Coregulatory protein–orphan nuclear receptor interactions in the human adrenal cortex

Sinead N Kelly, T Joseph McKenna¹ and Leonie S Young

Department of Surgery, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

¹Department of Endocrinology and Diabetes Mellitus, St Vincent's University Hospital, Elm Park, Dublin, Ireland

(Requests for offprints should be addressed to L S Young; Email: Leonie.Young@ucd.ie)

Abstract

The capacity of the adrenal to produce steroids is controlled in part through the transcriptional regulation of steroid enzymes. The orphan nuclear receptor steroidogenic factor 1 (SF-1) is central to the transcriptional regulation of all steroid hydroxylase enzymes, whereas *nur77* can preferentially regulate steroid enzyme genes relevant to cortisol production. We hypothesised that, in the presence of secretagogues, SF-1 and *nur77* may differentially interact with coregulatory proteins in the human adrenal cortex. Both coregulatory proteins, steroid receptor coactivator (SRC-1) and silencing mediator for retinoid and thyroid hormones (SMRT), were found to be expressed in the zona fasciculata and reticularis in the human adrenal cortex, but were largely absent from the zona glomerulosa. Both coregulatory proteins were colocalised with SF-1 and *nur77*. In the H295R adrenal

tumour cell line, SF-1 and *nur77* transcripts were increased in cells in the presence of forskolin, whereas *nur77* mRNA was also induced with angiotensin II (AII). The coactivator SRC-1 mRNA was increased in the presence of both forskolin and AII. Forskolin induced recruitment of SRC-1 to the SF-1 response element and induced SRC-1–SF-1 interactions, whereas AII increased recruitment of SRC-1 to the *nur77* response element and induced SRC-1–*nur77* interactions. The corepressor SMRT interacted with SF-1 in the presence of AII and with *nur77* in cells treated with forskolin. Orphan nuclear receptor–coregulatory protein interactions may have consequences for the regulation of key steroidogenic enzymes in the human adrenal cortex.

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Introduction

In the human adrenal gland, adrenocorticotrophic hormone (ACTH) modulates both cortisol and adrenal androgen production, but there are, however, conditions where the production of these steroids becomes divergent (McKenna & Cunningham 1991). This divergence is illustrated physiologically at the adrenarche and adrena-pause and in pathophysiological situations such as polycystic ovarian syndrome (PCOS) and anorexia nervosa (Winterer *et al.* 1985, McKenna & Cunningham 1991).

Steroid production of cortisol and androgens require the concerted action of a related group of cytochrome P450 steroid hydroxylase enzymes that convert the precursor cholesterol to biologically active products (Parker & Schimmer 1994). 21-Hydroxylase (CYP21) regulates cortisol and aldosterone production, whereas 17-hydroxylase/17,20-lyase (CYP17) is primarily concerned with androgen production. We have recently shown that modulation of these key steroidogenic hydroxylase enzymes is controlled, at least in part, by ACTH and angiotensin II (AII), signalling through specific transcription factors such as

steroidogenic factor-1 (SF-1) and *nur77*. We have previously demonstrated that *nur77* may preferentially regulate steroid enzyme genes relevant to cortisol production and thereby regulate differential cortisol and adrenal androgen production (Kelly *et al.* 2004).

Central to the efficient orchestration of events which lead to transcriptional activation of steroid enzyme genes is the recruitment by nuclear receptors of coregulators (coactivators and corepressors) which interact with and effect transactivation (McKenna *et al.* 1999a). It has been suggested that cofactors serve as a bridging apparatus between the nuclear receptor and the transcriptional machinery. Coactivator recruitment leads to the acetylation of histones, which destabilises nucleosomes and relieves transcriptional repression by allowing transcription factors access to recognition elements on the target gene promoter (McKenna *et al.* 1999b, Sterner & Berger 2000). On the contrary, corepressor binding results in recruitment of histone deacetylases, which stabilises the repressed state and inhibits gene transcription (Xu *et al.* 1999). A growing number of coregulatory proteins have been identified as mediators and modulators of steroid receptor

signalling. The most widely studied coactivators are steroid receptor coactivators (SRCs), members of the p160 family, which are critical components of the signalling pathway (Spencer *et al.* 1997). Members of the SRC family include SRC-1, SRC-2, transcription intermediary factor 2 (TIF-2)/glutamate receptor interacting protein-1) and SRC-3 (activator of thyroid and retinoic acid receptor (ACTR), AIB-I, CBP interacting protein). Conversely, corepressors such as silencing mediator for retinoid and thyroid hormone receptor (SMRT) interact strongly with nuclear receptors under basal conditions and this interaction is thought to be destabilised in the presence of a ligand. Thus, SMRT constitutively represses transcription (Horlein *et al.* 1995, Horwitz *et al.* 1996).

