

# Differential Regulation of Glucocorticoid Synthesis in Murine Intestinal Epithelial *Versus* Adrenocortical Cell Lines

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Glucocorticoids are steroid hormones with important functions in development, immune regulation, and glucose metabolism. The adrenal glands are the predominant source of glucocorticoids; however, there is increasing evidence for extraadrenal glucocorticoid synthesis in thymus, brain, skin, and vascular endothelium. We recently identified intestinal epithelial cells as an important source of glucocorticoids, which regulate the activation of local intestinal immune cells. The molecular regulation of intestinal glucocorticoid synthesis is currently unexplored. In this study we investigated the transcriptional regulation of the steroidogenic enzymes P450 side-chain cleavage enzyme and 11 $\beta$ -hydroxylase, and the production of corticosterone in the murine intestinal epithelial cell line mICcl2 and compared it with that in the adrenocortical cell line Y1. Surprisingly, we observed a reciprocal stimulation pattern in these two cell lines. Elevation of intracel-

lular cAMP induced the expression of steroidogenic enzymes in Y1 cells, whereas it inhibited steroidogenesis in mICcl2 cells. In contrast, phorbol ester induced steroidogenic enzymes in intestinal epithelial cells, which was synergistically enhanced upon transfection of cells with the nuclear receptors steroidogenic factor-1 (NR5A1) and liver receptor homolog-1 (NR5A2). Finally, we observed that basal and liver receptor homolog-1/phorbol ester-induced expression of steroidogenic enzymes in mICcl2 cells was inhibited by the antagonistic nuclear receptor small heterodimer partner. We conclude that the molecular basis of glucocorticoid synthesis in intestinal epithelial cells is distinct from that in adrenal cells, most likely representing an adaptation to the local environment and different requirements. (*Endocrinology* 148: 1445–1453, 2007)

**G**LUCOCORTICOIDS (GC) ARE steroid hormones with important functions in the regulation of metabolism, development, and immune responses (1–3). In particular, their antiinflammatory properties suggest that GC synthesis must be readily turned on and off because too little GCs produced may result in overactivation of immune cells, chronic inflammation, and immunopathology, whereas too much GC synthesis may render the host immunosuppressed and thus incapable of responding to pathogen invasion. The adrenal glands are the major source of GCs and release this hormone upon emotional, physical and immunological stress. The regulation of adrenal GC synthesis has been extensively studied and involves ACTH-induced transcription of a variety of steroidogenic enzymes. In particular, ACTH-mediated induction and activation of steroidogenic factor-1 (SF-1, NR5A1), a transcription factor of the nuclear receptor family, is closely involved in the regulation of adrenal GC synthesis through the induction of a variety of steroidogenic enzymes and cofactors. Consequently, SF-1-deficient mice lack adrenal glands and systemic GC (reviewed in Ref. 2).

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Abbreviations: GC, Glucocorticoid; LRH-1, liver receptor homolog-1; PMA, phorbolmyristate acetate; P450sc, P450 side-chain cleavage enzyme; SF-1, steroidogenic factor-1; SHP, small heterodimer partner.

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Recent years, however, have challenged the idea of the adrenal glands as the only source of GC. Initially described by Ashwell and colleagues (4, 5), it was found that the thymic epithelium can synthesize GCs in a paracrine manner and that thymic GCs contribute to the selection process in immature T cells. More recently skin, endothelium, and brain have been proposed to synthesize GCs (6–10). Our own studies revealed that the intestinal mucosa expresses steroidogenic enzymes and is an important source of GCs (11). Intestinal GCs were found to critically regulate local immune cell homeostasis because in the absence of intestinal GCs, T cell activation is impaired. As the primary source of intestinal GCs, we identified epithelial cells, more specifically cells in the crypt region of the epithelial layer. Interestingly, intestinal GC synthesis was induced by a strong immune response, *e.g.* as elicited by injection of anti-CD3 antibody or viral infection. Thus, most likely inflammatory signals initiate the synthesis of GC in epithelial cells, which in turn have a strong counterregulatory activity on the activation of immune cells.

Whereas the regulation of adrenal GC synthesis has been extensively studied and many of the molecular switches and signaling pathways have been identified, little is currently known regarding the signals that regulate extraadrenal GC synthesis. Circumstantial evidence suggests, however, that intestinal GC synthesis is regulated differently from that in adrenal glands. Whereas SF-1 is abundantly expressed in the

adrenal cortex and is critical for the transcriptional control of steroidogenic enzymes in cortical epithelial cells (2, 12, 13), SF-1 expression is absent in the intestinal mucosa (14). In marked contrast, a close homolog of SF-1, liver receptor homolog-1 (LRH-1, NR5A2), is expressed at high levels in the intestinal epithelium in which it contributes to crypt cell proliferation and epithelial cell renewal through the induction of cyclins D and E (14–16). Consequently, LRH-1 has been implicated in the development of colon carcinomas (16). More recently we found that LRH-1 is critical for the induction of intestinal GC synthesis *in vivo*. Both the expression of steroidogenic enzymes, such as P450 side-chain cleavage enzyme (P450<sub>scc</sub>) and 11 $\beta$ -hydroxylase, and corticosterone synthesis was markedly attenuated in LRH-1 haplodeficient mice (17).

In this study we investigated the differential regulation of steroidogenic enzyme expression and GC synthesis in an adrenocortical cell line (Y1) (18) and an intestinal epithelial cell line with a crypt like phenotype (mICcl2) (19). Surprisingly, we observed a reciprocal induction of steroidogenic enzymes in Y1 *vs.* mICcl2 cells. Whereas an intracellular increase of the second messenger cAMP strongly induced the expression of CYP11A1 (P450<sub>scc</sub>) and CYP11B1 (11 $\beta$ -hydroxylase) in Y1 cells, it reduced basal expression of these two genes in mICcl2 cells. In contrast, phorbol ester had an opposing effect on the expression of these genes in the two cells lines. Interestingly, LRH-1 and phorbol ester synergistically induced steroidogenic enzyme expression and GC synthesis in mICcl2 cells, which was inhibited by cAMP, dominant-negative LRH-1 and the LRH-1 corepressor small heterodimer partner (SHP) (20).

