600 U; GIBCO-BRL). One microliter of the reverse transcription mixture was used for polymerase chain reaction (PCR).[20] The PCR was performed in a total volume of 50 μL with a primer pair at 1 μmol/L in 50 mmol/L KCl; 10 mmol/L Tris-HCl (pH 8.3); 1.5 mmol/L $MgCl_2$; 200 μmol/L each of dATP, dCTP, dTTP, and dGTP; and 2.5 U Ampli *Taq* DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT). A primer pair used in the PCR to generate a GHRH-R–specific 251–base pair (bp) fragment was 5′-GGGTGAACTTTGGGCTTTTCTCAA-3′ (1s) and 5′-GCAGTAGAGGATGGCAACAATGAA-3′ (1As), whereas a primer pair for a splice-variant GHRH-R–specific 441-bp fragment was 5′-TAAGCACTCATGACCTCAGC-3′ (2s) and 5′-CTGTCGTGTGCACTTCATCT-3′ (2As) (Fig 1B). A primer pair to generate a β-actin–specific 661-bp fragment was commercially available (Stratagene, La Jolla, CA). PCRs were performed using the following amplification profile: a denaturation step at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes in an automated thermal cycler (Coy Laboratory Products, Ann Arbor, MI).

Statistical Analyses

All values are shown as the mean ± SD. When a significant difference is discussed, unpaired Student's *t* test or Welch's *t* test was used. A *P* value less than .05 was considered statistically significant.

RESULTS

COS-7 cells were transfected with the human GHRH-R cDNA expression vector (HPR3C) or the larger GHRH-R cDNA expression vector (HPR3C-L). To confirm their expression, RT-PCR was performed in RNA samples from the transfected cells. Two sets of primer pairs were designed to analyze the expression of the GHRH-R isoforms (Fig 1B). RT-PCR using the primer pair 1s-1As gave rise to 251-bp band from the cells transfected with HPR3C. From the cells transfected with HPR3C-L, an 811-bp band was yielded. In some experiments, both 251- and 811-bp bands were generated in HPR3C-L–transfected cells, but the intensity of the 251-bp band was consistently faint. By contrast, RT-PCR with the primer pair 2s-2As yielded a 442-bp band from cells transfected with HPR3C-L, while it yielded no amplified product from cells transfected with HPR3C (Fig 2). RT-PCR with each primer pair produced no amplified product from mock (pUC19)-transfected cells. Thus, COS-7 cells transfected with HPR3C expressed only the wild-type GHRH-R transcript, while cells transfected with HPR3C-L expressed mainly the variant GHRH-R transcript and small amounts of the wild GHRH-R transcript.

To examine the functional property of GHRH-R, COS-7 cells were transfected with either HPR3C or HPR3C-L expression vector and were stimulated with 10^{-6} mol/L GHRH. In cells transfected with HPR3C, GHRH increased intracellular cAMP

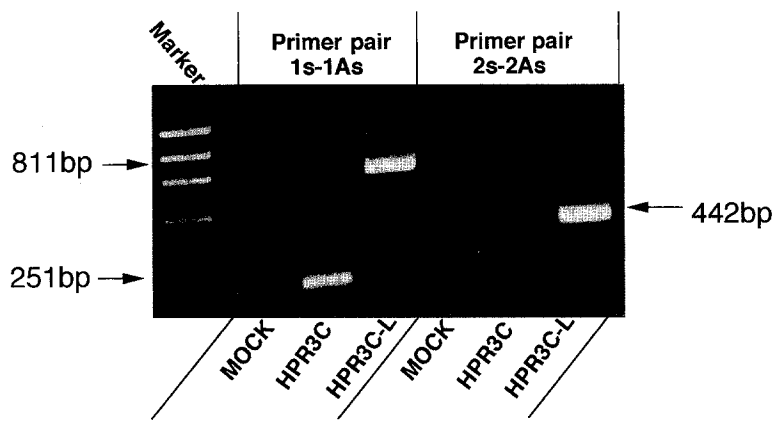

Fig 2. Demonstration of RT-PCR products using human GHRH-R–specific primers; 1 μg of total RNA from COS-7 cells transfected with pUC19 (MOCK), HPR3C, or HPR3C-L was reverse-transcribed and amplified with primer pairs 1s-1As or 2s-2As. The amplified products were stained with ethidium bromide. Data are representative of 3 experiments.

