RAPID COMMUNICATION

Steroidogenic factor-1 enhances basal and forskolin-stimulated transcription of the human glycoprotein hormone α-subunit gene in GH_3 cells

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Abstract

Steroidogenic factor-1 (SF-1) is a key regulator of endocrine development, and mediates expression of gonadotrophin-specific genes in the pituitary. Basal and hormone stimulated transcription of the human glycoprotein hormone α-subunit gene (αGSU) in gonadotrophs involves SF-1 and its cognate binding site, the gonadotroph-specific element (GSE). In this study, we demonstrate that SF-1 significantly enhances basal and forskolin-stimulated transcription of the human αGSU promoter in GH_3 cells. Mutation of the GSE abolished the SF-1-mediated transactivation of basal αGSU promoter activity, and significantly attenuated the forskolin effect by 50%. Mutation of the Ser203 residue in SF-1 to Ala blocked basal transactivation of αGSU promoter activity, and halved the forskolin effect. These data collectively reveal a direct role for SF-1 and the GSE in mediating basal and forskolin-stimulated transcription of the human αGSU promoter in GH_3 cells. The phosphorylation site at Ser203 appears to be required for these effects.

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Introduction

The orphan nuclear receptor, steroidogenic factor-1 (SF-1, NR5A1) is expressed in gonadal and adrenal tissues, where it plays an important role in endocrine development and maintenance of steroidogenic gene expression (Parker *et al.* 2002). However, SF-1 is also expressed in the ventromedial hypothalamus and in gonadotrophs of the anterior pituitary (Ingraham *et al.* 1994). In gonadotrophs, SF-1 regulates the expression of the gonadotrophin subunit genes, glycoprotein hormone α-subunit (αGSU), luteinising hormone-β (LHβ), follicle-stimulating hormone-β (FSH-β), as well as other important genes such as the gonadotrophin releasing hormone receptor (GnRH-R) and neuronal nitric oxide synthase (nNOS) (for review see Fowkes and Burrin 2003).

The human αGSU promoter is regulated by several response elements which reside in the proximal 435 bp of the promoter (Heckert *et al.* 1996). We have recently shown that SF-1 and its cognate binding site in the αGSU promoter (the gonadotroph-specific element, GSE) contribute to basal and hormone-stimulated αGSU transcription in αT3–1 and LβT2 gonadotroph cell lines (Fowkes *et al.* 2002, Fowkes *et al.* 2003). As these cell lines express high levels of endogenous SF-1, previous investigators have utilised the rat somatolactotroph GH_3 cell line as a surrogate gonadotroph model to examine the role of exogenous SF-1 on gonadotrophin gene expression (Halvorson *et al.* 1999, Kaiser *et al.* 2000). The regulation of αGSU by SF-1 in GH_3 cells has been poorly elucidated.

SF-1 transcriptional activity is regulated by post-translational modification, such as acetylation and phosphorylation (for review see Fowkes and Burrin 2003). The Serine 203 residue of SF-1 resides in a consensus mitogen activated protein kinase (MAPK) sequence, and has been shown to be phosphorylated by these kinases *in vitro* (Hammer *et al.* 1999, Gyles *et al.* 2001, Desclozeaux *et al.* 2002, Fowkes *et al.* 2003). Mutation of Ser203 to Ala (S203A) reduces SF-1 transcriptional activity and co-activator recruitment (Hammer *et al.* 1999), and S203A-SF-1 does not transactivate the αGSU promoter in αT3–1 cells (Fowkes *et al.* 2003). However, it is unclear whether this residue is important for mediating the effects of SF-1 in heterologous expression models such as GH_3 cells.

Our recent observations show that SF-1 can mediate pituitary adenylate cyclase-activating polypeptide (PACAP)-stimulated αGSU transcription in gonadotrophs (Fowkes *et al.* 2003). In this study, we show that SF-1 and

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the GSE mediate basal and forskolin-stimulated αGSU transcription in GH_3 cells, an effect which requires an intact Ser 203 residue in SF-1 for maximal activation.

Materials and Methods

Materials

All chemicals were purchased from Sigma (Sigma Chemicals, Poole, Dorset, UK) unless otherwise stated. Forskolin (FSK) was stored as a stock solution of 100 mM in DMSO at 4 °C, before dilution in culture medium.

