Activation of mitogen-activated protein kinases by a splice variant of GHRH receptor

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Abstract

Hypothalamic GHRH controls the release of GH from the pituitary gland and also acts as a growth factor in a variety of cancers. The mitogenetic activity of GHRH is exerted through the binding to the pituitary type receptor (pGHRH-R) and its splice variants, mainly SV1. The intracellular signaling pathways that are activated upon the binding of GHRH to the SV1 receptor have not been elucidated. HeLa cervical cancer cells do not express GHRH or GHRH receptors (GHRHRs) and thus do not respond to GHRH or GHRH antagonists. In order to elucidate the mechanism of action of SV1 receptor, we transfected HeLa cells with plasmids for pcDNA3-GHRHR or pcDNA3-SV1. The transfected cells responded to both GHRH (1–29)NH₂ and GHRH antagonist MZ-5-156, as shown by an increase or decrease respectively in the proliferation rate in vitro and the expression of proliferative cell nuclear antigen. We also demonstrated that when the cells transfected with SV1 plasmid are stimulated with GHRH (1–29)NH₂, SV1 receptor activates the mitogen-activated protein kinases pathway (MAPKs), as shown previously for the cells that express pGHRH-R. Our results show, for the first time, the activation of the MAPKs cascade by the SV1 receptor. Since SV1 receptor is found in various tumors and mediates the responses to GHRH and synthetic antagonists, our findings shed light on the mechanism of action of SV1 receptor in cancer cells.

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Introduction

Hypothalamic peptide GH-releasing hormone (GHRH) regulates the release of GH from the anterior pituitary gland and acts as a growth factor in various cancers. GHRH was first isolated from pancreatic tumors that caused paraneoplastic acromegaly, indicating its likely role in cancer, and only later was characterized from the hypothalamus (Guillemin et al. 1982, Rivier et al. 1982, Spiess et al. 1983). GHRH is a member of a family of related peptides that includes vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), secretin, glucagon, glucagon-like peptides-1 and -2, and gastric inhibitory peptide. Peptides in this family act to stimulate the intracellular accumulation of cAMP with the resultant activation of protein kinase A (Gaylinn 1999, Ramirez et al. 1999).

Pituitary type GHRH receptor, pGHRH-R, is a class II G protein-coupled receptor with seven transmembrane domains and is homologous with the receptors for VIP, PACAP, and calcitonin (Mayo et al. 2000). GHRHR is predominantly expressed in the pituitary but it is also found in a wide variety of established cancer cell lines and tumors (Schally et al. 2008). GHRHR is activated by GHRH and triggers the mitogen-activated protein kinases pathway (MAPKs), which is linked to cell proliferation and differentiation (Mayo et al. 2000).

The receptors that mediate the effects of GHRH on tumors were identified by our group (Rekasi et al. 2000). The isolation and sequencing of cDNAs, which correspond to the tumoral GHRHR mRNA, revealed that they are splice variants (SVs) of the pGHRH-R (Rekasi et al. 2000). SV1 of GHRHR is a functional receptor, which differs from the pGHRH-R only in the N-terminal extracellular domain. The first 89 amino acids of the pGHRH-R are replaced in SV1 receptor by a different 25-amino acid sequence. Besides its ligand-dependent activity, a ligand-independent activity of SV1 has also been demonstrated (Kiariis et al. 2003).

In an endeavor to develop new anticancer therapies, our group developed GHRH antagonists that can suppress the in vivo and vitro growth of a diverse variety of human experimental cancers. The inhibitory effect of these analogs is exerted in part by endocrine mechanisms in the suppression of GHRH-evoked
GH release from the pituitary. In turn, the inhibition of GH secretion results in the reduction of the hepatic production of insulin-like growth factor 1 (IGF1) and a decrease in serum IGF1 levels (Kiaris et al. 2005). Direct mechanisms are also involved in the antitumor effects of GHRH antagonists. One of these mechanisms is based upon the inhibition of the secretion of autocrine/paracrine IGF1 and IGF2 from the tumors. However, the likely main inhibitory pathway of GHRH antagonists appears to involve the blockade of action of autocrine GHRH in tumors (Schally et al. 2008, Stepien et al. 2009).