## Materials and Methods

### Cell and reagents

The murine intestinal epithelial cell line mICcl2 (19) and the adrenocortical tumor cell line Y1 (18) have been described previously. The culture medium consisted of DMEM/Ham's F-12 12 g/liter [1:1 (vol/vol)]; Invitrogen, Carlsbad, CA], NaHCO<sub>3</sub> 2.438 g/liter, 2% steroid-free fetal calf serum, 60 nmol/liter sodium selenate, 5  $\mu$ g/ml apo-transferrin, 10 ng/ml murine endothelial growth factor, 1 nmol/liter T<sub>3</sub>, 5  $\mu$ g/ml insulin, 2 mmol/liter L-alanyl-L-glutamine, 20 mmol/liter HEPES, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cell-permeable 8-bromo-cAMP, phorbolmyristate acetate (PMA), and forskolin were obtained from Calbiochem (VWR, Lucerne, Switzerland).

### Plasmids

The expression construct for wild-type murine LRH-1 (21), SF-1 (22), and SHP (21) and the dominant-negative expression vector for murine LRH-1 (DN-LRH-1) (17) have been described previously. The pCMX-SF-1 expression plasmid was kindly provided by Keith Parker (University of Texas, Southwestern, Dallas, TX). The murine CYP11A1 and CYP11B1 reporter constructs were generated by cloning 1.7 kb of the 5'-flanking region of the murine CYP11A1 (accession no. 13070) and CYP11B1 (accession no. NT 082172.1) genes into the HsLuc luciferase reporter construct (23) using *Bam*HI and *Sal*I restriction sites and the following primers: CYP11A1 forward, 5'-GCAAGGATCCCTTCCTCT-CACAATCCTAAG-3', reverse, 5'-GATCGTCGACAGTCTTTAGCC-AGCATAC-3'; CYP11B1 forward, 5'-CTAAGGATCCGTGATTCTCTG-ATGGTCTCT-3', reverse, 5'-CCTGTGCGACTTGTTCATCTTCCTCTCT-3' (17).

The predicted LRH-1/SF-1 core binding sites (AGG TCA) (24) at position -138 and -221 of the CYP11A1 and the CYP11B1 promoter, respectively, were mutated (to taa TCA) by site-directed mutagenesis

using a mutagenesis kit (QuikChange; Stratagene, La Jolla, CA) as previously described (17).

### CYP11A1 and CYP11B1 promoter assay

Wild-type or mutant CYP11A1 or CYP11B1 reporter constructs, and  $\beta$ -galactosidase expression vector for transfection control, were cotransfected into mICcl2 cells using the calcium phosphate precipitation method. Y1 cells were transfected using the Amaxa Nucleofection kit (Amaxa, Cologne, Germany). In some experiments cells were cotransfected with LRH-1 or SF-1 expression vector. After overnight transfection cells were washed, stimulated with indicated stimuli, and cultured for 16 h. Cells were then lysed, and  $\beta$ -galactosidase and luciferase activity assays were performed as described previously (25).

### Detection of CYP11A1, CYP11B1, SF-1, and LRH-1 mRNA by real-time RT-PCR

C57BL/56 mice were injected ip with PBS or 50  $\mu$ g of anti-CD3 for 4 h, and small intestinal tissue and adrenals (from control mice only) were isolated (11). All animal experiments were conducted according to the guidelines of the state of Bern. mICcl2 and Y1 cells were transfected as described above. Total RNA was isolated and cDNA was generated using a Taq man Gold RT kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a Applied Biosystems real-time PCR 7500 machine using SYBR green and the following primers: mCYP11A1 forward, 5'-CCAGCCCAACATTACCGAGAT-3', reverse, 5'-GACTTCAGCCCGCAGCAT-3'; mCyp11B1 forward, 5'-CAATAGAAGCTAGCCACTTTGT-3', reverse 5'-AGGGTGTGGAGGAACCTCAG-3'. For mLRH-1 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase amplification, Quantitec primer assays (QIAGEN, Valencia, CA) were used. Glyceraldehyde-3-phosphate dehydrogenase was used to normalize CYP11A1, CYP11B1 and LRH-1 expression levels. For the detection of SF-1 by real-time PCR an Assay-on-Demand (Applied Biosystems) was used.

### In vivo and ex vivo experiments

In some experiments C56BL/6 mice were injected with PBS control, 6  $\mu$ g ACTH (fragment 1–24; Sigma, St. Louis, MO), or 50  $\mu$ g anti-CD3 antibody ip (11, 17). After 3 h, mice were killed, serum was collected, and small intestinal tissue was cultured *ex vivo* as described previously (11, 17) for 6 h. After that, cell-free supernatant was collected, and corticosterone in serum samples and organ cultures was measured by RIA.

Alternatively, adrenal glands or small intestinal tissue were isolated from C57BL/6 mice and cultured *ex vivo* for 6 h, in the presence or absence of 1 mM cAMP. Cell-free supernatant was harvested and corticosterones were measured by RIA.

### Measurement of corticosterone

mICcl2 cells were transfected and stimulated as indicated. After overnight culture cell-free supernatant was harvested and corticosterone production was measured by RIA (11). The detection limit of the RIA was 5 pg/ml.

### Statistical analysis

In some experiments, differences between groups were analyzed by unpaired two-tailed Student's *t* test. Values of *P* < 0.05 were considered significant.

