Functional GHRH receptor carboxyl terminal isoforms in normal and dwarf (*dw*) rats

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ABSTRACT

GHRH plays a critical role in pituitary somatotroph development and function, actions which are mediated by a G-protein coupled receptor (GHRHr) that has been recently cloned. PCR amplification of rat pituitary mRNA using primers that span the GHRHr coding region resulted in two distinct products. When sequenced, the two isoforms were identical through bp 1278 of the GHRH coding region. However, the novel variant, which we have termed GHRHr β , contains a 131 bp deletion (1279–1408) and resumes at bp 1409 in the 3'UTR of the previously identified transcript (GHRHr α). The identical isoforms were present in pituitaries from dwarf (*dw*) rats. The predicted amino acid sequence for the alternate receptor isoform differs from the published amino acid sequence at the extreme carboxyl terminus, with the last 5 amino acids of the published sequence replaced and an additional 17 amino acids added to

the sequence. When translated *in vitro* or expressed as an epitope-tagged construct in non-GHRHr containing cell lines, the GHRHr β mRNA produces a 42 kDa protein product, appropriately larger than the 40 kDa product of GHRHr α mRNA. Furthermore, GHRHr β retains the ability to promote cAMP generation in response to GHRH when expressed in non-GHRHr containing cell lines. These results indicate the presence of a splice variant of rat GHRHr mRNA present in normal and *dw* rat pituitary that codes for a functional receptor protein with an alternate carboxyl terminal domain. These findings raise the possibilities of target cell regulation of GHRH response, modulation of response through receptor isoform interactions and the involvement of multiple intracellular signaling pathways.

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INTRODUCTION

The hypothalamic neuropeptide, growth hormone-releasing hormone (GHRH), plays a critical role in pituitary somatotroph development and function. Along with regulating growth hormone synthesis and secretion, GHRH acts as a trophic factor for pituitary somatotrophs (Asa *et al.* 1984, Barinaga *et al.* 1985, Billestrup *et al.* 1986, 1987, Cella *et al.* 1990, Struthers *et al.* 1991, Asa *et al.* 1992). In addition, GHRH is synthesized and secreted in a variety of extra-hypothalamic tissues, including testis, ovary, lymphocytes and intestines, where it is assumed to participate in autocrine and/or paracrine signaling (Bruhn *et al.* 1985, Pescovitz *et al.* 1990).

Partial cDNAs coding for the plasma membrane receptor for GHRH (GHRHr) in the rat, mouse, pig and human have been cloned (Lin *et al.* 1992, Mayo 1992, Gaylinn *et al.* 1993, Hsiung *et al.* 1993) and predict a 423 amino acid (46.8 kDa) member of the G-protein coupled receptor superfamily with strong sequence homology to members of the secretin/vasoactive intestinal polypeptide/glucagon receptor family. These cloned receptors promote cAMP production when expressed in non-GHRHr containing cell lines. Although only a single GHRHr gene has so far been identified, variant mRNA isoforms resulting from alternative splicing have been reported. In the rat, GHRHr mRNA species with and without a third intracellular loop insertion of either 130 bp (Mayo 1992) or 123 bp

(Lin *et al.* 1992) were described in the original reports of cloning. In the mouse and human, a number of similar internal isoforms have been isolated, particularly in human pituitary tumor tissue (Hashimoto *et al.* 1995, Tang *et al.* 1995). It is especially noteworthy that alternative isoforms of GHRHr mRNA are disproportionately expressed in human pituitary adenomas, suggesting that the biology of these isoforms may be distinct.

We report here the cloning of a novel alternate mRNA for rat GHRHr, present in pituitary and extra-pituitary tissues, that results from alternate splicing of the region coding for the carboxyl terminal intracellular domain of the receptor. In addition, we demonstrate that this novel mRNA is translated into a plasma membrane protein of the expected size and responds to GHRH *in vitro* when expressed in cells without endogenous GHRHr.

