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Restoration of Growth Hormone-Releasing Hormone (GHRH) Responsiveness in Pituitary GH3 Cells by Adenovirus-Directed Expression of the Human GHRH Receptor*

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ABSTRACT

GH-secreting GH3 cells lack GH-releasing hormone (GHRH) receptors. In this study we used adenoviral vectors to transfer the human GHRH receptor to GH3 cells in an effort to restore GHRH responsiveness. A replication-deficient recombinant adenovirus (AdGHRH-R) was designed to allow cytomegalovirus promoter-driven expression of the GHRH receptor messenger RNA. COS-7 cells and GH-producing GH3 cells infected with AdGHRH-R showed GHRH receptor expression on their membranes and exhibited specific GHRH binding. The addition of GHRH to GH3 cells infected with AdGHRH-R increased cAMP levels, induced cAMP response element-binding protein phosphorylation and restored GH secretory responsiveness. GHRH treatment also caused activation of mitogen-activated-protein kinase, induction of c-*fos*, stimulation of GH promotor activity, and increased cellular proliferation. These findings indicate that adenoviral vectors carrying human GHRH receptor are useful for *in vitro* studies of GHRH receptor biology and represent a first step toward the development of gene therapy for dwarfism caused by GHRH receptor mutations. (*Endocrinology* **142:** 414–420, 2001)

THE HYPOTHALAMIC peptide, GH-releasing hormone (GHRH) plays a critical role in somatotrope proliferation and GH synthesis and secretion. The GHRH receptor, a G protein-coupled cell membrane receptor, mediates GHRH action on pituitary somatotrope cells. The GHRH receptor is coupled to $G_s\alpha$, resulting in the activation of adenylate cyclase; cAMP can mimic most of the effects of GHRH, including stimulation of GH synthesis and secretion and proliferation of somatotrope cells in the pituitary (1–4). Inhibition of downstream effects of cAMP leads to somatotrope depletion and dwarfism in transgenic mice (5). A recent study revealed that inactivating mutations of the GHRH receptor gene cause somatotrope hypoplasia and profound dwarfism in the *little* mouse (*lit/lit*) (6). This mutant receptor fails to bind GHRH (7), precluding subsequent signaling by the peptide. Mutations in the human (h) GHRH receptor have recently been identified in families with dwarfism (8–10). These mutations generate a severely truncated receptor or impair receptor processing, leading to hypoplasia of pituitary somatotropes. Genetic treatment of such patients is theoretically possible by transfer and expression of a wild-type GHRH receptor.

GH3 cells, a rat pituitary GH- and PRL-producing cell line, have been valuable for studies of hormonal secretory physiology (11) and signal transduction pathways. However, GHRH fails to stimulate GH secretion or GH messenger RNA production in GH3 cells (12), suggesting the absence of GHRH receptors in this cell line. Replication-deficient, recombinant adenovirus vectors represent a highly efficient means for transferring genes *in vitro* and *in vivo* and are being used in a wide variety of applications in cell culture, experimental animals, and human gene therapy. We tested the feasibility of GHRH receptor gene transfer using a recombinant adenovirus and analyzed the functional effects of GHRH receptor transfer to GH3 cells, including the effects on signal transduction and cell proliferation.

Materials and Methods

Generation of recombinant adenoviral vectors

The recombinant adenoviral vectors were generated using adenovirus type 5 (Ad5 309/356), in which the E3 region has been deleted. A cassette containing the hGHRH receptor complementary DNA (provided by Kelly E. Mayo, Northwestern University, Chicago, IL), driven by the cytomegalovirus (CMV) promoter/enhancer (654 bp) with a simian virus 40 polyadenylation sequence, was inserted in place of the E1 deletion. Four different recombinant adenoviral vectors were generated as described previously (13): AdGHRH-R, AdAS, AdGHGal, and AdCMVGal (Fig. 1). AdAS, containing the antisense GHRH receptor, was used as a negative control. AdGHGal, which contains β-galactosidase driven by the hGH promoter (−610, +58), was used as a reporter. AdCMVGal (13) was used to determine the efficiency of gene transduction. Individual clones of the recombinant adenoviral vectors were purified and titrated by plaque assays. The sequences of the expression cassettes in the adenoviral vectors were confirmed by automated DNA sequencing of viral DNA using specific primers.

Cells cultures and infection with recombinant adenoviruses

GH and PRL producing GH3 cells, COS-7 cells, and HEK293 embryonic kidney cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEK293 cells and COS-7 cells were main-

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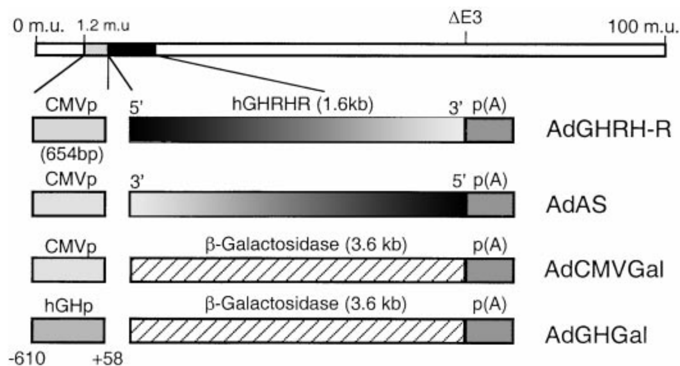

