The transcriptional regulating protein of 132 kDa (TReP-132) differentially influences steroidogenic pathways in human adrenal NCI-H295 cells

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Abstract

Steroid hormones synthesized from cholesterol in the adrenal gland are important regulators of many physiological processes. It is now well documented that the expression of many genes required for steroid biosynthesis is dependent on the coordinated expression of the nuclear receptor steroidogenic factor-1 (SF-1). However, transcriptional mechanisms underlying the species-specific, developmentally programmed and hormone-dependent modulation of the adrenal steroid pathways remain to be elucidated. Recently, we demonstrated that the transcriptional regulating protein of 132 kDa (TReP-132) acts as a coactivator of SF-1 to regulate human P450scc gene transcription in human adrenal NCI-H295 cells. The present study shows that overexpression of TReP-132 increases the level of active steroids produced in NCI-H295 cells. The conversion of pregnenolone to downstream steroids following TReP-132 expression showed increased levels of glucocorticoids, C_{19} steroids and estrogens. Correlating with these data, TReP-132 increases P450c17 activities via the induction of transcript levels and promoter activity of the P450c17 gene, an effect that is enhanced in the presence of cAMP or SF-1. In addition, P450aro activity and mRNA levels are highly induced by TReP-132, whereas 3β-hydroxysteroid dehydrogenase type II and P450c11aldo transcript levels are only slightly modulated. Taken together, these results demonstrate that TReP-132 is a *trans*-acting factor of genes involved in adrenal glucocorticoid, C_{19} steroid and estrogen production.

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Introduction

In the adrenal, cholesterol is converted into progesterone (Prog), mineralocorticoids, gluco-corticoids, dehydroepiandrosterone (DHEA) and small amounts of estrogens by a series of well-characterized steroidogenic enzymes (Fig. 1) (Hall 1984, Jamieson & Fraser 1994). The adult adrenal gland is divided into the zonæ glomerulosa, fasciculata and reticularis, which are three distinct zones involved in the secretion of mineralocorti-coids (mainly aldosterone (Aldo) in humans (Bartter *et al*. 1956, Tait & Tait 1979)), glucocorticoids (mainly cortisol in humans (Nelson *et al*. 1951)) and

C_{19} steroids as well as estrogens respectively. The specific pattern of steroid production depends on the relative activities of steroidogenic enzymes, which are mostly determined by their relative expression levels (Nonaka *et al*. 1992, Simpson & Waterman 1992, Hum & Miller 1993, Rainey *et al*. 1994, Parker & Schimmer 1995). Cytochrome P450scc, encoded by the *CYP11A1* gene, catalyzes the first enzymatic step of the steroid biosynthetic pathways by producing pregnenolone (Preg) from cholesterol. Next, the two branch-point enzymes P450c17 and type II 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-ene-isomerase (3β-HSDII) (Labrie *et al*. 1992*a*) determine the fate of Preg.

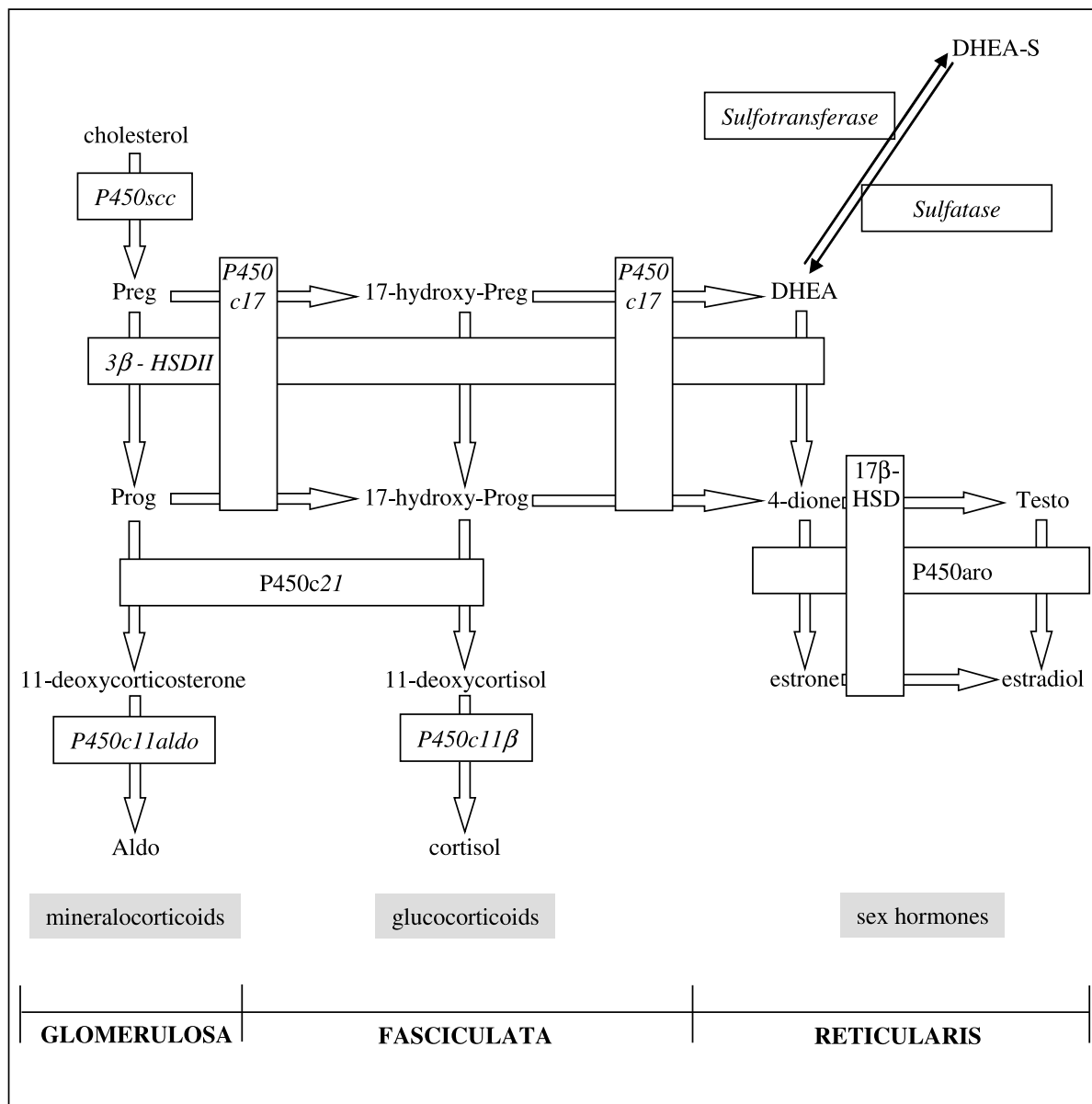**Figure 1** Human adrenal steroidogenic biosynthetic pathway, adapted from Rainey *et al.* (1994). P450 scc, cholesterol side chain cleavage; P450c17, 17α-hydroxylase and c17–20 lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c21, 21-hydroxylase; P450c11β, 11β-hydroxylase; P450c11 aldo, Aldo synthase. In human adrenal cortex, only type II 3β-HSD is expressed (Labrie *et al.* 1992*a*).

