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SREBP-1c mediates the retinoid-dependent increase in fatty acid synthase promoter activity in HepG2

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Abstract Treatment of HepG2 with all-*trans* retinoic acid (RA) induces expression of fatty acid synthase (FAS) mRNA and protein. Transfections show that the FAS promoter positively responds to retinoid X receptor (RXR) but not to RA receptor (RAR) agonists. Since RXR alone is capable of mediating the RA response of FAS, the existence of a classical RA-responsive element in the FAS promoter may be ruled out. Binding sites for NF-Y and SREBP-1 proved to be essential for the RA response. Exposure to all-*trans* RA increased mRNA and protein levels of SREBP-1, a transcriptional activator for FAS. Overexpression of a dominant-negative form of SREBP-1c diminished the RA-dependent increase in promoter activity. These data demonstrate that RXR ligands can stimulate the expression of a lipogenic gene solely by inducing transcription and cleavage of membrane-bound SREBP-1c.

Keywords: Fatty acid synthase; Retinoic acid; Sterol regulatory element-binding protein

1. Introduction

The mRNA level and transcription of fatty acid synthase (FAS), a major lipogenic enzyme, change in response to nutritional signals [1]. Response elements for insulin [2], cAMP [3] and sterols [4] were identified in the FAS promoter. A SREBP/USF-binding element at −65 is crucial for the response of the FAS promoter to diet [4,5]. An upstream NF-Y-binding CAAT-box is a prerequisite for this type of dietary response [4,6].

Retinoids exert their biological effects on development or differentiation [7] via the all-*trans* RA-binding RA receptor (RAR) and the 9-*cis* RA-binding retinoid X receptor (RXR), which influence gene expression by binding with or without their respective ligands to RA-responsive elements. RXRs are capable of activating transcription either as homodimers or as the heterodimeric partner of other nuclear receptors [8]. Two groups [9,10] reported a RXR ligand-dependent increase in triglyceride/fatty acid synthesis due to enhanced expression of the LXR/RXR target gene, SREBP-1c, a well-established activator of lipogenesis.

The goal of this study was to see how retinoids per se affect FAS expression in the human cell line, HepG2. For this purpose, we determined the FAS mRNA/protein levels and defined *cis*-elements responsible for the response of FAS to RA.

2. Materials and methods

2.1. Plasmids

FAS/luciferase plasmids were constructed as described [6]. Mini(−156/−43) contains the −156/−43 region upstream of the FAS minimal promoter(−49/+11)/luciferase fusion. Mutations/deletions were introduced in selected regions as described [6]. The expression plasmids containing the entire coding sequences of hRXRα, hRARβ and hLXRα were cloned into pSVSport1 (Invitrogen) and are referred to as RXRα, RARβ and LXRα, respectively. For monitoring transfection efficiency the β-galactosidase reporter plasmid pRSVlacZI was used.

2.2. Northern blot analysis

RNA from HepG2 cells grown for 24 h in serum-free medium containing all-*trans* RA or vehicle was extracted, electrophoresed and transferred onto nylon membranes. The AlkPhos labelling and detection system (GE Healthcare) was used to detect mRNA signals. Respective RNA and protein signals were quantitated by densitometry.

2.3. Real time RT-PCR

cDNA was synthesized using M-MLV RT (Promega). Samples contained 2 μl Master SYBR Green I reaction mix (Roche Applied Science™), 0.5 μM primers and 40 ng cDNA. qRT-PCR measurements were performed on a LightCycler (Roche Applied Science™). PCR cycles were as follows: 10 min, 95 °C and 40 cycles (15 s, 95 °C; 5 s, 58–63 °C; 20 s, 72 °C). The melting-curve analysis showed the specificity of the amplifications. The threshold cycle, which inversely correlates with the target mRNA level, was measured as the cycle number at which the reporter fluorescent emission appears above the background threshold. The relative mRNA levels were estimated by the standard method using glyceraldehyde-3-phosphate (GAPDH) as reference. Primer sequences will be made available upon request.