FIG. 1. Structures of recombinant adenoviruses. The adenoviral vector contains a backbone derived from adenovirus type 5 (Ad5 309/356), in which the E3 regions have been deleted. The genes of interest (*shaded area*) were inserted in the E1 region. Four different recombinant adenoviral vectors were generated: AdGHRH-R, AdAS, AdGHGal, and AdCMVGal. AdAS, containing antisense GHRH receptor complementary DNA, was used as a negative control. AdGHGal, which contains the β-galactosidase gene driven by the hGH promoter (−610 to +58), was used as a reporter. AdCMVGal was used to determine the efficiency of gene transduction. The CMV promoter contains 654 bp of the immediate-early enhancer region of the CMV gene.

tained in DMEM supplemented with 10% FBS. GH3 cells were grown in DMEM/Ham's F-12 containing 10% FBS. All media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37 C with 5% CO_2.

Twenty-four-hour after plating cells, infections were carried out by the addition of viral solutions to cell monolayers and incubation at 37 C for 1 h with brief agitation every 15 min. After the addition of new culture medium, infected cells were returned to the 37 C incubator, and medium was changed 24 h later. Transduction efficiency of adenoviral vectors was tested in cell lines using AdCMVGal. β-Galactosidase gene expression was detected in 95–100% of GH3 and COS-7 cells at 48 h after infection [5 plaque-forming units (PFU)/cell; data not shown]. Subsequent experiments were performed using the same concentration of the other recombinant adenoviral vectors.

For measurement of cAMP and pituitary hormones and for ligand binding studies, cells were plated in 12-well culture plates at a density of 2×10^5 cells/well. Triplicate wells were infected with adenoviral vectors at 5 PFU/cell. For Western blot analysis, cells were plated in 10-cm culture plates at a density of 5×10^6 cells/well. Cells were analyzed 48 h after infection of adenoviral vectors.

Immunofluorescence of GHRH receptor

Cells were plated on fibronectin-coated chamber slides (Becton Dickinson and Co., Bedford, MA) and infected with each virus at 5 PFU/cell. Forty-eight hours after infection, cells were washed with PBS twice and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5 min. The slides were incubated with rabbit anti-hGHRH receptor (1:1000; provided by Dr. Bruce Gaylinn, University of Virginia, Charlottesville, VA) at room temperature for 1 h. After washing with Tris-buffered saline/0.025% Tween, staining was performed using biotinylated secondary antibodies (ABC kit, Vector Laboratories, Inc., Burlingame, CA), and streptavidin-fluorescein isothiocyanate (1:100; Vector Laboratories, Inc.). The photomicrographs were taken using a Carl Zeiss microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) and Fuji Photo Film Co., Ltd. color film (1600 Super HG, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Measurement of ligand binding

GH3 or COS-7 cells infected with adenoviral vectors were rinsed with 1.0 ml binding buffer: 50 mM HEPES, 100 mM sucrose, 5 mM $CaCl_2 \cdot 6H_2O$, 0.1% (wt/vol) ovalbumin. ^{125}I-labeled (50,000 dpm) hGHRH-(1–44) amide (Amersham Pharmacia Biotech, Piscataway, NJ) was added to each well with various amounts of unlabeled hGHRH-(1–44) amide (Sigma, St. Louis, MO) in binding buffer at the indicated concentration. Incubation was continued at room temperature for 3 h, and cells were rinsed twice with 1.0 ml binding buffer followed by the addition of 0.5 ml 1.0 N NaOH. After 1 h, the wells were scraped, and the contents were transferred to a glass tube and washed twice with 0.25 ml 1.0 N NaOH. ^{125}I radioactivity was measured in a γ-counter (United Technologies Packard, Downers Grove, IL), and binding curves were determined using Prism analysis software (GraphPad Software, Inc., San Diego, CA).

Measurement of intracellular cAMP levels

GH3 and COS-7 cells infected with adenoviral vectors were treated with 0.1 mM isobutylmethylxanthine for 20 min at 37 C. The hGHRH (1–44) amide was added in fresh warmed medium, and incubation was continued for 30 min at 37 C. The medium was removed, and 0.5 ml cold 0.1 M HCl was added to each well and harvested. Cell lysates were centrifuged for 10 min at 4 C to remove protein, and the supernatants were neutralized with an equal volume of 150 mM Tris-HCl (pH 8.6) containing 4 mM EDTA. The cAMP levels were measured using a RIA kit (Biomedical Technologies, Stoughton, MA) according to the manufacturer's instructions.

Measurement of inositol phosphate accumulation

Twenty-four hours after infection, triplicate wells of cells were labeled with tritiated myo-inositol (NEM Life Science Products, Inc., Boston, MA) for 24 h. The medium was replaced by Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 20 mM lithium chloride and hGHRH-(1–44) amide (10 nM) and incubated for 1 h at 37 C. Ice-cold methanol (0.75 ml) was added to each well, and cells were scraped into a tube containing 0.66 ml chloroform and diluted by the addition of 0.75 ml water. The tubes were sonicated for 10 sec and centrifuged at 10,000 rpm at 4 C. The tritiated inositol phosphates (IP) in the aqueous layer were isolated by ion exchange chromatography. The organic phase (0.2 ml), containing nonhydrolyzed phosphatidyl inositol (PI), was evaporated in a scintillation vial. Tritium was measured in a liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Inositol phosphate accumulation is expressed as a percentage tritiated IP divided by the sum of tritiated IP and PI.