P450c17, which possesses both 17α-hydroxylase and 17,20-lyase activities, catalyzes the first step in the formation of glucocorticoids and C_{19} steroids (Zuber *et al.* 1986), while the formation of Prog from Preg is catalyzed by the 3β-HSDII enzyme (Samuels *et al.* 1951, Labrie *et al.* 1992*b*).

The different ratios of P450c17 *vs* 3β-HSDII expression levels in the three adrenocortical zones can determine the relative production of mineralo-corticoids *vs* glucocorticoids and C_{19} steroids (for review see Conley & Bird 1997). This specificity correlates with the absence of P450c17 in the zona glomerulosa (Suzuki *et al.* 2000), its high expression in both the zonæ fasciculata and reticularis, as well as the low expression of 3β-HSDII in the zona reticularis after adrenarch (Gell *et al.* 1996) and in

the fetal zone of the developing adrenal (Nonaka *et al.* 1992, Simpson & Waterman 1992, Hum & Miller 1993, Rainey *et al.* 1994, Parker & Schimmer 1995). Following the conversion of Preg to Prog by 3β-HSDII, Prog is a substrate of both the P450c17 and P450c21 enzymes, and thus represents a second branch-point towards either the mineralocorticoid or glucocorticoid pathways. Moreover, the expression of genes encoding P450aro, P450c11aldo and P450c11β is specific to each adrenal zone (Malee & Mellon 1991, Ogishima *et al.* 1992). Hence, the control of adrenal steroidogenic pathways requires a coordinated transcriptional regulation of genes encoding these key enzymes. However, despite numerous studies aimed at understanding the regulation of adrenal steroidogenesis (Waterman & Simpson 1989, Brentano *et al.* 1990, Leers-Sucheta *et al.* 1997, Mason *et al.* 1997), the transcriptional mechanisms are not yet fully understood.

The orphan nuclear receptor steroidogenic factor-1 (SF-1/fushi-tarazu factor 1 or FTZ-F1/ adrenal 4-binding protein or Ad4 BP, NR5A1) was identified based on its ability to interact with a shared promoter element in steroid hydroxylase genes (Lala *et al.* 1992, Honda *et al.* 1993). Further studies have demonstrated its essential role in the induction of most steroidogenic genes, including human P450scc (Monte *et al.* 1998), P450c17 (Hanley *et al.* 2001), P450aro (Michael *et al.* 1995), 3β-HSDII (Leers-Sucheta *et al.* 1997) and P450c11 aldo (Clyne *et al.* 1997). As for many nuclear receptors, both basal and cAMP-responsive transcriptional activity of SF-1 can be modulated by different mechanisms, including specific interactions with various cofactors. The coactivators CBP/p300 (Monte *et al.* 1998, Gizard *et al.* 2001), the transcription factors Sp1 (Liu & Simpson 1999) and c-Jun (Huang *et al.* 2001), and the co-repressors COUP-TF-1 (Zhang & Mellon 1996, 1997) and DAX-1 (Hanley *et al.* 2001) have been shown to regulate steroidogenic gene expression in a species- and tissue-specific manner by interacting with SF-1. Moreover, recent reports have identified other proteins that modulate the expression of genes encoding steroidogenic enzymes in a cell and developmentally dependent manner. For instance, LBP-1B and the closely related factor LBP-9 have been demonstrated to activate *CYP11A1* transcription in placental JEG-3 cells (Huang & Miller 2000), while the StF-IT-1

and StF-IT-2 proteins regulate P450c17 gene expression and production of steroids in the embryonic central nervous system (Zhang & Mellon 1997, Compagnone *et al.* 2000). Thus, the identification and characterization of novel transcriptional regulators of steroidogenic genes, which interact with SF-1 or not, will be important to better understand the mechanisms involved in steroidogenesis.

The transcription-regulating protein of 132 kDa (TReP-132) was recently cloned and demonstrated to activate P450 scc gene expression (Gizard *et al.* 2001) via a direct interaction with SF-1 and the coactivator CBP/p300 to form a transcriptional complex in NCI-H295 cells (Gizard *et al.* 2002*b*). TReP-132 transcript is predominantly found in steroidogenic cell lines and tissues, including the adrenal gland; however, its expression is also found in cells and tissues that express very low levels or no P450scc (such as the MCF-7 and LNCaP cell lines, as well as the uterus, prostate, testis and brain) (Gizard *et al.* 2001, 2002*a*, Duguay *et al.* 2003). Thus, to further understand the role of TReP-132, it was determined in the present study if this factor could modulate other steroidogenic genes in addition to P450scc in the adrenal gland. To address this question, the effect of TReP-132 overexpression on steroid biosynthetic pathways was determined in human adrenal carcinoma NCI-H295 cells, which express many of the steroidogenic genes and secrete a wide panel of adrenal steroids in a hormone-responsive manner (Gazdar *et al.* 1990, Staels *et al.* 1993, Rainey *et al.* 1994).