Coregulatory proteins would appear to play a role in endocrine disorders and tumours. Differential expression of endometrial steroid receptor coregulatory proteins has been shown to play a role in the regulation of endometrium remodelling (Wieser *et al.* 2002). Overexpression of coregulatory proteins in PCOS patients is thought to be associated with poor reproductive performance and increased incidence of endometrium hyperplasia and cancer (Gregory *et al.* 2002). The coregulators SRC-1 and SMRT have been shown to associate/interact with SF-1 and nur77. SRC-1 has been shown to potentiate the activity of SF-1 (Ito *et al.* 1998) and has been shown to bind to nur77 (Sohn *et al.* 2001, Maira *et al.* 2003). The corepressor SMRT may interact indirectly with SF-1 and has been shown to interact directly with nur77 to cause inhibition of gene transcription (Sohn *et al.* 2001). Variations in the activity of coregulatory proteins are predicted to lead to changes in hormone responsiveness (Auger *et al.* 2000, Xu *et al.* 2000). However, to date, no clear role for coregulatory proteins in adrenal steroidogenesis has been defined.

A balance between transcriptional activation and repression is essential in the control of adrenocortical hormone biosynthesis. Expression and regulation of specific transcription factors and coregulatory proteins may therefore be central to the dissociation of adrenal androgens and cortisol seen in physiological and pathophysiological conditions. We have investigated the expression and differential interaction of coactivator SRC-1 and corepressor SMRT with transcription factors SF-1 and nur77 in the human adrenal gland and adrenal cell line under the influence of ACTH and AII.

Materials and Methods

Preparation and stimulation of cell suspensions and H295R cells

The human adrenal tumour cell line, H295R, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in supplemented RPMI (5 µg/ml insulin, 10 µg/ml transferin,

30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-oestradiol, 10 mM Hepes, 2 mM glutamine and 2% foetal calf serum (w/v)) and grown in 75 cm² flasks at 37 °C under an atmosphere of 5% CO₂. Cells were incubated for 2, 16, 20 or 24 h in the presence or absence of forskolin (10^{−7}M) and AII (10^{−7}M) in serum-free medium. The cells were removed and stored for protein or RNA extraction.

Immunohistochemistry

Following ethical and donor approval, human adrenal glands were surgically removed at the time of resection for renal transplantation. An immunohistochemical technique was employed for the identification and localisation of SRC-1 and SMRT in human adrenal gland cryo-sections. Adrenal cryo-sections were air dried and fixed in absolute alcohol (7 µm), immunoblocked in serum for 90 min and incubated with a primary goat anti-human SRC-1 (2 µg/ml) (sc-6097; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-human SMRT (2 µg/ml) (sc-1612; Santa Cruz Biotechnology Inc.) for 1 h at room temperature and subsequently with anti-goat horse-radish peroxidase secondary antibody (1:2000) for 1 h at room temperature. Sections were developed in 3,3'-diaminobenzidine tetrahydrochloride for 7 min and counterstained with Mayer's haematoxylin. Negative controls were performed where the primary antibody was substituted with matched IgG controls (Dako, Glostrup, Denmark) and no staining was detected.

