Asynchronous Rhythm of Steroidogenic Factor 1 and Period Homolog 2 mRNA Expression in Mouse Y1 Adrenocorticol Tumor Cells

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ABSTRACT

The relationship between the expression of Steroidogenic factor 1 (Sf1) and the circadian-related gene, period homolog 2 (Per2), in the adrenal cortex is still unknown. We show here that in Y1 adrenocortical tumor cells, expression of steroidogenic-related genes such as P450scc mRNA and Sf1 mRNA were asynchronous with Per2 mRNA. SF1 promoter analyses showed that the E-box element functions in a rhythmic pattern. Rhythmic expression of Upstream factor 1 mRNA, correlated well with Sf1 mRNA expression. We propose that tumorigenesis of adrenocortical lesions cause disruption of synchronous expression of steroidogenic-related and circadian-related genes.

INTRODUCTION

The primary adrenal form of Cushing's syndrome is characterized by ACTH-independent autonomous cortisol secretion from adrenocortical tumors with diminished diurnal rhythm. The normal adrenal gland produces cortisol, which is stimulated by rhythmic secretion of ACTH from the pituitary gland driven by CRH secretion from parvocellular neurons of the paraventricular nucleus of the hypothalamus. Circadian circuits stimulate suprachiasmatic nucleus (SCN) afferents, which stimulate CRH secretion (7),(36). In addition to the activity of the hypothalamus-pituitary-adrenal (HPA) axis, the peripheral clock of the adrenal gland is known to be activated by light via the suprachiasmatic nucleus-sympathetic nervous system in the absence of ACTH (23). A continuous loss of the rhythmic but robust and autonomous cortisol secretion from adrenal tumors is ACTH-independent. In some cases, ectopic hormone receptors are expressed in the tumors, which aberrantly stimulate cortisol secretion (24). Together with activating mutations of the gene coding for the stimulatory G-protein subunit (*GNAS*) in adrenocorticotropin-independent macronodular adrenocortical hyperplasia (AIMAH) (13) and mutations of protein kinase, cAMP-dependent, regulatory, type I, gene (*PRKAR1A*) in primary pigmented nodular adrenocortical disease (PPNAD), accumulating evidence has endorsed the idea that adrenocortical lesions of Cushing's syndrome harbor abnormalities of the cyclic AMP signaling pathway (35).

An extensively detailed evaluation of cortisol level in patients with adrenocortical lesions of Cushing's syndrome has revealed increased pulsatile secretion and a 3 h delay in phase shift (39). This is reminiscent of the Clock mutant mouse, where the circadian period was 27.3 h in constant darkness (40). It remains unclear whether the autonomous secretion of cortisol by the adrenal tumor is induced by the abnormal rhythm of circadian-related genes. Per2 is an important component of the circadian clock system and participates in both the input and the output pathways of the clock (3). The blunted response to ACTH of steroid secretion in *Per2/Cry1* mutant mice has suggested that the adrenal clock gates the sensitivity to ACTH (30).

Although rate-limiting components of steroidogenesis, such as P450scc, show no transcriptional circadian rhythm (30), a nuclear factor, Sf1/Ad4BP (Nr5a1) is known to respond to light through the suprachiasmatic nucleus-sympathetic nervous system (23).