TReP-132 activated the P450c17-dependent adrenal steroid pathway, where its overexpression led to the increased production of glucocorticoids, C_{19} steroids and estrogens. In correlation with these results, TReP-132 induced P450c17 activity by increasing P450c17 gene expression, an effect enhanced by activators of the protein kinase A (PKA)-dependent pathway, as well as cotransfection of SF-1. In contrast, TReP-132 did not modulate the transcript levels of the gene encoding 3β-HSDII. In addition, TReP-132 was shown to differentially regulate genes of the steroidogenic pathway downstream of 3β-HSDII and P450c17. The activity and transcript levels of P450aro were significantly increased by TReP-132, whereas it had only minor effects on P450c11aldo. Together, these data indicate that TReP-132 plays an

important role in the activation and the modulation of adrenal steroidogenesis.

Materials and methods

Cell culture

Human NCI-H295 adrenal tumor cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in monolayer as previously described (Rodriguez *et al.* 1997) in Dulbecco's modified Eagle's medium/F12 medium (DMEM-F12) supplemented with penicillin (50 mg/l), streptomycin (10^5 units/l) (Gibco, Burlington, ON, Canada), 5% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 1% of ITS (insulin, transferrin, selenious acid) (Roche), 1% red phenol, 10^{-8} M estradiol and 10^{-8} M hydroxycortisone.

Plasmids

The P450c17 $-2500/+63$c17 Luc reporter construct consisted of the 5′-flanking region of the human P450c17 gene from nucleotides -2500 to $+63$ cloned in the pGL3 vector. The TReP-132 expression construct containing the KpnI/XbaI fragment of the TReP-132 cDNA ligated in the pcDNA3 vector was made as previously described (Gizard *et al.* 2001).

Transfections and luciferase assays

NCI-H295 cells were cultured for 24 h in 12-well plates at a density of 3×10^5 cells per well and grown. The medium was then changed and transient transfections were carried out for 12 h with Effectene Transfection Reagent (Qiagen, Mississauga, ON, Canada) at a ratio of 1 μg DNA to 25 μl Effectene Reagent in serum-free medium. Following transfection, cells were washed and incubated in fresh medium containing FBS for 36 h and treated or not with forskolin or 8Br-cAMP. For luciferase assays, cells were harvested and 20 μl of the cell lysate was assayed for luciferase activity with the Dual-Luciferase reporter Assay System (Promega, Madison, WI, USA). Firefly Luciferase activities were normalized to *Renilla* Luciferase activity. The efficiency of transfection was $\geq 60\%$, as verified by transfection of a GFP expression plasmid. Transfection of the TReP-132 expression

vector led to a 60-fold increase of TReP-132 mRNA levels as assessed by quantitative RT-PCR.

Steroid extraction, separation by thin-layer chromatography (TLC) and quantification

NCI-H295 cells were seeded into six-well culture plates at a density of 8×10^5 cells per well 24 h prior to transfection with pcDNA3 or the TReP-132 expression vector as described above. Medium was removed 24 h later and cells were incubated with 1×10^{-7} M (15 000 c.p.m./ml) of ^{14}C-labeled or 1×10^{-9} M (500 000 c.p.m./ml) of ^{3}H-labeled steroids (Dupont, Inc., Mississauga, ON, Canada) in serum-free medium for periods indicated in Figure legends. In addition, the corresponding unlabelled steroid was co-incubated with the ^{3}H-labeled steroid to obtain a final concentration of 1×10^{-6} M of substrate. After the incubation, the steroids were extracted twice with 2 ml ether. The organic phases were evaporated. The steroids were solubilized in 50 μl dichloromethane, then applied to Silica Gel 60 TLC plates (Merk, Darmstadt, Germany) for separation. Migration was performed in ethyl acetate–hexane–H$_2$0–acetic acid (55:25:50:10, v/v, solvent system 1, 16 min) or in toluene–acetone (40:10, v/v, solvent system 2, 18 min). Detection of metabolites was carried out by autoradiography and quantified by PhosphoImager System (Molecular Dynamics, Inc., Sunnyvale, CA, USA). The identification of labeled metabolites was made by comparison with reference steroids.

Reverse transcription (RT) and quantitative PCR

NCI-H295 cells were first transfected with pcDNA3 or the TReP-132 expression vector as described above. Total RNA was isolated using Trizol reagent according to the manufacturer's protocol (Invitrogen, Cergy Pontoise, France). Total RNA was reverse transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies, Paisley, Strathclyde, UK). The levels of P450c17, 3β-HSDII, P450c11aldo and P450aro transcripts were then assessed by quantitative PCR. Quantification was performed on an MX 4000 apparatus (Stratagene, La Jolla, CA, USA), with specific pairs of oligonucleotide primers (P450c17: 5′-gactacagtgattgtcggccaccac-3′ and 5′-tgatcgccatcc ttgaacaggg-3′, NM_000102; 3β-HSDII: 5′-gaaggaa

ctgaaggagatcagggcc-3′ and 5′-agactggcacactggcttgg acac-3′, M67466; P450c11aldo: 5′-gctgccgtttgaag ccatgc-3′ and 5′-cgtggtcctcccaagttgtacctg-3′, NM_ 000498; P450aro: 5′-atcaccagcatcgtgcctgaagc-3′ and 5′-ctctccagagatccagactcgcatg-3′, XM_035593; TReP-132: 5′-gccatgctgagctcccatgg-3′ and 5′-ctgg ctgggctctcccatc-3′, NM_033502). β-Actin transcripts were simultaneously quantified for normalization using the following oligonucleotide primers: 5′-gatgacccagatcatgtttga-3′ and 5′-cggatgtcaacgtca cacttcatg-3′. PCR amplification was performed in a final volume of 25 µl containing 100 nM of each primer, 4 mM $MgCl_2$, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene) and SYBR Green 0·33X (Sigma, L'Isle d'Abeau Chesnes, France). PCR conditions were 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. Denaturing curves of steroidogenic transcripts and β-actin products, performed at the end of the PCR, confirmed the homogeneity of the DNA products. Fluorescence intensities were normalized against the reference dye, ROX. They were plotted against the number of cycles using an adaptive base-line algorithm provided by the manufacturer to determine the cycle threshold (Ct). The Ct result is the calculated PCR cycle at which products first become detectable and is inversely related to the abundance of transcripts in the initial RNA sample (Jinquan *et al.* 1999). The Ct values for β-actin transcripts in the RNA samples were not modulated in TReP-132-transfected cells compared with pcDNA3-transfected cells and were used as references. Since standard curves performed to validate experiments showed that the amplification efficiencies of the targets and the reference were approximately equal (with a slope of about − 3·2/ − 3·3), quantification was performed using the comparative $\Delta\Delta C_T$ method. In all cases PCR products were analyzed on 1% agarose gels to confirm that a single band of the correct size was obtained.

