Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 4895–4900, May 1997
Biochemistry

Activation of the orphan nuclear receptor steroidogenic factor 1 by oxysterols

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Communicated by Bert W. O'Malley, Baylor College of Medicine, Houston, TX, March 10, 1997 (received for review January 8, 1997)

ABSTRACT **Steroidogenic factor 1 (SF-1), an orphan member of the intracellular receptor superfamily, plays an essential role in the development and function of multiple endocrine organs. It is expressed in all steroidogenic tissues where it regulates the P450 steroidogenic genes to generate physiologically active steroids. Although many of the functions of SF-1 *in vivo* have been defined, an unresolved question is whether a ligand modulates its transcriptional activity. Here, we show that 25-, 26-, or 27-hydroxycholesterol, known suppressors of cholesterol biosynthesis, enhance SF-1-dependent transcriptional activity. This activation is dependent upon the SF-1 activation function domain, and, is specific for SF-1 as several other receptors do not respond to these molecules. The oxysterols activate at concentrations comparable to those previously shown to inhibit cholesterol biosynthesis, and, can be derived from cholesterol by P450c27, an enzyme expressed within steroidogenic tissues. Recent studies have shown that the nuclear receptor LXR also is activated by oxysterols. We demonstrate that different oxysterols differ in their rank order potency for these two receptors, with 25-hydroxycholesterol preferentially activating SF-1 and 22(R)-hydroxycholesterol preferentially activating LXR. These results suggest that specific oxysterols may mediate transcriptional activation via different intracellular receptors. Finally, ligand-dependent transactivation of SF-1 by oxysterols may play an important role in enhancing steroidogenesis *in vivo*.**

Steroidogenic factor 1 (SF-1), a monomer member of the orphan nuclear receptor family, is expressed from the inception of adrenal and gonadal development, and mice deficient in SF-1 lack these organs (1–4). SF-1 also plays an important role in regulating the expression of multiple components of steroidogenesis, including the cytochrome P450 steroid hydroxylases and the steroidogenic acute regulatory protein (5, 6). Finally, SF-1 is important for the expression of several key products of pituitary gonadotropes, including follicle-stimulating hormone, leutinizing hormone, and the receptor for gonadotropin-releasing hormone (7). These results have established a critical role for SF-1 at multiple levels of endocrine development and function. An unresolved question, however, has been whether SF-1-dependent transcriptional activity is modulated by a ligand—either endogenous to steroidogenic cells or supplied in an endocrine fashion. In the present study, we have begun to answer this question by examining several different steroidogenic intermediates or their derivatives for their ability to activate SF-1. Our results demonstrate that certain endogenous oxysterols such as 25-, 26-, or 27-hydroxycholesterol (OHC), known inhibitors of cholesterol biosynthesis (8, 9), selectively enhance SF-1 mediated transcriptional activity. These compounds may be generated *in vivo* through the actions of the enzyme P450c27 (10–12). Furthermore, activation by these molecules is observed at concentrations comparable to those previously shown to suppress cholesterol biosynthesis and suggests that SF-1 is a ligand-activated receptor.

MATERIALS AND METHODS

Transfections. Transient transfections were carried out in CV-1 cells using an SF-1 expression plasmid (pCMV5 SF-1) (4) a reporter plasmid containing the -65 SF-1 binding site from the 21-hydroxylase promoter, the prolactin minimal promoter and a luciferase reporter gene (p-65Luc) (4), and a β-galactosidase (β-gal) expression plasmid as an internal control. Cells were plated in 96-well dishes at a density of 5,000 cells per well and transfected with 20 ng of total DNA per well using calcium phosphate. Cells were incubated overnight with DNA, washed, and treated with the various compounds dissolved in ethanol and added to media containing 10% charcoal-absorbed or cabosil-treated delipidated fetal bovine serum. In all experiments, control cells were treated with an equal amount of solvent. Cells were harvested 24–36 hr later and analyzed for luciferase and β-gal activity. Data are presented as the luciferase values normalized for β-gal activities, and each value represents the mean of six separate wells. Where shown, error bars represent the standard error. All experiments were carried out at least three times with similar results.