Although various previous findings (Christodoulou et al. 2006, Barabutis & Schally 2008a, Hohla et al. 2008, Fu et al. 2009) have enhanced the important role of the SV1 receptor in carcinogenesis, the intracellular signaling pathways that are triggered upon the binding of GHRH to this receptor are incompletely understood. Since SV1 receptor is structurally closely related to pGHRH-R and also activates cell proliferation, in this study, we investigated whether SV1 can activate the ERK1/2 MAPKs, as this pathway is activated upon the binding of GHRH to GHRHR (Pombo et al. 2000, Zeitler & Siriwardana 2000, Lee et al. 2001, Siriwardana et al. 2006). Thus, HeLa cervical cancer cells that do not express GHRH, GHRHR (Zeitler & Siriwardana 2000), or SV1 receptor (Koster et al. 2009) were transfected with plasmids for pcDNA3, pcDNA3-SV1, or pcDNA3-GHRHR, and the influence of GHRH (1–29)NH₂ and its antagonist MZ-5-156 on the transfected cells was evaluated.

Materials and methods

Peptides and chemicals

GHRH antagonist MZ-5-156[PhAc-Tyr1, d-Arg2, Phe (4-Cl)6, Abu15, Nle27]hGHRH (1–28)Agm, where Abu is L-aminobutyric acid, Agm is agmatine, Nle is norleucine, and PhAc is phenylacetyl, was synthesized in our laboratory by solid-phase methods as previously described (Schally et al. 2008). GHRH (1–29)NH₂ and GHRH antagonist MZ-5-156 were dissolved in DMSO and diluted with incubation media. The final concentration of DMSO in medium was 0.1%.

Cell culture

Breast cancer cell line T47D and HeLa cervical cancer cell line were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C in a humidified 95% air/5% CO₂ atmosphere. These cells lines were cultured in DMEM supplemented with antibiotics/antimycotics and 10% FBS. Benign prostate hyperplasia cells (BPH-1) were kindly donated by Dr Simon Hayward, Vanderbilt University Medical Center, Nashville, TN, USA, and maintained in RPMI 1640 medium supplemented with 5% FBS and 1% antibiotics/antimycotics. The culture media as well as all the cell culture reagents were purchased from Gibco.

Protein isolation and western blot assay

The proteins were isolated from the cells using CelyticM Lysis Reagent (Sigma) according to the manufacturer’s instructions. Protein-matched samples were separated by 12% SDS, Tris–HCl gels. Wet transfer was used to transfer the proteins onto nitrocellulose membranes (Bio-Rad). The membranes were incubated for 1 h in 5% nonfat dry milk in PBS–0.1% (v/v) Tween 20. The blots were then incubated at 4°C overnight with the appropriate antibodies. The signal for the immunoreactive proteins was developed with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Cell Signalling, Danvers, MA, USA) and visualized by exposure to chemiluminescence substrate (Amersham Biotechnologies). The β-actin signal (1:1000, sc-7099, Santa Cruz Biotechnology) was used as a loading control unless otherwise stated. The proliferative cell nuclear antigen (PCNA) antibody was obtained from Cell Signaling (#208), the GHRH antibody (sc-10281) from Santa Cruz Biotechnology, and the GHRHR antibody from Abcam (ab 28692-100, Cambridge, MA, USA).

Activation of MAPK pathway by GHRH

HeLa pcDNA3, HeLa pcDNA3-SV1, and HeLa pcDNA3-GHRHR transfected cells were grown in DMEM with 10% FBS. Before this assay, the medium was replaced with DMEM without FBS, and the cells were treated or not with 1 μM GHRH (1–29)NH₂ for 5 or 10 min. The cells were harvested in Cell Lysis Buffer (Sigma) containing proteinase inhibitor cocktail. The cell lysates (100 μl) were separated by electrophoresis according to their molecular weight. The proteins were then transferred onto nitrocellulose membranes and probed for phospho-MAPK (#9101, Cell Signalling). The blots were stripped (Restore Plus Western Blot Stripping Buffer, Thermo Scientific, Waltham, MA, USA) and probed for ERK2 (sc-81457, Santa Cruz Biotechnology) and β-actin (Santa Cruz Biotechnology).

Plasmids and transfection

The construction of the pcDNA3-SV1 and pcDNA3-GHRHR plasmids has been previously reported (Kiaris et al. 2002, 2003). The transfections were performed by seeding 5 × 10⁵ cells in six-well tissue culture plates with
1 μg appropriate plasmid by using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. Stable lines were generated by the selection of resistant colonies with 1 μg/ml selection agent G418 (Invitrogen) for 4 weeks. Culture media were changed daily during selection.