Sf1 was originally identified as an essential transcription factor that controls the expression of most steroidogenic genes (31). Sf1 has also been revealed to be indispensable for the differentiation and development of the adrenals, gonads, and particular regions of the pituitary gland and hypothalamus region, based on the findings of phenotypes of Sf1 knockout mice (25). Thus the modulation of the expression level or activity of Sf1 in adrenals is essential for steroidogenesis. ACTH or protein kinase A (PKA) have been shown to increase Sf1-mediated transcriptional activity, thus leading to increased steroidogenesis (11). Several mechanisms for this phenomenon have been proposed, including phosphorylation of Sf1 via PKA itself (43) (6) and via its downstream signaling, extracellular signal-regulated kinase (ERK) 1/2 (19) or by a dephosphorylation mechanism via mitogen-activated kinase (MAPK) phosphatase-1 (32) and stimulation of DAX-1 dissociation from Sf1 (32). Interestingly, because cAMP abnormalities have been implicated in the pathogenesis of adrenocortical lesions of Cushing's syndrome (35), the PKA-related increase of Sf1 activity may be associated with the increased cortisol production. The expression level of Sf1 in adrenocortical lesions of Cushing's syndrome has been controversial; some reports show no change in Sf1 (33), but other studies show a moderate elevation (5). In the cultured adrenal cell system, no or a little increase in Sf1 level by PKA stimulation has been demonstrated (28). This controversy may be linked to Sf1 expression with respect to oscillation by circadian rhythm, thus resulting in different levels of Sf1 expression in adrenocortical lesions of Cushing's syndrome. Sf1 promoter is mainly regulated by an E-box element to which Usf1 is known to bind in cell lines (20), leaving the question of whether Usf1 displaces a different factor, (e.g., the E-box element is also a target for circadian-regulated genes) binding the E-box in normal adrenal tissue. This is particularly interesting as the functioning adrenocortical tumor autonomously secretes steroid hormones and the mechanism is not well clarified.

The circadian-related gene, Per2, has been implicated in the development of cancer (14). Downregulation of Per2 has been reported in breast cancer (8; 38; 41), endometrial cancer (34), lymphoma cell lines and acute myeloid leukemia patient samples (17) and chronic myeloid leukemia (42). Overexpression of Per2 induces cancer cell apoptosis (22). Since adrenocortical lesions of Cushing's syndrome show autonomous cortisol secretion, it is of particular interest to investigate whether Per2 is expressed and/or oscillates, and whether it directs the oscillation of steroidogenic enzyme genes in these tumors. To our knowledge, the glioma cell line is the only example to show tumor-related circadian rhythm of Per-2 promoter activity (15).

In the present study, using a well-established activation method of circadian rhythm of cultured cells, namely stimulation by 50% serum shock (4), we tested the rhythmicity of Per2, P450scc and Sf1 in Y1 adrenocortical tumor cells and found asynchronous rhythm of

expression of these genes which was synchronous in normal adrenal tissue.

MATERIALS AND METHODS

Cell culture and Mouse Adrenal Culture

Y1 adrenocortical tumor cells were cultured as previously described (29). For normal mouse adrenal tissue, C57BL/6J (B6) mice were purchased from Charles River (Yokohama, Japan). At all times the animals were treated according to National Institutes of Health guidelines. The normal mouse adrenal gland was dissected from these mice, cut in half (approximately 2 mg), and incubated in culture medium (Dulbecco's Modified Eagle's Medium (DMEM) + 10%(v/v) serum).

Measurement of corticosterone and progesterone level

The corticosterone and progesterone level were determined in collaboration with SRL Co. Ltd. (Tokyo, Japan) using commercial RIA kits (Diagnostic Products Corp., LA, USA)

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and primers

Total RNA was extracted by TRizol reagent (Invitrogen). Primers used for amplifying mRNA were as follows. Mouse Per2 (forward; 5'-CAGACTCATGATGACAGAGG-3', reverse; 5'-GAGATGTACAGGATCTTCCC-3'), mouse P450scc (forward; 5'-AGGACTTTCCCTGCGCT-3', reverse; 5'-GCATCTCGGTAATGTTGG-3'), mouse Sf1 (forward; 5'-CCAGGAGTTCGTCTGTCTC-3', reverse; 5'- GATCCCTAATGCAAGGAG-3') mouse Usf1 (forward; 5'-GAAAACAGCTGAAACCGAAGAGGGAACAGT-3', reverse; 5'-CATAGACTGAGTGGCAGGGTAACCACTGAT-3'). First-strand complementary DNA was synthesized using 1 g of total RNA as the template and PCR was carried out in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Threshold values were obtained, where fluorescent intensity was in the geometric phase of amplification, as determined with LightCycler Software Ver. 3.5. The expression level at each time-point was calibrated to those of -actin (18).

Plasmids

The promoter-luciferase (promoter-luc) constructs of human SF-1 and human P450scc (29) and mouse Per2 (1) are described previously.