Construction of SF-1 Ligand Binding Domain (LBD) Mutants and Green Fluorescent Protein (GFP) Fusion Proteins. The SF-1 LBD mutant was created by converting an *Esp*I/*Bpu*1102 restriction site within the putative SF-1 LBD to an *Eco*RI site. A 1.2-kb *Eco*RI fragment containing the truncated SF-1 cDNA was then cloned into the *Eco*RI site of pCMV5. The deletion was confirmed by sequencing using standard techniques and lacks amino acids 345–473, which includes the putative SF-1 LBD and activation function 2 (AF-2) domain, this plasmid was called SF-1 ΔMUT. The SF-1 AF-2 specific mutation has an altered AF-2 domain in which the amino acids EMLQAKQT are replaced with the amino acids GYHAYR. This construct specifically alters only the SF-1 AF-2 domain, leaving its putative LBD intact and is called SF-1 AF2REPMUT. A *Sac*II/*Bam*HI fragment containing either the entire SF-1 cDNA or truncated SF-1 (see above) was cloned into corresponding sites of the pEGFP-N vector (Clontech) to yield the SF-1/GFP fusion constructs GFPSF1 and GFPSF1ΔMUT, respectively.

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0027-8424/97/944895-6$2.00/0
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Abbreviations: SF-1, steroidogenic factor 1; AF-2, activation function 2; OHC, hydroxycholesterol; β-gal, β-galactosidase; GFP, green fluorescent protein; LBD, ligand binding domain; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor.

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Band-Shift Assay. Baculovirus expressed retinoid X receptor (RXR) was used in a gel-shift assay as previously described (1). Briefly, RXR was incubated with radiolabeled −65 element (5′-GACCTGAAGCAAAGGTCAGAG-3′) in binding buffer (1). The protein-DNA complex was separated from unbound oligonucleotide on a 6% nondenaturing polyacrylamide gel. For competition studies, a 100-fold excess of the various oligos was added in the incubation mixture.

Visualization of GFP or GFPSF-1 Fusion Proteins. Cells were transfected as described above, rinsed twice with PBS and fixed at 24–48 hr after transfection. Fluorescent cells were visualized using a Leica fluorescence microscope. Pictures were printed using a Sony color video printer.

RESULTS

Oxysterols Stimulate SF-1 Activity. To screen for SF-1 activating molecules, we tested several intermediates in the steroidogenic pathway, including cholesterol, for their ability to enhance SF-1 activity in a transient transfection assay using heterologous cells that do not endogenously express SF-1. Surprisingly, the oxysterol 25OHC (10 μM) activated SF-1 function 10-fold (Fig. 1*a*), whereas a number of other compounds, including known steroid hormones and biosynthetic intermediates, were without significant effect (Fig. 1*a*, data not shown). Pregnenolone also activated SF-1 (3–4-fold) but only at the higher concentration of ≈30 μM (Fig. 1 *a* and *b*). We next compared other related compounds with 25OHC for their ability to activate SF-1. Interestingly, a number of these compounds activate SF-1 3–7-fold (Fig. 1*b*). The highest activity, however, is achieved by 25OHC (Fig. 1 *b* and *c*), a potent inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis (9). This oxysterol exists *in vivo* (13–15) and 25-hydroxylase activity has been reported in the adrenal cortex (16). Other oxysterols that activate SF-1 significantly are the two naturally occurring sterols 26(S)OHC and 26(R)OHC (also known as 27OHC) (12, 17, 18) (Fig. 1 *b* and *c*). Both of these can be synthesized from cholesterol through the action of the enzyme P450c27 (26/27 hydroxylase) (12, 18, 19) an enzyme that also is present in steroidogenic tissues (10–12). Additionally, pure preparations of this enzyme can convert cholesterol to 25OHC (18). Modest activation is observed with some other, but not all, naturally occurring oxysterols that serve as intermediates in the biosynthesis of steroids and bile acids (8, 20) (Fig. 1 *b* and *c*). 21-Hydroxypregnenolone, an intermediate in the conversion of pregnenolone to mineralocorticoids, is also a weak activator (Fig. 1 *b* and *c*). Concentrations at which half-maximal activity (EC_{50}) are observed are as follows: 25OHC, 26OHC, and 27OHC, 5 μM; and 21-hydroxypregnenolone, 11