Results

### TReP-132 expression activates steroid synthesis dependent on cytochrome P450c17 activity

In mammalian adrenal cells, Preg is the common substrate of two branch-point enzymes, cytochrome

P450c17 and 3β-HSDII, which determine the conversion of the precursor steroid into either mineralocorticoids, or glucocorticoids and sex steroids (Fig. 1) (Labrie *et al.* 1992*b*, Katagiri & Kagawa 1998, and for review see Pikuleva & Waterman 1999). To determine the ability of TReP-132 to regulate Preg transformation towards these groups of steroids, the products formed in NCI-H295 cells following the incubation with [³H]Preg were analyzed. Control cells transfected with the pcDNA3 empty vector produced high levels of labeled 11-deoxycorticosterone and corticosterone, along with more modest concentrations of Prog, 17-hydroxy-Preg, 11-deoxycortisol and cortisol (Fig. 2A). In cells overexpressing exogenous TReP-132, Preg transformation was significantly increased, indicating the ability of TReP-132 to upregulate steroidogenesis downstream of P450 scc. TReP-132 induced a high production of 11-deoxycortisol, cortisol, DHEA, androstenedione (4-dione), estradiol and estrone and a much lower synthesis of 11-deoxycorticosterone and corticosterone. Together, these results demonstrate that TReP-132 overexpression increases the conversion of Preg to glucocorticoids, C_{19} steroids and estrogens, but not mineralocorticoids. One explanation for this differential production of steroids is an increase in P450c17 activity. Therefore to address this possibility, the effect of TReP-132 on the P450c17-catalyzed conversion of [¹⁴C]Prog to [¹⁴C]4-dione was determined. To simplify the measurement of P450c17 activity, Prog (which is downsteam of 3β-HSDII) was used as a direct substrate. In cells overexpressing TReP-132, the production of [¹⁴C]4-dione was significantly higher over the entire time period tested from 30 min to 18 h (Fig. 2B), which demonstrates an increase in P450c17 activity.

TReP-132 enhances human cytochrome P450c17, but not 3β-HSDII gene expression

Previous reports have indicated that the modulation of steroid levels in chronic response to hormonal stimulation is mainly due to transcriptional regulation of the genes encoding steroidogenic enzymes (Nonaka *et al.* 1992, Simpson & Waterman 1992, Hum & Miller 1993, Parker & Schimmer 1995). Thus, considering that TReP-132 has been identified and characterized for its ability to regulate gene transcription (Gizard *et al.* 2001),

we assessed whether the increased production of glucocorticoids, DHEA, 4-dione and estrogens in the presence of TReP-132 was correlated with a differential transcriptional regulation of the pivotal P450c17 and 3β-HSDII genes. Quantitative RT-PCR analyses demonstrated that the over-expression of TReP-132 in NCI-H295 cells resulted in a 2·2-fold induction of P450c17 mRNA levels, whereas the level of 3β-HSDII transcript was not affected (Fig. 3).
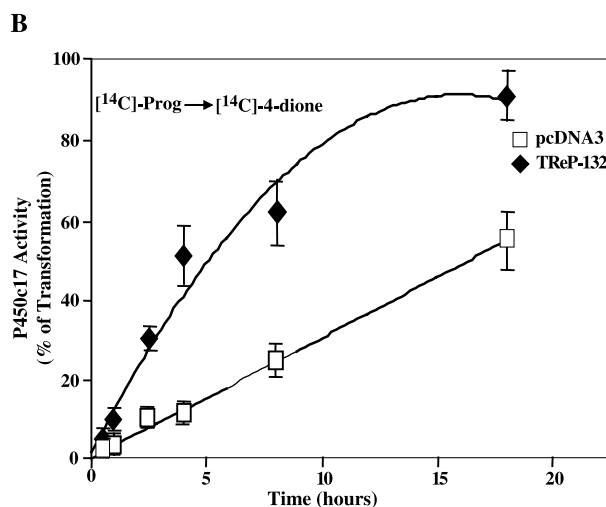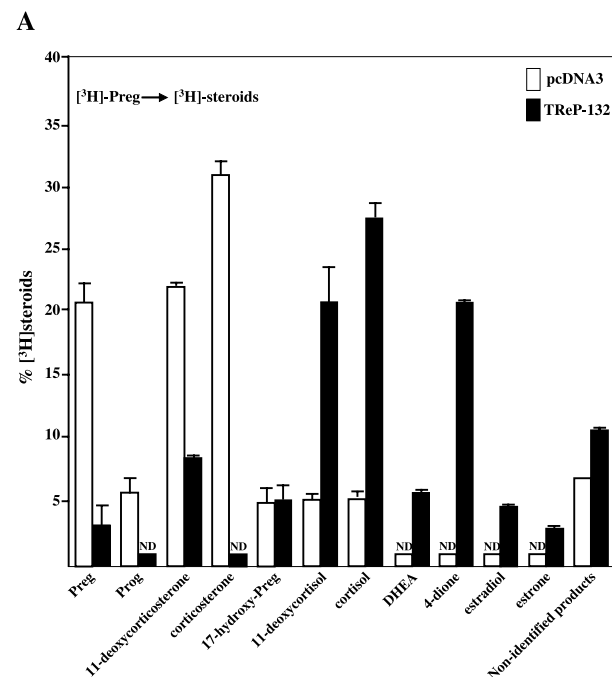
To determine if the increase in P450c17 transcript level was due to the regulation of gene expression, the TReP-132 expression plasmid was cotransfected with a reporter gene construct driven by the P450c17 gene promoter region encompassing nucleotides − 2500 to+63 (− 2500/ +63c17 Luc). The overexpression of exogenous TReP-132 was shown to increase basal promoter activity, thus demonstrating the ability of this factor to act at the level of P450c17 gene expression (Fig. 4).