Cell proliferation rate assay

The rate of cell proliferation was calculated by seeding 10,000 cells in six-well plates and after incubation for 72 h counting them under light microscope using the trypan blue assay.

Quantitative analysis of the immunoblot assay

The protein band signals were quantified by Adobe Photoshop and normalized to β-actin signal. In the case of the activation of the ERK1/2 pathway, the bands were normalized to ERK2. The intensity of the bands was equal to their mean value multiplied by their pixel value (absolute intensity). Relative intensity (RI) of each band was calculated by dividing its absolute intensity by the absolute intensity of the control band (β-actin).

Statistical analysis

The data are expressed as the mean ± s.e.m. Statistical evaluation of the results was performed by the two-tailed Student’s t-test. P values shown are expressed against the control group.

Results

Expression of GHRHR and SV1 receptor in the BPH-1, T47D, and HeLa cell lines

T47D breast cancer cell line (T47D), HeLa cervical cancer line, and BPH-1 benign prostate hyperplasia line were subjected to western blotting in order to detect whether HeLa cells express GHRHR(s). The antibody used can identify both SV1 and GHRHR (Schulz & Rocken 2006). T47D cells that express GHRHR (Zeitler & Siriwardana 2000) and SV1 (Barabutis & Schally 2008a) receptors, as well as BPH-1 cells that express GHRHR (unpublished results), were used as positive controls. Our results (Fig. 1) demonstrate that HeLa cervical cancer cell line does not produce receptors for GHRH.

Effect of GHRH (1–29)NH₂ and GHRH antagonist MZ-5-156 on the proliferation rate of the HeLa cells transfected with pcDNA3, pcDNA3-GHRHR, and pcDNA3-SV1 plasmids

When HeLa pcDNA3-GHRHR cells were treated with GHRH (1–29)NH₂ or GHRH antagonist MZ-5-156, 10⁻⁷ or 10⁻⁶ M GHRH increased the proliferation rate of these cells by 11.9 and 17.9% respectively, while 10⁻⁷ or 10⁻⁶ M GHRH antagonist decreased the proliferation by 11.9 and 29.8% respectively (Fig. 2).

Mechanism of action of SV1 receptor . N BARABUTIS and others 129

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HeLa pcDNA3-SV1 cells were similarly incubated with GHRH (1–29)NH₂ and MZ-5-156 at concentrations 10⁻⁷ and 10⁻⁶ M. GHRH (1–29)NH₂ stimulated the proliferation of the transfected cells by 15.5% and 19.6% respectively, while 10⁻⁷ and 10⁻⁶ M GHRH antagonist MZ-5-156 inhibited their proliferation rate by 21.6 and 30.9% respectively. HeLa cells transfected with empty vector pcDNA3 cells were not affected by the treatment with these peptides (Fig. 2).

**Expression of GHRH by human cancer cell lines**

Human breast cancer cell line T47D and BPH-1 were subjected to western blotting in order to compare them with HeLa cervical cancer cell line expression of GHRH. T47D and BPH-1 cells were used (unpublished results) as positive controls. The results show that HeLa cell line did not produce significant amount of GHRH protein (Fig. 3).

**Expression of PCNA by the transfected cells**

We examined the expression of PCNA by the HeLa cells in order to investigate whether it is influenced by the introduction of the GHRHR or the SV1 receptor. The results indicate that the expression of PCNA was

![Figure 4](image-url)  
**Figure 4** Expression of PCNA by HeLa pcDNA3 (control), pcDNA3-GHRHR, and pcDNA3-SV1-transfected cells. Protein levels were normalized to β-actin signal (loading control). The blot is representative of two independent experiments with similar results.

![Figure 5](image-url)  
**Figure 5** (A) Expression of PCNA by HeLa cells transfected with pcDNA3-SV1 plasmid after exposure to 10⁻⁷ and 10⁻⁶ M GHRH (1–29)NH₂ or 10⁻⁷ and 10⁻⁶ M GHRH antagonist MZ-5-156 and densitometric analysis of the protein bands. Protein levels were normalized to β-actin signal (loading control). The blot is representative of two independent experiments with similar results. (B) Expression of PCNA by HeLa pcDNA3-GHRHR cells after exposure to 10⁻⁷ M GHRH or 10⁻⁶ and 10⁻⁷ M MZ-5-156 and densitometric analysis of the protein bands. Protein levels were normalized to β-actin signal (loading control). The blot is representative of two independent experiments with similar results. (C) Expression of PCNA by HeLa cells transfected with pcDNA3 after exposure to 10⁻⁷ and 10⁻⁶ M GHRH or 10⁻⁷ M GHRH antagonist MZ-5-156 and densitometric analysis of the protein bands. Protein levels were normalized to β-actin signal (loading control). The blot is representative of two independent experiments with similar results.
higher in the cells transfected with pcDNA-GHRHR plasmid (RI: 1.22) and highest in the cells transfected with the pcDNA3-SV1 (RI: 1.48) compared to the empty vector (RI: 0.75) transfected cells. The results are shown in Fig. 4.