2.4. Western blot analysis

HepG2 cells were incubated for 24 h in serum-free medium prior to the addition of various ligands (1 μM) for a further 24 h. To suppress SREBP proteolysis, cholesterol (10 μg/ml) and 25-hydroxycholesterol (1 μg/ml) were added. Nuclear cell extracts were prepared as described

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Abbreviations: FAS, fatty acid synthase; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate; LXR, liver X receptor; NF-Y, nuclear factor-Y; RA, retinoic acid; atRA, all-*trans* retinoic acid; 9cRA, 9-*cis* RA; RAR, RA receptor; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein; TTNPB, 4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid; USF, upstream stimulatory factor

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[10]. Samples were separated by SDS–PAGE and blotted onto a PVDF membrane. Antibodies against SREBP-1 from mouse (BD Biosciences), FAS from rabbit and GAPDH from mouse (Abcam), peroxidase-labelled anti-mouse or anti-rabbit IgG and ECL + Plus™ (GE Healthcare) were used for detection.

2.5. Transfections

HepG2 cells were transfected as described [6]. Each 6-well transfection aliquot received 2 μg reporter plasmid, 0.5 μg pRSVlacZI, 0.5 μg RAR, RXR or LXR expression plasmid. To rule out transfection effects, empty expression plasmid was used for controls. Glycerol-shocked cells were incubated in serum-free medium containing ligand for 36 h.

3. Results

3.1. Effect of all-trans RA on FAS mRNA/protein levels

Since hypervitaminosis A increased triglyceride synthesis in liver [11] and HepG2 cells are able to metabolise vitamin A to retinoic acid [12], we examined the FAS mRNA level in RA-treated HepG2 cells. To exclude side effects caused by growth factors/hormones found in FCS, serum-free medium was used. After 24 h incubation with all-*trans* RA, the FAS mRNA amount was increased up to 3.5-fold in a dose-dependent manner (Fig. 1A). Similarly, FAS protein levels were elevated 3-fold (Fig. 1B). GAPDH mRNA/protein levels did not change after RA treatment and served as controls.

3.2. Effect of retinoids on the FAS promoter

To find out whether FAS was transcriptionally regulated by retinoids, the luciferase construct −2206/+67 was transfected into HepG2 with or without RARβ or RXRα (Fig. 1C and D). When transfected cells were treated with all-*trans* RA, luciferase activity increased 2-fold (Fig. 1C). All-*trans* RA concentrations up to 0.5 μM were not effective (data not shown). Due to isomerization of the RAR ligand, all-*trans* RA, to the RXR ligand, 9-*cis* RA [13], RXR activates transcription after the addition of 9-*cis* RA or high levels of all-*trans* RA, whereas RAR responds to low, more physiological levels of all-*trans* RA. Two RXR agonists, 9-*cis* RA and methoprene acid, increased promoter activity 2.7- and 1.5-fold. The RAR agonist,