Plasmids used in transfection studies

An expression vector encoding mouse SF-1 was prepared by sub-cloning the mouse full-length SF-1 cDNA sequence (from Prof. K L Parker, UT SouthWestern, TX, USA) into pCIneo (Promega Corporation, Southampton, UK), an expression vector containing a cytomegalovirus promoter for high levels of constitutive expression and imparting neomycin resistance. Restriction endonuclease digests verified correct orientation of the SF-1 coding sequence, and the vector was termed pCI-SF-1 neo. The reporter construct –517αLUC contains 517 bp of the 5′ flanking sequence and 44 bp of exon 1 of the human αGSU gene, linked to the luciferase (LUC) reporter gene in the plasmid pA3 LUC. The promoter-less LUC expression vector pA3 LUC was used as an internal control plasmid for basal luciferase expression. The internal control plasmid BosβGal contains the promoter of the human elongation factor 1 gene driving expression of β-galactosidase, and was used as an internal control to normalize transfection efficiencies. The expression vector encoding the mutant SF-1, S203A-SF-1 in pCIneo plasmid, and the prolactin promoter containing 5000 bp of the human PRL promoter (PRL-LUC) have been described previously (Hammer *et al.* 1999, Takasuka *et al.* 1998). All constructs were verified for orientation and correct sequence by restriction endonuclease digests and the dideoxy-DNA sequencing method. Large-scale preparation and purification of plasmids were performed by alkaline lysis and resin purification (Qiagen).

Site-directed Mutagenesis

The –517αLUC construct was sub-cloned in to the HindIII site in pBluescript and site-directed mutagenesis using the QuikChange kit (Stratagene, Cambridge, UK) was conducted to introduce a 2 bp mutation within the GSE region. Mutagenesis was performed according to manufacturer's instructions and using the following primers:

forward: 5′-CTCTCTTTTCATGGGCTGA**TT**TTG TCGTCACCATCACCTG-3′, reverse: 5′-CAGGTG ATGGTGACGACAA**AA**TCAGCCCATGAAAAGAG AG-3′, with the mutation highlighted. Following sequence analysis, the mutated –517αLUC (named –517αMUT) was cloned back in to the HindIII site of pA3 LUC.

Generation of stable cell lines

Sub-confluent GH_3 cells were transfected with pCIneo or pCI-SF-1 neo by electroporation. Briefly, 1×10^6 cells were placed in 0·8 ml of ice-cold DMEM containing 10 μg of either pCIneo (GH_3neo cells) of pCI-SF-1neo (GH_3SF-1 cells). The cell suspension containing the DNA to be transfected was transferred to an electroporation cuvette (Biorad Laboratories Ltd, Herts., UK). The cells received a single electrical pulse of 250 V from a total capacitance of 1080 μF with a 1000 msec discharge interval using the PG200 Progenitor II electroporator (Hoefer Scientific Instruments, Staffs., UK). Transfected cells were then selected with the Neomycin analogue Geneticin G418 (Invitrogen, Paisley, UK) at a concentration (250 μg/ml) shown in preliminary experiments to kill all untransfected GH_3 cells (data not shown). Continuous G418 exposure selected cells carrying the stably integrated Neomycin resistance gene and surviving colonies were propagated as a mixed population of cells and maintained in tissue culture medium containing G418.

Transient expression assays

GH_3SF-1 and GH_3NEO cells (3×10^6 cells/cuvette) were transfected by electroporation as described above with 10 μg of the expression vectors pA3 LUC, –517αLUC, –517αMUT or PRL-LUC in the presence of 5 μg of the control plasmid BosβGal. Forty hours after electroporation, medium was changed and cells were treated with or without FSK (10 mM) for a further 8 h. Cells were harvested after washing the cell monolayer with ice-cold phosphate buffered saline (PBS), and the cellular extracts were assayed for luciferase and β-galactosidase activity as previously described (Holdstock *et al.* 1996). In some experiments, 5 μg of SF-1, S203A-SF-1 or pCIneo were co-transfected with the –517αLUC and BosβGal. Luciferase data from separate experiments were pooled by normalizing the data to the level of β-galactosidase activity.

Statistical analysis

All graphical data were prepared using GraphPad Prism 3·0 (GraphPad, San Diego, USA) and analyzed using pre-programmed analysis equations within Prism. Transfection data are presented as normalized data pooled from multiple experiments (each in triplicate, and performed at least twice). Where appropriate, an ANOVA was

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performed on data followed by Student's *t*-test or Tukey's multiple comparisons test, accepting $P<0{\cdot}05$ as significant.

