Acta Biochimica Polonica

Vol. 52 No. 2/2005, 485–491

on-line at: www.actabp.pl

Temporal pattern of the induction of SF-1 gene expression by the signal transduction pathway involving 3′,5′-cyclic adenosine monophosphate✪

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Received: 18 August, 2004; revised: 15 December, 2004; accepted: 24 January, 2005
available on-line: 15 May, 2005

The objective of our study was to investigate the effect of stimulation of the cAMP-dependent pathway on the expression of an orphan nuclear receptor, SF-1/Ad4BP in mouse adrenal tumour, Y-1 cells in culture. We evaluated the temporal pattern of the effects of corticotropin (ACTH) and the adenylyl cyclase activator forskolin on the level of SF-1 mRNA, and compared the time course of induction of *SF-1* with that of *CYP11A1.* Forskolin, corticotropin and 8-Br-cAMP significantly elevated the level of the *SF-1* transcript, after 1.5 h of incubation, with a concomitant increase of SF-1 protein level, observed after 6 h. The *CYP11A1* transcript increased gradually over the incubation period, and reached the maximal level after 12 to 24 h. The steady-state level of the *SF-1* transcript was unaffected by forskolin when the cells were incubated with actinomycin D, indicating that stimulation of the cAMP pathway results in enhanced transcription of the gene. The effect of forskolin was augmented by cycloheximide, suggesting that an inhibitory protein, whose synthesis was inhibited by cycloheximide, could be involved in negative regulation of *SF-1* expression. It is concluded that *SF-1* expression is positively regulated by the cAMP pathway at the transcriptional level, and can represent the primary event in cAMP-mediated induction of steroid hormone synthesis in Y-1 cells.

Keywords: SF-1, *CYP11A1*, cAMP pathway, Y-1 cells, steroidogenensis

Steroidogenic factor 1 (SF-1/Ad4BP/NR5A1) is an orphan nuclear receptor first identified as a transcription factor with limited tissue distribution, recognising a regulatory motif (PyCAAGGTCA) in the proximal promoters of CYP genes encoding cytochromes P450, components of steroid hydroxylase enzyme complexes (Parker & Schimmer, 1997). It has been demonstrated that *SF-1* knockout mice exhibit adrenal and gonadal agenesis, male-to-female sex reversal, impaired gonadotrope function and ablation of ventromedial hypothalamic nucleus. Studies in the knockout mice have indicated the essential role of SF-1 in the regulation of the hypothalamic-pituitary and steroidogenic organ axis at various levels, as well as in the process of differentiation of steroidogenic tissues (Hammer & Ingraham, 1999). In the adult adrenal and gonad, SF-1 plays an important role in the maintenance of the differentiated functions of these organs, and is essential for the expression of all steroidogenic *CYP*s (Parker & Schimmer, 1997). It is also required for the expression of other genes involved in steroid hormone synthesis such as steroidogenic acute regulatory protein (*StAR*), 3β-hydroxysteroid dehydrogenase /Δ^5-Δ^4 isomerase (*HSD3B*) and the ACTH receptor (*ACTH-R*, melanocortin-2 receptor *MC2R*) (Hammer & Ingraham, 1999). SF-1 has also been linked to the regulation of target gene expression in response to tropic hormones acting *via* the cAMP/protein kinase A (PKA) pathway. SF-1 has been shown to take part in cAMP-induced expression of *StAR* (Wooton-Kee & Clark, 2000), P450scc (*CYP11A1*) (Liu & Simpson,

✪The results were presented at the 6th European Congress of Endocrinology, Lyon, 26-30 April, 2003, France.

Abbreviations: AcD, actinomycin D; ACTH-R, ACTH receptor; 8-Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monphosphate; cAMP,: 3′,5′-cyclic adenosine monophosphate; CBP, CREB-binding protein; CHX, cycloheximide; CYP, cytochromes P450; Fsk, forskolin; HSD3B, 3β-hydroxysteroid dehydrogenase; MC2R, melanocortin-2 receptor; PKA, protein kinase A; PNRC, proline-rich nuclear receptor coregulatory protein; SF-1/Ad4BP/NR5A1, steroidogenic factor 1; SRC, steroid receptor co-activator; StAR, steroidogenic acute regulatory protein.

1999), P450c17 (*CYP17*) (Zhang & Mellon, 1996), aromatase P450 (*CYP19*) (Carlone & Richards, 1997) as well as ACTH-R (*MC2R)* (Sarkar *et al.*, 2000).