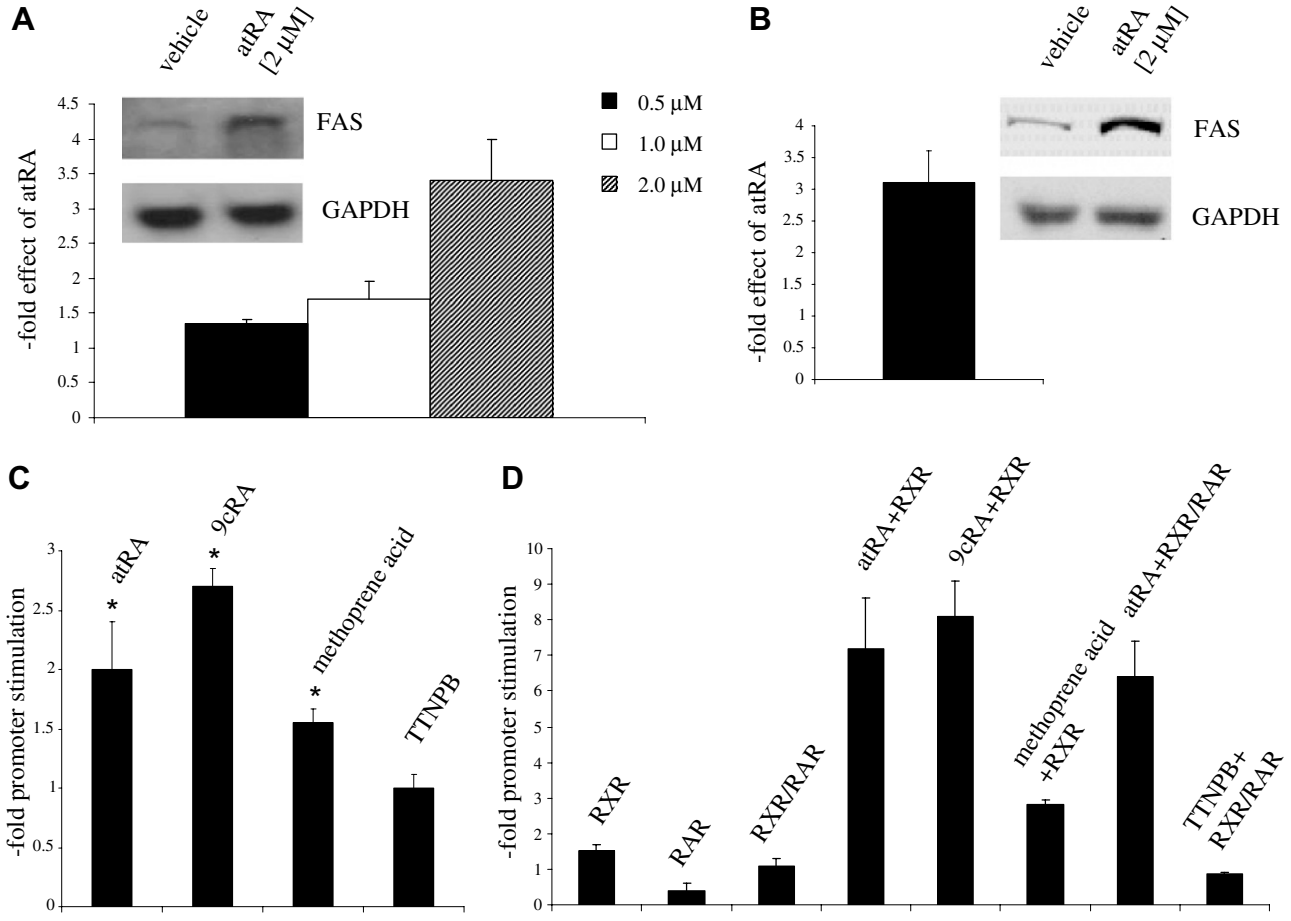

Fig. 1. RA-dependent increase in FAS mRNA, protein and promoter activity. (A) Regulation of FAS mRNA levels by all-*trans* RA (atRA). HepG2 cells were treated with various concentrations of RA. A representative Northern blot is shown. Values (means ± S.D.; $n = 3$) refer to the relative -fold changes in FAS mRNA levels after RA treatment normalized to GAPDH signals. (B) Regulation of FAS protein levels by all-*trans* RA. An immunoblot is depicted. Values ($n = 5$) refer to the relative -fold changes in normalized FAS protein levels after RA treatment. (C) FAS promoter activation by RXR ligands. The construct −2206/+67 was transfected into HepG2 cells treated with 1 μM all-*trans* RA, 1 μM 9-*cis* RA (9cRA), 25 μM methoprene acid, 1 μM TTNPB or vehicle. The -fold promoter stimulation represents the -fold increase in luciferase activity versus the control after normalizing to β-galactosidase values. Data shown are the means ± S.D. ($n = 4$). (D) Promoter activation by RXR and its ligands. −2206/+67 was cotransfected with pSVSport1 (Invitrogen) or the expression plasmids for RXRα and/or RARβ into ligand-treated cells. The -fold promoter stimulation represents the -fold increase in luciferase activity versus the vehicle-treated control ($n = 4$). Statistically significant differences between transfected samples are indicated as $^{*}P < 0.01$.