Measurement of GH and PRL in cell culture medium

GH3 cells infected with adenoviral vectors were washed twice with prewarmed DMEM/Ham's F-12 without serum, and the same medium containing hGHRH-(1–44) amide (1 nM) was added. Aliquots were collected at 5, 15, 30, and 60 min after the addition of ligand. The samples were frozen at −20 C until assayed for GH and PRL. The levels of GH and PRL were measured with a RIA kit provided by the National Hormone and Pituitary Program (NIDDK, NIH).

Western blot analysis

GH3 cells and COS-7 cells were grown in a 10-cm plate and infected with adenoviral vectors at 5 PFU/cell. Forty-eight hours after infection, cells were treated with hGHRH-(1–44) amide (1 nM) for various time intervals. Cells were washed with ice-cold PBS and harvested with 5 ml PBS^+ (PBS, 1 mM EDTA, 1 mM phenylmethysulfonylfluoride and 1 mM dithiothreitol). Nuclear extracts were prepared by the Shapiro method (14) modified by the addition of protease inhibitor cocktail tablets, Complete (Roche Molecular Biochemicals, Indianapolis, IN), and 25 mM NaF. In the experiments that involved GHRH receptor and mitogen-activated protein kinase (MAPK), whole cell lysis was prepared with lysis buffer [25% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES (pH 7.9), 1 mM phenylmethysulfonylfluoride, 0.2 mM EDTA, 25 mM NaF, and protease inhibitor cocktail tablets, Complete]. Proteins were solubilized in 1% Nonidet P-40. Equal amounts of proteins were resolved by 10% SDS-PAGE and transferred onto nitrocellulose filters. The membranes were blocked with 3% nonfat milk in PBS for 1.5 h and then incubated overnight at 4 C with rabbit polyclonal antibodies against either total cAMP response element-binding protein (CREB) or Ser^{133}-phosphorylated CREB (Upstate Biotechnology, Inc., Lake Placid, NY). To detect Pit-1, c-Fos, MAPK, or GHRH receptor, membranes were incubated with rabbit anti-Pit-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit

anti-c-Fos (Santa Cruz Biotechnology, Inc.), rabbit anti-phospho-p44/p42 MAPK (New England Biolabs, Inc., Beverly, MA), or rabbit anti-hGHRH receptor antibody (provided by Dr. Bruce Gaylinn). Immunoreactive proteins were detected using an antirabbit horseradish peroxidase-conjugated antibody (1:5000) and the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, IL). Bands were detected with Kodak (Rochester, NY) X-Omat film and quantitated using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA).

GH promoter activation by GHRH in AdGHRH-R-infected GH3 cells

AdGHGal, an adenoviral vector containing the β-galactosidase gene driven by 0.67 kb of the hGH promoter, was used as a reporter to evaluate the GHRH activation of GH promoter. Twenty-four hours after coinfection (5 PFU/cell of each virus) of AdGHGal with AdGHRH-R or AdAS, hGHRH-(1–44) amide was added, and the cells were incubated overnight. Culture medium was aspirated, and cell lysis solution was added. Triplicate wells of infected cells were used to measure β-galactosidase activity using *O*-nitrophenyl β-D-galactopyranoside as a substrate (Sigma, St. Louis, MO). Cell lysates were mixed with the *O*-nitrophenyl β-D-galactopyranoside substrate solution and incubated in 37 C for 1 h. The reaction was stopped with 100 μl 1 M Na_2CO_3. Absorption was measured at 405 nm, and β-galactosidase activity was calculated using a standard curve.

Cell proliferation assays of GH3 cells infected with AdGHRH-R

Cell proliferation was measured using a nonradioactive cell proliferation assay according to the manufacturer's protocol (Cell-Titer 96 Aqueous NonRadioactive Cell Proliferation Assay, Promega Corp., Madison, WI). GH3 cell cultures were depleted of estrogen for 4 days using phenol red-free DMEM/Ham's F-12 containing 10% dextran/charcoal-stripped FBS. One day after plating 1×10^4 cells in quadruplicate wells of 96-well plates, adenoviral vectors were infected at 5 PFU/cell. Fresh medium containing 1 nM hGHRH-(1–44) amide was added every day for 4 days, at which time cell proliferation was measured. The growth of GH3 cells was expressed relative to the growth (mean ± SD) of cells infected with AdGHRH-R without addition of GHRH.

Results

GHRH receptor expression and functional characterization

GH3 and COS-7 cells were infected with adenoviral vectors carrying the GHRH receptor (5 PFU/cell) to investigate the efficacy of GHRH receptor expression. Immunofluorescence was performed using an anti-hGHRH receptor antibody to assess GHRH receptor expression in cell membranes. GHRH receptor expression was readily detected in the membranes of COS-7 and GH3 cells infected with AdGHRH-R, but not in cells infected with the antisense construct (AdAS). Expression of GHRH receptor was seen in 95–100% of GH3 and COS-7 cells infected with 5 PFU/cell AdGHRH-R (Fig. 2A).

