The transcription factor GHF-1, but not the splice variant GHF-2, cooperates with thyroid hormone and retinoic acid receptors to stimulate rat growth hormone gene expression

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Abstract The rat growth hormone (GH) promoter was significantly activated in non-pituitary cells by the expression of unliganded trioodothyronine (T3) and retinoic acid (RA) receptors. Furthermore, a strong ligand-dependent activation was found in the presence of the pituitary-specific transcription factor GHF-1. When compared with GHF-1, the splice variant GHF-2 showed a decreased ability to bind the cognate site in the GH promoter. As a consequence, expression of GHF-2 had little stimulatory effect on the GH promoter and did not show cooperation with T3 or RA receptors even in the presence of ligands. Furthermore, over-expression of GHF-2 inhibited the response to T3 and RA in pituitary cells. These results show that alternative splicing of the GHF-1 gene gives rise to two isoforms that differ in their transactivating properties and in their ability to synergize with the nuclear thyroid hormone and retinoic acid receptors on GH gene expression.

Key words: Thyroid hormone; Retinoic acid; Growth hormone gene; GHF-1; GHF-2

1. Introduction

The pituitary-specific transcription factor GHF-1 (also called Pit-1) is a member of the homeobox POU family of DNA-binding proteins [1,2]. Binding of GHF-1 to two sequences (−65/−95 and −107/−137 base pairs) in the rat growth hormone (GH) promoter is required for the expression of this gene in the somatotrophs [3,4].

Differential splicing of the GHF-1 primary transcript gives rise to a functionally distinct isoform called either GHF-2 [5], Pit-1b [6] or Pit-1a [7] in prolactin and GH-producing cells. In GHF-2, 26 additional amino acids are inserted into the activation domain of the protein. It has been described that this insertion alters the properties of the transcription factor so that GHF-2 can activate the GH promoter but cannot activate the prolactin and GHF-1 promoters [5,7].

Rat GH gene transcription is known to be stimulated by triiodothyronine (T3) and retinoic acid (RA) [8–11]. A common responsive element located close to the distal GHF-1 binding site (at −170/−190) mediates regulation of the rat GH promoter by T3 and RA receptors (TR and RAR) [12]. Our laboratory and others have shown that the retinoid X receptor RXR, which heterodimerizes with TR and RAR, enhances transactivation of the rat GH gene by T3 and RA [12,13].

In this report we show that T3 and RA receptors produce both a ligand-dependent and a ligand-independent activation of the rat GH promoter. These receptors activate the promoter in the absence of GHF-1, and act synergistically with the pituitary-specific factor. However, GHF-2 binds DNA with a lower affinity than GHF-1, and as a consequence shows a markedly decreased ability to transactivate the rat GH promoter and does not cooperate with the nuclear receptors.

2. Materials and methods

2.1. Plasmids

GH-CAT constructs containing −530 and −145 bp of the rat GH promoter have been described [10]. The plasmid -3000Prl-CAT contains the 5′-flanking sequences of the rat prolactin promoter. Expression vectors for the rat GHF-1 and GHF-2, the chick TR c-*erbA*α , the human RARα, and the human RXRα have been described previously [5,14–16].

2.2. Transfections

GH4C1 cells were transfected by electroporation, and Cos-7 and HeLa cells were transfected with calcium phosphate as previously described [12,17]. The cells from each transfection were split into different culture plates in Dulbecco's modified Eagle's medium containing 10% AG1×8 resin-charcoal stripped newborn calf serum and treatments with RA and T3 were administered in this hormone-depleted medium. CAT activity was determined by incubation of the cell extracts with [^{14}C]chloramphenicol as previously described [12,17]. Each experiment was repeated at least two or three times with similar relative differences in regulated expression.