Results

SF-1 regulation of αGSU promoter activity in transfected GH_3 cells requires the GSE

To examine the role of SF-1 and the GSE in GH_3 cells, we created a stable GH_3 cell-line that expressed SF-1. Use of an SF-1 deficient cell line allowed us to investigate FSK effects on basal as well as SF-1 stimulated αGSU promoter activity. Our initial studies compared basal luciferase expression following transient transfection of the GH_3SF-1 and GH_3NEO cells with –517αLUC and BosβGal, before harvesting 48 h later. Basal αGSU promoter activity was $2{\cdot}6 \pm 0{\cdot}6$-fold greater in GH_3SF-1 cells compared with GH_3NEO cells ($P<0{\cdot}05$, Fig. 1A). To establish whether the presence of SF-1 could alter the transcriptional response to FSK, the same transfection was performed but with 0 or 10 μM FSK present for the last 8 h. In the absence of SF-1, FSK stimulated αGSU promoter activity by $4{\cdot}3 \pm 0{\cdot}8$-fold over basal (Fig. 1B, $P<0{\cdot}001$). However, the FSK effect in GH_3SF-1 cells was still significantly greater, by $2{\cdot}2 \pm 0{\cdot}2$-fold ($P<0{\cdot}001$). To determine whether this effect was promoter specific, the FSK effect on the prolactin promoter (PRL-LUC) was compared between cell lines, and showed no significant difference in the absence or presence of SF-1 (Fig. 1C).

To examine the role of the GSE in mediating the observed effects of SF-1 in GH_3 cells, we transiently transfected –517αLUC or –517αMUT constructs into both GH_3SF-1 and GH_3NEO cells. Basal expression of –517αLUC was $3{\cdot}4 \pm 1{\cdot}0$-fold greater in the stably transfected GH_3SF-1 cell line as compared with GH_3NEO ($P<0{\cdot}05$), but mutation of the GSE completely abolished this increase in basal luciferase activity (Fig. 2A). Thus, the basal enhancement of αGSU expression achieved by stable transfection of SF-1 is mediated by the GSE. The expression of the promoter-less control pA3 LUC was not significantly different in either cell line (data not shown).

Following transient transfection of GH_3SF-1 or GH_3NEO cells with either -517αLUC or –517αMUT, cells were stimulated in the presence or absence of FSK (10 μM) for 8 h. As expected, –517αLUC promoter activity following FSK stimulation was significantly greater in GH_3SF-1 cells compared with GH_3NEO cells ($13{\cdot}3 \pm 2{\cdot}2$-fold cf $6{\cdot}3 \pm 1{\cdot}5$-fold, $P<0{\cdot}05$). However, in the SF-1 expressing cells, mutation of the GSE reduced FSK-stimulated αGSU promoter activity to $2{\cdot}8 \pm 1{\cdot}2$-fold when using –517αMUT, further indicating that SF-1 acts at the GSE to mediate the response to cAMP ($P<0{\cdot}01$, Fig. 2B). In GH_3NEO cells, a similar FSK response was observed with the wild-type ($6{\cdot}3 \pm 1{\cdot}5$-fold) and mutated −517αMUT constructs ($5{\cdot}8 \pm 1{\cdot}8$-fold). These data suggest that the enhanced basal and FSK-responsiveness seen in GH_3SF-1 cells was specific to SF-1 binding at the GSE.