MATERIALS AND METHODS

Animals

Homozygous *dw* rat breeding stock and adult Sprague–Dawley rats were purchased from Harlan, Inc. (Indianapolis, IN, USA). Animals were housed under controlled environmental conditions with food and water provided freely. Animals were killed by rapid decapitation after CO₂ administration. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and a protocol approved by the Children's Hospital Research Foundation Animal Care Committee.

Tissue extraction

Pituitary glands from adult Sprague–Dawley and *dw* rats were rapidly removed, immediately frozen in liquid nitrogen and ground by mortar and pestle in liquid nitrogen. Total RNA was extracted from powdered tissue by the single-step acid-guanidinium–phenol–chloroform extraction using RNeasy (Molecular Research Center, Cincinnati, OH, USA). Resulting total RNA was quantitated by spectrophotometry and the quality of the RNA verified by formaldehyde gel electrophoresis, transfer to nitrocellulose membrane and visualization of ribosomal bands with methylene blue.

Genomic DNA was prepared from rat liver frozen in liquid nitrogen and ground. Tissue powder was suspended in buffer (50 mM Tris pH 7.8, 50 mM EDTA, 100 mM NaCl; 2% SDS) and treated with 50 µg/ml Proteinase K at 65 °C for 3 h. The suspension was then extracted twice against phenol and once against chloroform. DNA was

precipitated in 100% ethanol, spooled onto glass pipettes, washed in 70% ethanol and allowed to air dry. The dry DNA was resuspended in 50 mM Tris pH 7.8, 50 mM EDTA, quantitated by spectrophotometry and visualized by ethidium bromide after agarose gel electrophoresis.

Reverse transcription and PCR

Total RNA from adult Sprague–Dawley and *dw* rat pituitaries was reverse transcribed using (dT)17 primer and avian myeloblastosis virus reverse transcriptase as previously described (Zeitler *et al.* 1993). The coding sequence of the GHRHr was amplified from reverse transcribed mRNA or directly from genomic DNA by PCR using a combination of *Taq* and *Vent* polymerase, as previously described (Zeitler *et al.* 1993), utilizing primer pairs spanning the entire GHRH coding sequence (Fig. 1A). The identity of PCR products was confirmed by Southern blot hybridization as previously described (Zeitler *et al.* 1993) with a ³²P-labeled GHRHr cDNA probe synthesized by random primer labeling of RPR64 cDNA (Mayo 1992) (encodes 3' end of the GHRHr cDNA; a gift of K Mayo, Northwestern University). Resulting amplification products were separated by agarose gel electrophoresis and visualized with ethidium bromide. Products of interest were excised from the gel and isolated with Prep-A-Gene (Bio-Rad, Richmond, CA, USA), quantitated by spectrophotometry, verified by electrophoresis and cloned into the TA cloning vector (Invitrogen, San Diego, CA, USA). Double-stranded PCR products in plasmids were sequenced using Sequenase 2 (Amersham Life Sciences, Arlington Heights, IL, USA). All sequences were confirmed in the opposite direction and sequences were determined for a minimum of two independent product clones in all cases. If discrepancies existed between the two independent clones, additional clones were examined until an unambiguous sequence was determined. The verified cDNAs were subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen).

Construction of epitope tagged cDNA vectors

Antibodies to the rat GHRHr have not yet become available, limiting the examination of receptor protein expression. Therefore, to verify that the alternate constructs direct translation of variant receptor isoforms, we constructed plasmids expressing each of the alternate mRNAs carrying the FLAG epitope tag (DYKDDDDK) (Kodak, Rochester, NY, USA) at the carboxyl terminal end of the protein. Plasmids containing the individual