Real-time monitoring of luciferase activity

Promoter activity was monitored using an *in vitro* real-time monitoring system (2). In brief, transfection by lipofectamine was performed 24 h prior to serum shock (medium exchanged for

serum-rich medium [DMEM, supplemented with 50%(v/v) serum] for two hours and replaced with normal medium [DMEM + 10%(v/v) serum] thereafter). After serum shock, light emission from the transfected cells, in the presence of 0.1 mM luciferin was measured and integrated for 1 min at intervals of 15 min, with a photomultiplier tube (Hamamatsu Photonics).

RESULTS

Steroid production after serum shock in normal mouse adrenal tissue and Y1 cells.

To determine whether adrenal tumors possess intrinsic circadian rhythm of *de novo* steroid hormone synthesis, tissues and cells were stimulated with 50% serum shock for 2 h and steroid hormones were measured every 4 h thereafter (Fig. 1). Primary culture from normal mice adrenal tissue (approximately 2 mg in 10 ml medium) showed small peaks of corticosterone at 16, 24, 32, 40, 48, 56 h and an overall (circadian-like) tendency of peaks at 32 and 60 h (Fig. 1A). Corticosterone was undetectable in mouse Y1 adrenocorticol tumor cells because these cells do not express steroid 21-hydroxylase (37). Instead, we measured progesterone secretion from Y1 cells and found an almost linear increase of progesterone up to 32 h, which was kept constant for up to 60 h (Fig. 1B). These results correlate well with clinical features, where adrenocortical tumors show autonomous secretion of steroids and normal adrenal tissue does not. Next we sought whether the expression of the circadian-related gene, Per2, was impaired or not in Y1 cells.

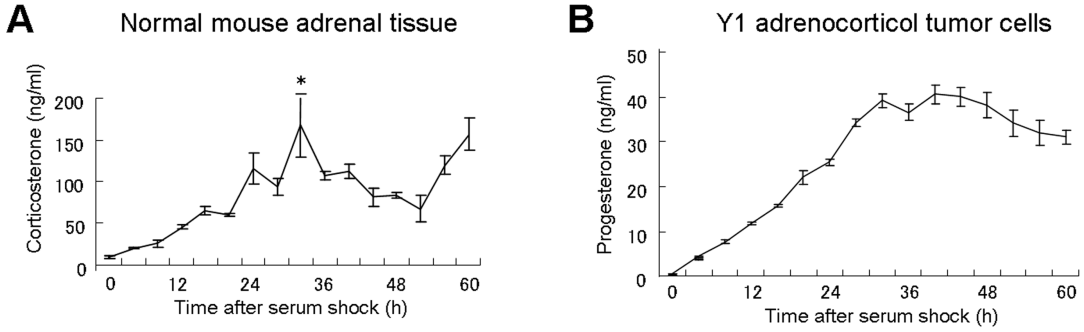

FIG. 1. Steroid secretion from normal mouse adrenal gland and Y1 adrenocortical cells after 50% serum shock. *En bloc* tissue cultures of normal mouse adrenal glands (A) and Y1 cells (B) were stimulated with 50% serum for 2 h, and corticosterone was measured by collecting aliquots of medium every 4 h. Data are the mean ± SEM of three experiments. * represents significant difference from neighboring time points ($p<0.05$).

mRNA expression of Per2, Sf1 and P450scc after serum shock.

Total RNA was extracted from tissue and cells at the time-points at which steroid production was assessed (Fig. 2). Real-time RT-PCR was performed and the relative expression of target gene to -actin was examined. Peak expression of Per2 and Sf1 after serum shock in normal

mouse adrenal tissue were quite variable but synchronous, with peaks in Per2 mRNA and Sf1 mRNA levels at 8 and 20 h (Fig. 2A). On the other hand, Y1 cells showed a different profile. While Per2 mRNA showed a peak expression at 4, 28 and 52 h, with a 24-h interval (Fig. 2B, upper panel), Sf1 mRNA showed peaks at 4, 32, 56 h (Fig. 2B, middle panel), and P450scc mRNA at 12, 36, 52 h (in Fig. 1B, lower panel). With regard to the highest peak for Ad4BP/SF-1 mRNA (32 h) was 4 h later than for P450scc mRNA (28 h). This asynchronous pattern prompted us to test the promoter activity of these three genes in Y1 cells.