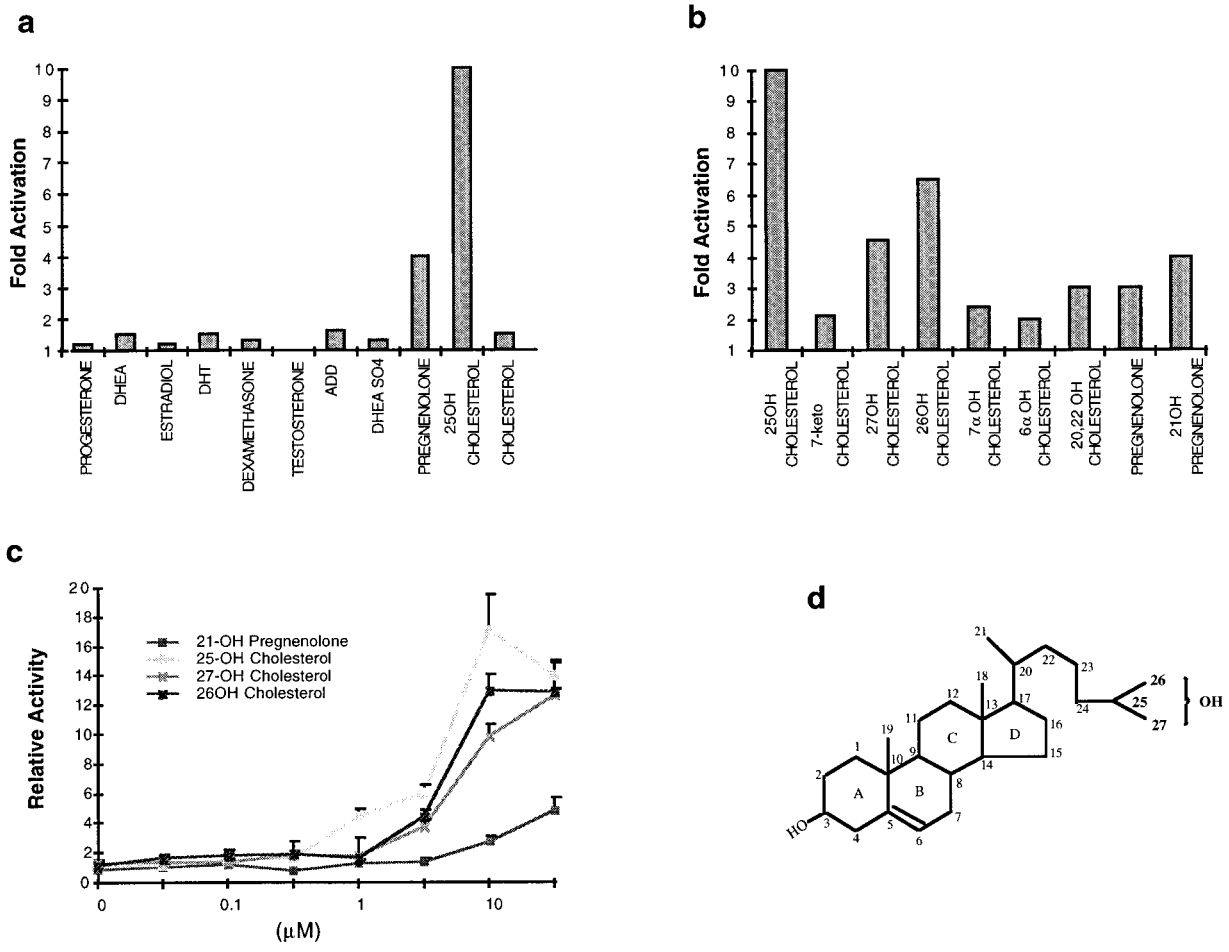

FIG. 1. (*a*) 25OHC (10 μM) specifically activates SF-1. Transfected cells were incubated with the indicated compounds, and the fold-activation was determined. 25OHC (10 μM) markedly activated SF-1, whereas pregnenolone (30 μM) was a weak activator. (*b*) Activation of SF-1 by oxysterols and pregnenolones. 25OHC, 26OHC, and 27OHC activated SF-1 10-, 7-, and 5-fold, respectively at 10 μM. Other oxysterols were weaker activators (2–3-fold) at the same concentration. Pregnenolone and 21-hydroxypregnenolone activated SF-1 3–4-fold at 30 μM. (*c*) Dose response profile of SF-1 activation. Shown are the complete dose response curves of SF-1 with the indicated compounds. The EC_{50} values calculated from these curves for each compound are: 25OHC, 5 μM; 26OHC, 5 μM; 27OHC, 5 μM; and 21-hydroxypregnenolone, 11 μM. (*d*) Structure Activity Relationship. Based on their activities, hydroxyl groups at positions 25, 26, or 27 of the cholesterol molecule appear critical for up-regulating SF-1 function suggesting a specific structure activity relationship.

μM (Fig. 1*c*). These values are well within the postulated physiological concentrations for oxysterols (9, 17, 21, 22). The presence of a hydroxyl group at either the 25 or 26(S) /(R) position appears to be important for activation, as hydroxyl groups at any of the other positions, or cleavage of the cholesterol side chain considerably diminishes activation (Fig. 1 *b* and *c*). This suggests a distinct structure activity relationship for these molecules (Fig. 1*d*), with the following rank order: 25OHC > 26OHC > 27OHC > 21-hydroxypregnenolone.