isoform cDNAs were amplified using an upstream PCR primer (GGGGCGGACTACAGGCACCACT) in the 5' untranslated region (UTR) of the receptor mRNA sequence and downstream primers (α :CCGCTCGAGCTATTTATCGTCATCGTCTTGTAGTCTGCTGCGCACTCAGAGGTGAG) and (β :CCGCTCGAGCTATTTATCGTCATCGTCTTGTAGTCTGCTGCGATAGGAAGAAAGAA) containing 15 bp of sequence complementary to the last 18 bases of each alternate receptor mRNA (italics) upstream from its respective stop codon, two codons for Ala, 24 bases coding for the FLAG epitope (bold), a new stop codon (underlined) and terminal restriction sites to facilitate subcloning. The resulting amplification products contained the alternate receptor mRNA with its stop codon removed and the FLAG epitope along with a new stop codon added. The epitope-tagged cDNA constructs were verified by direct sequencing of both strands and subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen).

In vitro transcription and translation

Protein products of the GHRHr isoforms were translated from the isoform cDNAs cloned as described above by coupled *in vitro* transcription and reticulocyte lysate translation (TnT coupled reticulocyte lysate system, Promega, Inc., Madison, WI, USA) according to the manufacturer's instructions using [³⁵S]methionine (New England Nuclear, Boston, MA, USA). Resulting labeled products were separated by electrophoresis on 12% acrylamide and visualized by autoradiography. Transcription and translation of *Ets-1* (Bradford *et al.* 1995) served as a positive control.

Expression of receptor isoforms in COS cells

The alternate receptor mRNA species were expressed in COS-7 and HeLa cells by transient transfection as previously described (Conrad *et al.* 1994, Bradford *et al.* 1995). COS and HeLa cells obtained from the ATCC were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (Gibco, New York, NY, USA) according to standard cell culture procedures. Media were changed 6 h prior to each transfection and cells were harvested at 50–70% confluence with 0.05% trypsin, 0.5 mM EDTA and resuspended in DMEM supplemented with 10% FCS. Aliquots of approximately 2×10^6 cells in 200 μ l medium were added to 10 μ g plasmid DNA and transfected by electroporation at 220 V and 500 μ F using a Bio-Rad gene pulser with 0.4 mm

cuvettes. Total transfected DNA was kept constant and non-specific effects of viral promoters were monitored through the use of empty vectors in all experiments. Following transfection, cells were plated in DMEM with 10% FCS for 24 h to allow GHRHr expression.

For experiments examining receptor responsiveness to GHRH, cells were subsequently serum-starved for 12 h prior to treatment. GHRH (Sigma Chemical Co., St Louis, MO, USA) in 0.1% BSA, 0.01 M acetic Acid, 0.1 mM ascorbic acid or vehicle alone was added to the medium at time 0. At the appropriate times, the medium was removed and replaced with 0.1 M HCl in 95% ethanol, placed at -20°C overnight, removed, lyophilized and resuspended in assay buffer. cAMP was determined by ELISA according to the manufacturer's instructions (Amersham Life Sciences). All transfections were performed in triplicate for each condition within an experiment and experiments were repeated a minimum of three times.

For Western blot analysis of receptor protein expression, transfected cells were incubated for 24 h in medium containing 10% FCS, washed in PBS and harvested in RIPA (50 mM Tris pH 8.0, 166 mM NaCl, 0.1% Triton X-100, 0.05% sodium deoxycholate, 0.1% SDS) on ice for 30 min. Proteins were separated by electrophoresis on 12% acrylamide, electro-transferred to nitrocellulose and probed with an antibody to the FLAG epitope (M2, Kodak) followed by chemiluminescent visualization (ECL, Amersham).

RESULTS

Total pituitary RNA from adult male wild-type and *dw* rats (2 μ g each) was amplified using primer pairs spanning the entire GHRHr coding region as shown in Fig. 1A (primer pair A) and resulted in the appearance of two amplification products that differed by ~ 150 bp (Fig. 1B). The quantity of the smaller of the two products ranged from 20 to 40% of the larger product based on ethidium bromide staining. Except for a decrease in the amount of product obtained from amplification of *dw* pituitary RNA, as recently reported (Carmignac *et al.* 1996), there were no gross differences in the amplification products between the normal and *dw* pituitaries (Fig. 1; lane 1 vs lane 2). In addition, no differences were noted between male and female Sprague-Dawley pituitaries (not shown). Both amplification products hybridized to RPR64 (Mayo 1992) by Southern blot (data not shown), indicating significant overlap of sequence with the 3' GHRHr coding region cDNA.