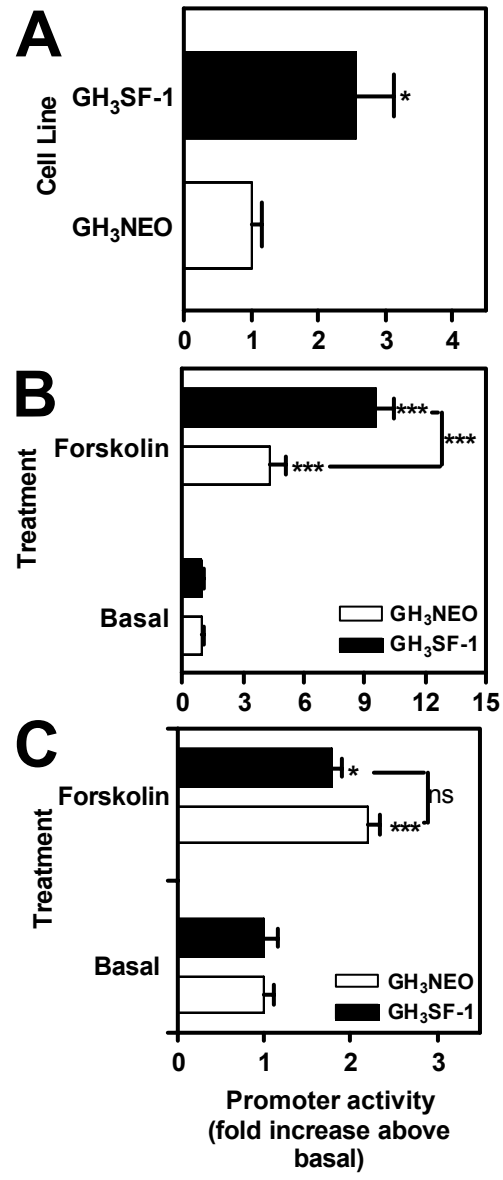

Figure 1 Effect of SF-1 on basal and FSK-stimulated αGSU promoter activity. A, GH_3SF-1 and GH_3NEO cells were transiently transfected with –517αLUC and BosβGal and harvested 48 h later. B, GH_3SF-1 and GH_3NEO cells were transfected as above, and stimulated with 0 or 10 μM FSK for 8 h. C, GH_3SF-1 and GH_3NEO cells were transfected with PRL-LUC and BosβGal, and stimulated with 0 or 10 μM FSK for 8 h. All data represents the mean ± S.E.M. of at least 3 independent experiments each performed in triplicate. *, $P<0{\cdot}05$, ***$P<0{\cdot}001$, significantly different as indicated.

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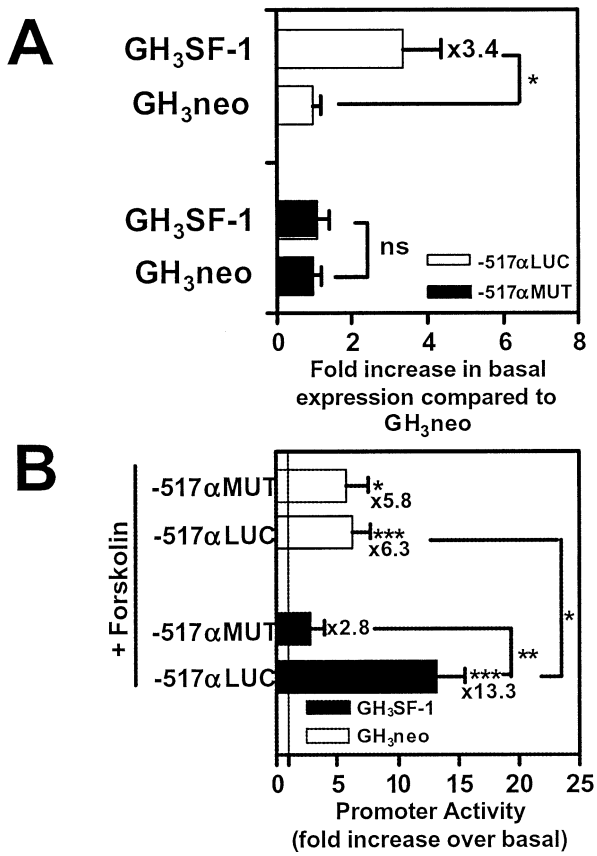

Figure 2 Role of the GSE in mediating the SF-1 effect on basal and FSK-stimulated αGSU promoter activity. A, GH_3SF-1 and GH_3NEO were transiently transfected with either –517αLUC or –517αMUT and harvested 48 h post-transfection. The data are normalized to basal –517αLUC promoter activity in GH_3NEO cells. Mean fold increase over basal is indicated above the relevant bar. *, $P<0{\cdot}05$, significantly different from GH_3NEO. B, GH_3SF-1 and GH_3NEO cells were transfected as above, and stimulated with 0 or 10 mM FSK for 8 h. The data are normalized to basal –517αLUC or –517αMUT promoter activity. Mean fold increase over basal is indicated above the relevant bar. *, $P<0{\cdot}05$, **, $P<0{\cdot}01$, ***, $P<0{\cdot}001$, significantly different from the relevant basal, except where indicated by brackets.