Considering that P450c17 transcription is up-regulated by adrenocorticotropin (ACTH) acting via the cAMP second messenger pathway in the human adrenocortex and NCI-H295 cells (Zuber *et al.* 1985, Di Blasio *et al.* 1987, Rainey *et al.* 1990, 1993), it was next determined whether TReP-132 can also enhance the cAMP-dependent induction of P450c17 gene promoter activity. As previously reported by Rodriguez *et al.* (1997), incubation of cells with forskolin and 8Br-cAMP only slightly stimulated the − 2500 to +63 P450c17 promoter region in the reporter gene studies. However, the overexpression of TReP-132 significantly enhanced the induction of the promoter obtained with the stimulators of the PKA pathway (Fig. 4A). These data demonstrate the ability of TReP-132 to

Figure 2 TReP-132 induces P450c17 activity in NCI-H295 cells. NCI-H295 cells were transfected with the expression plasmid encoding TReP-132 or the empty vector pcDNA3. (A) Twelve hours following transfection, cells were incubated with [³H]Preg for 6 h and steroid products were separated by TLC. The results are expressed as percentage of [³H]steroid initial, with respect to the ±SD amount of [³H]Preg incubated. The results are an average of two independent experiments performed in duplicate. The majority of the radioactivity noted as 'non-identified products' were not separated during migration and stayed at the application points on the TLC plate, and may correspond to sulfated or glucuronylated steroids. Aldo was not detected in this experiment. ND denotes 'not detected'. (B) Twelve hours following transfection, cells were incubated with [¹⁴C]Prog to assess the 17α-hydroxylase and 17,20-lyase activities of P450c17 in the transfected cells. After incubation for the indicated periods at 30 min and 1, 2·5, 4, 8 and 18 h, the media were collected and labeled products were analyzed on TLC. Results are expressed as percentage of transformation of [¹⁴C]Prog into 4-dione, represented as the average ±SD of two independent experiments performed in triplicate.
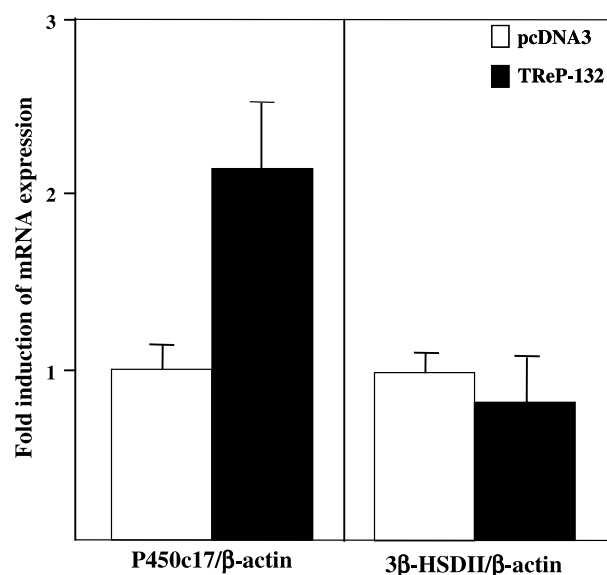
Figure 3 TReP-132 increases P450c17 but not 3β-HSDII mRNA levels. P450c17 and 3β-HSDII mRNA levels from NCI-H295 cells transfected with pcDNA3 or the TReP-132 expression plasmid were measured by quantitative RT-PCR and normalized to β-actin mRNA levels as control. Values are expressed as means of fold induction±S.D. (*n*=3) of mRNA levels relative to the levels obtained in cells transfected with the pcDNA3 control, which were arbitrarily set to 1.

cooperate with the cAMP-dependent pathway to activate the *CYP17* gene promoter.

Since human *CYP17* is a target gene of SF-1 (Hanley *et al.* 2001) and considering that TReP-132 is able to act as a coactivator of SF-1 (Gizard *et al.* 2002*b*) in NCI-H295 cells, we next addressed if TReP-132 influences SF-1 regulation of the human *P450c17* gene. To this end, the − 2500/ +63c17Luc reporter construct, which contains three functional SF-1 binding sites (Hanley *et al.* 2001), was cotransfected with the expression plasmids encoding SF-1 and TReP-132 in NCI-H295 cells. The expression of exogenous SF-1 or TReP-132 alone increased the luciferase activity over the basal value obtained from the reporter construct, and the coexpression of both proteins led to the greatest increase in promoter activity (Fig. 4B). These results indicate therefore a cooperation between SF-1 and TReP-132 to increase *CYP17* promoter activity.

To further demonstrate the relevance of the interaction between TReP-132 and SF-1 in *CYP17* promoter activity, the nuclear receptor box (NR box) LRQLL found at residue 181 of TReP-132

was mutated. This NR box has previously been shown to be required for SF-1 interaction involved in activation of the P450 scc gene promoter (Gizard *et al.* 2002*b*). Similarly to these results, the present study demonstrates that mutation of this sequence in the full-length TReP-132 protein decreases the ability of TReP-132 to activate P450c17 promoter activity in both the presence or absence of exogenous SF-1 (Fig. 4B). These results further suggest the importance of the SF-1 interaction via the LRQLL motif in the function of TReP-132 as activator of steroidogenic gene expression.

TReP-132 modulates differentially the final steps of active steroid hormone synthesis

Following the observation that TReP-132 increases the expression of P450c17 transcript but not that of 3β-HSDII, and that it increases the level of C_{19} steroids and estrogens but not mineralocorticoids, it was next determined if TReP-132 modulates the activities of P450aro and P450c11aldo, which catalyze the last step in the formation of estrogens and Aldo respectively. When NCI-H295 cells were incubated with [^{3}H]corticosterone, with or without the overexpression of TReP-132, there was no significant change in the level of [^{14}C]Aldo, thus indicating that the level of P450c11aldo activity was unchanged by TReP-132 (Fig. 5A). In contrast, although P450aro activity is very weak in NCI-H295 cells transfected with pcDNA3 alone, the transformations of [^{14}C]4-dione into [^{14}C]estrone and of [^{14}C] testosterone (Testo) into [^{14}C]estradiol were significantly upregulated by TReP-132 overexpression (Fig. 5B).