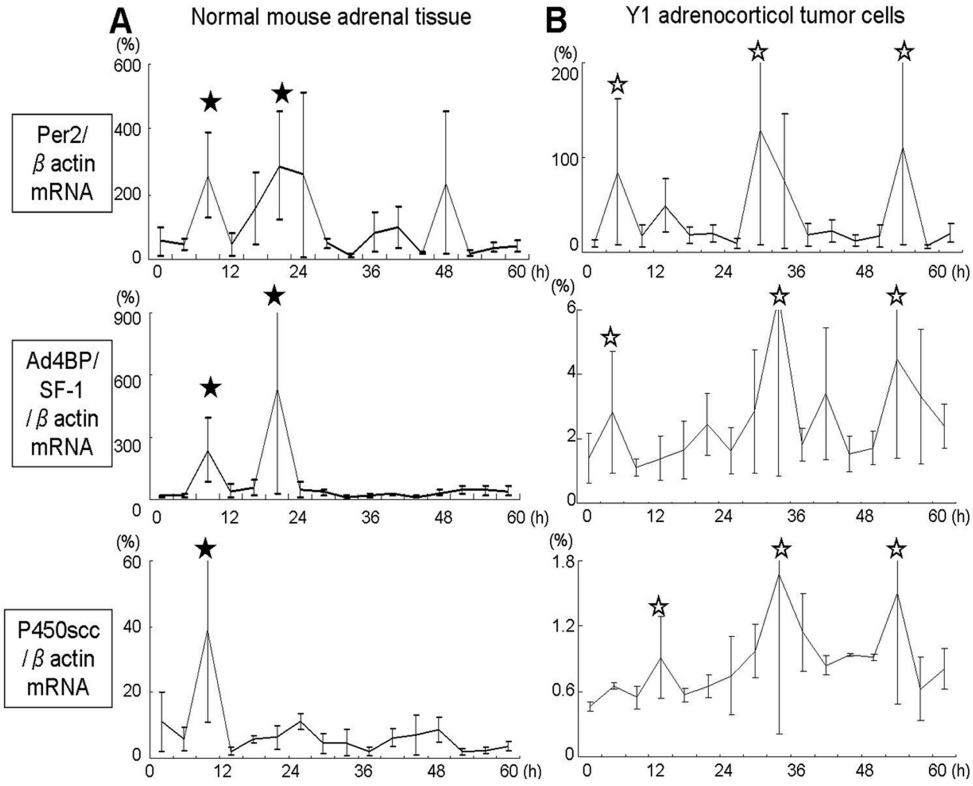

FIG. 2. mRNA expression of Per2, Ad4BP/SF-1, P450scc relative to - actin after serum shock in *en bloc* tissue cultures of normal mouse adrenal glands (A) and Y1 cells (B) were evaluated in triplicates. Total RNA at each time-point in Fig. 1 was extracted and subjected to real-time RT-PCR. Data are the mean ± SEM of three experiments. and indicated where the value was significantly different from time 0 ($p<0.05$).

Per2, SF1 and P450scc promoter activity after serum shock in Y1 cells.

To determine whether the asynchronous mRNA expression of Per2 and Sf1, P450scc in Y1 cells were due to asynchronous promoter activity, Per2-luc, P450scc-luc (–400 bp) and SF-1-luc were transfected and promoter activity was monitored using an *in vitro* real-time luciferase system (Fig. 3). In Y1 cells, Per2-luc showed an interval of 16.5 h, but diminished thereafter (Fig. 3A). P450scc-luc showed a dip in promoter activity, which was restored to the same value after 19.75 h but continued to increase (Fig. 3B). For the SF1 promoter, we used deletion constructs (–