concentrations as a function of the amount of the expression vector: The maximal stimulation was observed at 0.4 μg of HPR3C per dish (Fig 3A). In contrast, cells transfected with HPR3C-L did not increase cAMP levels in response to GHRH, even when 8 μg of HPR3C-L per dish was introduced (data not shown). As shown in Fig 3B, GHRH increased cAMP levels in HPR3C-transfected cells in a concentration-dependent manner, reaching the maximum at a concentration of 10^{-7} mol/L. Again, GHRH failed to stimulate cAMP production in HPR3C-L–transfected cells.

Next, to determine the function of the splice-variant GHRH-R when it is coexpressed with the wild GHRH-R, HPR3C-L or control vector was introduced to COS-7 cells together with

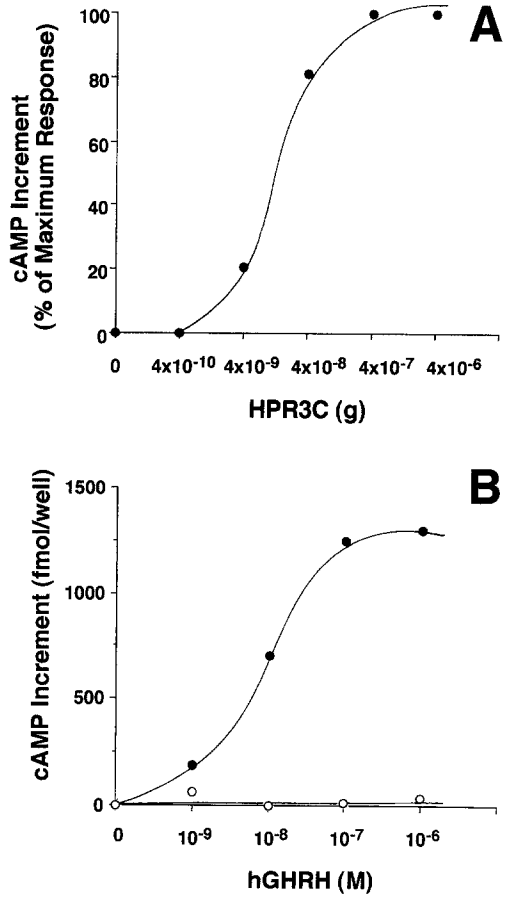

Fig 3. (A) Stimulatory effect of GHRH on intracellular cAMP production of COS-7 cells transfected with HPR3C. The cells were transfected with the indicated amounts of HPR3C. The transfected cells were stimulated with 10^{-6} mol/L GHRH. After 15 minutes, intracellular cAMP levels were determined. Results are shown as % of the maximum response. The absolute value corresponding to 100% is 143 fmol/well. Data are the mean of duplicate samples in a typical of 3 experiments. (B) Concentration dependence of the stimulatory effect of GHRH on intracellular cAMP production of COS-7 cells transfected with HPR3C (●) or HPR3C-L (○). The amount of each expression vector was 4 μg/dish. The transfected cells were stimulated for 15 minutes with the indicated concentration of GHRH. Intracellular cAMP were determined. The results are shown as cAMP increase with GHRH treatment. Data are the mean of duplicate samples in a typical of 3 experiments.

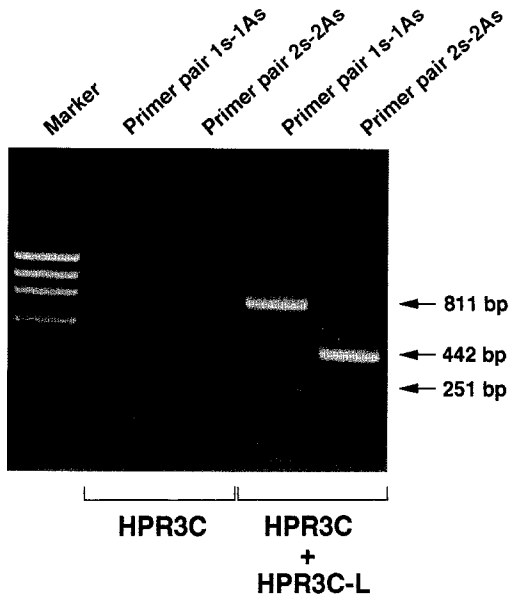

Fig 4. Expression of the wild and the larger GHRH-R transcripts in COS-7 cells transfected with HPR3C in the presence or absence of HPR3C-L. The cells were transfected with HPR3C (0.4 μg/dish) together with HPR3C-L or control vector (3.6 μg/dish). RT-PCR products using human GHRH-R–specific primer pairs (1s-1As and 2s-2As) were demonstrated. Data are representative of at least 4 experiments.