We next analyzed if the ability of TReP-132 to differentially affect steroidogenic steps catalyzed by P450c11aldo and P450aro was correlated with a specific role of TReP-132 at the level of transcript expression encoding these enzymes. To address this question, P450c11aldo and P450aro mRNA levels were determined by quantitative RT-PCR in NCI-H295 cells transfected with the TReP-132 expression plasmid or pcDNA3. The level of P450c11aldo mRNA was only slightly increased following TReP-132 overexpression; however, the level of P450aro transcript was markedly increased by 4·3-fold (Fig. 6). Together these data indicate that TReP-132 can regulate the final steps of adrenal steroid synthesis by differentially

modulating the expression of specific steroidogenic enzymes.

Discussion

Steroid hormones produced by the adult human adrenal cortex are important regulators of many physiological processes, and abnormalities in their synthesis lead to various pathologies of varying degrees of severity (Baxter 1988, Bolander 1994). While mineralocorticoids are major regulators of sodium homeostasis (Booth *et al.* 2002), glucocorticoids are involved in lipid and carbohydrate metabolism, as well as in the modulation of immune and stress responses (Hasselgren 1999, Cavagnini *et al.* 2000, Engelhardt 2000). Mineralocorticoid resistance, known as pseudohypoaldosteronism type 1, and generalized inherited glucocorticoid resistance lead to several consequences such as hyponatremia, hyperkalemia, and metabolic acidosis (Cheek & Perry, Malchoff *et al.* 1990, Lamberts *et al.* 1992, Zennaro 1998). Furthermore, the abnormal production of glucocorticoids or mineralocorticoids results in several disease states, such as Cushing's or Conn's syndrome, respectively (Grell *et al.* 1984, Miyachi 2000), which are in particular characterized clinically by hypertension. As well, C_{19} steroids (mainly 4-dione, DHEA and DHEA-sulfate) play essential roles as precursors of androgen and estrogen synthesis in peripheral tissues (Parker & Schimmer 1995, Labrie *et al.* 2001). An imbalance in C_{19} steroid and sex hormone levels, that occurs for instance with age, is associated with numerous pathologies such as osteoporosis, cancer and deficiencies of the nervous system (Labrie 1992, Kroboth *et al.* 1999, Berga 2001). Of clinical relevance, the numerous studies aimed at finding hormonal therapies have led to marked progress in the prevention and treatment of these endocrine diseases (Labrie *et al.* 1985, Lamberts *et al.* 1992).