## Results

### Differential induction CYP11A1 and CYP11B1 expression in Y1 *vs.* mICcl2 cells

ACTH-induced elevation of cAMP is an important trigger of GC synthesis in adrenal cells. Similarly, treatment of cells with cell-permeable 8-bromo-cAMP or forskolin, which induces an increase of cellular cAMP levels, can promote the expression of steroidogenic enzymes and GC synthesis via

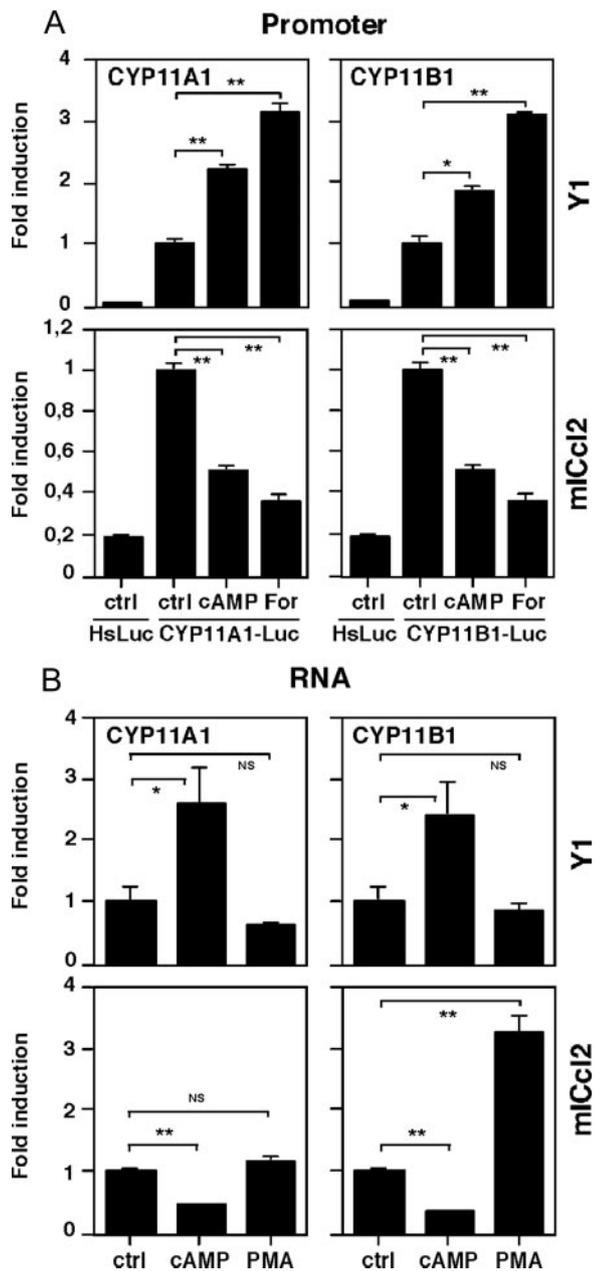


FIG. 1. cAMP- and PMA-induced expression of CYP11A1 and CYP11B1 in Y1 and mICcl2 cells. A, Y1 cells and mICcl2 cells were transfected with either the empty reporter construct (HsLuc) or the CYP11A1 or CYP11B1 luciferase reporter constructs (1  $\mu$ g each). Cells were then treated with medium control (ctrl), cAMP (1 mM), or forskolin (For; 100  $\mu$ M), and induction of luciferase was measured. Mean values  $\pm$  SD of triplicates of a typical experiment of four are shown. Luciferase values were normalized to untreated controls. B, Y1 cells and mICcl2 cells were treated with medium control (ctrl), cAMP (1 mM), or the phorbol ester PMA (25 ng/ml) for 8 h. Induction of CYP11A1 and CYP11B1 mRNA was measured by real-time RT-PCR. Mean values  $\pm$  SD of triplicates of a typical experiment of three are shown. Expression levels were normalized to untreated control cells. NS, Not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ .

the activation of cAMP response elements in the promoter of steroidogenic enzymes (26, 27). To assess whether this pathway is also operative in intestinal epithelial cells, we investigated the cAMP-mediated induction of the genes for

P450<sub>scc</sub> (CYP11A1) and 11 $\beta$ -hydroxylase (CYP11B1), two crucial enzymes in the synthesis of corticosterone from cholesterol. Figure 1A demonstrates that both cAMP and forskolin induced an increase in CYP11A1 and CYP11B1 promoter activity in the adrenocortical tumor cell line Y1. This finding is in agreement with previous reports demonstrating that cAMP can induce the activation of these promoters (18, 26–29). In marked contrast, we observed that elevation of intracellular cAMP failed to enhance CYP11A1 and CYP11B1 promoter activity in intestinal mICcl2 cells but rather led to an inhibition of basal promoter activities (Fig. 1A).

Similar to the results described above, treatment of Y1 cells with 8-bromo-cAMP resulted in increased the expression of endogenous CYP11A1 and CYP11B1 mRNA (Fig. 1B). In mICcl2 cells, however, cAMP caused a reduced expression of these genes. We next examined the effect of phorbol ester and associated protein kinase C activation on CYP11A1 and CYP11B1 expression. As reported previously (18, 29), we failed to detect an increase in gene expression in Y1 cells. Surprisingly, however, we observed that PMA substantially promoted CYP11B1 expression in intestinal mICcl2 cells (Fig. 1B). Thus, cAMP and PMA have opposing effects on the expression of steroidogenic enzymes in adrenal *vs.* intestinal epithelial cells.

#### LRH-1 and SF-1 expression in the intestinal mucosa

The expression of steroidogenic enzymes is critically regulated by members of the nuclear receptor family, in particular SF-1 (NR5A1) and LRH-1 (NR5A2). Interestingly, the expression profile of SF-1 and LRH-1 appears to be mutually exclusive. Whereas SF-1 is abundantly expressed in the adrenals, it is absent in the intestinal epithelium (Fig. 2). In contrast, LRH-1 is preferentially expressed in the intestinal crypt cells, and only minimal levels are observed in the adrenal glands (reviewed in Refs. 2 and 14). This suggests that these nuclear receptors might have distinct functions in the regulation of adrenal and intestinal GC synthesis, respectively.