Western blot analysis was also performed to assess GHRH receptor expression (Fig. 2B). A GHRH receptor-specific band of approximately 52 kDa was detected in GH3 and COS-7 cells infected with AdGHRH-R. This band was not observed in cells infected with AdAS. AdGHRH-R-infected GH3 cells also showed a higher molecular mass band, presumably corresponding to different form of glycosylated GHRH receptor.

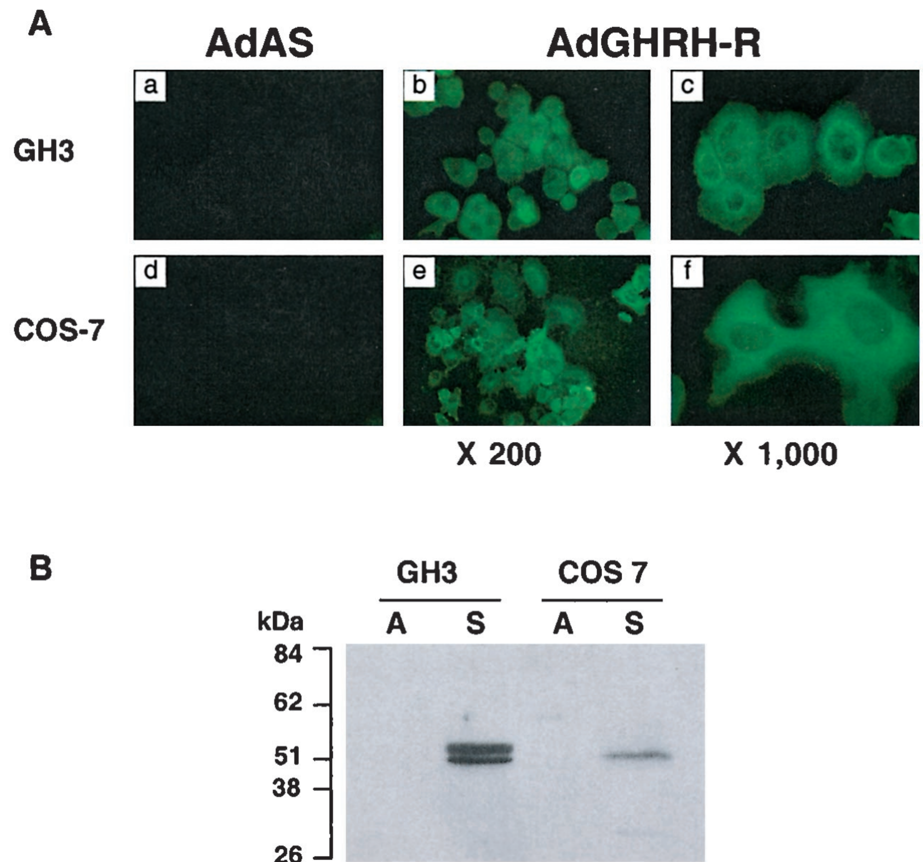

FIG. 2. Expression of the hGHRH receptor in COS-7 and GH3 cells infected with AdGHRH-R. A, Immunofluorescent localization of the GHRH receptor. Indirect immunofluorescence of GH3 (A–C) and COS-7 cells (D–F) infected with AdGHRH-R (B, C, E, and F) or AdAS (A and C) was performed using rabbit anti-hGHRHR receptor. The pictures were taken using a Carl Zeiss (model) microscope and Fuji Photo Film Co., Ltd. 1600 film. B, Western blot analysis of the GHRH receptor. Membrane extracts of GH3 (lanes 1 and 2) and COS-7 cells (lanes 3 and 4) infected with AdAS (lanes 1 and 3) or AdGHRH-R (lanes 2 and 4) were analyzed using the same antibody.

GHRH binding to receptors was analyzed in cells infected with adenoviral vectors (Fig. 3). GHRH bound to AdGHRH-R-infected GH3 cells with a K_d of 3.46 nM. The number of expressed receptors expressed ranged from 186,000–277,000/cell. No GHRH binding was detected in cells infected with AdAS. COS-7 cells infected with AdAS exhibited a low amount of specific GHRH binding, suggesting that these cells may have a small number of endogenous GHRH-binding sites.

cAMP levels were measured to determine whether the expression of GHRH receptors by adenoviral vectors mediates signal transduction in response to GHRH. An increase in intracellular cAMP was detected at 10^{-11} M GHRH and reached maximal values at 10^{-9} M GHRH in GH3 and COS-7 cells infected with AdGHRH-R (Fig. 4A). GH3 cells infected with AdAS showed no cAMP increase in response to GHRH, whereas AdAS-infected COS-7 cells showed a slight increase (24.5 ± 0.9 pM/well) in cAMP at high concentrations of GHRH (10^{-7} M).