As a result of covalent modifications (phosphorylation, dephosphorylation and acetylation) (Zhang & Mellon, 1996; Jacob *et al.*, 2001; Sewer & Waterman, 2002) SF-1 could recruit co-regulators to trigger cAMP-dependent expression of target genes. It has been shown that SF-1 interacts with: CREB binding protein (CBP), steroid receptor co-activator 1 (SRC-1), cJun, TF-IIB, DEAD box protein (DP103), glucocorticoid receptor-interacting protein 1 (GRIP1), silencing mediator of retinoid and thyroid receptors (SMRT), p300/CBP/cointegrator-associated protein (p/CIP) and proline-rich nuclear receptor coregulatory protein (PNRC) (Ito *et al.*, 1998; Li *et al.*, 1999; Zhou *et al.*, 2000; Ou *et al.*, 2001; Borud *et al.*, 2002). It has been shown that *SF-1* expression is regulated by stimulation of the cAMP pathway in adrenocortical cells and postulated that this regulation could in part be responsible for the observed stimulation of CYP genes by corticotropin (Enyeart *et al.*, 1996; Aesoy *et al.*, 2002; Osman *et al.*, 2002).

The present study was designed to investigate regulation of *SF-1* expression by cAMP and to determine whether this regulation takes place at the transcriptional level, or concerns the stability of the transcript. This investigation also aimed to verify the hypothesis that accumulation of SF-1 protein precedes the expression of *CYP11A* thus mediating the long-term effect of ACTH.

MATERIALS AND METHODS

Materials. ACTH, forskolin, 8-Br-cAMP, actinomycin D and cycloheximide were obtained from Sigma (St Louis, MO, USA) and [α-^{32}P]dCTP (>3000 Ci/mmol) was from Perkin-Elmer Life Sciences Products (Boston, MA, USA). Bovine StAR cDNA was provided by Dr. R. Ivell and an anti-SF-1 antibody was generously donated by Dr. K. Morohashi.

Cell culture and treatment. Y-1 adrenal tumour cells were grown to confluence in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DME/F-12, 1:1 mixture) (Sigma), supplemented with 10% foetal calf serum (FCS), L-glutamine (2.5 mM), penicillin (100 U/ml), streptomycin (1 mg/ml) and nystatine (100 U/ml) (Invitrogen, Carlsbad, CA, USA). The medium was removed and the cells were subsequently incubated with test substances. After incubation the cells were washed with phosphate buffered saline and lysed.

Northern blot analysis. Total RNA was extracted from the Y-1 cells using a single-step guanidinum-thiocyanate/phenol-chloroform procedure (Chomczynski & Sacchi, 1987). Twenty micrograms of total RNA was electrophoresed in 1% agarose-formaldehyde gel containing ethidium bromide, transferred to a Hybond™-N+ membrane (Amersham, Little Chalfont, UK), and the membrane was exposed to UV light in a cross-linker (Vilber Lourmat, France). Photographs of the membrane stained with ethidium bromide were taken with a digital camera and 28S rRNA quantified with the Bio1D software (Vilber Lourmat). The blots were hybridised with α^{32}P-labelled cDNA probes, generated by PCR, using as a template reverse transcripts of total RNA extracted from Y-1 cells. The PCR products were purified on agarose gels and eluted with the GelElute™ Agarose Spin Column Kit (Sigma). The identity of the probes was confirmed by digestion with restriction enzymes. The probe for SF-1 was sequenced. Probes were labelled using [α-^{32}P]dCTP and the Ready-To-Go™ DNA Labelling Beads (-dCTP) kit (Amersham).

Prehybridisation was carried out at 42°C for 16 h in a solution containing 50% deionised formamide, 10% dextran sulfate, 0.1% SDS, 5 × Denhardt solution, 1.25 mM pyrophosphate, 0.75 M NaCl, 1 mM EDTA and 100 μg/ml denatured salmon sperm DNA (Roche Molecular Biochemicals, Indianapolis, IN, USA). For hybridisation, 10^7 c.p.m. of labelled DNA probes was added to 15 ml of the hybridisation solution containing 0.5% SDS and the membranes were incubated at 42°C for 16 h.