We initially described GC synthesis in the intestinal epithelium in response to anti-CD3 injection (11). To test whether this strong immune cell activation induces LRH-1 or SF-1 expression, we assessed LRH-1 and SF-1 expression by quantitative real-time PCR. In the intestinal mucosa of con-

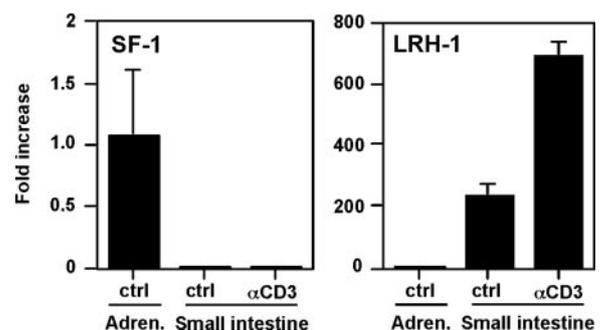


FIG. 2. Expression of SF-1 and LRH-1 in adrenal glands and intestinal mucosa. RNA was isolated from adrenal glands and small intestinal tissue from control and anti-CD3-treated mice, and SF-1 and LRH-1 expression was measured by real-time RT-PCR. Mean values  $\pm$  SD of triplicates are shown. Expression levels were normalized to that in adrenals. \*\*,  $P < 0.005$ .

trol mice, LRH-1 was expressed at levels more than 200-fold higher than those in adrenal glands. In addition, its expression was further induced in the small intestine of anti-CD3-injected mice (Fig. 2). In marked contrast, we failed to observe SF-1 expression in the intestinal mucosa (Fig. 2).

*LRH-1 and SF-1 strongly induce expression of CYP11A1 and CYP11B1 in mICcl2 cells*

To assess whether SF-1 and LRH-1 differentially regulate CYP11A1 and CYP11B1 expression in Y1 and mICcl2 cells, we examined the induction CYP11A1 and CYP11B1 reporter constructs upon ectopic expression of SF-1 and LRH-1. Interestingly, although SF-1 and LRH-1 bind to the same consensus sequence in the promoter region of these two genes (14, 30–33), LRH-1 failed to significantly enhance basal CYP11A1 and CYP11B1 promoter activity in Y1 cells. Similarly, CYP11A1 was only marginally induced by SF-1, whereas the CYP11B1 promoter was more responsive to SF-1 (Fig. 3A). This relatively weak response of Y1 cells to ectopic expression of these two nuclear receptors was also observed when analyzing endogenous CYP11A1 and CYP11B1 mRNA expression (Fig. 3B). No more than a 3-fold increase over basal levels was detected upon transfection with either SF-1 or LRH-1. This low responsiveness of Y1 cells to SF-1 and LRH-1 overexpression may in part be explained by the already very high endogenous SF-1 expression levels (data not shown).

In marked contrast to Y1 cells, both promoters were strongly induced by LRH-1 and even more pronounced by SF-1 overexpression in mICcl2 cells (Fig. 3, A and B). In particular, the CYP11B1 promoter appeared to be highly responsive to LRH-1 and SF-1 overexpression, and up to 7500-fold higher mRNA levels were observed in SF-1-transfected cells (Fig. 3B). This strong increase in CYP11A1 and CYP11B1 transcription was also reflected by substantial corticosterone production in LRH-1- and SF-1-transfected cells, supporting an important role of these nuclear factors in the regulation of GC synthesis.

*SF1 and LRH-1 induce CYP11A1 and CYP11B1 promoter activity via identical response elements*

To identify whether SF-1 and LRH-1 regulate CYP11A1 and CYP11B1 promoter activity in mICcl2 cells via identical binding sites, putative SF-1/LRH-1 binding sequences in the two promoters were mutated and reporter constructs were tested for induction by SF-1 and LRH-1. Figure 4 shows that both wild-type promoters were strongly induced by SF-1 and LRH-1 overexpression, whereas mutant promoters were only minimally or not at all induced. This indicates that SF-1 and LRH-1 use identical binding sites in the CYP11A1 and CYP11B1 promoter.

*Antagonistic and synergistic effects of cAMP and PMA in LRH-1-transfected mICcl2 cells*

The data described above indicated an agonistic role of PMA and LRH-1 and an antagonistic activity of cAMP in steroidogenic enzyme expression in intestinal mICcl2 cells. We thus next examined LRH-1, PMA, and cAMP for syner-