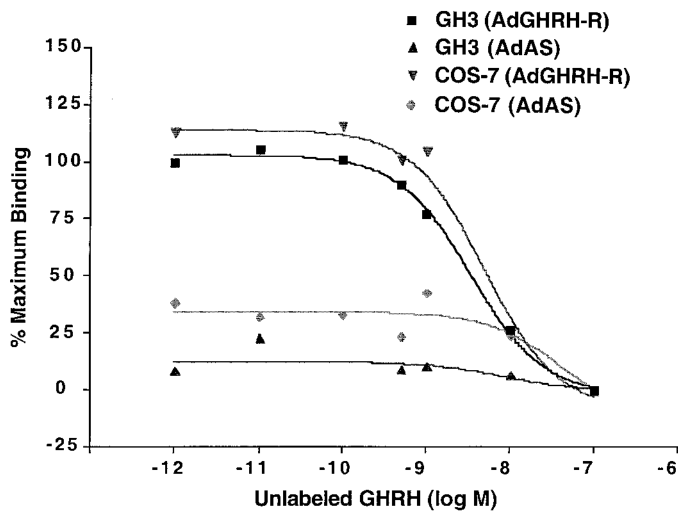

FIG. 3. Binding of GHRH to GHRH receptors in GH3 or COS-7 cells infected with AdGHRH-R or AdAS. ^{125}I-Labeled and increasing concentrations of unlabeled hGHRH-(1–44) amide were added to plated cells. Results are expressed as a percentage of the maximum specific binding.

The cAMP response to GHRH plateaued at 5 PFU/cell AdGHRH-R in GH3 cells and 10 PFU/cell in COS-7 cells (Fig. 4B). This suggests that the cellular capacity to generate cAMP may be saturated by these high levels of GHRH receptor expression.

To investigate the possibility of involvement of protein kinase C (PKC) pathway in GHRH receptor signal transduction, inositol phosphate accumulation was measured in cells expressing the GHRH receptor. TRH was used as a positive control because the endogenous TRH receptor in GH3 cells is linked to the PKC pathway. GHRH did not stimulate inositol phosphate accumulation in GH3 or COS-7 cells infected with AdGHRH-R (Fig. 5). In contrast, TRH stimulated high levels of inositol phosphate accumulation in GH3 cells. This suggests that GHRH does not activate the PKC pathway in somatotrope cells.

GH and PRL secretion of GH3 cells by GHRH

To evaluate whether GHRH receptor expressed by adenoviral vector reconstitutes GH or PRL secretion, GHRH was added to GH3 cells infected with AdGHRH-R. The secretion of GH and PRL increased significantly at 15, 30, and 60 min after GHRH addition in AdGHRH-R-infected GH3 cells. AdAS-infected GH3 cells showed no GH or PRL secretory response to GHRH stimulation (Fig. 6, A and B).

CREB phosphorylation in AdGHRHR-infected GH3 and COS-7 cells

GH3 and COS-7 cells were infected with AdGHRH-R or AdAS and treated with GHRH to determine whether it alters CREB phosphorylation. GHRH increased the level of pCREB more than 10-fold within 5 min in GH3 cells and 2- to 3-fold in COS-7. This level of phosphorylation was sustained for at least 1 h (Fig. 7). There was no significant change in the pCREB level in cells infected with AdAS. The total amount of CREB was unchanged throughout this time course (Fig. 7).

Pit-1 expression, MAPK activation, and c-Fos induction in GH3 cells

Levels of Pit-1, MAPK, and c-Fos, all potential targets of GHRH receptor, were also measured. As shown in Fig. 8A,

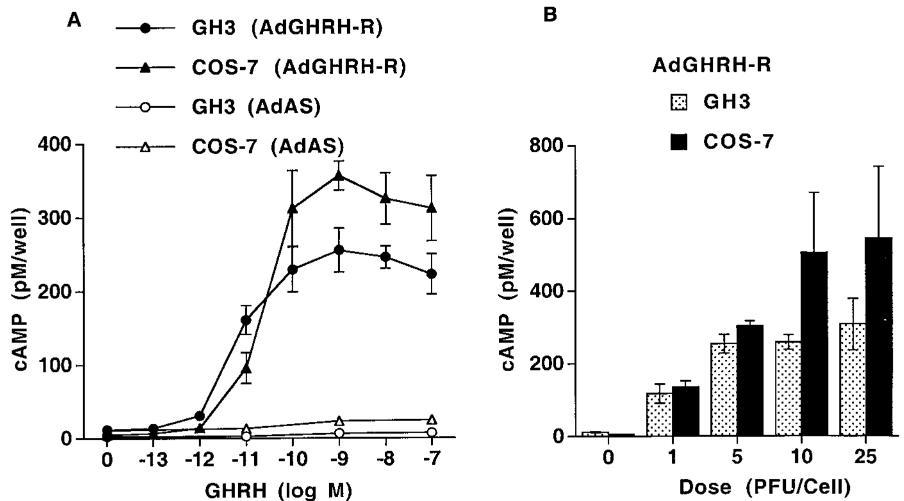

FIG. 4. GHRH-stimulated cAMP accumulation in GH3 or COS-7 cells infected with AdGHRH-R or AdAS. A, Intracellular cAMP accumulation in response to different doses of GHRH. The EC_{50} value is 30 pM. B, The maximal cAMP response at 10^{-9} M GHRH was tested using increasing doses of adenoviral vectors.

Pit-1 was abundantly expressed in the absence of GHRH in both GH3 cell groups infected with AdAS or AdGHRH-R. GHRH did not alter Pit-1 expression. In contrast, GHRH induced MAPK activation within 5 min and dramatically increased the level of c-Fos by 2 h in GH3 cells infected with AdGHRH-R (Fig. 8C).