After hybridisation, the membranes were washed once at room temperature with 2 × SSC/0.1% SDS (15 min), followed by washing twice at 65°C with 1 × SSC/0.1% SDS (15 min) and twice at 65°C with 0.5 × SSC/0.1% SDS (15 min). The probes were visualised in a β-imager (PhosphorImager, Molecular Dynamics, Inc., Sunnyvale, CA, USA) and quantified using the ImageQuant 5.0 software (Molecular Dynamics). The membranes were also exposed to Biomax MR films (Eastman Kodak, Rochester, NY, USA) and the quantities of mRNA were normalised to the amount of 28S rRNA.

Western blot analysis. The cell extracts for Northern blot analysis were also used for protein isolation according to the TRIZOL® Reagent protocol (Invitrogen). The protein samples were subjected to electrophoresis in 10% SDS/polyacrylamide gels and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were then incubated with an anti-SF-1 antibody (Morohashi *et al.*, 1993) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO, Carpinteria, CA, USA). The membranes were subsequently reprobed with an anti-β-actin monoclonal antibody (Sigma) using the ECL detection kit (Amersham), exposed to X-Omat AR (Eastman Kodak) film and analysed with the NIH Image densitometric software (ver 1.62).

Statistical analysis. Data were expressed as the mean of the fold of control in each experiment.

The results were analysed by the Student's *t*-test and $P < 0.05$ was taken as the level of significance.

RESULTS

Effects of cAMP signalling on the level of StAR, CYP11A1, SF-1, CBP and SRC-1 mRNAs in Y-1 cells

After 6 h of incubation with 25 μM forskolin or 10 nM ACTH, StAR mRNA levels increased 4.3- and 3.8-fold, respectively (Fig. 1A), while CYP11A1 mRNA levels increased 2.2- and 1.5-fold, respectively (Fig. 1B). Forskolin at 25 μM and 8-Br-cAMP at 50 μM concentrations induced *SF-1* expression stronger than 10 nM ACTH, whereas there was no significant effect of forskolin and ACTH on the abundance of CREB-binding protein (CBP) and steroidogenic receptor co-activator 1 (SRC-1) mRNAs (Fig. 1, C and D). Since the response to the adenylyl cyclase activator forskolin was higher than that to ACTH, in further experiments forskolin was used as the stimulator.

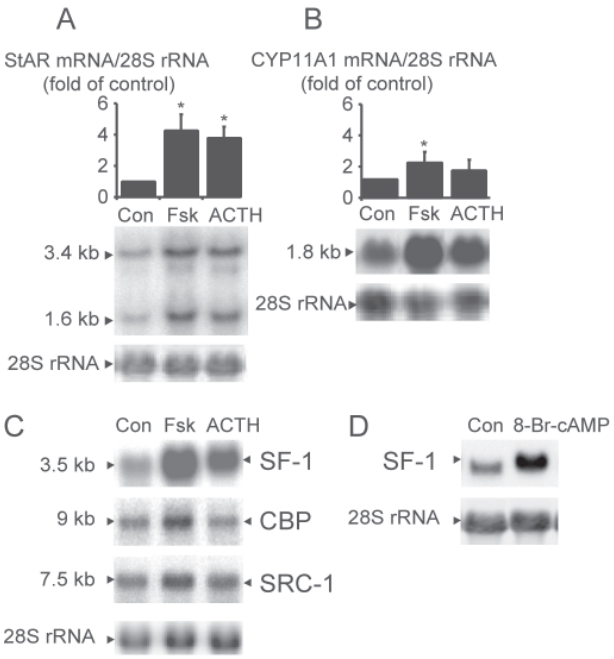

Figure 1. Effects of cAMP signalling on the level of StAR, CYP11A1, SF-1, CBP and SRC-1 mRNAs in Y-1 cells.

Y-1 cells were either untreated (Con) or treated with 25 μM forskolin (Fsk), 10 nM corticotropin (ACTH) or 50 μM 8-Br-cAMP and specific transcript content was determined by Northern blotting. Blots were hybridised with ^{32}P-labelled cDNA probes specific for *StAR*, *CYP11A1*, *SF-1*, *CBP* or *SRC-1* transcripts. Typical results are shown for *StAR* and *CYP11A1* after 6 h of incubation (A and B, respectively). The signals were quantified as described in Materials and Methods, and normalised to 28S rRNA. Results are presented as mean ± SEM from at least three separate experiments. Asterisks designate values significantly different from controls, $P < 0.05$. The levels of *SF-1* transcript after 12 h of incubation with Fsk and ACTH (C) and 8-Br-cAMP (D) are shown. For comparative purposes, the levels of *CBP* and *SRC-1* transcripts after 12 h of incubation are also presented (C).