The Oxysterol Activity Depends on SF-1. To ascertain that the observed oxysterol activity was mediated through SF-1, we examined the ability of 25OHC to augment reporter activity with or without SF-1 in the presence or absence of its binding site. Fig. 2*a* shows that 25OHC is only able to activate in the presence of both SF-1 and its DNA binding element. All ligand-activated nuclear receptors contain an AF-2 domain, a motif essential for ligand-dependent transactivation, that is present at their C terminus (23). Its main features are conserved between all known ligand activated members of the nuclear receptor superfamily including RA receptor, RXR, thyroid hormone receptor, and estrogen receptor. Interestingly, this AF-2 motif is present at the extreme C terminus of SF-1 (24) (Fig. 2*b*). If the oxysterols mediate their activity through SF-1 as ligands, one prediction would be that deletion of its AF-2 domain would lead to a decrease in the response of SF-1 to 25OHC. To test this, we created a mutant that lacked a broad region (amino acids 345–473) of the SF-1 cDNA including its putative LBD and its AF-2 domain (SF-1 ΔMUT, Fig. 2*c*). We compared the activity of the mutant with wild type SF-1 in the presence or absence of 25OHC. As shown, basal activity and inducibility is drastically reduced in the mutant compared with wild-type receptor (Fig. 2*c*), demonstrating that amino acids 345–473 are essential for activation of SF-1