2.3. Protein preparations

The coding regions of GHF-1 and GHF-2 cloned in Bluescript SK$^-$, and the vectors for TR, RAR, and RXR cloned in pSG5 were used for in vitro transcription and translation. For this purpose 1 μg of the different vectors was transcribed and translated using the TNT kit (Promega) following the manufacturer's recommendations. All reactions were split into two aliquots, one was translated in the presence of 4 μCi of [^{35}S]methionine (Amersham), and the other in the presence of the same amount of the unlabeled amino acid. Three μl of the reaction product were resolved in 10% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis. The gel was dried and autoradiographed overnight. Recombinant GHF-1 and GHF-2 proteins expressed in the bacterial strain BL21(DE3) were also used [5]. An aliquot of the bacterial extracts used in the DNA binding studies, run in SDS-PAGE gels and stained with Coomassie blue, is shown in Fig. 2D.

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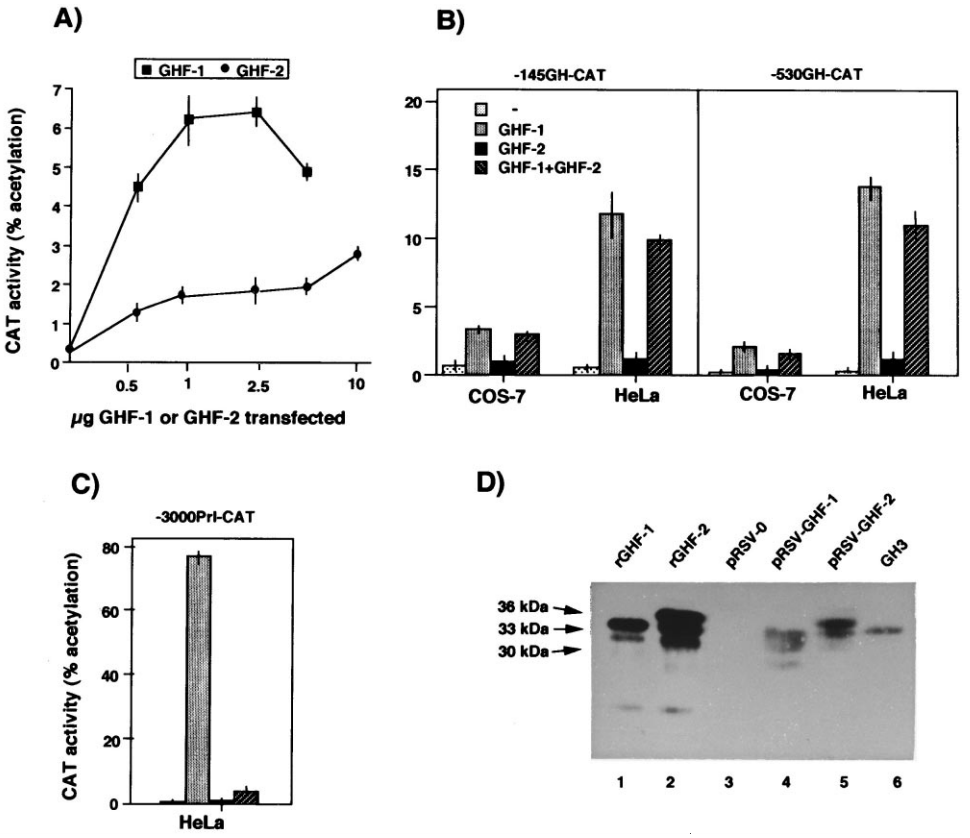