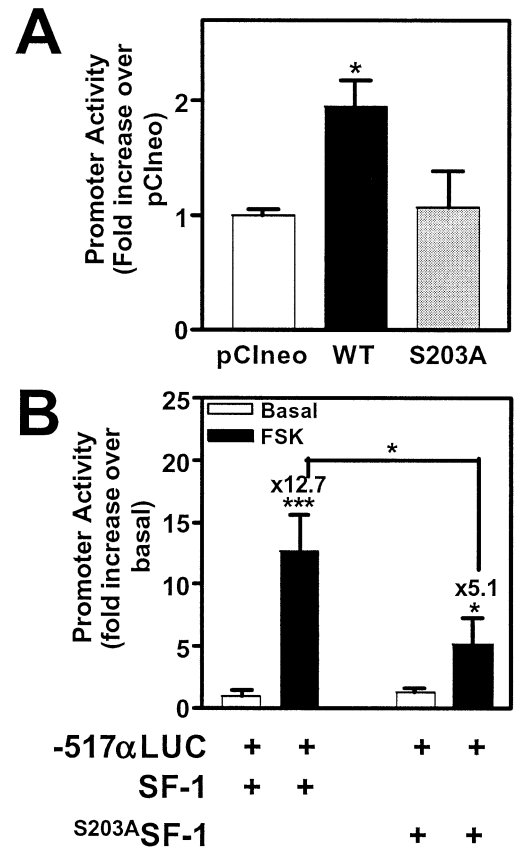

Figure 3 Role of Ser 203 in SF-1 on basal and FSK-stimulated αGSU promoter activity in GH_3 cells. A, GH_3 cells were transiently co-transfected with –517αLUC and either wild-type SF-1, pCIneo or S203A-SF-1 and harvested 48 h after transfection. The data are normalized to –517αLUC activity with pCIneo. *, $P<0{\cdot}05$, significantly different from pCIneo. B, Transiently co-transfected GH_3 cells were cultured for 48 h post-transfection and stimulated with 0 or 10 μM FSK for the last 8 h before harvesting. The data are normalized to the basal expression of each promoter construct to account for differences in basal activity. Mean fold increase over basal is indicated above the bar. *, $P<0{\cdot}05$, ***, $P<0{\cdot}001$, significantly different from pCIneo or as indicated.

The Ser203 residue in SF-1 is required for full basal and FSK-stimulated transcriptional activity

The MAPK phosphoryation site on Ser203 in SF-1 has been shown to regulate SF-1 activity *in vitro* (Hammer *et al.* 1999, Desclozeaux *et al.* 2002, Fowkes *et al.* 2003). To assess the effect of mutating this residue in SF-1 on the regulation of the human αGSU promoter, we performed a series of transient co-transfections in wild-type GH_3 cells. We co-transfected 5μg of the wild-type –517αLUC promoter with 5 μg of a wild-type SF-1 expression vector (the maximally effective concentration as determined in preliminary experiments, data not shown), 5 μg of empty expression vector (pCIneo) or 5 μg of the S203A-SF-1 expression vector in which the Ser203 residue had been converted to alanine to prevent MAPK-mediated phosphorylation. As shown (Fig. 3A), wild-type SF-1 transactivated the αGSU promoter by $1{\cdot}9 \pm 0{\cdot}2$-fold ($P<0{\cdot}05$) compared with empty vector. This effect was absent when S203A-SF-1 was co-transfected with the αGSU promoter ($1{\cdot}0 \pm 0{\cdot}3$-fold). When these co-transfected GH_3 cells were stimulated for 8 h with 10 μM FSK, the $12{\cdot}7 \pm 2{\cdot}9$-fold increase seen in the presence of wild-type SF-1 was reduced to $5{\cdot}2 \pm 2{\cdot}0$-fold in the presence of mutant SF-1 ($P<0{\cdot}05$, Fig. 3B). Collectively, these data suggest that an intact MAPK phosphorylation site at Ser203 is required for the SF-1 enhancement of basal and FSK-stimulated αGSU promoter activity in GH_3 cells.