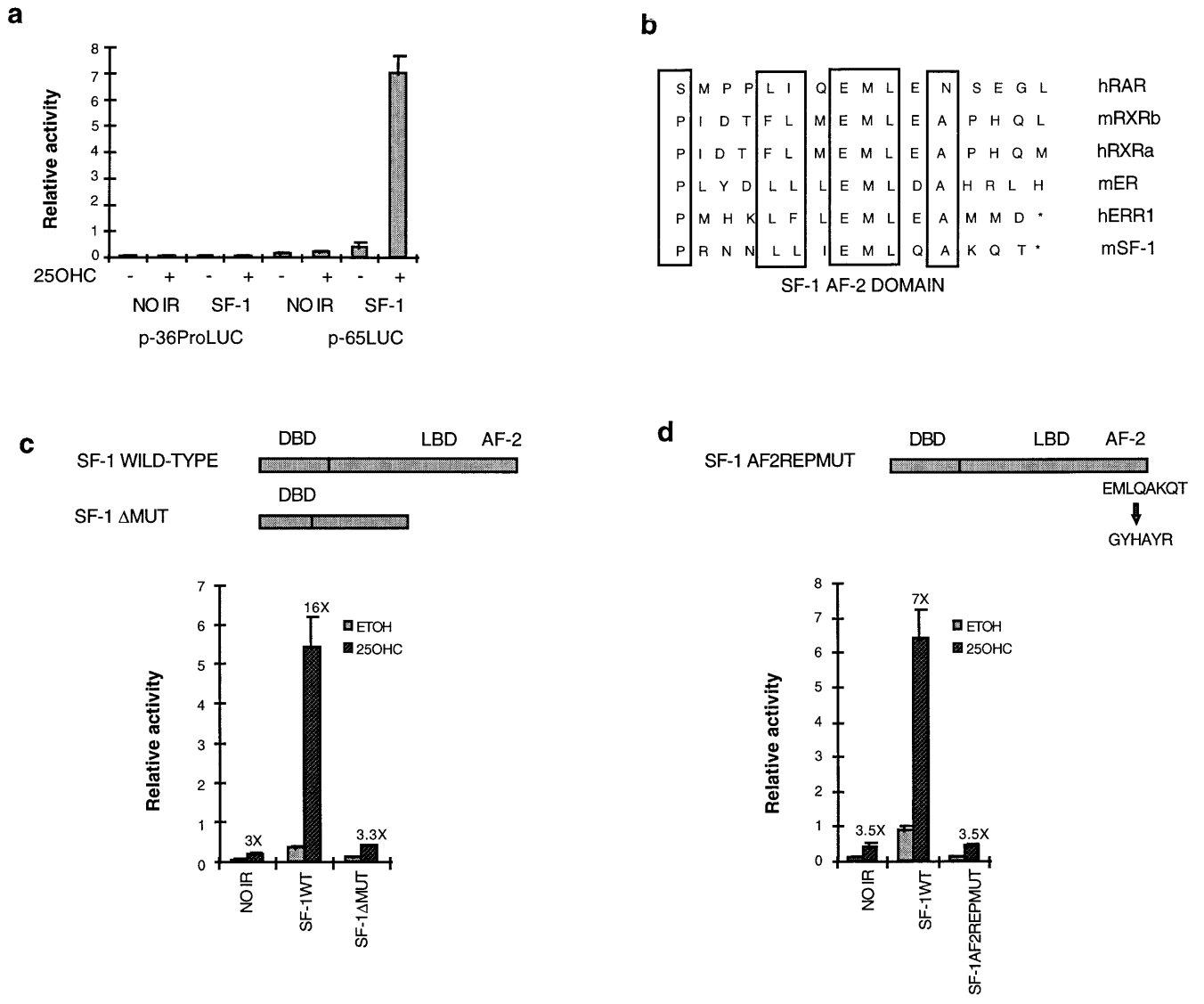

FIG. 2. (*a*) Activation by 25OHC requires both the SF-1 binding site and SF-1. p-36ProLuc (4) is identical to p-65Luc (4) except it lacks the SF-1 binding sites. This plasmid showed no response to SF-1. (*b*) The AF-2 domain of SF-1 is compared with other receptors, with boxes denoting conserved amino acids. (*c*, *Top*) Schematic diagram illustrating the deletion of the putative SF-1 LBD including the AF-2 domain. Construction of the plasmids is described in *Materials and Methods*. (*c, Bottom*) 25OHC activation requires an intact SF-1 ligand binding domain. The SF-1 wild-type (SF-1WT) or SF-1 mutant lacking amino acids 345–473 (SF-1 ΔMUT), were cotransfected with the reporter plasmid as described in *Materials and Methods*. NO IR indicates no SF-1 was added. SF-1 ΔMUT has lower basal activity than wild-type SF-1 and is induced poorly by 25OHC (≈3-fold induction) relative to cells transfected with wild-type SF-1 (≈16-fold induction). (*d*, *Top*) Schematic diagram illustrating a specific AF-2 mutant. (*d, Bottom*) The oxysterol activity requires an intact AF-2 domain. Alteration of amino acids EMLQAKQT to GYHAYR (SF-1 AF2REPMUT) within the SF-1 AF-2 domain leads to a decrease in oxysterol activity (3.5×, similar to the NO IR control) when compared with SF-1 WT (7×). NO IR indicates no SF-1 was added.

by 25OHC. We next determined whether a more specific AF-2 mutant would also lead to reduced 25OHC activation. For this, we created a mutant that replaced the amino acids EMLQAKQT of the SF-1 AF-2 domain with the unrelated amino acids GYHAYR (Fig. 2 *b* and *d*) while keeping its presumed LBD intact (SF-1 AF2REPMUT). As shown (Fig. 2*d*), a decrease in oxysterol induction is observed suggesting that an intact SF-1 AF-2 domain is required for the oxysterol effect. Similar results were obtained with 26OHC (data not shown).