Fig. 1. A: Different potency of GHF-1 and GHF-2 on the activation of the GH promoter. Duplicate culture of Cos-7 cells were co-transfected with 5 µg of the -530GH-CAT construct and the indicated amounts of vectors expressing GHF-1 or GHF-2. CAT activity was determined 48 h after transfection. The data represent the mean ± S.D. of two independent experiments. B: Influence of GHF-1 and GHF-2 on the expression of the GH promoter in heterologous cells. The -530GH-CAT plasmid (right) and a construct containing a shorter fragment (-145GH-CAT) (left) were transfected into Cos-7 and HeLa cells alone or in combination with 10 µg of vectors expressing GHF-1 and/or GHF-2. CAT activity was determined 48 h after transfection and represents the mean of 3 to 5 independent experiments. C: Dominant negative effect of GHF-2 in HeLa cells. Ten micrograms of a construct containing the rat prolactin promoter (-3000Prl-CAT) was transfected alone or with 10 µg GHF-1, 10 µg GHF-2 or both as indicated. The data represent the mean ± S.D. of CAT activity obtained from triplicate cultures. D: Detection by Western blot analysis of GHF-1 and GHF-2 in transiently transfected cells. HeLa cells were transfected with 10 µg of vectors expressing GHF-1 (lane 4) or GHF-2 (lane 5), or with the same amount of an empty non-coding vector (lane 3). Transfection efficiency determined by co-transfection with a luciferase vector (CMV-luc) was similar in all cases. The cell extracts (150 µg) were probed with a polyclonal antibody that recognizes both recombinant GHF-1 and GHF-2 (lanes 1 and 2). Lane 6 shows the immunoblot obtained using an extract from pituitary GH3 cells. The sizes of the proteins are indicated by arrows.

2.4. Western blot analysis

Extracts from HeLa cells were run in parallel with GH3 pituitary cell extracts, and with recombinant GHF-1 or GHF-2 in a 12% acrylamide gel. The proteins were transferred to a nitrocellulose membrane and incubated with a 1:2000 dilution of a polyclonal antibody that recognizes both isoforms. The proteins were identified by chemoluminescence as previously described [5].

2.5. Mobility shift assays

Gel retardation analysis was performed with recombinant or in vitro translated proteins and an oligonucleotide corresponding to the proximal GHF-1 binding site of the rat GH promoter (5′-CCAGCCATGAATAAATGTATAAGGG-3′). For the binding assay, the proteins were incubated on ice for 15 min in a buffer (20 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM dithiothreitol, 5 mg/ml bovine serum albumin, 13% glycerol) containing 3 µg Poly(dI-dC) and then for 15–20 min at room temperature with approximately 50 000 cpm double-stranded oligonucleotide end-labeled with [^{32}P]ATP, using T4-polynucleotide kinase. Each binding reaction contained the same amount of extract or proteins which was obtained by adding the appropriate amount of mock lysate or bacterial proteins. DNA–protein complexes were resolved on 7.5% polyacrylamide gels in 0.5% TBE buffer. The gels were then dried and autoradiographed at −70°C.

3. Results and discussion

We have examined the ability of GHF-1 and its splice variant GHF-2 to activate the rat GH promoter in pituitary and non-pituitary cells. Fig. 1A shows the influence of increasing amounts of GHF-1 and GHF-2 expression vectors on the activity of the -530GH-CAT construct in Cos-7 cells. Basal promoter activity was very low and GHF-1 enhanced this activity in a dose-dependent manner. However, GHF-2 was much less effective in stimulating the GH promoter even when high concentrations of GHF-2 plasmid were used. Fig. 1B compares the effect of transfection with the same amount of expression vectors for GHF-1 and GHF-2 in Cos-7 and HeLa cells. The degree of GHF-1 dependent induction was much greater in HeLa than in Cos-7 cells, and in both the effect of GHF-2 was negligible in comparison with that produced by GHF-1. The influence of both isoforms was also examined in the -145GH-CAT construct. GHF-1 increased the activity of -145GH-CAT by about 4- and 15-fold in Cos-7 and Hela cells, respectively, whereas GHF-2 was practically ineffective. There was the possibility that the GHF-2 isoform was not