GH promoter activation in GH3 cells by GHRH

AdGHGal, an adenoviral vector containing the β-galactosidase gene driven by 0.67 kb of the hGH promoter, was used as a reporter to evaluate the ability of AdGHRH-R to activate the GH promoter. Basal β-galactosidase activity of AdGHGal was greater in GH3 cells than in COS-7 cells. GHRH induced a 2- to 3-fold increase in β-galactosidase activity in GH3 cells infected with AdGHRH-R. GHRH did not increase β-galactosidase activity in GH3 cells infected with AdAS. In COS-7 cells infected with AdGHRH-R there was no increase in the β-galactosidase activity in response to GHRH (Fig. 9), presumably because these cells lack the specific transcription factors required for GH promoter function.

Effect on cellular proliferation of GH3 cells

Cell proliferation of GH3 cells expressing the GHRH receptor was examined after GHRH addition. Estrogen-depleted medium was used to avoid a possible proliferative effect of estrogen in these estrogen-transformed cells. GHRH (1 nM) significantly stimulated the proliferation of AdGHRH-R-infected GH3 cells (Fig. 10).

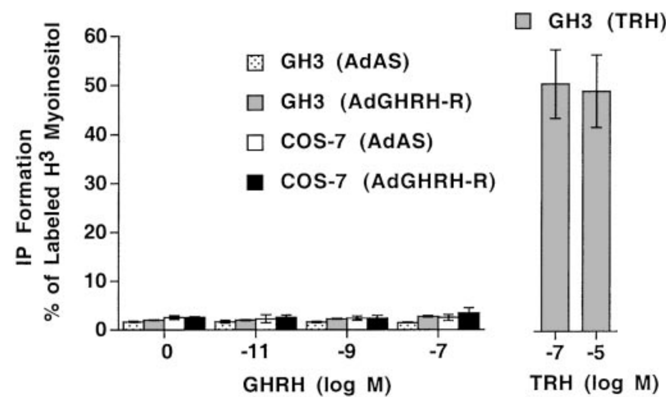

FIG. 5. Inositol phosphate (IP) accumulation in GH3 or COS-7 cells infected with AdGHRH-R or AdAS. IP accumulation in GH3 cells stimulated by TRH was measured as a positive control.

Discussion

Adenovirus is an attractive vehicle for gene transfer and transgene expression in mammalian cells because high efficiency gene transduction can be achieved in a wide variety of quiescent and proliferating cells (15, 16). Using a recombinant adenoviral system, a functional hGHRH receptor was expressed in COS-7 cells and GH3 cells. Infection with the AdGHRH-R resulted in a high level of GHRH receptor expression, specific GHRH binding, cAMP responsiveness, and CREB phosphorylation after stimulation by GHRH. We also demonstrated the restoration of GHRH responsiveness in GH3 cells infected with AdGHRH-R, as demonstrated by GH gene stimulation, increased GH secretion and cellular proliferation.

The GHRH receptor is an *N*-linked glycoprotein, and photoaffinity cross-linking studies have revealed three distinct receptor glycosylation forms, two complex and one core-glycosylated (high mannose) form (7, 17). In Western blot analyses, two GHRH receptor bands were seen in GH3 cells, whereas only one band was detected in COS-7 cells, suggesting that different forms of glycosylation may occur in various cell types.

It is notable that COS-7 cells, which are derived from kidney, exhibit low levels of GHRH binding and respond to high doses of GHRH. Mayo *et al.* (5) reported GHRH receptor messenger RNA expression in rat kidney using a RT-PCR assay. The biological role of the GHRH receptor in kidney, if any, is unknown.

The efficiency of infection combined with the strong activity of the CMV promoter presumably resulted in supraphysiological receptor expression in excess of available G protein. These conditions may result in uncoupled, low affinity receptors, as suggested by the relatively high K_d for GHRH. In contrast, cAMP production, which requires the coupled receptor, showed the expected higher affinity EC_{50}.

An established function of the GHRH receptor in pituitary somatotrope cells is to mediate the release of GH in response to GHRH. Increased cAMP levels mediate the phosphorylation of ion channels, leading to the opening of a Na^+-permeable ion channel, depolarization of the cell membrane, and influx of Ca^{2+} through the L-type Ca^{2+} channels. The increase in intracellular Ca^{2+} levels promotes GH release through the process of exocytosis (18). We have shown that adenovirus-mediated expression of GHRH receptor in GH3 cells confers GH secretion in response to GHRH. The ability