To ensure that the absence of the C-terminal region of SF-1 (SF-1 ΔMUT) did not alter its expression or cellular localization, we prepared fusion proteins linking the GFP with either wild-type SF-1 (GFPSF1) or the large deletion mutant (GFPSF1ΔMUT). Although both wild-type and mutated SF-1 localized to the nucleus (Fig. 3 *a–d*), only the wild-type fusion protein is functionally active and responds to 25OHC (Fig. 3*e*).

Oxysterol Activity Is Mediated Preferentially Through SF-1. To examine whether 25OHC activity was mediated specifically through SF-1, we examined its effect on several other receptors acting through their cognate response elements. As shown (Fig. 4*a*), 25OHC activity is mediated preferentially through SF-1. Next, we sought to examine whether another receptor binding to the same element used in our studies could also be activated by 25OHC. As shown the RXR (23) binds this element (Fig. 4*b*), and importantly, responds to its own ligand 9-*cis* retinoic acid (23) (9-*cis* RA), acting through this element in a cotransfection assay (Fig. 4*c*). As expected, SF-1 does not respond to 9-*cis* RA (Fig. 4*c*). Conversely, RXR responds poorly to 25OHC while SF-1 activity is greatly enhanced (Fig. 4*d*). It is important to note that RXR appears to bind this element as a homodimer and SF-1 and RXR are unable to cooccupy or heterodimerize on this site (data not shown). Therefore, the activity of 25OHC is mediated through SF-1 and requires its AF-2 domain for function, suggesting it is a ligand-activated receptor.

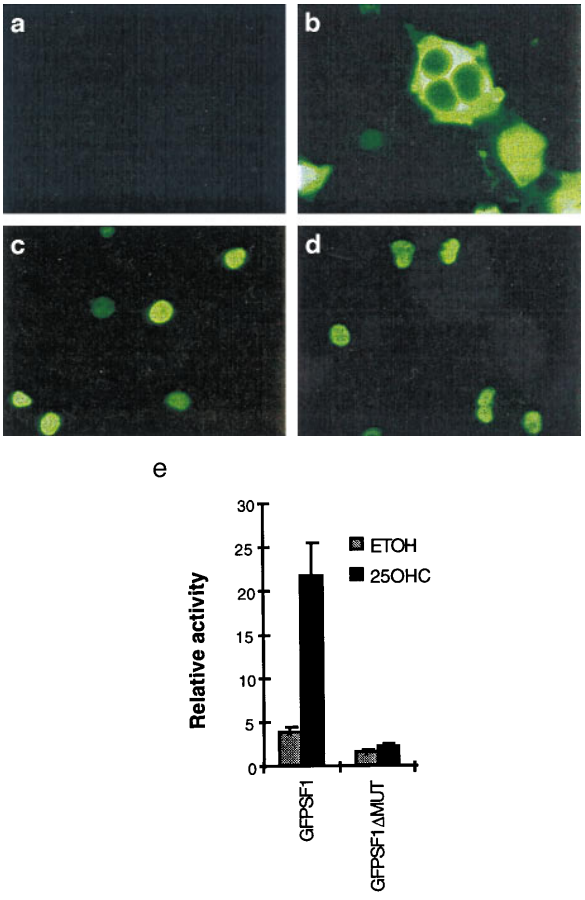

FIG. 3. SF-1 (wild-type) and the deletion mutant are expressed in transfected cells at comparable levels and localize to the nucleus: (*a*) negative control, (*b*) GFP alone, (*c*) wild-type SF-1-GFP fusion (GFPSF1), and (*d*) SF-1 deletion mutant-GFP fusion (GFPSF1ΔMUT), (*e*) The SF-1-GFP fusion protein is functional and responds to 25OHC whereas the mutant is nonresponsive.