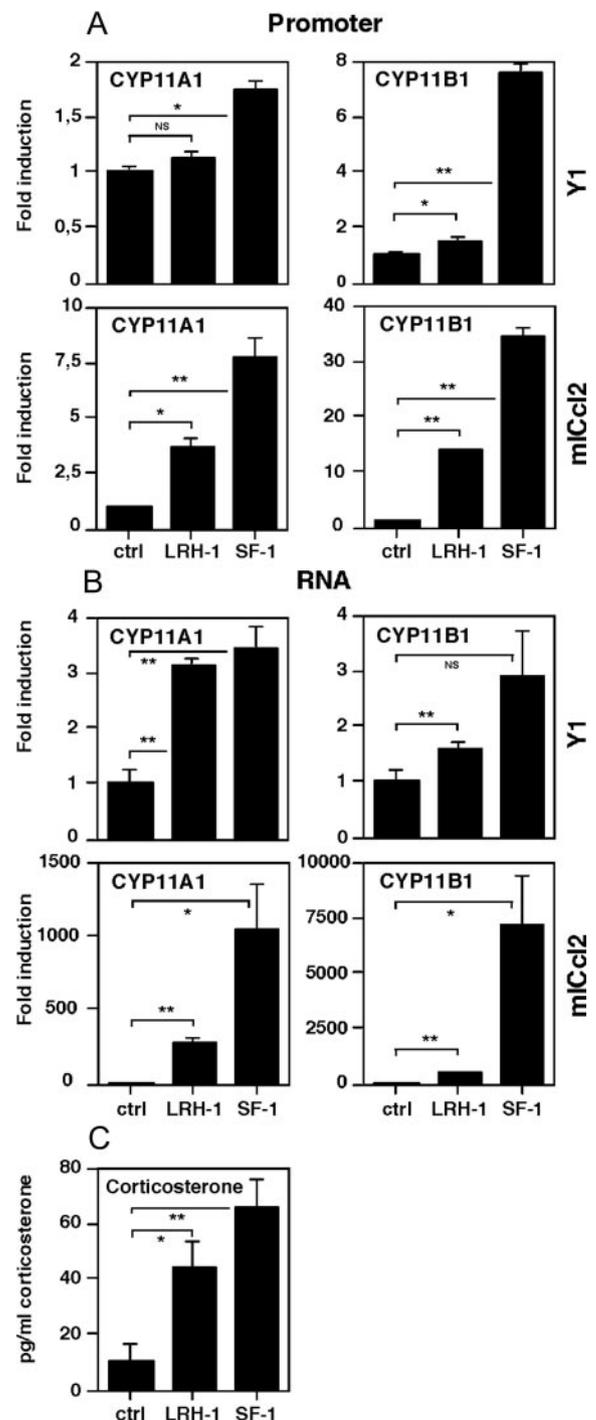


FIG. 3. LRH-1 and SF-1 promote expression of steroidogenic enzymes. A, Y1 cells and mICcl2 cells were cotransfected with CYP11A1 or CYP11B1 reporter constructs (1  $\mu$ g each), and vector control (ctrl), LRH-1 or SF-1 expression vectors (1.5  $\mu$ g each). Luciferase activity was normalized to levels in control vector transfected cells. Mean values of triplicates  $\pm$  SD of a typical experiment of three are shown. B, Y1 cells and mICcl2 cells were transfected with vector control (ctrl), LRH-1, or SF-1 expression vector (1.5  $\mu$ g each) and induction of CYP11A1 or CYP11B1 mRNA was measured by RT-PCR. Expression levels were normalized to that in control transfected cells. Mean values of triplicates  $\pm$  SD of a typical experiment of three are shown. C, mICcl2 cells were transfected as described above and corticosterone production was measured by RIA. Mean values of triplicates  $\pm$  SD of a typical experiment of two are shown. NS, Not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ .

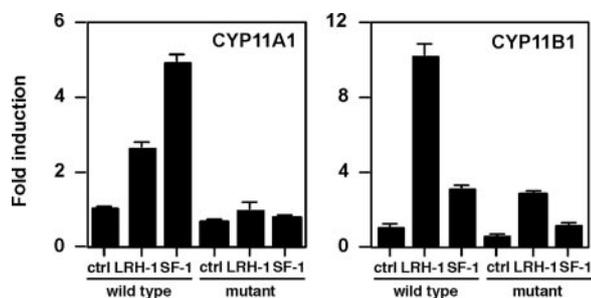


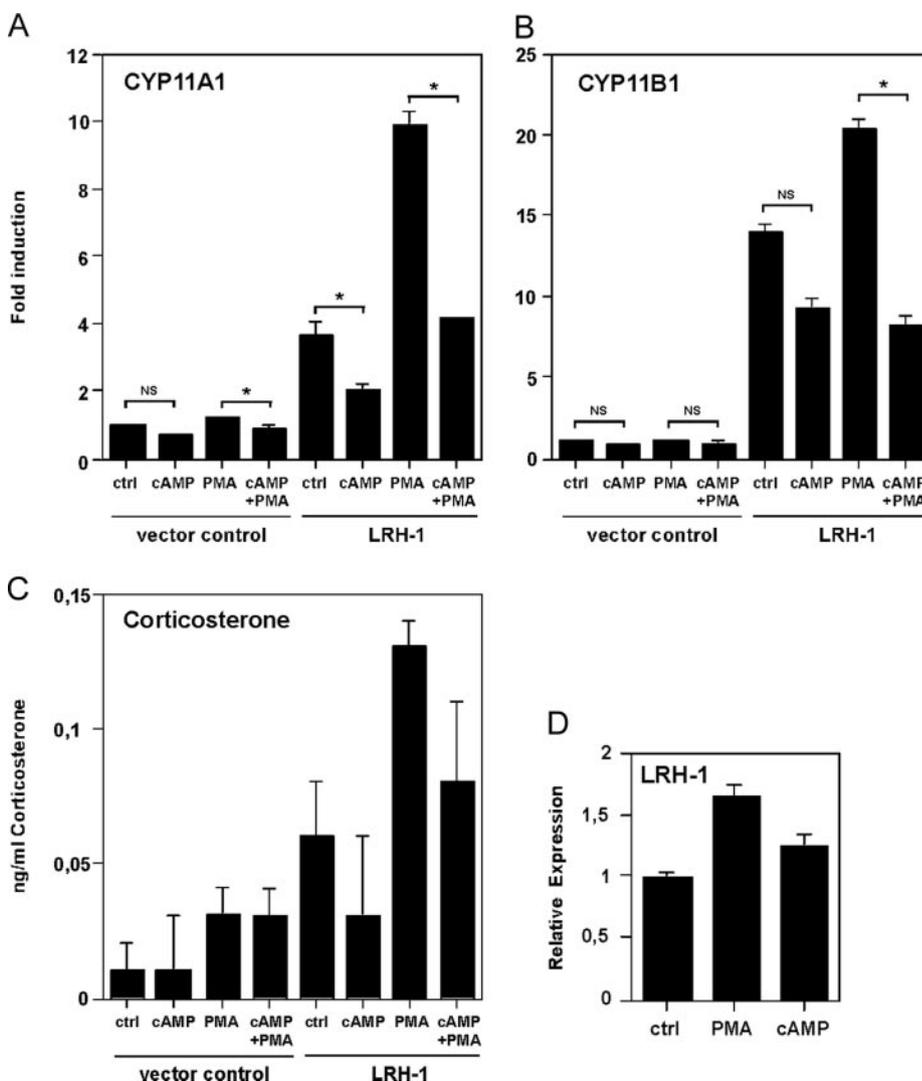
FIG. 4. Mutation of the putative SF-1/LRH-1 response element inhibits SF-1- and LRH-1-mediated CYP11A1 and CYP11B1 promoter activity in mICcl2 cells. mICcl2 cells were cotransfected with wild-type or mutant CYP11A1 or CYP11B1 reporter constructs (1  $\mu$ g each), respectively, and control plasmid, SF-1 or LRH-1 expression plasmid (1  $\mu$ g each). Luciferase activity was measured and normalized to wild-type CYP11A1, respectively CYP11B1 reporter construct alone. Mean values of triplicates  $\pm$  SD of a typical experiment of two are shown.