SF-1 and CYP11A1 mRNAs accumulation in response to forskolin

At 1.5 h of incubation with 25 μM forskolin, the steady state level of SF-1 mRNA increased approx. 2.3-fold ($P < 0.05$) above the untreated control, with a maximal 3.4-fold induction achieved after 6 h of treatment. After 9 h, the expression of SF-1 was slightly reduced and, after 24 h, the level of SF-1 mRNA was still significantly increased (Fig. 2, A and B). The steady-state level of *CYP11A1* transcript increased about 1.8-fold ($P < 0.05$) after 6 h of treat-

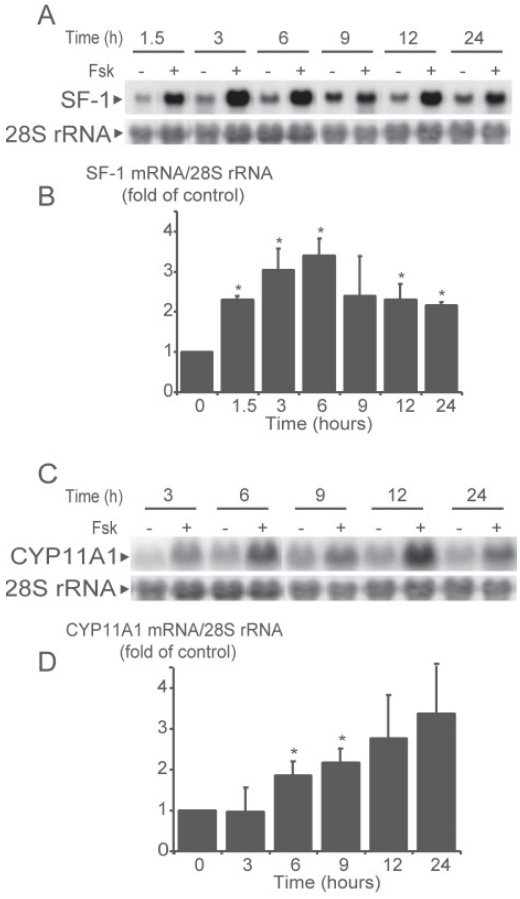

Figure 2. Time-course of SF-1 and CYP11A1 mRNA accumulation in response to forskolin.

The cells were either untreated or treated with 25 μM forskolin (Fsk) for the time indicated. The relative abundance of SF-1 (A and B) and CYP11A1 (C and D) mRNA was determined by Northern blotting and typical blots are shown. The signals were quantified as described in Materials and Methods and normalised to 28S rRNA. Results are presented as mean ± SEM from at least three separate experiments. Asterisks designate values significantly different from controls, $P < 0.05$.

ment with forskolin, followed by the maximal 3.3-fold induction ($P < 0.05$) after 24 h of treatment (Fig. 2, C and D).

Effects of actinomycin D and cycloheximide on the kinetics of SF-1 mRNA decay

In order to investigate a possible contribution of mRNA stability to the cAMP-induced increase in the abundance of SF-1 transcript, the cells were incubated with 5 μg/ml actinomycin D in the presence or absence of 2.5 μM forskolin. After 6 h of treatment with actinomycin D, SF-1 mRNA level was reduced by about 50% and the rate of disappearance of SF-1 mRNA was independent of the treatment of the cells with forskolin (Fig. 3, A and B).

The role of protein synthesis in maintaining the basal or forskolin-induced level of SF-1 mRNA was estimated by incubating the cells with a protein synthesis inhibitor, cycloheximide (CHX). After 6 h of incubation with 2.5 μM forskolin, a significant 1.6-fold ($P < 0.05$) increase in SF-1 mRNA level was observed, while, after the same time of treatment with forskolin and 10 μM cycloheximide, a significant 2.8-fold stimulation was noted. In the presence of cycloheximide the stimulatory effect of forskolin increased gradually and after 24 h of treatment it reached 5.3-fold activation, which was over 4-times higher than in the cells incubated with forskolin alone (1.3-fold). In the cells treated with cycloheximide alone, no appreciable difference in the level of SF-1 mRNA was noted for up to 24 h of incubation (Fig. 3, A and C).