The Oxsterols Differentially Activate SF-1 and LXR. Surprisingly, another member of the orphan nuclear hormone receptor superfamily, LXR, has recently been shown to be activated by the oxysterol 22(R)OHC (25). Although SF-1 and LXR show distinct patterns of expression, they also are coexpressed in certain tissues (e.g., adrenal, gonads, and brain (26)). Like SF-1, LXR also is expressed at the earliest stages of organogenesis (26), suggesting that it also may play an important developmental role. Although no target gene or function for LXR has been identified, it potentially may play a role in steroidogenesis and bile acid synthesis (25). It is thus possible that both SF-1 and LXR play coordinate roles in cholesterol metabolism. To look for potential differences in the way SF-1 and LXR respond to this class of compounds, we compared their abilities to respond to the different oxysterols (Fig. 4 *e* and *f*). Interestingly, 25OHC strongly activates SF-1 relative to 22(R)OHC, whereas 22(R)OHC strongly activates LXR relative to 25OHC. Thus, it appears that different oxysterols have distinct preferences for activating the two receptors. Our studies and those of Janowski *et al.* (25) suggest that oxysterols can induce activation of gene transcription through at least two members of the nuclear receptor superfamily.

DISCUSSION

The above studies are the first demonstration that the nuclear receptor SF-1 can be activated by oxysterols and suggest that SF-1 is a ligand activated receptor. This is an important step forward in our understanding of how this important transcription factor functions to promote the development and function of multiple endocrine tissues. An unanswered question, however, is the mechanism by which this occurs. Several mechanisms are plausible. First, oxysterols may act as ligands and bind directly to SF-1. Second, an oxysterol metabolite or oxysterol-induced metabolite may serve as the true ligand. Third, oxysterols may bind and recruit other protein(s) that might interact with SF-1 and activate it indirectly. Another question is whether the oxysterol activation of SF-1 is biologically significant. Oxysterols are involved in maintaining cholesterol homeostasis through two sterol regulated factors, SREBP-1 and -2 (27). In a sterol deficient environment, SREBPs are cleaved to release a transactivation domain, ultimately leading to enhanced cholesterol biosynthesis and uptake from plasma lipoproteins (27). Cholesterol can be converted within the mitochondria to 27OHC, which then down-regulates cholesterol biosynthesis (11, 18). Our results raise the intriguing possibility that, in a sterol rich environment, oxysterols generated through P450c27 or as side products of other enzymatic activities (15, 16) act as signals to increase steroid biosynthesis. This may be accomplished through SF-1, which could then initiate a cascade of events by increasing its own expression (28), as well as that of the cytochrome P450 steroid hydroxylases and steroidogenic acute regulatory protein. Furthermore, steroidogenic acute regulatory protein may also increase P450c27 activity (29), which converts cholesterol to 26(S) and 26(R)OHC and is expressed in a number of tissues including the adrenal glands and gonads (10–12). All of these events could lead to a powerful and novel feed-forward signaling mechanism leading to enhanced steroidogenesis. In conclusion, the demonstration that SF-1 can be activated by oxysterols provides yet another example of gene regulation by intracellular metabolites. Other examples include FXR, a nuclear receptor activated by farnesol deriv-