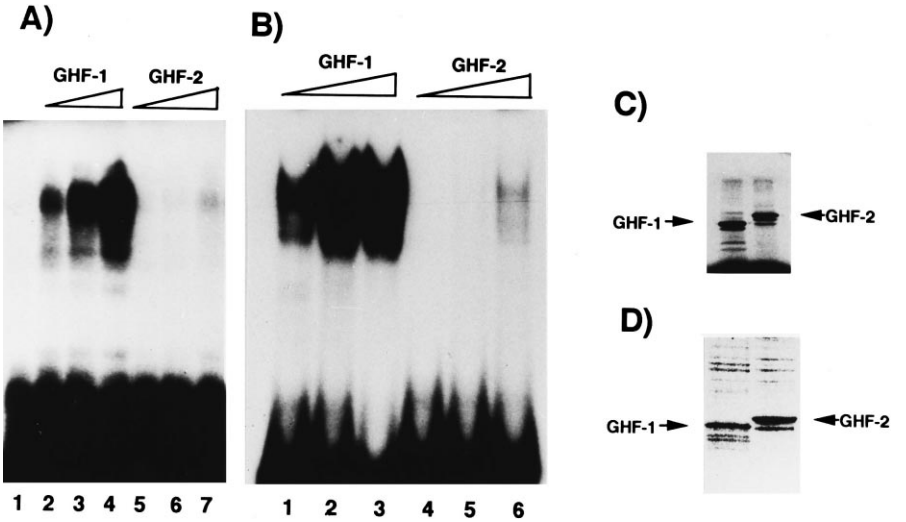

Fig. 2. DNA binding of GHF-1 and GHF-2. A: Mobility shift assays with the proximal GHF-1 binding site of the rat GH promoter and 'in vitro' translated GHF-1 and GHF-2. The ^{32}P-labeled oligonucleotide was used for gel retardation with 1, 2 and 4 μl of in vitro translated GHF-1 (lanes 2–4) and GHF-2 (lanes 5–7). Lane 1 shows that 4 μl of unprogrammed transcription–translation mixture did not produce retardation. B: The oligonucleotide was incubated with increasing amounts (0.1 μl, 0.25 μl and 0.5 μl) of recombinant GHF-1 (lanes 1–3) and GHF-2 (lanes 4–6). C: The in vitro translated proteins used in panel A (1 μl) were subjected to SDS-PAGE. The expected sizes for GHF-1 (33 kDa) and GHF-2 (36 kDa) are indicated by arrows. In panel D the Coomassie Blue staining of 12 μl of the recombinant preparations of GHF-1 and GHF-2 used in panel B is shown.

present in the cells transfected with its expression vector or that the factor was inactive in these cells. However, as shown in Fig. 1C, GHF-2 is expressed in sufficient amounts in HeLa cells to abolish the activation of the prolactin promoter by GHF-1 as described [5]. To further analyze whether differences in GHF-1 and GHF-2 levels could account for the decreased activity of GHF-2 on the GH promoter, we examined the levels of both isoforms by Western blot in HeLa cells transfected with equal amounts of expression vectors of GHF-1 and GHF-2 under the same conditions as those shown in panel B. Fig. 1D shows that the antibody used in the assay recognizes both recombinant GHF-1 (lane 1) and GHF-2 (lane 2). Neither isoform was detected in HeLa cells transfected with the empty expression vector (lane 3, RSV-0), but GHF-1 (lane 4) and GHF-2 (lane 5) were detected in roughly similar amounts in cells transfected with the corresponding expression vectors. Lane 6 illustrates the results obtained in pituitary cells in which GHF-1 is the predominant isoform [5].

These data confirm that the GH promoter can be significantly stimulated by expression of GHF-1 in cell types that lack the endogenous protein. In contrast, we observe that GHF-2 is markedly less efficient than GHF-1. These results differ from others which state that GHF-2 stimulates the GH promoter in other non-pituitary cell lines [5,7], but correlate with more recent data obtained in a deficient α-TSH cell line in which GHF-2 also failed to stimulate this promoter [18]. These observations would suggest the existence of additional cell-specific factors required for efficient activation by GHF-2.