HPR3C. In this experiment, 0.4 μg of HPR3C per dish was transfected, since this amount is enough to obtain the maximal stimulation (Fig 3A). In cells transfected with both HPR3C and HPR3C-L, RT-PCR using the primer pair 1s-1As generated 251-bp and 811-bp bands, while RT-PCR using the primer pair 2s-2As gave rise to a 442-bp band (Fig 4). Thus, in these cells, both the wild and the larger GHRH-R transcripts were found to be expressed. By contrast, in cells transfected with HPR3C, but not HPR3C-L, only a 251-bp band was yielded by RT-PCR using the primer pair 1s-1As and no amplified product was obtained by RT-PCR using the primer pair 2s-2As, showing the expression of the wild GHRH-R alone. The transfected cells were stimulated with 10^{-6} mol/L GHRH and intracellular cAMP levels were determined (Fig 5A). The results demonstrated that in cells expressing both the wild and the splice-variant GHRH-R transcripts (HPR3C and HPR3C-L–transfected cells), the cAMP increment was decreased to 39% of that in cells expressing only the wild GHRH-R transcript (HPR3C-transfected cells). The extent of the inhibition of cAMP induction was irrespective of the concentrations of GHRH added: it was almost the same as with treatment with 10^{-8} mol/L through 10^{-5} mol/L GHRH (Fig 5B).

DISCUSSION

In the present study, the expression vectors for human GHRH-R were introduced to COS-7 cells. RT-PCR analyses using specific primer pairs were performed to differentiate the wild GHRH-R from the larger GHRH-R transcript. The results showed that COS-7 cells transfected with HPR3C expressed

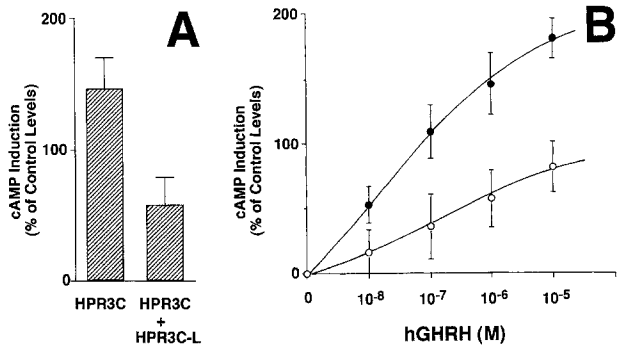

Fig 5. (A) Stimulation of intracellular cAMP production in response to GHRH in COS-7 cells cotransfected with HPR3C and HPR3C-L. The cells transfected with 0.4 μg per dish of HPR3C and 3.6 μg per dish of HPR3C-L or control vector were stimulated for 15 minutes with 10^{-6} mol/L GHRH. Induction of intracellular cAMP is shown as % of control levels. The absolute value of control levels in HPR3C-transfected cells is 657 ± 99 fmol/well, while that in HPR3C and HPR3C-L-transfected cells is 360 ± 104 fmol/well. Data represent the mean ± SD in triplicate assays in a typical of 3 experiments. (B) Concentration dependence of the stimulatory effect of GHRH on intracellular cAMP induction. COS-7 cells were transfected with 0.4 μg/dish HPR3C and 3.6 μg/dish HPR3C-L (○) or control vector (●). Cells were treated for 15 minutes with increasing concentrations of GHRH. Induction of intracellular cAMP is shown as % of control levels. Absolute values of control levels are the same as in A. Data show the mean ± SD in triplicate assays in a typical of 3 experiments.

only the wild GHRH-R transcript. By contrast, cells transfected with HPR3C-L mainly expressed the larger GHRH-R transcript. In some experiments, these cells expressed both the wild and the larger GHRH-R transcripts, but the band intensity of the PCR product for the wild GHRH-R transcript was somewhat variable, but consistently much less than that for the larger transcript. Thus, in COS-7 cells transfected with HPR3C-L, a splicing mechanism generating the wild GHRH-R transcript from the larger transcript appears to occur to a small extent.