4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB), had no impact on luciferase activity of HepG2 cells with or without overexpressed RXRα and RARβ, confirming previous results by Tall and colleagues [14] who showed that the livers of TTNPB-fed mice did not show any changes in FAS mRNA expression. Untreated, RXRα-expressing cells showed a slight induction of promoter activity, whereas cells cotransfected with RARβ, but not RA-treated, had an approximately 50% decreased FAS promoter activity (Fig. 1D). Currently, we do not know the reason for this observation. Extracts from HepG2 cells cotransfected with RXRα and incubated with all-*trans* RA or 9-*cis* RA showed a 7- to 8-fold increase in promoter activity. Since neither RARβ, TTNPB, nor physiological concentrations of all-*trans* RA stimulated the FAS promoter, we concluded that all-*trans* RA does not induce the promoter via a RAR/RXR-binding RA-responsive element.

3.3. Identification of a RA-responsive region

To define the RA-responsive region, 5′-deletion/luciferase constructs were cotransfected with a RXRα-expressing plasmid. Deletions from −2206 to −157 gave an approximately average 7-fold induction by all-*trans* RA (Fig. 2A). A sharp drop in stimulation was observed between −157 and −50 implying a fundamental role of this region.

The −156/−43 section contains three major *cis*-elements (Fig. 2B): a SREBP/USF-binding element at −65 [2,4], a CAAT-box at −100 [3,6] and a SRE at −150 [4]. To determine if these elements confer the RA signal to the FAS gene, deletions between −156 and −43 were created (Fig. 2C). RA treatment led to a clear stimulation of luciferase activity in cells with constructs harbouring the deletions, ΔSRE(−151/−141), Δ(−140/−131), Δ(−130/−122) and Δ(−118/−108). In contrast, luciferase activity in HepG2 cells transfected with FAS/luciferase vectors containing a deleted CAAT-box (−99/−83) or