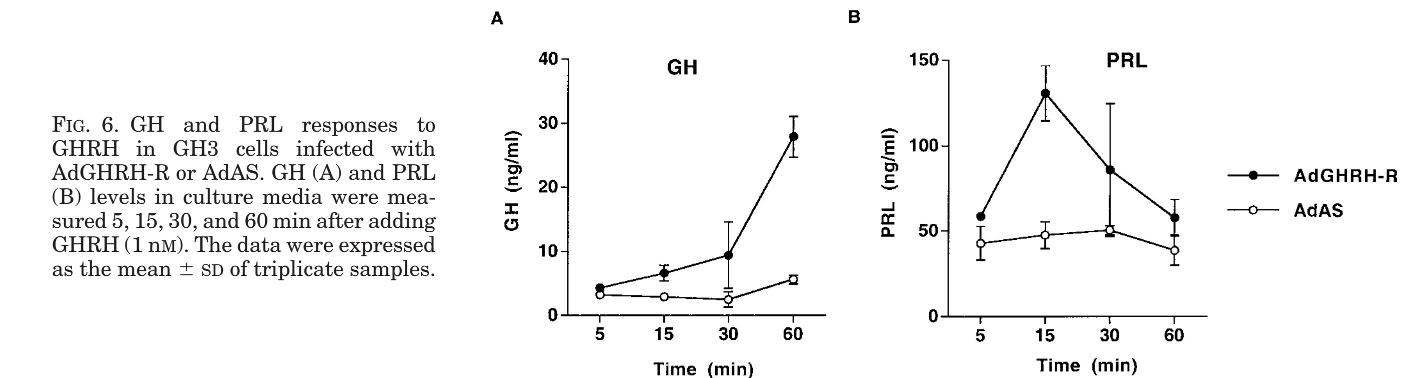

FIG. 6. GH and PRL responses to GHRH in GH3 cells infected with AdGHRH-R or AdAS. GH (A) and PRL (B) levels in culture media were measured 5, 15, 30, and 60 min after adding GHRH (1 nM). The data were expressed as the mean ± SD of triplicate samples.

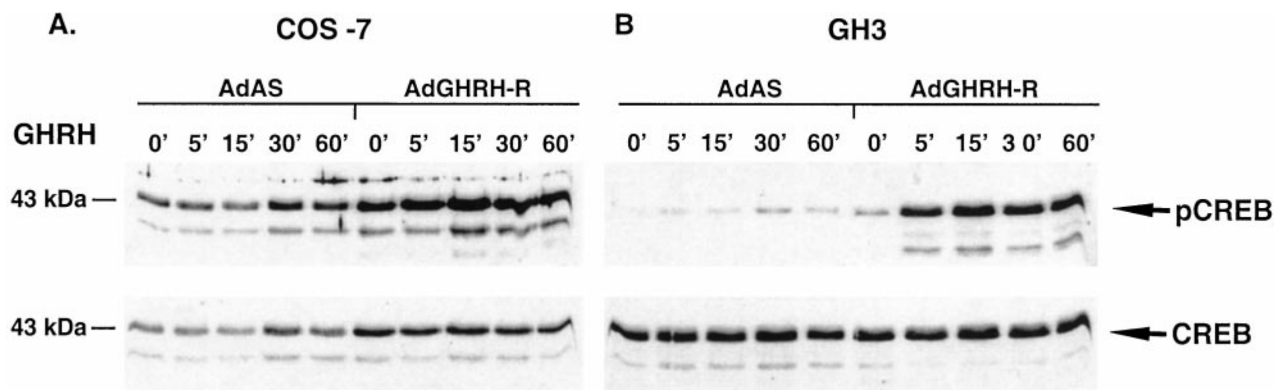

FIG. 7. CREB phosphorylation in COS-7 (A) or GH3 (B) cells infected with AdGHRH-R or AdAS. Western blot analysis was performed using rabbit polyclonal antibodies against either total CREB or Ser$^{133\text{-phosphorylated CREB}}$.