The larger GHRH-R transcript contains premature stop codon in frame,[16] assumed to yield a variant receptor truncated at the carboxyl terminal of the third intracytoplasmic loop. Functional expression in COS-7 cells showed that the splice-variant GHRH-R failed to respond to GHRH in terms of intracellular cAMP induction. The GHRH-R belongs to a superfamily of seven-transmembrane segment receptors, which mediate the actions of extracellular signals via guanine nucleotide-binding protein and its effector.[6-8] The third intracytoplasmic loop of the seven-transmembrane segment receptors has been shown to be important for activating G proteins, ligand discrimination, and receptor internalization.[21] Some mutations in this segment confer constitutive activation of α_{1B} adrenergic receptor[22] and thyrotropin receptor.[13] In contrast, other mutations resulting in premature termination in this region are associated with defects of vasopressin V_2 receptor[10,23,24] and corticotropin (ACTH) receptor,[11] although functional expression analyses of these mutated receptors have not been demonstrated.

It has been shown that alternative RNA processing is used to generate isoforms of various seven-transmembrane segment receptors. In rat GHRH-R, an isoform with a 41–amino acid insertion at the third intracytoplasmic loop can be generated, although its expression and function remain obscure.[7] In the case of dopamine D2 receptor, alternative RNA splicing generates the two isoforms, which differ by an insertion of 29 amino acids at the third intracytoplasmic domain.[25,26] The two isoforms of dopamine D2 receptor have been reported to interact with different G proteins.[26] Five splice variants of pituitary adenylyl cyclase–activating polypeptide (PACAP) type-I receptor have been isolated with insertions at the third intracellular loop.[27] They are differently coupled to adenylyl cyclase and phospholipase C, which is linked to functional diversity. Other splice variant forms have been shown in cases of thyrotropin-releasing hormone receptor[28] and ACTH-releasing factor receptor.[29]

Our study clearly demonstrated that coexpression of the splice variant GHRH-R significantly inhibited GHRH-induced cAMP accumulation mediated through the wild receptor. It suggests that the splice variant form decreases GHRH-induced cellular signaling in a dominant-negative fashion. The mechanisms for this dominant-negative inhibition by the splice variant GHRH-R are uncertain. It has been shown that the mutated β_2-adrenergic receptor truncated at the carboxyl terminal of the third intracytoplasmic loop entirely lacks ligand-binding ability.[30] It leads us to suppose marked reduction in the binding activity of the variant GHRH-R, although we have not tested the ligand binding assay of the variant GHRH-R. In addition, variant GHRH-R–mediated inhibition of the wild GHRH-R signaling was not restored by treatment with 10^{-5} mol/L GHRH (Fig 5B). Thus, it is unlikely that the variant receptor sequesters GHRH available for binding to the wild receptor. Receptor oligomerization, inactivation of G-proteins, or some other mechanisms might account for the dominant-negative effect of the variant GHRH-R.

A recent study[31] has shown that a naturally occurring splice variant of gonadotropin-releasing hormone receptor (GnRH-R) also inhibits GnRH-induced cellular signaling. The splice variant of GnRH-R is truncated at the C-terminal end of the fourth transmembrane domain, and is incapable of ligand binding.[31] The variant GnRH-R is shown to impair the insertion of the wild GnRH-R into the plasma membrane.[31] Thus, such regulatory mechanisms may also exist in the other seven-transmembrane segment receptor systems that splice-variant receptor downregulates the function of the wild receptor.

In conclusion, the splice-variant GHRH-R preferentially expressed in some GH-producing pituitary adenomas functions as a dominant-negative inhibitor in GHRH-induced cellular signaling. It should be determined whether the variance of the expression levels of the two GHRH-R isoforms in GH-producing pituitary adenomas causes the variance of GH response to GHRH stimuli. In addition, it would be desirable to examine the mechanisms by which differential RNA processing occurs in normal pituitaries and GH-producing pituitary adenomas.

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