Immunofluorescence

Adrenal gland cryosections were prepared as above and incubated in goat serum for 60 min. Rabbit anti-human SF-1 (50 µg/ml in 10% human serum; PA1-800; Affinity Bioreagents, Golden, CO, USA) or rabbit anti-human nur77 (50 µg/ml in 10% human serum; sc-5569; Sigma, Poole, Dorset, UK) were placed on each slide for 90 min. The sections were rinsed in phosphate-buffered saline (PBS) and incubated with the corresponding secondary tetramethylrhodamine isothiocyanate chloride-conjugated antibody (1:100) for 60 min. Sections were rinsed in PBS, blocked in rabbit serum for 90 min and washed with PBS. Each slide was incubated with either goat anti-human SRC-1 (sc-6097; Santa Cruz Biotechnology Inc.) or goat anti-human SMRT (sc-1612; Santa Cruz Biotechnology Inc.) (both 10 µg/ml in 10% human serum) for 90 min, followed by a wash in PBS. The slides were incubated with the corresponding fluorescein isothiocyanate-conjugated antibody (1:100) for 60 min. Sections were rinsed in PBS and mounted using fluorescent mounting media (Dako). Sections were examined under fluorescent light. Negative controls were performed where the primary antibody was substituted with a matched IgG control and no staining was detected. Sections were counterstained with diamidino-2-phenylindole (DAPI) to identify the nucleus.

Protein extraction and quantification

Total protein (whole cell lysates) was extracted from H295R cells by incubation in lysis buffer (PBS, 1% Igepal and 0.5% deoxycholic acid) supplemented with phenylmethylsulphonyl fluoride (10 µg/ml) and aprotinin (1.5 µg/ml) for 30 min. Protein was quantified using the BCA assay kit (Pierce Chemicals Co., Rockford, IL, USA). Cytoplasmic and nuclear protein extractions of H295R were performed using NE-PER nuclear and cytoplasmic extraction kits (Pierce Chemicals Co.) according to the manufacturer's instructions.

Northern blot analysis

Total RNA was isolated from stimulated H295R cells using RNeasy extraction kit (Qiagen, Crawley, W Sussex, UK) at specific times after treatment. RNA was quantified by u.v. absorption. RNA (10 µg) was electrophoresed on a standard Northern gel and transferred to a nylon membrane (Biorad, Hercules, CA, USA) by pressure blotting overnight and incubated for 3 h at 80 °C.

Prehybridization was carried out at 42 °C for 3 h in a final buffer composition of 45% formamide, 4 × SSC, 0.1 M NaPhosphate, 0.1% napyrophosphate, 0.1% SDS and 250 µg/ml herring sperm. Hybridisations were performed in the same buffer at 42 °C overnight with a DNA probe for SF-1 (forward 5'-AGATGACGCTGCA GAAGT-3', reverse 5'-GAGTCCTCTTTTGGTTG CGG-3') and nur77 (forward 5'-ATACACCCGTGA CCTCAACCA-3', reverse 5'-GAGGAGTGAAGGGA CCAGGC-3') amplified by PCR and antisense probes to SRC-1 (IMAGE clone; PT7T3D-PACI; Invitrogen) and SMRT (Professor R M Evans, Salk Institute for Biological Studies, La Jolla, CA, USA) using [α -³²P]dCTP and a random primer labelling system (Promega, Madison, WI, USA). The blots were then washed in 2 × SSC containing 0.5% SDS at room temperature for 10 min, at 42 °C for 10 min and at 55 °C for 10 min before they were exposed to film at -80 °C using intensifying screens. Results were assessed directly by Eagle Eye (Stratgene, La Jolla, CA, USA) analysis and compared with levels of 28S RNA in the same lane.

Electrophoretic mobility shift assays (EMSA)

Nuclear protein extracts were prepared as described above. For EMSA, 5 µg nuclear extract was incubated for 20 min in the presence of 20 mM Hepes, pH 7.9, 5 mM MgCl₂, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 8% Ficoll, 600 mM KCl, 500 ng/µl poly deoxyinosinic-deoxycytidylic acid, 50 mM dithiothreitol and [α -³²P]dCTP-labelled double-stranded oligonucleotide. For supershift experiments, antibodies against SF-1 (PA1-800; Affinity Bioreagents), nur77 (sc-7014X; Santa Cruz Biotechnology Inc.), SRC-1 (sc-6097X; Santa Cruz