SF-1 protein accumulation in response to forskolin

In the cells incubated with 25 μM forskolin for 6 h, a significant increase of SF-1 protein level above the untreated control was observed. After 12 h, a 2.5-fold increase was reached, and the level of SF-1 protein remained unchanged up to 24 h of treatment. Under these conditions, no significant differences were observed in the level of β-actin between the forskolin-treated and untreated cells (Fig. 4, A and B).

In order to analyse the induction of SF-1 protein by forskolin, the decay of the pre-existing protein was determined by Western blot analysis after inhibition of either RNA synthesis by actinomycin D or protein synthesis by cycloheximide (Fig. 4, C, D, E and F). It was noticed that when RNA synthesis was blocked by 5 μg/ml actinomycin D, 2.5 μM for-

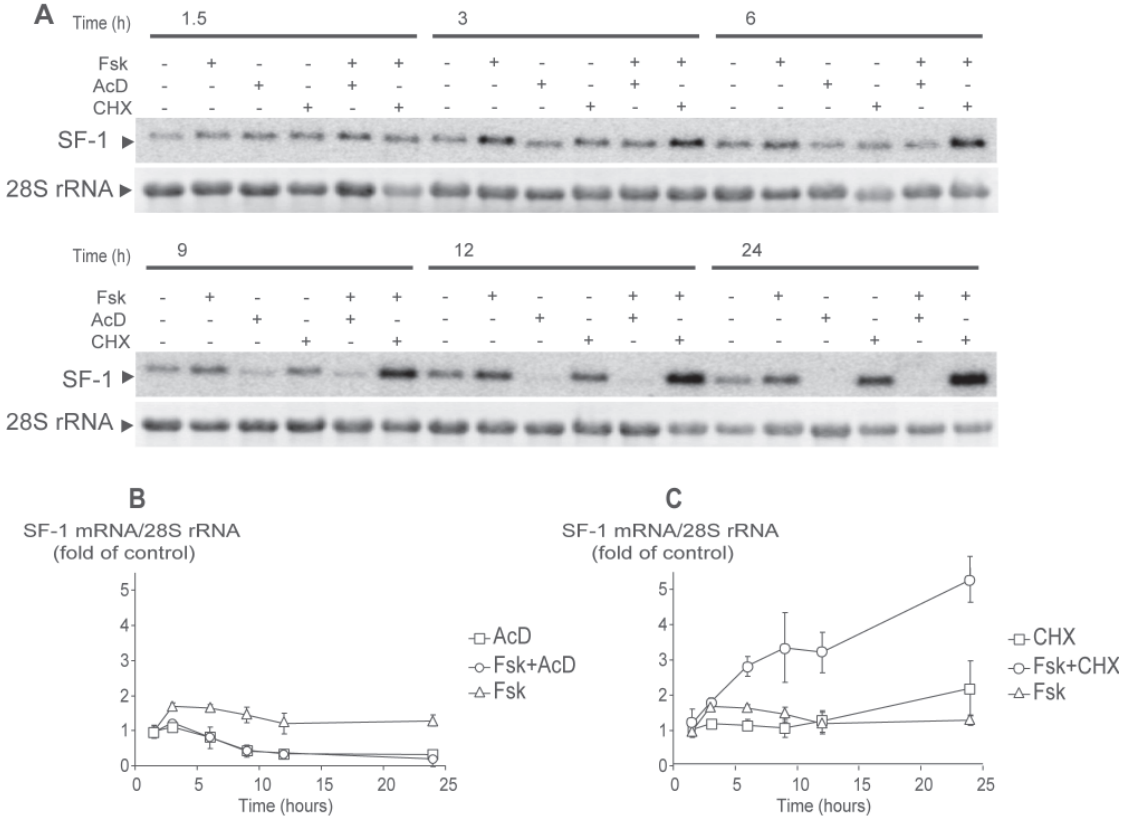

Figure 3. Time-course of actinomycin D and cycloheximide effect on SF-1 mRNA.

The cells were treated for the times indicated with: 2.5 μM forskolin (Fsk); and either 5 μg/ml actinomycin D (AcD) (B) or 10 μg/ml cycloheximide (CHX) (C). The relative abundance of SF-1 transcript was determined by Northern blotting and typical blots are shown (A). The signals were quantified as described in Materials and Methods and normalised to 28S rRNA. Results are presented as mean ± SEM from at least three separate experiments.

skolin did not affect SF-1 protein decay. Similarly when the protein synthesis was blocked by 10 μg/ml cycloheximide, forskolin did not change SF-1 protein steady-state levels.