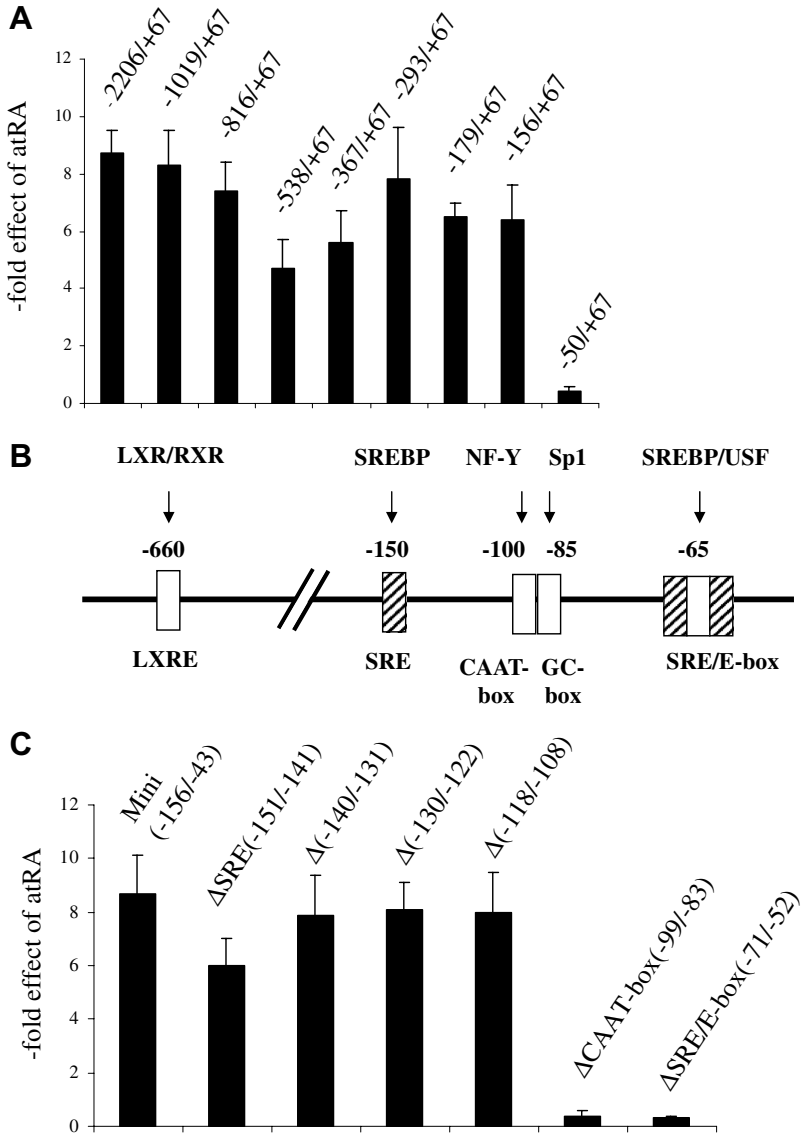

Fig. 2. FAS promoter deletion analysis to define RA-responsive regions. (A) 5′-deletion analysis. Cells transfected with pSVSport1hRXRα and the indicated promoter constructs were incubated in the presence or absence of 1 μM all-*trans* RA (atRA) ($n = 5$). (B) Schematic representation of binding sites in the FAS promoter. (C) Deletion analysis of the −156/−43 region. The effect of RA was analyzed by transfection using deletion mutants of the −156/−43 promoter in the Mini(−156/−43) background ($n = 5$).

SRE/E-box (−71/−52) was barely affected by RA. Although the aforementioned constructs −50/+67, ΔCAAT-box (−99/−83) and ΔSRE/E-box (−71/−52) no longer responded to RA treatment, they still had significant basal promoter activity (4- to 8-fold above the vector-only control), making it unlikely that a severely compromised transcription initiation is the cause of the reduced RA response.

The SRE/E-box (−68/−52) and CAAT-box (−99/−94) were mutated in the context of the −1019/+67 and −816/+67 constructs, respectively. Whereas 9-*cis* RA stimulated the promoter of the transfected wild-type plasmid approximately 5-fold, a construct with the SRE/E-box mutation no longer responded to the stimulus (Fig. 3A). Furthermore, the SRE/E-box mutation had only a minor effect on the promoter activation by the LXR agonist, TO-901317. This is in accordance with a study [15] reporting that a mutated SRE/E-box marginally influenced the upregulation of the FAS promoter by an LXR ligand. Additionally, our data demonstrate that RXR and LXR additively activate the promoter. The mutated CAAT-box abolished the RA response of the promoter but had no effect on its responsiveness to LXR (Fig. 3B). Although using two different plasmid backgrounds for the mutations, these data clearly underline the importance of the two regulatory elements, SRE/E-box and CAAT-box, in their synergistic activation of the RA-induced promoter, a scenario not unexpected in light of our [6] and others' work [4] on the regulation of FAS by SREBP-1 and NF-Y.

3.4. The role of SREBP-1 in the RA regulation of the FAS promoter

SREBP-1a, SREBP-1c and SREBP-2 are inserted into the ER membrane as precursors and following cleavage their N-termini enter into the nucleus where they act as transcription factors [16]. Interestingly, RXR agonists induced SREBP-1 expression in mouse [9] and HepG2 cells [10]. However, the latter study was performed with 10% FCS, which may interfere with the RA treatment. To see whether all-*trans* RA would affect expression of SREBP-1 and/or SREBP-2 under our experimental conditions, we quantified SREBP mRNA levels by qPCR (Fig. 4A). In agreement with the aforementioned studies, RA stimulation caused an 8-fold increase in SREBP-1c mRNA. Furthermore, SREBP-1a and SREBP-2 mRNA levels are increased 2-fold. Immunoblots with antiserum recognizing SREBP-1a and SREBP-1c revealed a 3- to 5-fold increase in both the membrane-bound and nuclear SREBP-1 (Fig. 4B) following RA treatment and reflect the increase of SREBP-1c mRNA. For control purposes, cells were incubated with TO-901317, which led to a several-fold increase in both SREBP-1 forms. Addition of sterols to the medium could not prevent the 9-*cis* RA-dependent increase in processed SREBP-1. These