FIGURE 1. PCR amplification of rat pituitary GHRHr. (A) PCR primer pairs used for amplification of GHRHr from rat pituitary RNA. Black box indicates GHRH coding sequence and flanking lines indicate 5' and 3' untranslated regions. The black triangle indicates the location of the third intracellular loop insertion previously proposed (Mayo 1992). Primer pairs are indicated by arrows with matching letters. Location of the cDNA probe (RPR64) used in Southern hybridization confirmation of amplification products is shown. (B) Agarose gel electrophoresis of products from PCR amplification of reverse transcribed total RNA (2 µg) using primer pair A in normal (lane 2) and *dw* (lane 1) pituitary RNA. (C) Agarose gel electrophoresis of products from PCR amplification using primer pairs B (lane B), C (lane C) and D (lane D). Primer pairs (base pair numbers according to sequence of Mayo (1992)): A: 3-GGGGCGGACTACAGGCACCACT; 1422-GGAAGTGAAGTTCGGAGGTTG; B: 64-TTGCTGAACCTGTGGGGAGTTG; 270-GGGTCTGAGCCAAAATGAGAGAAG; C: 64 (as above); 863-TTGATGATCCACCAGTAGGGGG; D: 1166-ATCAAGAGGTGAGGACGGAGATT; 1597-AAGTCGGAGGTTGGTAT.

The presence of amplification isoforms was confirmed using additional pairs of primers spanning various regions of the GHRHr mRNA as shown in Fig. 1A. As seen in Fig. 1C, amplification of pituitary RNA with primers (pairs B and C) spanning the 5' portion of the receptor sequence upstream from bp 863 resulted in a single product. However, primers (pairs A and D) spanning the 3' portion of GHRHr including the carboxyl terminal resulted in the amplification of two distinct

FIGURE 2. 3' GHRHr genomic DNA sequence, 3' GHRHr cDNA sequence and predicted carboxyl terminal amino acid sequence for the two receptor isoforms. Boxes indicate consensus splice donor and acceptor sequences. Dashed line indicates splicing of the GHRHr variant. Translation stop sites are underlined.

products differing by ~150 bp. It is important to note that separation of each of the products obtained with primer pair A and reamplification using primers present within the 130 bp insert proposed by Mayo (1992) (triangle in Fig. 1A) leads to a product of a size that indicates the presence of the insert (data not shown). This result indicates that the proposed insert is present in both of the receptor isoforms isolated here. The alternate product appears exclusively when amplification spans the distal 3' region of the RNA. These results indicate that both forms of the receptor amplified in these studies contain the 130 bp third intracellular loop insert, but differ in their carboxyl termini.

The two receptor mRNA species were gel purified, cloned and sequenced as described in Materials and Methods. The resulting sequences are shown in Fig. 2. The two mRNAs are identical through bp 1278 (a consensus splice donor **AAGGT**) of the GHRH coding region. However, the novel isoform contains a 131 bp deletion and resumes at bp 1409 (a consensus splice acceptor **AGGTCCC**), which lies in the 3'UTR of the

previously identified (α) transcript, 110 bases downstream from the α receptor translation stop site. The remaining downstream bases are identical in the two mRNAs. Figure 2 also compares the predicted translation products of the two mRNA species. As shown, the amino acid sequence for the alternate receptor isoform is predicted to differ from the published amino acid sequence at the extreme carboxyl terminus, with the last 5 amino acids of the published sequence replaced and an additional 17 amino acids added to the sequence.