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Discussion

Investigation of the role of SF-1 in regulating gonadotrophin gene transcription is often hampered by the high levels of endogenous SF-1 expressed in gonadotroph cell lines. In this study, we have used GH_3 cells that have previously been reported as lacking SF-1 expression but have been used by others as a useful heterologous model to study regulation of expression of the gonadotrophin subunit genes (Halvorson *et al.* 1999, Kaiser *et al.* 2000). We used two approaches to examine the role of SF-1 in regulating αGSU promoter activity; generation of a stably transfected GH_3 cell lines (GH_3SF-1 and GH_3NEO), and transient co-transfection of wild-type and mutant SF-1 and αGSU-LUC in native GH_3 cells. Both these approaches yielded comparable results. We observed a 50% decrease in activity with the mutant GSE in GH_3SF-1 cells, similar to that reducation seen in αT3–1 gonadotropes (Fowkes *et al.* 2003). Furthermore, when a mutant form of SF-1 (S203A-SF-1) was co-transfected with –517αLUC in GH_3 cells, the observed increases in basal and FSK-stimulated promoter activity were blocked (basal) or reduced by at least 50% (FSK-stimulated).

The failure to completely abrogate the effects of FSK on αGSU promoter activity by either mutating the GSE or SF-1 are not surprising given that multiple response elements can be found within the proximal –435 bp of the human αGSU promoter (Heckert *et al.* 1996). Indeed, tandem cAMP response elements (CRE) reside proximally of the GSE at –146 bp. Our earlier studies revealed that mutation of these elements could significantly blunt basal and PACAP or 8-Br-cAMP-stimulated αGSU promoter activity in αT3–1 cells (Burrin *et al.* 1998) similar to our more recent observations with GSE mutations (Fowkes *et al.* 2002, Fowkes *et al.* 2003). However, mutation of the CRE was not sufficient to completely block the effect of PACAP on the αGSU promoter (Burrin *et al.* 1998). Instead, PACAP responsiveness was only lost when the promoter was deleted at –195 bp, removing the GSE-containing region, even though the CRE's remained intact. It is likely that in addition to the GSE and CREs, other factors are required for maximal hormonal regulation of pituitary αGSU expression.

The GSE has previously been implicated in the basal regulation of αGSU promoter activity (Barnhart and Mellon 1994, Ingraham *et al.* 1994), and similar CC to TT mutations have displayed a comparable 50% inhibition in basal promoter activity. We have recently showed that this same mutation abolishes binding of SF-1 in αT3–1 and LβT2 gonadotrophs by electrophoretic mobility shift assays (Fowkes *et al.* 2002, Fowkes *et al.* 2003), and inhibits both GnRH and PACAP-stimulated αGSU promoter activity when introduced within the context of the –517αLUC promoter. Our current observations that FSK-stimulated αGSU transcription is partially mediated by SF-1 and the GSE in GH_3 cells adds to a growing list of target gene promoters where hormone-responsiveness maps to the SF-1 site. For example, FSK and cAMP stimulation in adrenocortical Y-1 cells increases StAR transcription via a MAPK and SF-1-dependent mechanism (Gyles *et al.* 2001). Furthermore, in αT3–1 cells, PACAP and protein kinase A (PKA)-stimulation of the GnRH-R promoter requires an intact SF-1 response element, similar to the GSE (Pincas *et al.* 2001). Thus, the propagation of peptide hormone signalling to gene transcription via SF-1 is likely to involve a number of intracellular signalling pathways that ultimately impinge upon mechanisms of post-translational modification.

Phosphorylation of SF-1 has previously been shown to occur on Ser203 *in vitro*, and is mediated via the activity of extracellular signal-regulated kinase (ERK) members of the MAPK family (Hammer *et al.* 1999, Desclozeaux *et al.* 2001). We recently have shown that PACAP utilises the same ERK/MAPK pathway to enhance phosphorylation of SF-1 in αT3–1 cells (Fowkes *et al.* 2003). Our current observations that basal and FSK-stimulated αGSU promoter activity are impaired in the presence of the S203A mutant further support the requirement for this MAPK phosphorylation site. Furthermore, we have observed that FSK stimulates ERK and CREB phosphorylation, and ERK and CREB-mediated transcription with similar kinetics and magnitude in GH_3 cells (data not shown) compared with PACAP effects in αT3–1 cells (Fowkes *et al.* 2001, Fowkes *et al.* 2003*b*). It remains to be seen whether S203A is as influential *in vivo* as it clearly is *in vitro*.

The regulation of orphan nuclear receptor activity by peptide hormone signalling is becoming a commonplace occurrence in endocrine tissues (Hammer *et al.* 1999, Desclozeaux *et al.* 2002). In the absence of identified ligand, it is possible that a class of nuclear receptors, similar to SF-1, are predominantly mediated by the propagation of signalling mechanisms from the cell membrane to the nucleus. Our current observations suggest that this is an important mechanism in the regulation of SF-1-mediated gene transcription in pituitary cells.

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