DISCUSSION

Although the transcriptional regulation of target genes by SF-1 has been extensively investigated, the results of studies on SF-1 expression are not fully consistent. It has been demonstrated that SF-1 mRNA levels are unaffected by forskolin in murine adrenocortical tumour Y-1 cells (Zhang & Mellon, 1996). It has been reported, however, that SF-1 mRNA level is increased by forskolin and corticotropin in bovine adrenocortical cells, with a maximal effect observed after 6 h of treatment (Enyeart *et al.*, 1996; Osman *et al.*, 2002).

The present study demonstrated that, in Y-1 cells, which derive from a mouse adrenal cortex tumour, both the *SF-1* transcript and SF-1 protein synthesis were increased after forskolin treatment. The induction of SF-1 gene expression was rapid, since a significant effect was observed already after 1.5 h of incubation with forskolin. Our results were fully consistent with experiments carried out in primary cultures of bovine adrenocortical cells (Enyeart *et al.*, 1996; Osman *et al.*, 2002), but sharply contrasted with previous investigations conducted in Y-1 cells, showing insensitivity of SF-1 gene expression to cAMP treatment (Nomura *et al.*, 1998). This discrepancy could be explained by the known variability of Y-1 cell responsiveness to cAMP (Schimmer *et al.*, 2002). Y-1 cells used in the present study were highly responsive to cAMP, judging from the increased expression of StAR and CYP11A1 genes in response to ACTH and forskolin. Induction of *SF-1* by forskolin or ACTH was specific, since under these conditions the mRNA levels of two SF-1 co-regulators, CBP (Monte *et al.*, 1998) and SRC-1 (Crawford *et al.*, 1997), were unaffected (Fig. 1 C).

The results of experiments with the use of actinomycin D (Fig. 3) indicated that in Y-1 cells *SF-1* expression was positively regulated by cAMP at the transcriptional level, as reported in adrenal glomerulosa cells treated for 6 h with forskolin (Osman *et al.*, 2002). We also excluded that the increase in *SF-1* transcript was caused by an enhanced stability of SF-1 mRNA, since in the cells treated with actinomycin D forskolin did not change the steady-state level of *SF-1* transcript. Although 25 μM forskolin

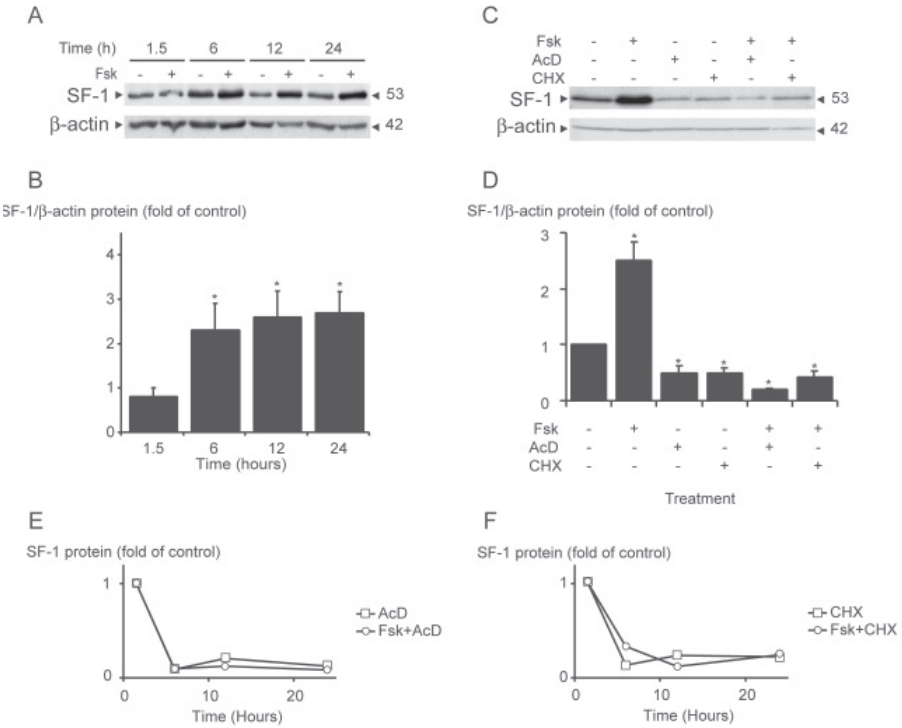

Figure 4. The effect of forskolin on SF-1 protein content.