Biotechnology Inc.) and SMRT (sc-1612X; Santa Cruz Biotechnology Inc.) were added following the initial 20-min incubation, then incubated for a further 20 min. The samples were electrophoresed through a 5.5% non-denaturing polyacrylamide gel in 0.5 × Tris borate-EDTA buffer. For competition studies, the reaction was performed as described with 50 × molar excess of unlabelled probe. The following oligonucleotides were designed to incorporate the native human CYP21 (for nur77 binding) and CYP17 (for SF-1 binding) promoter sequences: SF-1 (CYP17, NM_000102) 5'-TTGCAAGCCTTGACTCC TGAGCCCAGATACCA-3' and nur77 (CYP21, NM_000500) 5'-CTGGATGCAGGAAAAAGGTCAG GGTTGCACTT-3'. The consensus sequence for the SF-1- and nur77-binding elements is underlined (Wilson *et al.* 1991, Hanley *et al.* 2001).

Immunoprecipitation

Complex formation between coregulators and SF-1 and Nur77 was examined by using H295R cell lysates. Whole cell lysates were prepared as described above. Lysate (100 µg) was immunoprecipitated with 2 µg of either anti-SRC-1 (sc-6097; Santa Cruz Biotechnology Inc.) or anti-SMRT (sc-1612; Santa Cruz Biotechnology Inc.) for 60 min at 4 °C. The precipitates were collected for 1 h on protein A/G-agarose (Santa Cruz Biotechnology Inc.). After washing with RIPA buffer (PBS, 1% Igepal and 0.5% deoxycholic acid), precipitates were resuspended in Laemmli SDS sample buffer and resolved in 12% SDS-PAGE. After transfer to nitrocellulose, the proteins were probed with either anti-SF-1 (PA1-800; Affinity Bioreagents) or anti-nur77 (sc-7014; Santa Cruz Biotechnology Inc.), followed by the corresponding peroxidase-conjugated secondary antibody (1:2000). Labelled bands were detected using chemiluminescence (Santa Cruz Biotechnology Inc.).

Chemicals

All chemicals were obtained from Sigma unless otherwise specified.

Results

Localisation of SRC-1 and SMRT in human adrenal gland

SRC-1 and SMRT were localised within the human adrenal gland using immunohistochemistry. Strong positive staining was detected for SRC-1 and SMRT in the zona fasciculata and zona reticularis but was largely absent from the zona glomerulosa. Both coregulatory proteins were found to be expressed principally in the nuclei of adrenal cells. Specific staining was absent when the primary antibody was substituted with matched IgG (Fig. 1).

Figure 1 Immunohistochemical localisation of (A and C) SRC-1 and (B and D) SMRT counterstained with haematoxylin (left) and matched IgG-negative controls (right) in human adrenal gland tissue. Positive cells are indicated by a brown stain. G, glomerulosa; F, fasciculata; R, reticularis.

Colocalisation of SF-1 and nur77 with coregulatory proteins

Given the evidence that SRC-1 and SMRT interact directly with nuclear receptors, the coregulatory proteins would be expected to be expressed in the same cell as the transcription factors, SF-1 and nur77. Immunofluorescence staining was undertaken to confirm subcellular localisation of SRC-1 and SMRT and to identify coexpression with SF-1 and nur77 (Fig. 2). Negative controls were performed where the primary antibody was substituted with matched IgG and no staining was observed (data not shown). SRC-1 and SMRT were found to be expressed in the nucleus and, to a lesser extent, in the cytoplasm of adrenocortical cells. Though the transcription factors and coregulatory proteins were expressed independently, both SRC-1 and SMRT were found to be colocalised with SF-1 and nur77 within a subset of cells in the human adrenal gland (Fig. 2).

Regulation of SF-1 and nur77 mRNA in the H295R cell line

We have previously shown that SF-1 and nur77 protein expression can be regulated by forskolin and AII. To

examine whether these secretagogues have a similar effect at a transcriptional level we assessed the changes in mRNA expression of the transcription factors. SF-1 mRNA was expressed under basal conditions. Following treatment with forskolin an increase in SF-1 mRNA expression was observed. There was no increase in SF-1 mRNA expression in the presence of AII. Nur77 mRNA expression was absent at a basal level but was induced in the presence of forskolin and in particular AII (Fig. 3A and B).