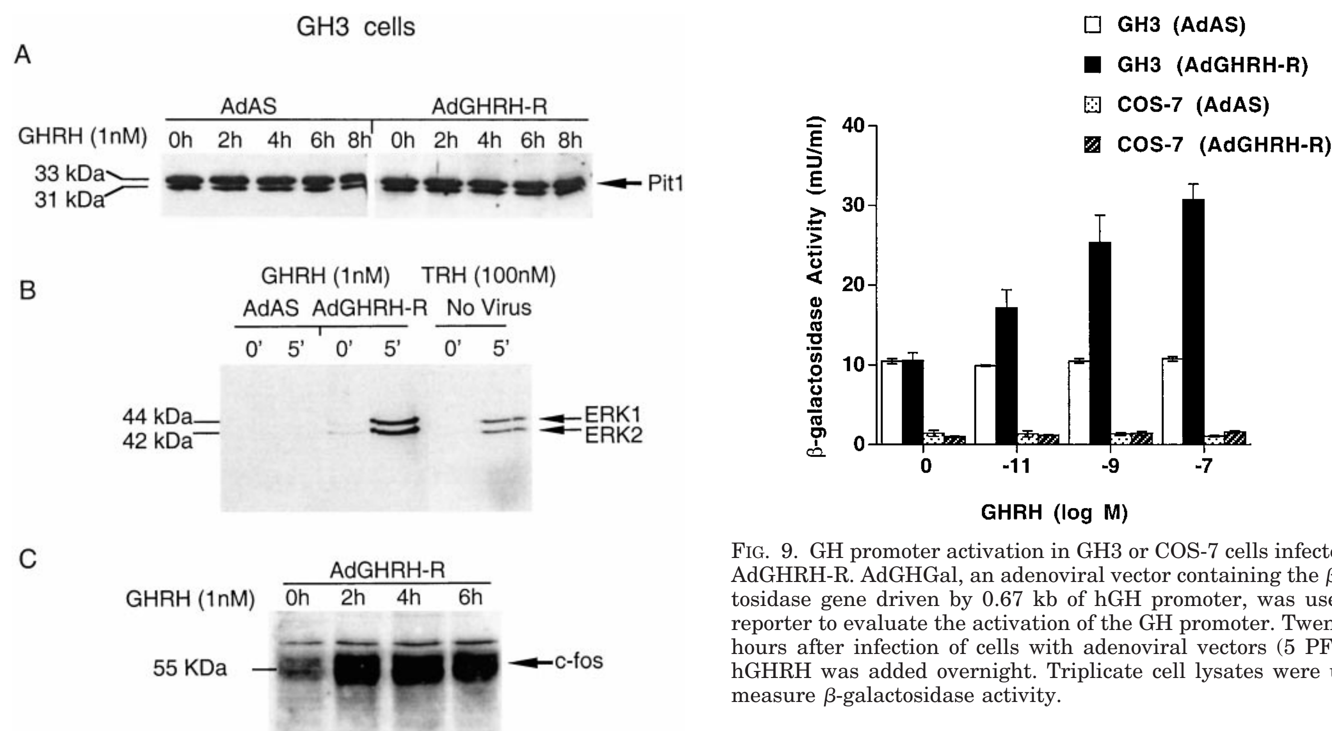

FIG. 8. Pit-1 expression, MAP kinase activation, and c-Fos induction in GH3 cells infected with AdGHRH-R. Western blot analysis was performed using rabbit anti-Pit-1, rabbit anti-c-Fos, or rabbit anti-phospho-p44/p42 MAPK.

FIG. 9. GH promoter activation in GH3 or COS-7 cells infected with AdGHRH-R. AdGHGal, an adenoviral vector containing the β-galactosidase gene driven by 0.67 kb of hGH promoter, was used as a reporter to evaluate the activation of the GH promoter. Twenty-four hours after infection of cells with adenoviral vectors (5 PFU/cell), hGHRH was added overnight. Triplicate cell lysates were used to measure β-galactosidase activity.

to reconstitute the GHRH receptor signaling system using the adenovirus system will allow further investigation of GH secretory pathways.

GHRH stimulates transcription of the GH gene in pituitary somatotrope cells (19, 20). Stimulation of the cAMP pathway and activation of protein kinase A lead to phosphorylation and activation of the transcription factor CREB. It has been suggested that CREB induced synthesis of Pit-1 or perhaps other action of phospho-CREB lead to a subsequent increase in GH gene expression. In this study, we observed rapid CREB phosphorylation and GH gene stimulation after the addition of GHRH to GH3 cells expressing the GHRH receptor. However, the amount of Pit-1 protein did not increase significantly during the time of GHRH treatment. In agreement with previous studies (21, 22), basal expression of Pit-1 (in the absence of GHRH stimulation) is relatively high in these cells. These observations may explain the high basal activity of the GH promoter in GH3 cells, but the basis for further activation of the GH promoter by GHRH remains to be determined. Possible explanations include 1) phosphorylation-mediated activation of Pit-1 protein; 2) cAMP responsiveness through nonclassical cAMP response element motifs in the hGH promoter; 3) CREB-independent regulation by CREB binding protein; or 4) MAPK activation.

It has been demonstrated that GHRH induces c-Fos expression (23, 24), a factor that may be involved in the proliferation of somatotrope cells. Somatostatin inhibits the proliferation of GH3 cells, and it inhibits GHRH induced c-fos expression (25). The mitogenic effect of GHRH has been known to be mediated by the activation of the cAMP pathway; increased cAMP stimulates c-Fos expression through its cAMP response element. In GH3 cells, Pit-1 also enhances c-Fos promoter activity by binding to the serum response

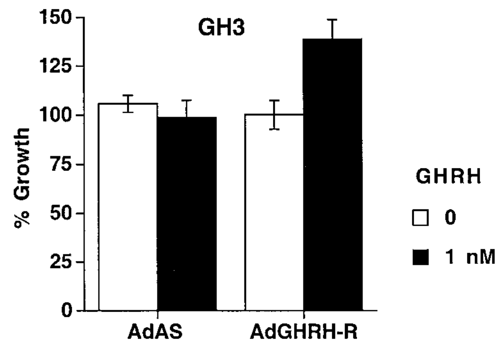

FIG. 10. Effect of GHRH administration on growth of GH3 cells infected with AdGHRH-R. Cell proliferation was measured with a nonradioactive cell proliferation assay. GH3 cells were depleted of estrogen for 4 days. Cells (1×10^4) in quadruplicate wells of 96-well plates were infected with adenoviral vectors at 5 PFU/cell. Fresh medium (dextran/charcoal-stripped FBS) containing 1 nM hGHRH was added every day, and cell proliferation was assayed on day 4 after the first GHRH dose.

element in the c-Fos promoter (26). In this study we observed that GHRH stimulated the proliferation of GH3 cells expressing GHRH receptor. We also demonstrated activation of MAPK and an increase in c-Fos expression after treatment with GHRH. Recently, Pombo *et al.* (27) demonstrated MAPK activation by GHRH in CHO cells stably expressing GHRH receptor. It has been demonstrated that cAMP analogs and TRH activate MAPK, leading to cellular proliferation of GH3 cells (28, 29). MAPK is instrumental in several signal transduction pathways involved in cell. Once activated, MAPK translocates to the nucleus, where it induces transcription factors, including c-Fos and c-Jun. It is likely that MAPK activation plays a role in mediating the mitogenic action of GHRH.

In summary, we demonstrated restoration of GHRH responsiveness of pituitary GH3 cells in response to GHRH after expression of functional hGHRH receptor using recombinant adenoviral vectors. These results indicate that adenoviral vectors carrying the hGHRH receptor gene are useful for *in vitro* studies of GHRH receptor biology and represent a first step toward the long-term goal of gene therapy for dwarfism caused by GHRH receptor mutations.

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