Despite accumulated evidence on the abnormal expression of steroidogenic enzymes in several of these diseases (Ogo *et al.* 1991, Shibata *et al.* 1993, Davies *et al.* 2001, Inglis *et al.* 2001), novel
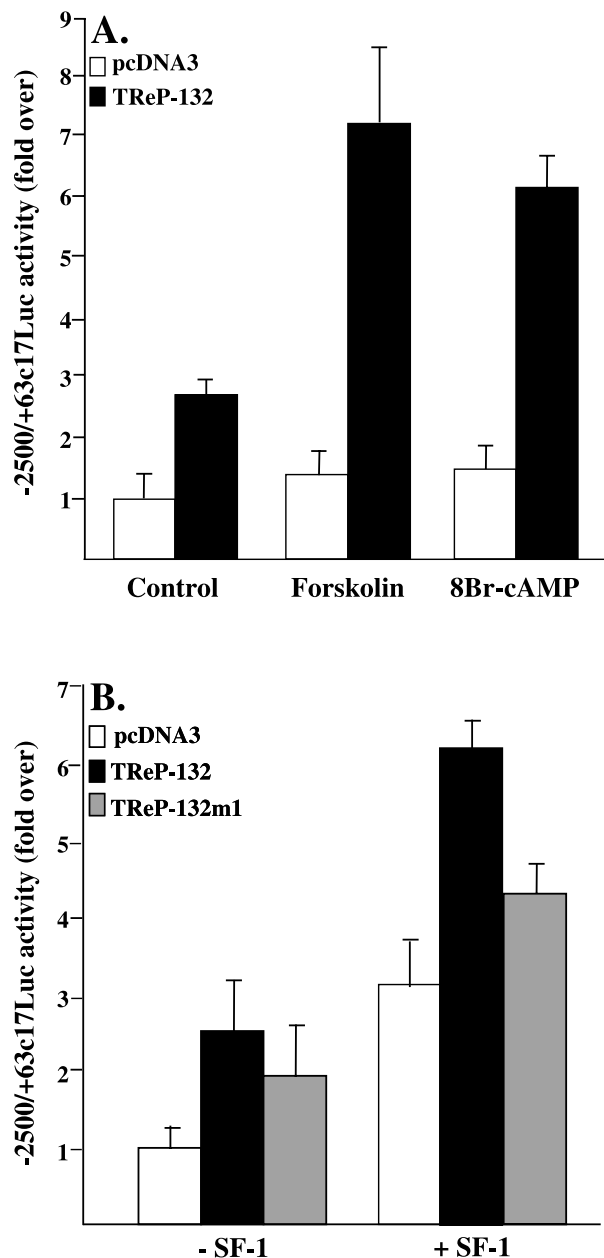
Figure 4 TReP-132 stimulates basal as well cAMP- and SF-1-induced P450c17 promoter activity. NCI-H295 cells were transiently cotransfected with the expression plasmid and the −2500/+63c17 Luc luciferase reporter construct. (A) Twelve hours after transfection, cells cotransfected with pcDNA3 or the TReP-132 expression plasmid were incubated with forskolin (25 μM), 8Br-cAMP (100 μM) or vehicle as control. (B) Cells were cotransfected with SF-1 and/or TReP-132 that was wildtype or mutated at the LRQLL motif (TReP-132 m1). In (A and B) luciferase activities are expressed relative to the value obtained in control cells transfected with the pcDNA3 vector alone, which was arbitrarily set to 1. Transfection experiments were each performed four times in triplicate, and values represent the means±S.D. of a representative experiment.
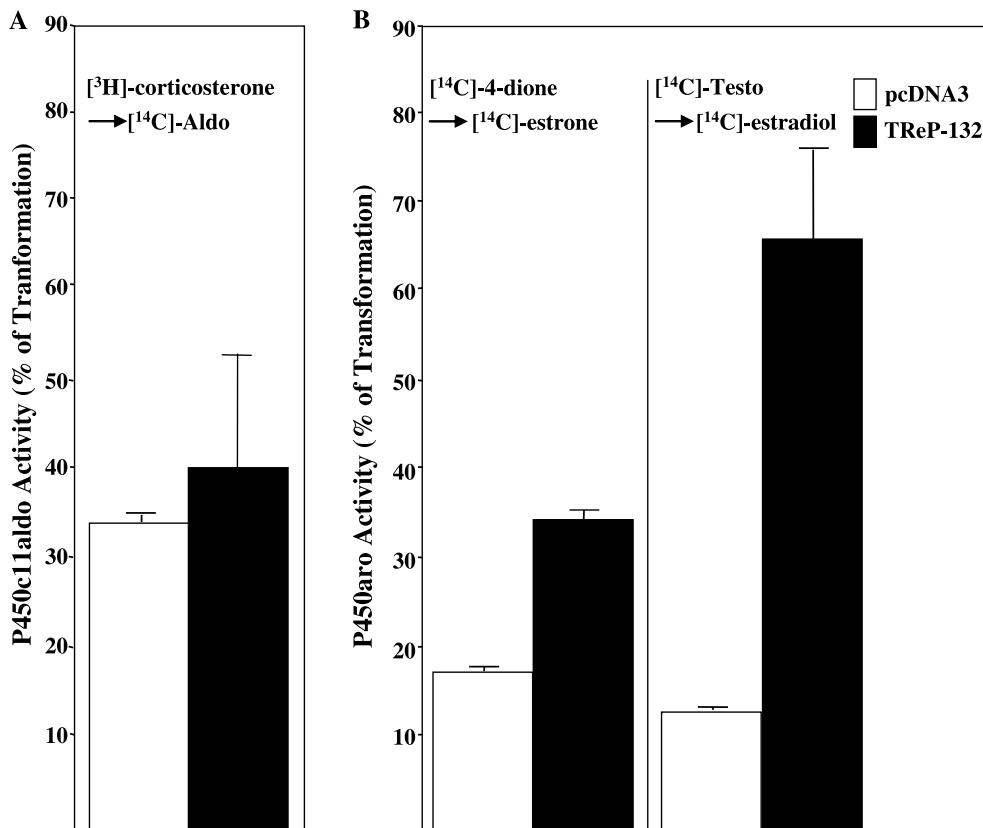
Figure 5 TReP-132 modulates differently the last steroidogenic steps involved in the formation of estrone and estradiol compared with that of Aldo. Cells were transfected with pcDNA3 or the TReP-132 expression plasmid for 12 h. [^{3}H]corticosterone (A) or [^{14}C]4-dione or Testo (B) was then added to fresh serum-free culture medium to determine the activities of P450aro and P450c11aldo in transfected cells. After 4 h, radiolabeled steroids in the media were separated on TLC and quantified. Results are expressed as the percentage of transformation of each labeled substrate, and represent the average of two independent experiments±SD performed in triplicate.

regulatory factors and transcriptional mechanisms modulating steroidogenesis remain to be studied. Thus, it is clear that a thorough understanding of steroidogenesis regulation will require an improved knowledge of the transcription factors involved. In order to address this question, the present study investigated the effect of the transcription factor TReP-132 on several steps of steroidogenesis. Given that this protein has recently been shown to interact with SF-1 and is expressed in different steroidogenic tissues not expressing P450scc (Gizard *et al.* 2001, 2002*b*), we examined whether it regulates the expression of additional steroidogenic genes downstream of P450scc in the steroidogenic pathway. The effect of TReP-132 on the conversion of [^{3}H]Preg into downstream steroid products

was determined in NCI-H295 cells. In fact, TReP-132 overexpression was found to increase the transformation of Preg into glucocorticoids (cortisol and 11-deoxycortisol), C$_{19}$ steroids (4-dione and DHEA) and estrogens (estradiol and estrone), while decreasing the formation of Prog and mineralocorticoids (11-deoxycorticosterone and corticosterone) (Fig. 2A). Together, these data suggest a specific positive effect of TReP-132 on the P450c17-dependent pathway, but not on 3β-HSDII-dependent pathway. In agreement with this, the conversion of Prog to 4-dione was highly increased following TReP-132 overexpression in NCI-H295 cells, which implicates both 17α-hydroxylase and c17–20 lyase activities of the endogenous P450c17 enzyme (Baron *et al.* 1972)
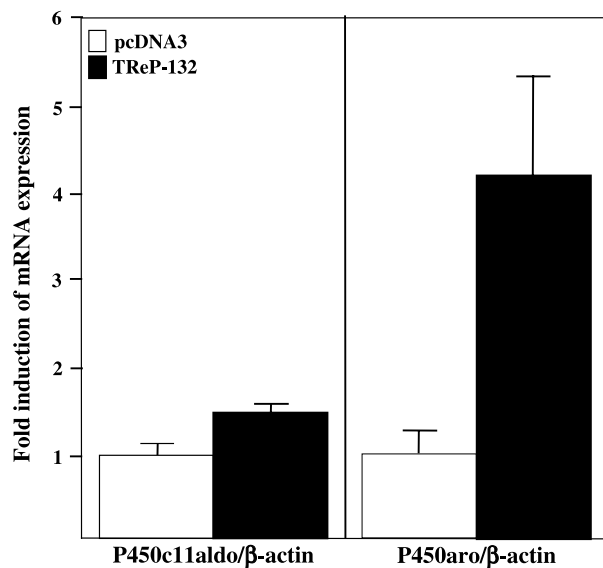
Figure 6 TReP-132 increases P450 aro but not P450c11 aldo mRNA levels. NCI-H295 cells were transfected with pcDNA3 or the TReP-132 expression plasmid. Forty-eight hours after transfection, cells were harvested and RNA extracted. P450c11 aldo and P450 aro mRNA levels were measured by quantitative RT-PCR and normalized to β-actin RNA levels as control. Values are expressed as fold induction of mRNA levels compared with control cells and represent the means±S.D. of three separate experiments performed in triplicate.