The identity of the POU and homeobox domains of GHF-1 and GHF-2 suggested that they could bind similarly to DNA, and binding of both isoforms to DNA has been previously reported [5]. However, a careful examination by gel retardation analysis demonstrates a different binding potency of GHF-1 and GHF-2. Fig. 2A shows binding of in vitro translated GHF-1 and GHF-2 to the GHF-1 binding site of the GH promoter, and Fig. 2C shows that translation efficiency of the two isoforms was similar. Although the amounts of GHF-1 and GHF-2 applied for the gel retardation assays were identical, GHF-1 (lanes 2 to 4) produced a much stronger retardation than GHF-2 (lanes 5 to 7) showing that the affinity of GHF-2 is markedly reduced. This lower affinity was also demonstrated by using recombinant GHF-1 and GHF-2. Panel D in Fig. 2 shows Coomasie blue staining of the recombinant preparations and panel B shows the retardation produced by increasing amounts of GHF-1 and GHF-2. Although the concentration of GHF-2 used for the DNA binding assays was similar to that of GHF-1, the later isoform produced a much stronger retardation with the DNA elements. These differences in binding affinity must contribute

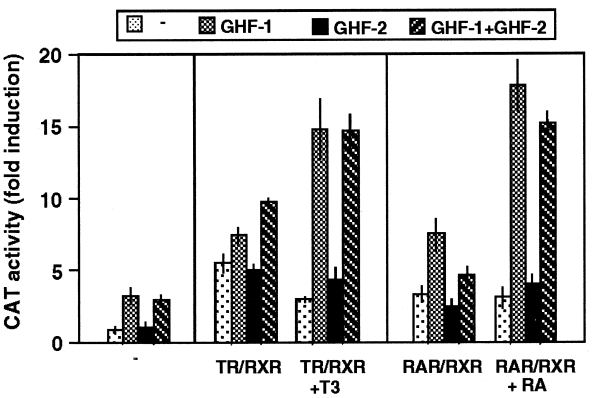

Fig. 3. GHF-1 but not GHF-2 cooperates with T3 and RA receptors in regulating GH promoter activity in Cos-7 cells. The cells were transfected with the -530GH-CAT construct in the absence (–) or presence of 5 μg of expression vectors for TR or RAR in combination with 1 μg of RXR vector (TR/RXR and RAR/RXR, respectively). The cells were also transfected with 10 μg of GHF-1, GHF-2, or both. Each transfection was split into different plates and CAT activity was determined after 48 h in control cells (C) and in cells incubated with 5 nM T3 or 1 mM RA.

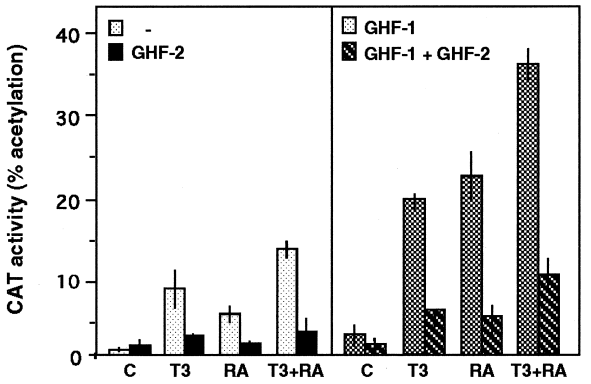

Fig. 4. GHF-2 blocks the response of the GH promoter to T3 and RA in GH4C1 cells. CAT activity was determined in GH4C1 cells transfected with the -530GH-CAT construct alone or with 10 mg GHF-1, 10 mg GHF-2 or both as indicated. The cells were incubated with medium alone (control, C) or containing 2 nM T3 and/or 1 mM RA for 48 h and CAT activity determined. The data represent the mean of two independent experiments performed in duplicate.

to the different transactivation potency of both splice variants. On the other hand, GHF-1 binds as a homodimer [19,20], and it can also heterodimerize with other proteins such as the transcription factor Oct-1 [21] and most likely GHF-2 itself. We have detected the existence of factors in Cos-7 cells that produce a more efficient GHF-1 binding (unpublished results). GHF-2 could compete with GHF-1 for these proteins and/or could form less active GHF-1/GHF-2 heterodimers.