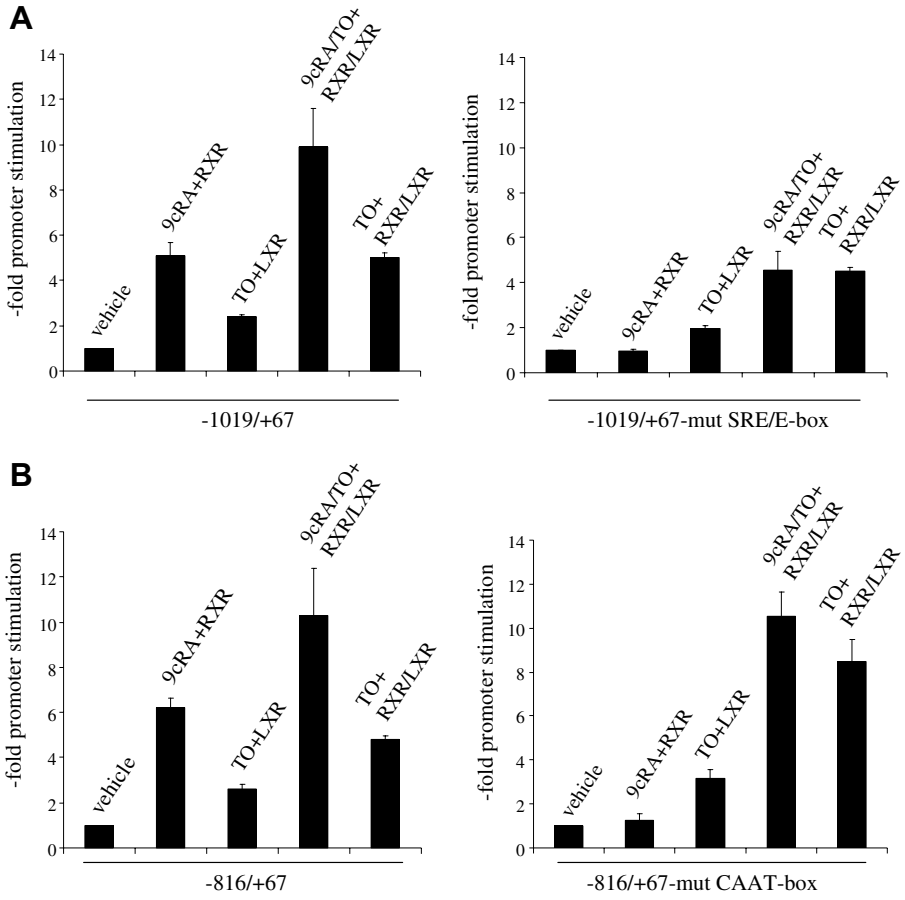

Fig. 3. SRE/E-box and CAAT-box mediate the RA response. (A) Point mutation of the SRE/E-box (mut SRE/E-box). HepG2 cells were co-transfected with the construct, −1019/+67 or −1019/+67-mutSRE/E-box, and vectors expressing RXR and/or LXR. Cells were treated with vehicle, 1 μM 9-*cis* RA and/or 1 μM TO-901317. For each panel, the -fold promoter stimulation represents the -fold increase in luciferase activity versus the vehicle-treated control after normalization ($n = 4$). (B) Point mutation of the CAAT-box (mut CAAT-box).