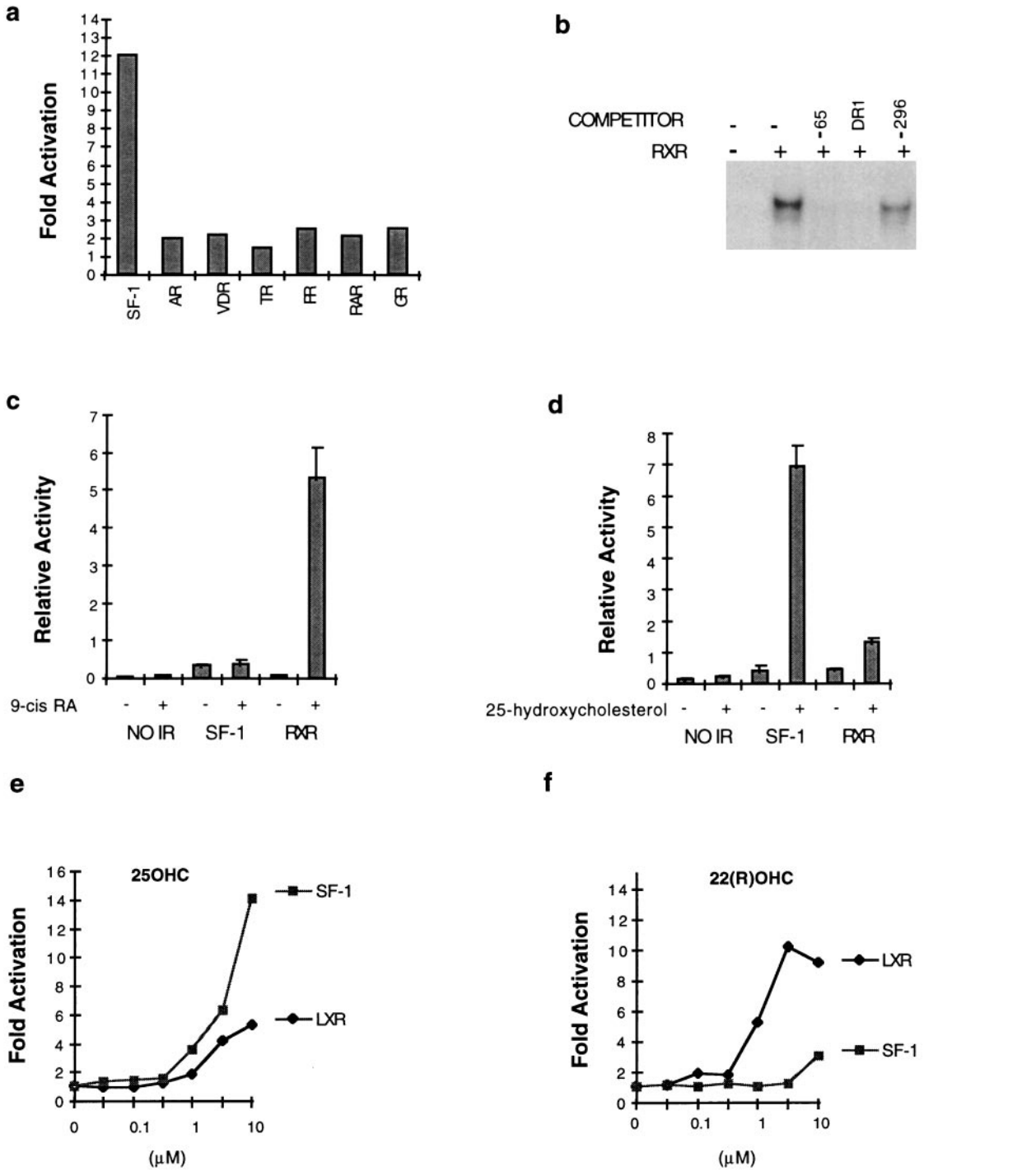

FIG. 4. (*a*) SF-1 is preferentially activated by 25OHC. The indicated nuclear receptors were analyzed in the cotransfection assay with 25OHC. All receptors were tested on their respective response elements, and results are given as fold-activation relative to cells without 25OHC. AR, androgen receptor; TR, thyroid hormone receptor; RAR, RA receptor; PR, progesterone receptor; VDR, vitamin D receptor; and GR, glucocorticoid receptor. (*b*) RXR specifically binds the 21-hydroxylase −65 element. One hundred × excess of unlabeled −65 oligos (1) and a classical RXR binding site (DR-1) abolish RXR binding whereas another SF-1 binding site (−296) (1) is a weaker competitor. (*c*) RXR responds to 9-*cis* (1 μM) whereas SF-1 does not. (*d*) SF-1 activity is enhanced in response to 25OHC (10 μM) whereas RXR responds weakly. (*e*) 25OHC is a strong activator of SF-1 but weak for LXR. (*f*) Conversely, 22(R)OHC is a strong activator of LXR but weak for SF-1.

atives and PPAR, a fatty acid-activated receptor (30). Furthermore, the observation that two members of the nuclear receptor family, SF-1 and LXR, are activated by oxysterols suggests the possibility that different oxysterol molecules may mediate some of their activities through distinct members belonging to a unique class of nuclear receptors.

We thank Drs. Bill Lamph, Ira Schulman, Peter Hornsby, Geoff Rosenfeld, Chris Glass, and Bernard Schimmer for critically reading the manuscript and/or helpful discussion. We thank Edward Kim for the construction of the SF-1 mutant, SF-1 AF2REPMUT.

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