(Fig. 2B). Moreover, quantitative RT-PCR analyses demonstrated that TReP-132 induces P450c17, but not 3β-HSDII mRNA levels (Fig. 3). As previously demonstrated for the P450scc promoter (Gizard *et al.* 2001), reporter gene assays demonstrated the ability of TReP-132 to increase expression of a reporter plasmid driven by the 5′-flanking region of the P450c17 gene (Fig. 4).

To further study the role of TReP-132 in steroidogenic gene regulation at steps further downstream of P450c17, we determined whether the factor can also differentially modulate the last steps of active steroid synthesis. TReP-132 was shown to increase the level of P450aro transcript, which is consistent with the observed increase in the conversion of Testo and 4-dione into estradiol and estrone, respectively. However, it had only a minor effect on P450c11aldo mRNA levels and did not modulate P450c11aldo activity (Figs 5 and 6).

In adrenal tissues, steroidogenic genes involved in mineralocorticoid synthesis are induced by

angiotensin II and Ca^{2+} via the protein kinase C (PKC) signaling pathway (Bird *et al.* 1993, 1996, Holland *et al.* 1993, Clyne *et al.* 1997, Pezzi *et al.* 1997) in the zona glomerulosa (Bolander 1994). Glucocorticoids and C_{19} steroid synthesis, in contrast, is specifically activated by ACTH (Rainey *et al.* 1993, Denner *et al.* 1996) via the PKA-dependent pathway in the zonæ fasciculata and reticularis (Mapes *et al.* 1999 Sep). Thus, it is tempting to speculate that TReP-132 is involved specifically in cAMP-responsive gene expression in the zonæ fasciculata and reticularis, considering that TReP-132 regulates P450c17 expression in response to activators of the PKA signaling pathway, but does not regulate P450c11aldo, which is responsive to the PKC pathway. Interestingly, preliminary experiments indicate that the transcript levels of TReP-132 in NCI-H295 cells is also upregulated following 48 h of treatment with activators of the PKA signaling pathway (data not shown). Thus, an increased level of TReP-132 potentially constitutes a mechanism contributing to the ACTH stimulation of genes encoding steroidogenic enzymes.

Taken together, the results in this study demonstrate that TReP-132 acts differentially on several genes of the steroidogenic pathway where it regulates specific genes such as P450scc, P450c17 and P450aro, and not others such as 3β-HSDII and P450c11aldo. These results suggest that the expression of TReP-132 can influence the synthesis of steroids along different pathways, for example by increasing the expression of genes (such as P450c17 and P450aro) required for sex steroid production without regulating the genes (such as 3β-HSDII and P450c11aldo) required for mineralocorticoid production. As a consequence, it is possible that TReP-132 plays a role in the relative regulation of each zone-specific steroidogenic pathway. Adrenocortical development is characterized by the growth and differentiation of the fetal adrenal cortex, which is initially able to produce the three types of steroids (mineralocorticoids, glucocorticoids and sex steroids) (Mesiano & Jaffe 1997). During the development of the definitive zone, which is the precursor of the zona glomerulosa, the levels of 3β-HSDII increase and those of P450c17 decrease. By contrast, the transitional zone formation, which leads to the zonæ fasciculata and reticularis, is associated with increased levels of the P450c17 enzyme (Mesiano & Jaffe 1997). As well, P450c11

aldo and P450aro expression is specific to the zonæ glomerulosa and reticularis, respectively (Malee & Mellon 1991, Ogishima *et al.* 1992). Thus, the ability of TReP-132 to increase transcription of P450c17 and P450aro in contrast to the weak effect on 3β-HSDII and P450c11aldo in NCI-H295 cells, raises the possibility that TReP-132 represents a marker of the zonæ fasciculata and reticularis cell phenotype during fetal adrenal development. However, it is also important to consider that the expression of TReP-132 may directly influence cell differentiation independently of its effects on steroidogenic gene expression. Further studies examining the influence of TReP-132 on steroidogenic capacity, as well as study of the adrenal phenotype of TReP-132 knock-out mice, will be obviously useful to decipher the role of TReP-132 in adrenal development and differentiation.

Previous experiments have demonstrated that TReP-132 can interact with SF-1 to regulate *CYP11A1* gene expression (Gizard *et al.* 2002*b*), and the present study also shows a functional cooperation between both proteins on the *CYP17* promoter. However, it is probable that additional mechanisms are involved in TReP-132-dependent activation of selective steroidogenic genes. To date, several proteins have been shown to interact with SF-1. For instance, Sp1 (Liu & Simpson 1999), c-Jun (Huang *et al.* 2001), DAX-1 (Lalli *et al.* 1998) and CBP/p300 (Monte *et al.* 1998) have each been shown to interact with SF-1 to modulate the *CYP11A1* promoter in a species- and cell-dependent manner. In addition, the expression level of the cofactors DAX-1, COUP-TF and N-CoR, which form a repressor complex of SF-1 activity (Zhang & Mellon 1997, Hanley *et al.* 2001), has been inversely correlated with P450c17 transcript level and steroidogenic potential of various adrenal adenomas (Shibata *et al.* 2000, Hanley *et al.* 2001). By analogy, it is probable that TReP-132 interacts with different proteins in transcriptional complexes with or without SF-1, to activate the expression of specific steroidogenic genes such as *CYP17* and *CYP19*. It is interesting to note that the expression of the 3β-HSDII and P450c11aldo genes, which are regulated by SF-1, are not influenced by TReP-132. Therefore further studies aimed at understanding the transcriptional mechanism(s) by which TReP-132 specifically regulates the expression of different steroidogenic genes will be important to decipher the role of this factor involved in the regulation of adrenal steroidogenesis.

Acknowledgements

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