**Effect of GHRH (1–29)NH₂ and GHRH antagonist MZ-5-156 on the expression of the PCNA in HeLa cells transfected with pcDNA3-SV1 and pcDNA3-GHRHR plasmids**

When HeLa pcDNA3-SV1 cells were exposed to 0.1 or 1 μM GHRH (1–29)NH₂, the expression of the PCNA was increased (RI: 1.50, 1.59), while 0.1 and 1 μM MZ-5-156 decreased the expression of this marker (RI: 0.95, 0.92) compared to the control pcDNA3-SV1 (RI: 1.01). These results are shown in Fig. 5A. The addition of 0.1 μM GHRH to the HeLa pcDNA3-GHRHR cells resulted in an increase (RI: 0.43) in the expression of PCNA compared to the control cells. Conversely, the addition of 0.1 or 1 μM MZ-5-156 resulted in a decrease (RI: 0.36, 0.30) in the PCNA expression in these cells compared to the control pcDNA3-GHRHR cells (RI: 0.39). These results are shown in Fig. 5B. The addition of GHRH or MZ-5-156 to the media of the HeLa cells transfected with the pcDNA3 empty vector did not influence the expression of this protein (PCNA). The results are shown in Fig. 5C.

**Activation of the MAPKs pathway by addition of GHRH to the transfected cells**

We investigated whether GHRH (1–29)NH₂ can activate the ERK1/2 pathway of the HeLa cells transfected with pcDNA3-SV1. GHRH (1–29)NH₂ was added to the media of these cells as well as to the media of the HeLa pcDNA3-GHRHR and HeLa pcDNA3 transfected cells for 5 and 10 min. The activation of ERK1/2 pathway in the SV1-transfected cells was the strongest at the end of the 5-min incubation (RI: 3.70) followed by the 10-min incubation (RI: 2.58) compared to the control (RI: 1.01). GHRH also activated the ERK1/2 cascade in the cells transfected with GHRHR in a time-dependent manner. The activation of this pathway in the GHRHR-transfected cells was strongest in the 10-min incubation (RI: 2.35) followed by the 5-min incubation (RI: 1.06) compared to the control cells (RI: 1.06). The addition of the GHRH to the empty vector transfected cells did not affect this pathway. These results are shown in Fig. 6.

![Figure 6 Activation of ERK1/2 by 1 μM GHRH in HeLa cells transfected with empty vector pcDNA3, pcDNA3-GHRHR and pcDNA3-SV1 plasmids after a period of 0, 5, or 10 min and densitometric analysis of the protein bands. Protein levels were normalized to ERK2 signal (loading control). The blot is representative of two independent experiments with similar results.](image-url)
Discussion

GHRHR is a G protein-coupled receptor that is expressed predominantly in the anterior pituitary gland. This receptor is also known to activate the MAPKs pathway upon the binding of GHRH to it (Pombo et al. 2000, Zeitler & Siriwardana 2000, Siriwardana et al. 2006). A SV of GHRHR, defined as SV1, has also been isolated and sequenced by our group (Schally et al. 2008). SV1 includes the seven membrane spanning motifs of a G protein-coupled receptor, but it lacks a small extracellular domain of the pGHRH-R (Rekasi et al. 2000).

SV1 receptor has been shown to possess a ligand-independent activity, since its transfection to MCF7 cells strongly increased their proliferation rate. This response of unstimulated cells was independent of the response to the mitogen GHRH (1–29)NH₂ (Barabutis et al. 2007). Furthermore, the proliferation rate of cancer cells that express high levels of SV1 receptor is not strongly suppressed after the knocking down of the GHRH gene expression. This is contrary to the cells that express low amounts of SV1 receptor (Barabutis & Schally 2008a). The activation of SV1 receptor by GHRH has also been shown to activate the accumulation of cAMP (Kiaris et al. 2002). In the present study, we elucidated whether the SV1 receptor can activate the p44/42 kinase pathway. This pathway is activated by growth factors and is associated with cell proliferation (Mebratu & Tesfaigzi 2009).