To confirm that the alternate receptor isoform is a consequence of alternate splicing, we examined the genomic structure of the GHRHr in this region. PCR amplification using identical primers with genomic DNA as template resulted in a single product. This product was cloned and sequenced and the nucleotide sequence of the genomic DNA is compared with that of the two alternate mRNA species in Fig. 2.

Since our results agree with previous reports demonstrating no decrease in GHRHr expression in *dw* rats, we determined whether differences in sequence of the GHRHr mRNA sequences are responsible for the impaired response to GHRH in *dw* rats by sequencing both receptor mRNAs from *dw* rat pituitaries. The sequences of both the α and β receptor mRNAs were identical to the wild-type receptor.

To confirm that the alternate mRNA species are appropriately translated into receptor protein, we examined translation of these constructs by two independent approaches. First, the two constructs were used as templates for *in vitro* transcription and translation. As shown in Fig. 3, linked transcription and translation of the α and β receptor mRNAs resulted in single translation products of 40 and 42 kDa respectively, while the control *Ets-1* plasmid resulted in the translation of the appropriately sized 66 kDa product.

In addition, we examined the products produced by these mRNAs when expressed in COS cells under the control of a mammalian expression vector. As shown in Fig. 4, expression of either mRNA species in COS cells results in translation of protein products that can be visualized with anti-epitope antibody. The α mRNA variant results in expression of an epitope-tagged protein product of an apparent molecular mass of approximately 42 kDa, somewhat larger than the product seen in the linked transcription/translation above as a result of the added FLAG epitope. This product sometimes appears as a doublet with an epitope-positive band approximately 2.5 kDa larger. Since this band increases as a function of transfected DNA and incubation time (not shown), it may

FIGURE 3. Coupled *in vitro* transcription reticulocyte lysate translation of the GHRHr mRNA isoforms. Plasmids containing the GHRHr α and β cDNAs were used as templates for transcription and translation in the presence of [35 S]methionine, the products separated by PAGE and visualized by autoradiography. Transcription and translation of *Ets-1* (see Materials and Methods) served as a positive control.

FIGURE 4. Expression of epitope-tagged GHRHr isoforms in COS cells. Protein extracts of COS cells transiently transfected with mammalian expression plasmids containing the epitope-tagged receptor isoforms as described in Materials and Methods with FLAG were separated by PAGE, transferred to nitrocellulose and immunoblotted with anti-FLAG (M1) antibody, followed by chemiluminescent visualization. Three independent samples for each receptor isoform are shown.

represent pro-GHRHr without the signal peptide cleaved or post-translational modification of GHRHr. As predicted from the cloned mRNA sequence, the β mRNA variant runs with an apparent molecular mass approximately 2 kDa larger than the α isoform, occasionally occurring as a doublet as seen in the Figure.

Both the α and β cDNAs, when transfected into COS and/or HeLa cells, result in expression of

receptors with intact responsiveness to GHRH *in vitro* as reflected in generation of cAMP. As shown in Fig. 5A, when transfected into COS cells, the α and β isoforms elicited a nearly identical cAMP response to 10 μ M GHRH, peaking at approximately 15 min. However, as shown in Fig. 5B, when transfected into HeLa cells, the receptor isoforms mediated robust cAMP generation in response to GHRH (10 μ M), reaching a maximum between 7 and 12 min. In HeLa cells, GHRH (10 μ M) elicited a 71% greater maximum cAMP production in cells transfected with the β isoform than the α isoform. There were no differences in expression level of the two receptor isoforms in either cell type (data not shown).

DISCUSSION

We have demonstrated the presence of a functional 3' GHRHr mRNA isoform (GHRHr β) expressed in the pituitary that appears to be a consequence of alternate splicing of the receptor gene and predicts an amino acid sequence that is 17 amino acids longer than the previously published receptor. The identical isoform was amplified from several non-pituitary tissues of the rat previously shown to express GHRHr (Matsubara *et al.* 1995), including testis, ovary, kidney and peripheral blood lymphocytes (not shown).