4000, –781, –480, –92, –84 and –77 bp) (Fig. 3C) (29). A clear rhythm, with 14–16-h intervals, was seen in the –92-luc and longer constructs, which disappeared in the –82-luc and –77-luc constructs. An E-box element is known to be located between –87 and –82 bp of the human SF1 promoter, which is the main regulatory element (29) to which USF is known to bind (20). The intervals of the Per-2 promoter activity was slightly longer compared with the SF1 promoter activity (16.5 and 14–16 h, respectively). Though a mutational analyses of the E-box element has not been performed, it is conceivable that promoter activity of Per-2 and Sf1 have different rhythms in Y1 cells. Since elements other than the E-box element seemed not to influence the rhythm seen in the SF1 promoter, we tested the expression of Usf1 mRNA (Fig. 3D) and found that it correlated well with that of Sf1 mRNA (Fig. 2B, middle graph) compared with Per2 mRNA and Sf1 mRNA. Although the relationship between the two variables is not linear, the correlation values are as follows: Sf1 mRNA and Usf1 mRNA, 0.738; Sf1 mRNA and Per2 mRNA, 0.541. This is consistent with our hypothesis that the Usf1 rhythm regulates Sf1 in Y1 cells.

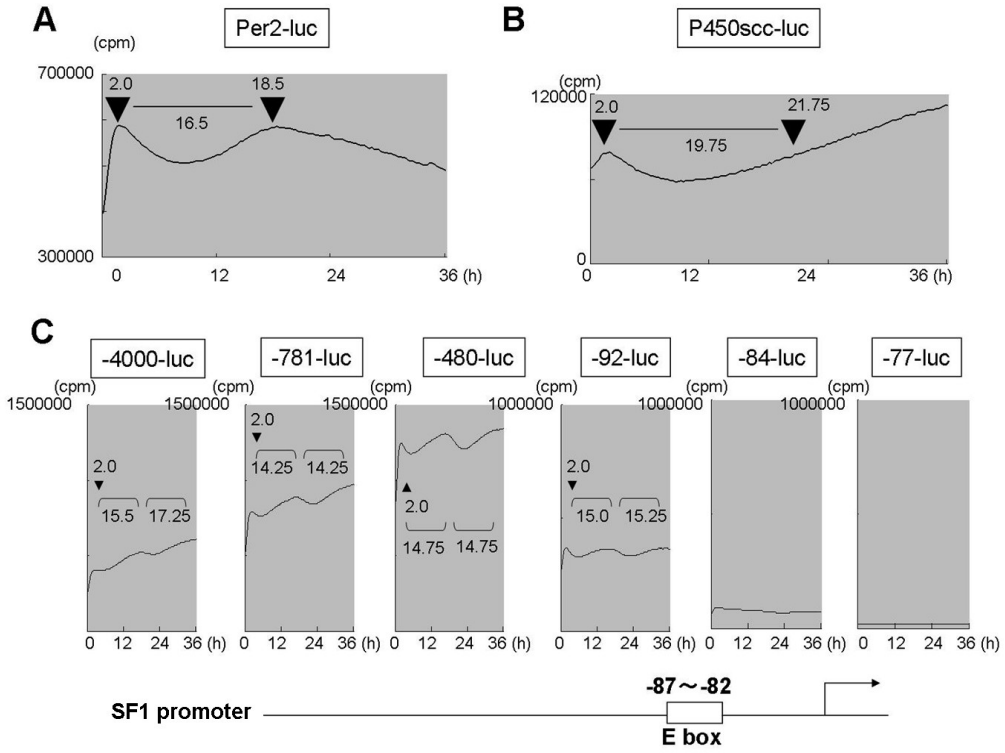

FIG. 3. Luciferase activity of Per2 (A), P450scc (B) and deletions of the Ad4BP/SF-1 (–4000, –781, –480, –92, –84 and –77 bp) (C) promoter after serum shock in Y1 cells and evaluated by real-time luciferase monitoring system. A representative result of four independent experiments is shown. The position of the E-box element is illustrated in a cartoon of SF-1 promoter in the bottom panel. (D) mRNA expression of USF-1 after serum chock in Y1 cells. Total RNA at each time-point in Fig. 1 was extracted and subjected to real-time RT-PCR. Data are the mean ± SEM of three experiments.