Regulation of SRC-1 and SMRT mRNA expression in the H295R cell line

The ability of forskolin and AII to regulate SRC-1 and SMRT mRNA following 16, 20 and 24 h of incubation was examined. Under basal conditions, mRNA expression of SRC-1 and SMRT was low. Treatment with forskolin caused an increase in SRC-1 mRNA expression at all time-points compared with basal levels, with maximal increase seen at 16 h. Following treatment with AII, SRC1 mRNA expression reached a maximum at 20 h. SMRT mRNA expression remained low in the presence of both secretagogues with a small increase in SMRT

Figure 2 Immunofluorescent colocalization of SF-1 with SRC-1, SF-1 with SMRT, Nur77 with SRC-1 and Nur77 with SMRT in the zona fasciculata of the human adrenal gland tissue. Sections were counterstained with DAPI to identify the nucleus.

mRNA transcripts seen at 24 h following treatment with forskolin (Fig. 3C and D).

SF-1 and nur77 binding and recruitment of SRC-1 and SMRT

To determine the ability of SF-1 and nur77 to bind to their respective response elements in the presence of forskolin and AII and, once bound, to identify SRC-1 and SMRT in the transcription factor–DNA complex, gel shift analysis were performed. Using published oligonucleotide sequences that incorporate the native human CYP17 (for SF-1 binding) and CYP21 (for nur77 binding) (Wilson *et al.* 1991, Hanley *et al.* 2001), the ability of nuclear extracts from unstimulated H295R cells to bind to the DNA-binding motif was compared with cells treated with forskolin and AII. DNA protein binding was induced in the presence of both secretagogues at the response element for SF-1 and nur77, in particular AII induced protein interaction at the nur77 response element (NBRE) (Fig. 4). A supershift induced by preincubation of nuclear extracts with anti-SF-1 and anti-nur77 established that these transcription factors were present in the protein–DNA complex. The presence of the coregulatory proteins

Figure 3 Representative Northern blot of (A) SF-1 and (B) nur77 mRNA expression in the H295R cells following 24 h of stimulation with 10^{-7} M forskolin (Forsk) and 10^{-7} M AII. Representative Northern blot of (C) SRC-1 and (D) SMRT mRNA expression in the H295R cells over a 16, 20 and 24 h time-course following stimulation with 10^{-7} M forskolin and 10^{-7} M AII. The presence and integrity of the 28S major RNA component for each lane was examined and found to be similar.

was investigated by preincubation with antibodies directed against SRC-1 and SMRT. SRC-1 was found to be recruited to the SF-1 response element in the presence of forskolin and to the NBRE under basal conditions and in particular in the presence of AII. Conversely, the corepressor SMRT was recruited to the SF-1 response element primarily in the presence of AII and to a lesser extent forskolin, and to the NBRE in the presence of forskolin (Fig. 4).

The ability of the response elements to differentially recruit SRC-1 and SMRT in the presence of AII and forskolin was confirmed by coimmunoprecipitation. SF-1 interacted with SRC-1 in the presence of forskolin but not AII, whereas AII but not forskolin induced SF-1–SMRT interactions. Conversely, nur77 preferentially interacted with SRC-1 in the presence of AII and forskolin induced nur77–SMRT interactions (Fig. 5).

Discussion

Specific regulation of the adrenal steroids cortisol and adrenal androgen is achieved in part through the

Figure 4 Electrophoretic mobility shift analysis of nuclear extracts from H295R cells. Nuclear protein extracts from H295R cells in the presence and absence of forskolin (10^{-7} M) and AII (10^{-7} M) were compared for increased binding to (A) [α^{32} P]dCTP-labelled SF-1 response element and (B) [α^{32} P]dCTP-labelled NBRE. DNA protein interactions were assayed in the presence of 50 $\times$ molar excess of homologous oligonucleotide (+50X). Nuclear protein extracts were preincubated in the presence of anti-SF-1, -SRC-1, -SMRT or -nur 77. Results are representative of those obtained in three separate experiments.

differential expression of key steroidogenic enzymes, under the influence of secretagogues (Hu *et al.* 2001b). In the last 10 years studies have shown that the transcription factor SF-1 participates in the expression of all steroidogenic enzymes in the adrenal cortex (Leers-Sucheta *et al.* 1997, Hu *et al.* 2001a, Bassett *et al.* 2002, Sewer & Waterman 2002). We have previously shown that ACTH and AII can specifically regulate CYP21 by signalling through nur77 (Kelly *et al.* 2004), signalling through discrete transcription factors may therefore enable the differential regulation of key steroidogenic enzymes. More recently, nerve growth factor inducible β has been further implicated in cortisol production through its ability to

increase the mRNA expression of 3 β -hydroxysteroid dehydrogenase (Bassett *et al.* 2004).