The cells were incubated in the absence or presence of 25 μM forskolin (Fsk) for the time indicated followed by cellular protein extraction and SF-1 and β-actin detection by Western blotting. Typical blot is shown (A). In order to determine protein stability, the cells were incubated in the absence or presence of 2.5 μM forskolin (Fsk) and either 5 μg/ml actinomycin D (AcD) or 10 μg/ml cycloheximide (CHX). Typical blot is shown for 6 h of treatment (C). Diagrams below (B and D) represent the relative level of SF-1 protein normalised to β-actin, as described in Materials and Methods. Results are presented as mean ± SEM from at least three separate experiments. Asterisks designate values significantly different from control, $P < 0.05$. The graphs represent the time course of the effects of actinomycin D (E) and cycloheximide (F).

was applied in the time course experiment (Fig. 2), in the case of treatment with forskolin and actinomycin D or cycloheximide, a lower concentration of forskolin was used (2.5 μM). This concentration was sufficient to increase SF-1 mRNA level and resulted in the highest effect of the combined treatment with forskolin and cycloheximide (5.3-fold).

The influence of ongoing protein synthesis on the transcription of *SF-1* had not been previously investigated. In the present study, we blocked translation with cycloheximide and observed that treatment with forskolin resulted in much greater SF-1 mRNA accumulation than incubation with forskolin or cycloheximide alone. This suggested that newly synthesised protein(s) might inhibit the accumulation rate of SF-1 mRNA in the presence of forskolin. Since *SF-1* expression was activated by forskolin at the transcriptional level, we suggest that cycloheximide leads to the disappearance of a labile repressor of *SF-1* transcription.

It has been postulated that an increase of SF-1 protein level in response to PKA activation was caused by an increased stability of the protein (Aesoy *et al.*, 2002). Our results imply that the observed increase in protein concentration was a direct consequence of the transcriptional activation of *SF-1*. Furthermore, co-treatment of the cells with actinomycin D and forskolin did not increase the level of SF-1 protein, suggesting that the stability of this protein was not affected. The discrepancies between our results and those obtained in Y-1 cells transfected with a PKA expressing vector suggest that the increase in SF-1 protein stability by PKA is mediated by a labile protein (Aesoy *et al.*, 2002).

The maximal level of SF-1 transcript, after 6 h of treatment with forskolin, was thus reached about 18 h earlier than that of *CYP11A1* (Fig. 2), which is also true for other target genes: *CYP17*, *CYP21*, (Staels *et al.*, 1993) as well as *ACTH-R* and *HSD3B* (Le Roy *et al.*, 2000). In addition, we showed that a significant increase of SF-1 protein was observed already after 6 h of treatment with forskolin (Fig. 4). This characteristic temporal pattern of cAMP-induced *SF-1* expression suggests that, at least in the Y-1 cells, SF-1 could participate in the regulated expression of target genes, and mediate chronic response to cAMP stimulation.

In conclusion, our studies provide evidence that, in Y-1 cells, *SF-1* expression is stimulated by cAMP at the transcriptional level and could contribute to positive regulation of SF-1 target genes. The time-course of SF-1 induction suggests that this protein could participate in the long-term action of cAMP on steroidogenesis. However, the mechanism of activation of *SF-1* transcription remains to be determined, and it should be established to what extent the level of *SF-1* expression *in vivo* plays a role in the regulation of the co-ordinated expression of genes involved in steroid hormone synthesis in the adrenal cortex.

Acknowledgements

The work was supported in part by grants from the State Committee for Scientific Research (KBN, Poland, No. P05 A 060 22) and from the French Ministry of Health (INSERM - Action Specifique PECO).

T.P.L. is a recipient of a scholarship from the Postgraduate School of Molecular Medicine affiliated with the Medical University of Warsaw, and of a scholarship from the French Government.

We thank Dr. K. Morohashi for anti-SF-1 antibodies, Dr. Ivell for StAR cDNA. Editorial assistance of Prof. J. Kuhn is gratefully acknowledged. We thank Dr. K. Koscinski for his help.

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