gistic and antagonistic effects on CYP11A1 and CYP11B1 expression in mICcl2 cells. Figure 5, A and B, illustrates that PMA alone did not increased basal CYP11A1 and CYP11B1 promoter activities, whereas ectopic LRH-1 expression

strongly induced both promoters. Interestingly, when LRH-1-transfected cells were also stimulated with PMA, a strong synergistic induction of both genes was observed. The simultaneous activation of mICcl2 cells by LRH-1 and PMA also resulted in a pronounced and synergistic induction of corticosterone production (Fig. 5C), suggesting that increased steroidogenic enzyme expression results in increased GC synthesis.

We further assessed the effect of cAMP on PMA-stimulated and LRH-1-transfected cells. Treatment of mICcl2 cells with cAMP inhibited not only basal CYP11A1 and CYP11B1 promoter activities but also those induced by PMA stimulation, LRH-1 overexpression, and the synergistic activity of both signals (Fig. 5, A and B). In agreement with an antagonistic role of cAMP in mICcl2 cells, we also observed a profound inhibition of PMA- and LRH-1-induced corticosterone synthesis (Fig. 5C). To exclude that cAMP might inhibit the expression of steroidogenic enzyme expression and GC synthesis by lowering the levels of endogenous LRH-1, we examined the expression of endogenous LRH-1 by real-time PCR in mICcl2 cells after treatment with PMA and cAMP. Figure 5D illustrates that only minimal changes

FIG. 5. cAMP inhibits PMA and LRH-1-induced steroidogenesis in mICcl2 cells. A and B, mICcl2 cells were cotransfected with CYP11A1 (A) or CYP11B1 (B) reporter constructs (1  $\mu$ g each) and control vector or LRH-1 expression vector (1.5  $\mu$ g each). Cells were then stimulated with medium control (ctrl), cAMP (1 mM), PMA (25 ng/ml), or cAMP plus PMA. Luciferase activity was normalized to levels of unstimulated control vector transfected cells. Mean values of triplicates  $\pm$  SD of a typical experiment of two are shown. C, Cells were transfected with control vector or LRH-1 and stimulated with medium control (ctrl), cAMP, PMA, or cAMP plus PMA. Corticosterone production was measured by RIA. Mean values of triplicates  $\pm$  SD of a typical experiment of two are shown. D, mICcl2 cells were treated with medium control (ctrl), PMA, or cAMP and LRH-1 expression levels were measured by RT-PCR. Expression levels were normalized to those in untreated cells. Mean values of triplicates  $\pm$  SD are shown. NS, Not significant. \*,  $P < 0.05$ .



in LRH-1 expression were induced by PMA or cAMP, and rather an increase than a decrease was noted. Thus, the inhibitory activity of cAMP on expression of steroidogenic enzymes and GC synthesis in mICcl2 cells appears to be LRH-1-independent.

#### LRH-1 inhibitors block basal and PMA-induced steroidogenic enzyme expression

Nuclear orphan receptors, such as SF-1 and LRH-1, are regulated by a variety of cofactors and inhibitors. SHP is an atypical nuclear receptor, which lacks a DNA binding domain and therefore transcription factor activity (20). SHP can heterodimerize with LRH-1 and thereby antagonize its transcriptional activity (33–35). We thus examined the role of SHP in basal and induced steroidogenic enzyme expression in mICcl2 cells. Cells were transfected with expression plasmid for dominant-negative LRH-1 or SHP, and CYP11A1 and CYP11B1 activity was monitored. Figure 6, A and B, illustrates that dominant-negative LRH-1 substantially inhibited basal CYP11A1 promoter activity and to a lesser extent CYP11B1 activity. This finding indicates that endogenous levels of LRH-1 may be responsible for basal expression of CYP11A1 and partially of CYP11B1. Interestingly,

ectopic expression of SHP also resulted in a pronounced inhibition of basal CYP11A1 activity. In contrast, CYP11B1 activity was only marginally affected by SHP. These findings indicate that basal CYP11A1 activity in mICcl2 cells is critically regulated by endogenous LRH-1, whereas basal CYP11B1 expression may be additionally regulated by other factors.

We next addressed whether SHP could also inhibit the synergistic activity of LRH-1 and PMA in inducing CYP11A1 promoter activity. Figure 6C demonstrates that simultaneous activation of mICcl2 cells with LRH-1 and PMA resulted in an up to 6-fold induction of basal promoter activity, which was almost completely abolished by SHP. Interestingly, co-expression of dominant-negative LRH-1 inhibited CYP11A1 induction only to a lesser extent. In conclusion, these data illustrate that PMA and LRH-1 can synergistically induce CYP11A1 induction, which is strongly antagonized by the inhibitory nuclear receptor SHP.