This novel isoform became apparent in a somewhat serendipitous manner as a consequence of a fortunate, but unforeseen, choice of primer pairs. The original primer pair (A in Fig. 1) that led to the visualization of the novel PCR amplification product has a downstream primer just 3' to the 131 bp deleted in the β isoform, thereby leading to amplification of a noticeably shorter product. Previous PCR-based reports of GHRHr expression (Matsubara *et al.* 1995) have utilized primer pairs within upstream regions of the GHRHr coding sequence that would not identify the alternate mRNA species.

As noted above, both receptor isoforms described here contain the third intracellular loop insertion originally reported as an alternate RNA species found during the library screen that led to cloning (Mayo 1992). A similarly located, though slightly smaller, insertion was described in an independent report (Lin *et al.* 1992). However, except for a comment that the insertion appears to be more abundant in older estrogen-treated animals (Lin *et al.* 1992), there have been no further studies examining the expression of these alternate third intracellular loop isoforms *in vivo*. Thus, the question of which form of the receptor predomi-

FIGURE 5. cAMP response to GHRH in COS-7 cells and HeLa cells expressing GHRHr isoforms. Cells were transiently transfected with mammalian expression plasmids containing the α and β isoforms and plated in serum-containing medium for 24 h, followed by serum starvation for 12 h. Cells were subsequently exposed to GHRH or vehicle for the indicated times and the cell extract assayed for cAMP by RIA as described in Materials and Methods. Results are expressed as means $\pm$ S.E.M., $n=3$.

nates remains unresolved. The studies described here would suggest, based on the PCR amplification results above, that the common forms of the GHRHr in young adult rats contain the third intracellular insertion. However, the current studies were not designed to determine the relative abundance of these isoforms.

The presence of an alternately spliced mRNA species does not necessarily indicate expression of the associated protein product. However, since there is no antibody to the GHRHr currently available, it is not presently possible to document the endogenous presence of the alternate receptor isoform. Therefore, we examined the ability of the protein isoform to be expressed by three inter-related approaches *in vitro*. First, we examined the

ability of the alternate constructs to be translated *in vitro*. Next, we utilized epitope-tagged versions of the constructs to demonstrate expression of alternate forms in non-pituitary cell lines. Finally, we examined the ability of the alternate constructs to promote intracellular cAMP generation in response to GHRH.

When transcribed and translated *in vitro*, or expressed by transient transfection into COS cells, the alternate mRNA isoform is fully capable of directing translation of a protein product with an apparent molecular mass approximately 2.5 kDa larger than the previously reported α isoform, as predicted from the mRNA sequence. *In vitro* transcription/translation gave GHRH α and β products of apparent molecular masses of approximately 40 and 42 kDa respectively. When expressed in transfected cells and visualized by immunoblotting with antibody to the epitope tag, the α and β isoforms have apparent molecular masses of approximately 42 and 44 kDa respectively, with the increased size relative to the *in vitro* translation a consequence of the added FLAG epitope. This molecular mass estimate for GHRH α is somewhat smaller than predicted from the cDNA sequence (46.8 kDa) and than previous estimates of the human GHRHr (52 kDa) from binding studies (Zysk *et al.* 1996). However, when completely deglycosylated, the human receptor has an apparent molecular mass of 45 kDa (Gaylann *et al.* 1994), not too different from that seen here. The reason for the discrepancy between the size predicted based on an open reading frame (Mayo 1992) and that seen after *in vitro* transcription/translation and expression of epitope-tagged receptor in COS cells is unclear. However, the observation that the transfected receptors expressed in this study are functional supports the notion that these epitope-tagged receptor proteins are full length. The difference in apparent size may reflect oddities in migration on SDS gel, or differences in post-translational modification of the receptor between the *in vivo* binding studies and the transfection experiments described here. It is interesting to note that a protein size of 42 kDa can be accounted for if the receptor is translated beginning at Met 53 of the putative sequence reported by Mayo (1992). Resolution of this discrepancy will need to await isolation of the receptor protein and direct determination of amino acid sequence, which has not yet been reported.