A functional cooperation between the T3 receptors and GHF-1 has been previously reported [22]. Induction of the rat GH promoter by both isoforms of the pituitary factor in the presence of transfected T3 and RA receptors was also examined in Cos-7 cells (Fig. 3). Expression of TR/RXR caused a ligand-independent increase of CAT activity (5- to 6-fold over control uninduced levels) even in the absence of GHF-1. Interestingly, T3 did not activate the promoter in the presence of TR/RXR unless GHF-1 was co-transfected. However, in cells transfected with the receptors plus GHF-1 incubation with the hormone increased maximally (almost 15-fold over control levels) the promoter activity. Fig. 3 also illustrates the influence of RAR/RXR. In the absence of GHF-1, the retinoid receptors by themselves increased CAT activity although with a lower potency than thyroid hormone receptors. Co-transfection with GHF-1 further increased promoter activity, and a clear synergistic effect was observed when RA was added to cells expressing both the receptors and GHF-1.

These results may be significant in vivo since it has been reported that the two GHF-1 binding sites are necessary, but not sufficient, for efficient transcriptional activation of the rat GH gene promoter in transgenic mice. The inclusion of additional sequences which contain the T3/RA response element results in much higher levels of transgene expression suggesting the existence of synergistic interactions between GHF-1 and this element [23]. On the other hand, the finding that GHF-1 is required for the ligand-dependent stimulation of the GH promoter not only supports a role for synergism with the nuclear receptors on GH gene expression, but also contributes to explain the strict pituitary-specific expression of this gene, since only in pituitary cells where GHF-1 is expressed the potent stimulatory effect of T3 and RA could be observed.

In contrast to the results obtained with GHF-1, the combination of TR/RXR or RAR/RXR and GHF-2 exhibits no synergistic effect on the GH promoter, and this isoform was ineffective even in the presence of the ligands. A clear demonstration of the different transactivating properties of GHF-1 and GHF-2 was obtained in pituitary GH4C1 cells. Fig. 4 shows that T3, RA and the combination of both increased the activity of the GH promoter in GH4C1 cells. In these cells, which already contain endogenous GHF-1 and nuclear receptors, over-expression of this factor produced a 4- to 5-fold increase in CAT basal levels and markedly enhanced the response to T3 and RA. These results confirm the existence of functional cooperation between the receptors for both ligands and GHF-1. In contrast with the effect of GHF-1, transfection with an expression vector for GHF-2 did not increase the response to T3 and RA, but rather almost totally blocked the response to both ligands. The specificity of this inhibition is shown by the finding that GHF-2 did not decrease basal GH promoter activity. It should be noted that GHF-2 did not significantly block the effect of T3 or RA in Cos-7 cells expressing GHF-1. It is possible that a different relative concentration of both GHF-1 and GHF-2 could be involved in the differences in the behaviour of both isoforms in pituitary and non-pituitary cell lines. That the ratio GHF-1/GHF-2 is important in determining the response to T3 and RA was shown by the finding that when GHF-1 and GHF-2 were transfected together, the response to both ligands in GH4C1 cells was partially restored (Fig. 4).

Alternative splicing has been shown to generate diversity in the expression of different transcription factors with different transcriptional effects. Our results show a synergism of GHF-1 with thyroid hormone and RA receptors. However, the decreased DNA binding affinity in GHF-2 substantially decreases its transcriptional activity and prevents the cooperation with the nuclear receptors. The lower binding and activity of GHF-2, together with the fact that GHF-1 is the predominant form expressed in both normal pituitaries and in pituitary cell lines [5], suggests that the larger splice variant does not play a dominant regulatory role in GH gene expression. However, a significant role for GHF-2 on the GH promoter under physiological or pathological conditions in which this isoform could be preferentially synthesized cannot be dismissed. On the other hand, GHF-2 could specifically regulate other, still unidentified, pituitary-specific genes.

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