Orphan nuclear receptors activate their target gene transcription through interaction with coactivators and corepressors that link receptors to the transcriptional machinery (McKenna *et al.* 1999a). SF-1 has been previously shown to interact with numerous coactivators such as SRC-1 (Ito *et al.* 1998), nuclear receptor interacting protein 1 (Zhou *et al.* 2000), TIF-2 and p/CIP (Hammer *et al.* 1999, Borud *et al.* 2002). These interactions, independent of endogenous ligand, are dependent on the activation function 2 (AF-2) and have been implicated in the transduction of cAMP signal (Val *et al.* 2003). To date,

Figure 5 The ability of SF-1 and nur77 to differentially recruit SRC-1 and SMRT was determined by coimmunoprecipitation of the coregulators SRC-1 and SMRT with the transcription factors SF-1 and nur77. Cell lysates were immunoprecipitated with either anti-SRC-1 or anti-SMRT and subsequently immunoblotted with anti-SF-1 or anti-nur77. Results are representative of those obtained in three separate experiments. forsk, forskolin.

the only coregulatory proteins to interact with nur77 that have been identified are activating signal cointegrator 2, p160 proteins and SMRT. Unlike SF-1 and most other nuclear receptors AF-2 is thought not to be crucial for nur77 transactivation (Wansa *et al.* 2002). To date, there is little information regarding the recruitment and potential functional role of SRC-1 and SMRT in the adrenal gland. We propose that, in the adrenal, SF-1 and nur77 can differentially interact with specific coregulatory proteins in the presence of forskolin and AII. In this study, we have investigated the recruitment of coregulatory proteins SRC-1 and SMRT to orphan nuclear receptor response elements and determined secretagogue-dependent interactions with SF-1 and nur77.

The mechanisms behind the differential choice of cofactor recruitment by different nuclear receptors are not known, although different expression profiles of the cofactors in different cell types has been suggested to be essential in cofactor recruitment preferences (Wansa *et al.* 2002). There have been conflicting reports regarding the subcellular localisation of coregulators. SRC-1 has been reported to be localised either in the nucleus (Nazareth *et al.* 1999, Stenoien *et al.* 2001) or in the cytoplasm (Chen *et al.* 2001), depending on the cell type and whether it is endogenous or transfected. The present study represents the first immunohistochemical attempt to elucidate the localisation of coregulators SRC-1 and SMRT in the human adrenal gland. Both SRC-1 and SMRT were localised primarily within the nuclei of the human adrenal cortex. Both coregulatory proteins were expressed in the

zona fasciculata and zona reticularis but were absent from the zona glomerulosa. Subcellular localisation and dynamic movements of transcription factors has been shown to be one of the primary methods of regulating their transcriptional activity. Given the ability of SRC-1 and SMRT to interact directly with steroid receptors, we expected that these coregulatory proteins would be expressed in the same cells as SF-1 and nur77. SF-1 has recently been colocalised with the coactivators general control of amino-acid synthesis 5-like 2 (yeast)/transformation/transcription domain associated protein in KGN cells following stimulation of the protein kinase A (PKA) pathway with forskolin (Fan *et al.* 2004). In this study, both SRC-1 and SMRT were coexpressed with both nur77 and SF-1 in the human adrenal cortex. These data suggested that transcription factors may assemble in the nucleus with an accompanying recruitment of coregulatory proteins, SRC-1 and SMRT, and therefore may have a potential role on the functional expression of SF-1 and nur77 target genes in adrenal steroid biosynthesis.