The β isoform, despite the substitution of the carboxyl terminal sequence, retains full responsiveness to GHRH, as reflected in its ability to promote cAMP generation *in vitro*. When transfected into COS cells, the β isoform had essentially the same

signaling characteristics as the α isoform. Curiously, the cAMP response to GHRH appears to be somewhat cell-type specific, since the β isoform elicited a stronger cAMP response than the α isoform when the alternate receptors were transfected into HeLa cells. This does not appear to be a result of differences in expression of the two isoforms, since, if there are any differences in expression levels in the transfected cells, the β isoform would appear to be somewhat less well expressed (Fig. 4). Detailed analysis of the signaling characteristics of the two isoforms, with respect to generation of cAMP as well as other intracellular signaling pathways, is currently underway.

The presence of multiple receptor isoforms with varying physiological actions is a widespread phenomenon (Gingrich & Caron 1993, Reisine *et al.* 1993, Satoh *et al.* 1993). In particular, similar carboxyl terminal isoforms have been reported for other members of the G-protein linked receptor superfamily, including the thyrotropin-releasing hormone (Satoh *et al.* 1993, Jones *et al.* 1996), somatostatin type 2 (Vanetti *et al.* 1992, 1993, Reisine *et al.* 1993), glucagon (Maget *et al.* 1994) and the closely related PACAP receptor (Spengler *et al.* 1993). The somatostatin type 2B receptor differs from the type 2A receptor in a manner highly reminiscent of the differences between GHRH α and β , with the type 2B receptor being shorter in the carboxyl terminus, analogous to GHRH α . Of interest, the type 2B receptor is less efficiently coupled to cAMP inhibition than 2A, a relationship similar to that seen between the two GHRHr isoforms.

The binding of GHRH to cloned GHRHr transfected into non-pituitary cells has previously been demonstrated to result in production of intracellular cAMP (Lin *et al.* 1992, Mayo 1992), an important mediator of GHRH action in the somatotroph (Frohman *et al.* 1992, Wong *et al.* 1995). However, elevation of cAMP does not account for all actions of GHRH on pituitary cells (Frohman *et al.* 1992). Additional pathways have been suggested based on physiological studies, including activation of the phosphatidylinositol/protein kinase C pathway and alterations in intracellular calcium concentration (Frohman *et al.* 1992, Naumov *et al.* 1994). The carboxyl terminus of the novel transcript reported here contains a proline-rich region suggestive of a binding site for SH3-domain peptides (Williamson 1994). Furthermore, a number of potential phosphorylation sites are present in this region, indicating a potential link with additional intracellular signaling pathways (Gonzalez *et al.* 1991, Kennelly & Krebs 1991). The presence of multiple receptor isoforms could

provide the infrastructure for the involvement of multiple intracellular pathways in GHRH response. Since GHRH has multiple effects on the pituitary gland (Asa *et al.* 1984, 1992, Barinaga *et al.* 1985, Billestrup *et al.* 1986, 1987, Cella *et al.* 1990, Struthers *et al.* 1991), it is intriguing to consider the possibility that these actions are mediated by more than one receptor isoform, each of which might have its own profile of cellular responses. If this were the case, then the target organ effects of GHRH could be regulated at the level of receptor isoform expression, allowing for differences in GHRH target organ response under different physiological or developmental circumstances.

In summary, we have demonstrated the presence of a functional GHRHr isoform that differs from the previously reported receptor by having an altered intracellular carboxyl terminus. We have also demonstrated that the sequence of GHRHr isoforms in the *dvw* rat are identical to those found in the wild-type rat, thus excluding a GHRHr defect as a cause of the *dvw* phenotype. Further investigation of the signal transduction mechanism associated with the novel receptor isoform and evaluation of developmental and physiological regulation of the relative abundance of the two isoforms is currently underway.

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