#### Intestinal corticosterone synthesis is inhibited by cAMP

To investigate the *in vivo* relevance of our findings, *i.e.* that ACTH and cAMP have the opposite effect on adrenal *vs.* intestinal glucocorticoid synthesis, we analyzed adrenal and intestinal corticosterone synthesis *in vivo* and *in ex vivo* organ cultures. As predicted, injection of ACTH and anti-CD3 antibody resulted in an increase of serum corticosterone levels, indicating that both triggers had been able to induce corticosterone synthesis and its release from the adrenal glands (Fig. 7C). In marked contrast, only anti-CD3 injection, but not ACTH injection, was able to promote corticosterone synthesis in the small intestine (Fig. 7D). In agreement with this finding we further observed that *ex vivo* cultured adrenal glands could be stimulated with cAMP to release corticosterone (Fig. 7A), whereas cAMP treatment resulted in reduced corticosterone synthesis in organ cultures of the small intestine (Fig. 7B). These findings confirm that adrenal and intestinal glucocorticoid synthesis are differentially regulated.

#### Discussion

We recently identified the intestinal epithelium as an important source of extraadrenal GCs in the intestinal mucosa. Interestingly, we observed induction of GC synthesis in response to immune cell stimulation, supporting a role of intestinal GCs as a negative feedback mechanism in the regulation of intestinal immune responses (11). Whereas the enzymatic synthesis pathways of adrenal and intestinal GCs from cholesterol seem to be identical, our present data provide first-time evidence that adrenal and intestinal GC synthesis may be regulated by distinct signaling events and molecules. A most obvious difference is the differential expression of SF-1 and its close homolog LRH-1. SF-1 is abundantly expressed in the adrenal glands and exhibits a critical role in the adrenal GC synthesis. Although we observed that ectopic expression of SF-1 in mICcl2 cells can induce GC synthesis, it is unlikely that this nuclear receptor plays a role in intestinal GC synthesis *in vivo* because it is not expressed in the intestinal epithelium. In marked contrast, LRH-1 is strongly expressed in the crypt region of the intestinal epi-

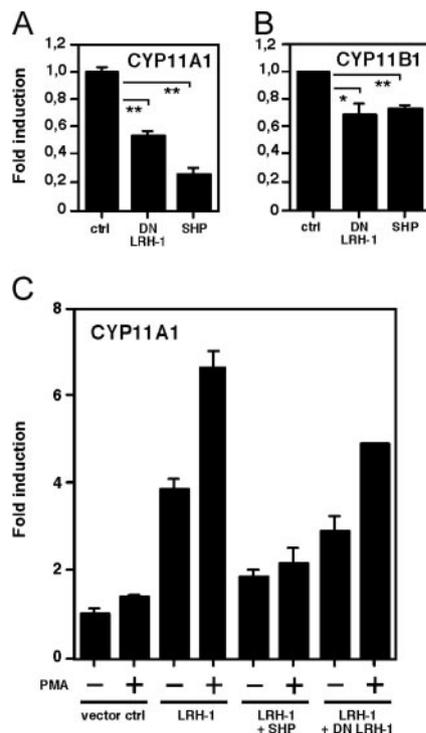


FIG. 6. Inhibition of CYP11A1 and CYP11B1 promoter activity in mICcl2 cells by SHP-1. A and B, mICcl2 cells were cotransfected with the CYP11A1 (A) or CYP11B1 (B) luciferase reporter construct (1  $\mu$ g each) and control vector (ctrl), dominant-negative LRH-1 (DN LRH-1), or SHP-1 (1.5  $\mu$ g each). Luciferase levels were normalized to those measured in control vector transfected cells. C, Cells were cotransfected with the CYP11A1 reporter construct (1  $\mu$ g) and control vector, LRH-1 (0.3  $\mu$ g), LRH-1 plus SHP-1 (1.2  $\mu$ g), or LRH-1 plus dominant-negative LRH-1 (DN LRH-1, 1.2  $\mu$ g). Luciferase levels were normalized to those measured in control vector transfected cells. Mean values of triplicates  $\pm$  SD of a typical experiment of two are shown. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ .