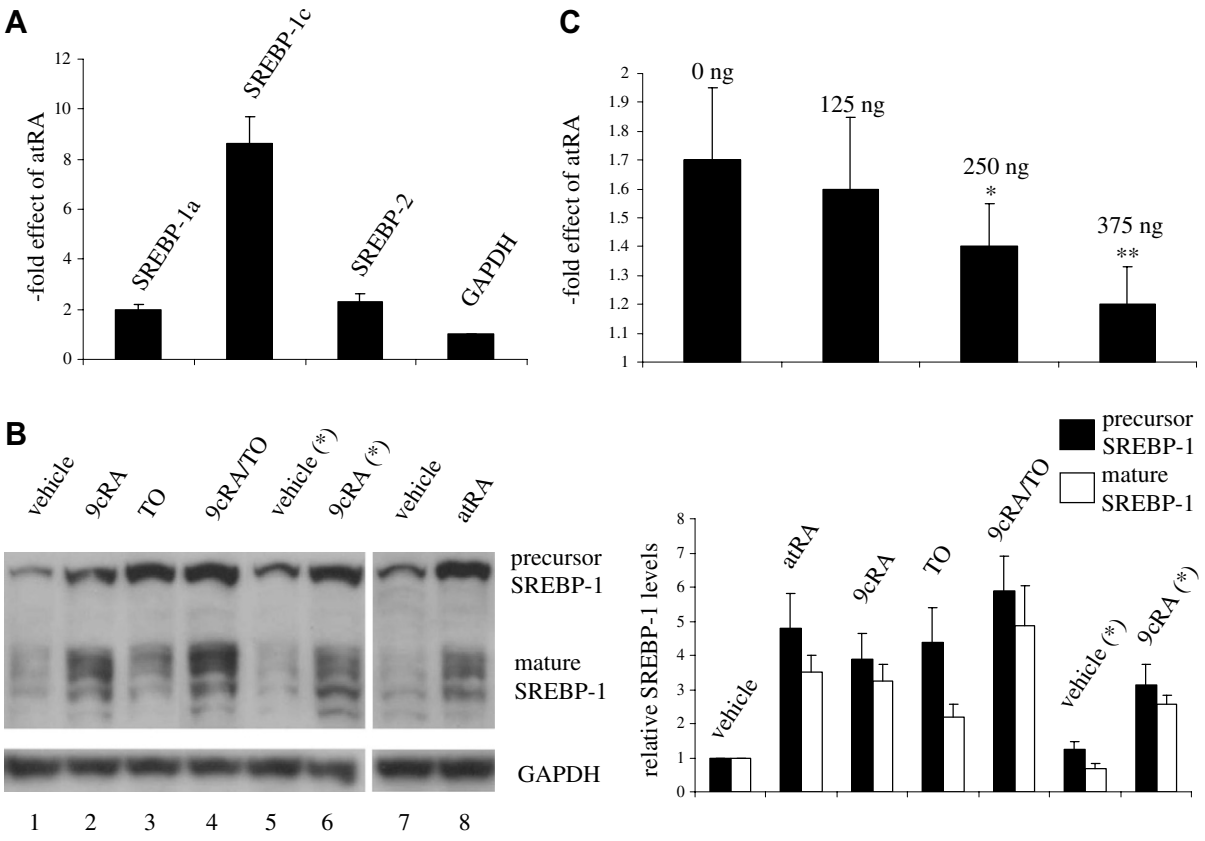

Fig. 4. Involvement of SREBP-1c in the RA-dependent upregulation of FAS promoter activity. (A) Regulation of SREBP mRNA levels by all-*trans* RA (atRA). Respective mRNA levels were quantified by qPCR. Values ($n = 3$) refer to the relative -fold changes in SREBP and GAPDH mRNA levels after 2 μM all-*trans* RA treatment. (B) Immunoblot analysis of SREBP-1 protein levels after treatment with RXR and LXR ligands. *Left panel*: immunoblot. HepG2 cells were treated with vehicle, 1 μM 9-*cis* RA (9cRA), all-*trans* RA (atRA), TO-901317 (TO), or 9-*cis* RA/TO-901317 (9cRA/TO). Under sterol-suppressed conditions (*), cells were treated with vehicle or 1 μM 9-*cis* RA. SREBP-1 antiserum detected precursor and mature forms of SREBP-1a and SREBP-1c. The various bands of mature SREBP-1 are probably due to post-translational modifications [8]. *Right panel:* quantitated SREBP-1 forms were normalized to GAPDH signals ($n = 4$). (C) RA-dependent increase in promoter activity is attenuated by coexpression of a dominant-negative (DN) form of SREBP-1c. Increasing amounts of DN-SREBP-1c were cotransfected with the reporter plasmid −156/+67 into RA-treated HepG2 ($n = 3$). Statistically significant differences between transfected samples are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$.

findings imply a RXR-dependent, direct effect of retinoids on SREBP-1 processing in HepG2 cells and are in accordance with a previous study demonstrating SREBP-1, but not SREBP-2, processing in liver and intestine of mice fed diets containing the synthetic RXR ligand, LG268 [9]. Increased levels of nuclear SREBP-1 could, in turn, account for the upregulation of FAS expression.