DISCUSSION

We have shown here that adrenal tumor-derived cells respond to serum shock, which is known to induce the circadian expression of various genes (4). *Per2* as well as *Sf1* and *P450scc* genes responded to serum shock in adrenal tumor-derived cells, but these cells could not produce steroids in a circadian fashion. The rhythm of Per2 mRNA expression was synchronized with Sf1 mRNA in normal mouse adrenal tissue, but became asynchronous in Y1 tumor cells. This was confirmed by the promoter activity of Per2-luc and SF1-luc in Y1 cells. The similar expression rhythm of Per2 mRNA and Sf1 mRNA in normal adrenal tissue and the similar expression rhythm of Usf1 and Sf1 in the tumor-derived tissue, suggested that the E-box element in the SF1 promoter may be regulated by Clock/Bmal (21) (10) (16) in normal tissue and by Usf1 in tumors. Confirmation of this awaits direct evidence for Clock/Bmal heterodimeric protein to regulate Sf1 in normal adrenal cells.

With respect to the abnormalities of circadian-related genes in adrenocortical lesions of Cushing's syndrome, the identical 3-h shift in cortisol level of patients with Cushing's syndrome (39) and *Clock* mutant mouse (40) led us to assume that an abnormal *Clock* gene functions in these tumors. But because the rhythmicity of per2 mRNA was 24 h in Y1 cells (Fig. 2B upper panel), without a 3-h shift, the existence of a non-functional clock protein (Per2 is regulated by Clock/Bmal) seems unlikely.

The sequence of the E-box of Ad4BP/SF-1 promoter is 5'-(–92)AGAGT**CACGTG**GGGCA(–76)-3' (E-box denoted in bold letters). This is a perfect E-box sequence, which is also known as a site of action for Clock/Bmal. A study of the sequences that surround the E-box has revealed that they are important for the decision of the E-box to function in a circadian or non-circadian manner (27). In this context, the E-box of SF1 promoter is flanked with sequences resembling that for USF binding, RY-CACGTG-RY (R for purine and Y for pyrimidine) (27), which has already been shown in cell lines (20). But because USF factors are ubiquitous, other tissue specific factors have been implicated in the tissue-specific expression of Sf1. For example, in the testis, additional factors, besides USF, have been shown to bind to the E-box of Ad4BP/SF-1 promoter (9). In this respect, it is of interest to test the possibility that Clock/Bmal could bind to this element in normal adrenal tissue.

Adrenal tumors responsible for Cushing's syndrome are usually in benign forms, except that they secrete excess cortisol in an ACTH-independent fashion that can lead to death if left untreated. Recently, Cushing's syndrome caused by ACTH-independent bilateral adrenal tumors does not seem to be as rare as previously thought. In fact, recent studies have shown that rare inherited bilateral adrenal tumors and sporadic solitary adrenal tumors are linked to abnormalities in cAMP signaling (reviewed in (35)).

cAMP signaling and circadian rhythm are close related; for instance, it is well known that

circadian expression of cAMP-inducible factors such as cAMP response element modulator (CREM) (12), which is one of the most highly induced genes in the adrenal gland, is induced by light (23). Ad4BP/SF-1 is also induced by light (23), but is not induced by cAMP. An interaction between Ad4BP/SF-1 (or its post-translationally modified form) and CREB-binding protein seems to be important for coordinated regulation of steroidogenic enzymes by Ad4BP/SF-1 and cAMP (26).

Thus the asynchronous correlation (or maybe just a bystander effect) of cAMP-inducible genes, such as *Ad4BP/SF-1*, *P450scc* and circadian-related gene, *Per2*, in the adrenal tumors of Cushing's syndrome, may be due to a defect of the adrenal circadian clock to respond at the time window of ACTH responsiveness. This gating mechanism has been carefully studied (30), but whether this can be applied to tumorigenesis awaits further investigation into the underlying mechanism. We propose a disruption between cAMP-inducible genes and circadian-related genes may give us a clue to the pathogenesis of the loss of circadian rhythm in these tumors.

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