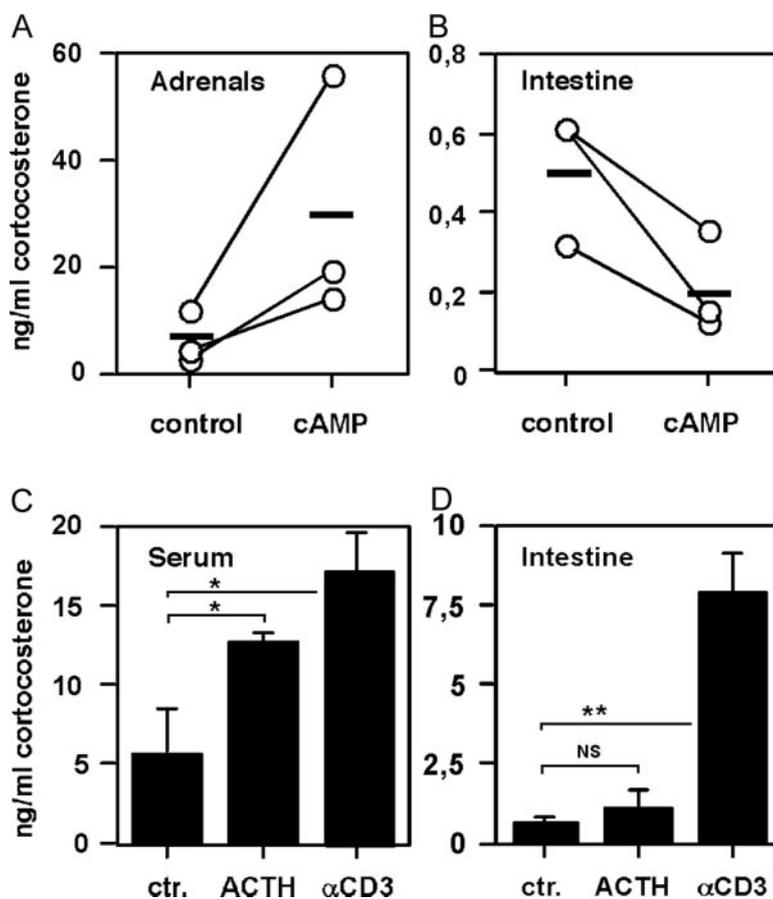


FIG. 7. Differential effects of ACTH and cAMP on adrenal *vs.* intestinal GC synthesis. Adrenal glands (A) or small intestinal tissue (B) from C57BL/6 ( $n = 3$ ) mice was isolated and cultured in the presence or absence of 1 mM cAMP for 6 h. Corticosterone released into the supernatant was measured by RIA. *Bullets* show values of individual samples, *bars* indicate mean values. C and D, C57BL/6 mice were injected with buffer control, ACTH, or anti-CD3 ( $\alpha$ CD3) antibody for 4 h. Serum (C) was collected, and small intestinal tissue (D) was isolated and cultured for 6 h *ex vivo*. Corticosterone in serum and released into the supernatant of organ cultures was measured by RIA ( $n = 3$  per group). NS, Not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ .

thelial layer, in which it promotes cell cycle progression (15, 16). Because we have shown in this study that LRH-1 can promote GC synthesis in mICcl2 cells *in vitro* and LRH-1 and steroidogenic enzymes are coexpressed in crypt cells (11, 15), it is likely that LRH-1 exhibits an important function in the regulation of intestinal GC synthesis. In support of this notion, we recently observed that LRH-1 is critical for immune cell-induced intestinal GC synthesis *in vivo* (17). Interestingly, we noticed that both SF-1 and LRH-1 overexpression only weakly promoted the expression of steroidogenic enzymes in Y1 cells. In part, this may be explained by the already high levels of SF-1 in adrenal cells (2, 36, 37). Thus, SF-1 expression may be saturated and SF-1/LRH-1 response elements in the promoter regions of responsive genes may be occupied.

Another major difference in the regulation of intestinal and adrenal GC synthesis is their differential response to cAMP and PMA. Clearly, cAMP-dependent signaling events have a critical role in the transcriptional control of steroidogenic enzymes in the adrenals (27). Stimulation of adrenals by ACTH causes a rise in cAMP and subsequent protein kinase A activation, which regulates the expression of a variety of steroidogenic enzymes via cAMP response elements in their promoters (38). Intriguingly, we repeatedly observed that in mICcl2 cells cAMP failed to trigger expression of CYP11A1 and CYP11B1 and GC synthesis and rather caused a profound inhibition of both basal and LRH-1-driven steroidogenesis. On the other hand, PMA synergistically in-

duced the expression of steroidogenic enzymes and GC synthesis in mICcl2 cells but not Y1 cells. The reason for this differential stimulation pattern is difficult to reconcile. Because the positive regulators of adrenal and intestinal GC synthesis, *i.e.* SF-1 and LRH-1, respectively, show differential expression profiles in adrenals *vs.* intestinal epithelium, it is likely that also other regulatory cofactors may be differentially expressed or induced in these tissues and associated cells and may thus be differently activated by cAMP and PMA. Similarly, protein kinase A- and protein kinase C-mediated signals may have differential effects on the activation or inhibition of these cofactors in different cell types. It is interesting to note that SF-1 and its inhibitor dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1) on one side and LRH-1 and its inhibitor SHP on the other side show almost mutually exclusive expression patterns (12, 13, 20), suggesting a clear dependency of agonistic and antagonistic nuclear receptor couples in the fine-tuning of tissue-specific GC synthesis. In addition, it has been recently shown that phorbol ester induces ERK-dependent phosphorylation of LRH-1, which leads to increased transcriptional activity (39). In agreement with this notion we observed that PMA only slightly induced LRH-1 levels in mICcl2 cells but strongly synergized with LRH-1 in promoting the expression of CYP11A1 and CYP11B1 (39).

Our data presented here clearly indicate that adrenal GC synthesis, as represented by the established adrenocortical

cell line Y1, and intestinal GC synthesis, represented by the intestinal crypt cell line mICcl2, are differentially regulated. The question remains why GC synthesis in these two steroidogenic organs is controlled by different molecular switches and signaling pathways. Adrenal GC synthesis is rapidly initiated upon physical, emotional and immunological stress in a hypothalamus-pituitary gland-regulated manner, and large quantities are distributed in the body via the circulation. In contrast, there is no evidence for ACTH-regulated GC synthesis in the intestinal epithelium. Elevation of intracellular cAMP, an important second messenger of ACTH receptor signaling, failed to induce expression of steroidogenic enzymes and GC synthesis in mICcl2 cells. In addition, we did not succeed in detecting an increase in glucocorticoid synthesis in the intestinal mucosa after ACTH injection *in vivo* (Fig. 7D and our unpublished data). Current evidence supports the idea that intestinal GC are induced upon activation of local immune cells, act locally, and are not released into the circulation (11). For example, in adrenalectomized animals, intestinal GC synthesis is strongly induced upon activation of T cells by anti-CD3 injection; however, no increase in serum GC is observed (11). Thus, whereas adrenal GCs may be required to regulate strong and systemic immune responses at distant sites, intestinal GC synthesis contributes to the fine-tuning of locally confined immune responses and the maintenance of intestinal immune homeostasis. We conclude that the molecular regulation of intestinal GC synthesis is well adapted to the different local requirements and represents an important tissue-specific immunoregulatory mechanism.

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