In line with other studies, we have previously shown that forskolin and AII can regulate the protein expression of SF-1 and nur77 (Enyeart *et al.* 1996, Kelly *et al.* 2004). The mRNAs encoding several members of the steroid hormone receptor family have been shown either to increase or decrease in response to treatment with their cognate hormones (Misiti *et al.* 1998). We studied the mRNA expression of orphan nuclear receptors SF-1 and nur77 following treatment with forskolin and AII. Although both their corresponding mRNAs were induced

by these secretagogues, forskolin was clearly more effective at increasing SF-1 while AII induced larger increases in nur77 mRNA. SF-1 was present in unstimulated cells confirming its constitutive expression, whereas nur77 was not present at a basal level. We next addressed whether coregulatory mRNA levels are hormonally regulated. There has been marked variation in the tissue-specific expression and differential hormonal regulation of the mRNAs encoding SRC-1 and SMRT described in the literature (Misiti *et al.* 1998). Oestradiol (E_2) down-regulates SRC-1 expression in the rat pituitary gland and up-regulates it in breast cancer cells (Thenot *et al.* 1999). In contrast, SRC-1 mRNA levels have been reported to be insensitive to E_2 in the uterus of the rat. SMRT mRNA expression is reported to be low and unchanged after secretagogue stimulation (Nephew *et al.* 2000). In this study, we found low levels of SRC-1 mRNA in untreated cells; however, in the presence of forskolin or AII, SRC-1 mRNA levels were increased. In contrast, SMRT mRNA was absent under basal conditions and no significant changes were observed following stimulation in the H295R cells. These results are in accordance with the theory that different cell types have different relative compositions of these coregulators associated with nuclear receptors.

Treatment of adrenocortical cells with forskolin and AII induced binding of SF-1 and nur77 to their respective response elements. A dual binding motif was observed for SF-1, in particular in the presence of AII. Sewer *et al.* (2002) have demonstrated similar findings where they observed multiple bands resulting at the SF-1 response element in the promoter of CYP17. We determined the ability of forskolin and AII to differentially recruit SRC-1 and SMRT to the DNA-binding elements of SF-1 and to the NBRE and looked at the ability of secretagogues to induce transcription factor coregulatory protein interaction. SRC-1 and SMRT were not present at the SF-1 response element under basal conditions. Binding and recruitment of SRC-1 to SF-1 was induced in the presence of forskolin, whereas in the presence of AII and, to a lesser extent, forskolin, corepressor binding was observed. In line with this, forskolin induced SRC-1–SF-1 interactions and AII induced moderate interactions between SMRT and SF-1. *In vitro*, SF-1–SRC-1 interactions have been previously described (Crawford *et al.* 1997); however, direct interaction between SF-1 and SMRT are thought not to occur (Ito *et al.* 1998). SF-1 may therefore recruit SMRT by heterodimerising with other nuclear receptors in a similar manner to its recruitment of the corepressor nuclear co-repressor (Crawford *et al.* 1998).

Both SRC-1 and SMRT were observed at the nur77–DNA complex under control conditions. AII, but not forskolin, induced SRC-1 recruitment to the NBRE and initiated nur77 and SRC-1 interactions. Conversely, SMRT recruitment to the NBRE and transcription factor

interactions were seen when cells were treated with forskolin. Previous studies have shown that PKA-induced recruitment of SRC-1 occurs exclusively to nur dimers at the nur response element (Maira *et al.* 2003). Nur77, however, binds an NBRE site in the promoter region of 21-hydroxylase as a monomer (Wilson *et al.* 1993), which may explain the poor forskolin induction of SRC-1 recruitment reported here. Forskolin-induced nur77 signalling may therefore be through the recruitment of alternative coactivator proteins. In contrast, AII-induced SRC-1 recruitment and binding to the NBRE suggests that the protein kinase C pathway may promote monomeric transactivation of nur77 target genes.

Overall, these results have described differential expression and regulation of coregulatory proteins in the adrenal gland. In this study, the ACTH mimetic forskolin induced interactions between the SF-1–DNA complex in the promoter region of CYP-17 and the coactivator SRC-1, and conversely recruited the corepressor SMRT to the NBRE response element in the promoter of CYP21. By contrast, AII induced SRC-1–nur77 interactions and SMRT recruitment to SF-1. These data provide evidence that secretagogues can utilise coregulatory proteins to discretely regulate expression of key steroidogenic enzymes in the human adrenal cortex.

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