To clarify the involvement of SREBP-1c in the RA response, a dominant-negative (DN) form of SREBP-1c was used: a point mutation was introduced into the N-terminal fragment of SREBP-1c (amino acids 1–403), replacing tyrosine at amino acid 320 by alanine. The resulting protein no longer binds to SRE/E-boxes but is still able to dimerize resulting in decreased availability of endogenous SREBP-1c [17]. Cotransfection with the construct −157/+67 and the DN-SREBP-1c mutant diminished RA stimulation of the promoter up to 30% in a dose-dependent manner (Fig. 4C) corroborating SREBP-1c as a mediator of the RA-induced stimulation of FAS expression.

4. Discussion

The RA response appears to be mediated by two elements in the proximal FAS promoter: the NF-Y-binding CAAT-box (−100) and the SREBP/USF-binding SRE/E-box (−65). The NF-Y-binding site is required for the sterol regulation of the FAS promoter in cell lines and carbohydrate activation in primary hepatocytes [4]. Whereas in the first scenario the NF-Y-binding site but not its neighbouring Sp1-binding site (−85) is important, full response to carbohydrates has an absolute requirement for both sequences to be intact. Since SREBP-1a levels are higher than SREBP-1c levels in cell lines and SREBP-1c is the major isoform in liver [16], the authors proved that SREBP-1c uses NF-Y and Sp1 as indispensable co-regulators, while SREBP-1a requires mainly NF-Y. Interestingly, we observed that the Sp1-binding motif (−85) does play a role in the RA response since its mutation diminished upregulation of FAS promoter activity by almost 50% (data not shown), thus pointing towards an involvement of the SREBP-1c isoform in the RA response. Our findings are in agreement with studies that show that RXR agonists enhance SREBP-1c expression [9,10]. Two LXR/RXR-binding sites in the SREBP-1c promoter mediate not only the response of SREBP-1c to retinoids but also to LXR ligands, coordinating cholesterol homeostasis with lipogenesis. A recent report [18] described a central role for LXR in insulin-mediated activation of SREBP-1c transcription and stimulation of fatty acid synthesis in liver. Interestingly, the activation of LXR by

TO-901317 leads to the induction of SREBP-1c expression and precursor protein, but not of its mature nuclear form [19]. The LXR-induced SREBP-1c precursor, however, is rapidly cleaved on acute exposure to insulin via a phosphatidylinositol 3-kinase-dependent mechanism. It remains to be shown whether insulin is also necessary for processing immature SREBP-1c induced by retinoids *in vivo*. Since we used serum-free medium, we can rule out the involvement of insulin during processing of SREBP-1c in RA-treated HepG2 cells. Our data suggest a direct effect of RA treatment on the proteolysis of SREBP-1. Indeed, preliminary data show a decrease of Insig-2 mRNA after RA treatment [Lei Zhang, unpublished data]. We also present evidence that not only SREBP-1c but also SREBP-1a or SREBP-2, whose mRNA levels are increased approximately 2-fold after RA treatment, could convey the RA signal to the FAS promoter. Similarly, increased SREBP-1a/SREBP-1c mRNA levels were noticed after stimulating CaCo-2 cells with an LXR ligand [20].

Due to the aforementioned studies it is reasonable to assume that retinoids affect FAS expression via formation of LXR/RXR heterodimers binding to their recognition sequences in the SREBP-1c promoter. However, recent data support the existence of a well-conserved LXR/RXR-binding site in the FAS promoter [15] since synthetic LXR ligands induced FAS expression without changes in the SREBP-1c mRNA levels. The authors concluded that the coregulation of FAS by both SREBP and LXR might serve to balance its expression under fluctuating sterol conditions. Indeed, we noticed a minor contribution of the LXR/RXR-binding site at −650 to the RA response (data not shown).

In summary, our results support an essential contribution of the SREBP-binding motif to the stimulatory effect of retinoids on the FAS promoter and together with other studies [9,10] help to explain the cause of elevated triglyceride levels after administering retinoids to animals at the molecular level.

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