In order to address this question, we transfected HeLa cells that do not produce GHRHR (Zeitler & Siriwardana 2000), SV1 receptor (Koster et al. 2009) or GHRH with plasmids for pcDNA3-SV1 and pcDNA3-GHRHR. While the HeLa cells do not respond to GHRH, HeLa transfected with pcDNA3-GHRHR and HeLa pcDNA3-SV1 responded to GHRH in a dose-dependent manner. This was shown not only by the in vitro proliferation rate, but also by the expression of the PCNA by these cells.

Furthermore, when SV1 and pGHRH-R transfected cell lines were exposed to the GHRH antagonist MZ-5-156, the proliferation rate of both lines was decreased in a dose-dependent manner, and the expression of the PCNA proliferation marker was also decreased.

Interestingly, the transfection of the SV1 receptor to the HeLa cell line resulted in an increased proliferation rate of the transfected cells, which was higher than the baseline proliferation of the cells transfected with pcDNA3-GHRHR or the empty vector pcDNA3 cells (Fig. 2). This was also confirmed by the expression of the PCNA (Fig. 4). These results are concordant with previous findings that also reported a ligand-independent activity of the SV1 receptor in MCF7 pcDNA3-SV1 transfected cells (Barabutis et al. 2007). HeLa cells transfected with the empty vector did not show activation of the p44/42 MAPK pathway when exposed to GHRH (1–29)NH₂, in contrast to HeLa pcDNA3-SV1 and HeLa pcDNA3-GHRHR transfected cells (Fig. 6). Our results are in line with previous findings and confirm the activation of p44/42 MAPK by GHRHR (Pombo et al. 2000, Zeitler & Siriwardana 2000, Siriwardana et al. 2006).

Our study reports for the first time that the activation of the p44/42 MAPK pathway can also be triggered upon the binding of GHRH (1–29)NH₂ to the SV1 of the GHRHR. The activation of ERK1/2 by SV1 receptor when the SV1-transfected cells were incubated for 5 min with GHRH (1–29)NH₂ is stronger than that triggered by GHRHR-transfected cells.

Activation of MAPKs by growth factors such as GHRH is involved in cell proliferation and differentiation and is strongly implicated in cancer (Mebratu & Tesfaigzi 2009, Wagner & Nebreda 2009).

Previous studies by other groups indicate that the ERK1/2 pathway is crucial for the proliferation of the HeLa cells, and that the inhibition of this pathway by MAPKs inhibitors is strongly linked with suppression of the proliferation of these cells (Holmstrom et al. 1999, Deva et al. 2003, Yin et al. 2005, Zhao et al. 2007). In addition, in this and previous studies, the GHRH antagonists have been shown to strongly inhibit the proliferation rate of cancer cells through the inhibition of the MAPKs pathway (Kanashiro et al. 2004, Schally et al. 2008, Annunziata et al. 2009). Thus, the ERK1/2 pathway plays a crucial role in the proliferation of the HeLa cervical cancer cells, which can be suppressed by compounds that inhibit the ERK1/2 pathway (Wang et al. 2009) such as GHRH antagonists. Our results support previous findings on the crucial role of GHRH and its receptor(s) in cancer, and underline the antitumor potential of GHRH antagonists.

The activation of the ERK1/2 pathway is closely related to changes in the redox status of cancer cell lines, i.e. the metabolism of reactive oxygen and reactive nitrogen species (Finkel & Holbrook 2000, Myhre et al. 2004, Ischiropoulos & Gow 2005, Schumacker 2006). We have recently shown that GHRH antagonists such as JMR-132 possess an antioxidant activity in the LNCAP prostate cancer cell line (Barabutis & Schally 2008b). New developments in the area of cell biology suggest that reactive oxygen species are also involved in the differentiation (Heng et al. 2004) and survival (Han et al. 2008) of stem cells (Rossi et al. 2008). Future applications of agonistic and antagonistic analogs of GHRH in this field cannot be excluded, since it is well known that growth factors as well as the activation of the ERK1/2 pathway are involved in the differentiation of the stem cells (Yao et al. 2003, Bost et al. 2005, Li et al. 2006, Rossi et al. 2008).
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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