Regulation of the Hyaluronan Synthase 2 Gene by Convergence in Cyclic AMP Response Element-binding Protein and Retinoid Acid Receptor Signaling*

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The human hyaluronan synthase 2 (HAS2) gene encodes for an enzyme making hyaluronan, altered concentrations of which are associated with many pathological situations including wounding, several inflammatory conditions, and malignant tumors. In this study we showed that HAS2 is a primary target of the cAMP activator forskolin and the nuclear hormone all-trans-retinoic acid (RA). The first 2250 bp of the promoter contain three response elements (REs) for the transcription factor CREB1 as well as two REs for the nuclear receptor RAR. Chromatin immunoprecipitation and re-chromatin immunoprecipitation assays using selected fragments of the promoter containing the putative REs showed that forskolin and all-trans-RA modulate the formation of complexes between CREB1 and RAR with various co-regulators at the predicted sites. Interestingly, CREB1 complexes are regulated by all-trans-RA as are RAR complexes by forskolin. Reporter gene assays using nested promoter fragments supported these findings. Forskolin and all-trans-RA co-stimulation reduced the binding of CREB1, RAR, and the co-repressor nuclear receptor co-repressor 1 (NCoR1), but enhanced the association of co-activators MED1 and CREB-binding protein (CBP). RNA interference experiments suggested that MED1 and NCoR1 are central for the all-trans-RA induction of the HAS2 gene and CBP dominates its forskolin response. In general, our findings suggest a convergence of CREB1 and RAR signaling, and demonstrate the individual character of each RE in terms of co-regulator use.

Hyaluronan is a high molecular mass linear non-sulfated polysaccharide that is a key component of the vertebrate extracellular matrix and has a variety of functions during (1) and following development (2). The unique physicochemical properties of this glycosaminoglycan and its interactions with specific cell surface receptors provide hyaluronan with a central role in cellular migration, adhesion, and proliferation (3). Hyaluronan is produced by hyaluronan synthase (HAS),2 an enzyme that resides at the plasma membrane and delivers the growing polysaccharide directly into the extracellular space (3). Of the three members of the vertebrate HAS gene family, HAS2 is vital, whereas no phenotype has been reported for mice with deletions of HAS1 and HAS3 (1). The expression levels of HAS genes, especially HAS2, undergo large and rapid fluctuations, often controlled by growth factors and external conditions (4–8). Although post-transcriptional regulation of HAS and its enzymatic activity has been reported (9–11), the majority of data available suggests that hyaluronan synthesis closely correlates with the expression of HAS. Therefore, detailed information on processes that control HAS2 transcription are important for understanding the biological functions of hyaluronan, and also for possible therapeutic interference in the clinical conditions involving hyaluronan.

We have characterized human HAS2 as a primary retinoic acid receptor (RAR) target gene in keratinocytes (12). In addition to RARs, the transcription factors STAT3, SP1, and NF-κB have also been shown to play a role in the control of hyaluronan synthesis via regulation of the HAS2 gene expression (12–15). The regulation of gene expression involves the execution of multiple events that cumulate in the initiation of mRNA transcription by activated RNA polymerase II (pPol II) (16). This process consists of a series of spatially and temporally regulated events including associations and dissociations of transcription factors with co-repressors, such as nuclear receptor co-repressor 1 (NCoR1) (17, 18), co-activators, such as CREB-binding protein (CBP) (19), and members of the mediator complex, such as MED1 (20, 21), on regulatory fragments of the respective genes. The access of transcription factors to their specific binding sequences, referred to as response elements (REs), is dependent on the density of chromatin packaging at these fragments (22). The latter is controlled by chromatin remodeling proteins and enzymes that control the levels of histone modifications, such as acetylation, phosphorylation, and methylation (23).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S4 and Fig. S1.

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The abbreviations used are: HAS, hyaluronan synthase; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; CRE, CREB response element; CREB, cAMP response element-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; DR, direct repeat; FBS, fetal bovine serum; MED, mediator; NCoR1, nuclear co-repressor 1; pPol II, phosphorylated RNA polymerase II; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RE, response element; RPLO, ribosomal protein large P0; RXR, retinoid X receptor; siRNA, small inhibitory RNA; TSS, transcription start site; DOTAP, N-(1,2-dioleoyl)propyl-N,N,N,N-trimethylammonium methylsulfate; ERK, extracellular signal-regulated kinase; STAT, signal transducers and activators of transcription.

2 The human hyaluronan synthase 2 (HAS2) gene encodes for an enzyme making hyaluronan, altered concentrations of which are associated with many pathological situations including wounding, several inflammatory conditions, and malignant tumors. In this study we showed that HAS2 is a primary target of the cAMP activator forskolin and the nuclear hormone all-trans-retinoic acid (RA). The first 2250 bp of the promoter contain three response elements (REs) for the transcription factor CREB1 as well as two REs for the nuclear receptor RAR. Chromatin immunoprecipitation and re-chromatin immunoprecipitation assays using selected fragments of the promoter containing the putative REs showed that forskolin and all-trans-RA modulate the formation of complexes between CREB1 and RAR with various co-regulators at the predicted sites. Interestingly, CREB1 complexes are regulated by all-trans-RA as are RAR complexes by forskolin. Reporter gene assays using nested promoter fragments supported these findings. Forskolin and all-trans-RA co-stimulation reduced the binding of CREB1, RAR, and the co-repressor nuclear receptor co-repressor 1 (NCoR1), but enhanced the association of co-activators MED1 and CREB-binding protein (CBP). RNA interference experiments suggested that MED1 and NCoR1 are central for the all-trans-RA induction of the HAS2 gene and CBP dominates its forskolin response. In general, our findings suggest a convergence of CREB1 and RAR signaling, and demonstrate the individual character of each RE in terms of co-regulator use.
CREB and RAR Convergence on the HAS2 Promoter

The transcription factor cAMP response element-binding protein 1 (CREB1) is implicated in a large number of biological processes, including long-term neuronal plasticity, cell survival, circadian rhythms, adaptation to drugs, and hormonal regulation of metabolism (24). CREB1 binds constitutively to cAMP response elements (CREs) that have the consensus sequence TGACGTCA (25–27). Mammalian genomes contain more than 10,000 copies of this sequence (28), but their access is under cell-specific epigenetic control (29). CREB1 is activated by phosphorylation at residue Ser-133 via protein kinase A and a variety of other kinases. Experimentally, CREB1 activation can be obtained by forskolin, a compound that induces cAMP levels (30). This phosphorylation is essential for the recruitment of the co-activator CBP (31), which in turn via its acetylase activity, leads to transcriptional initiation. Thus, CREB signaling is mainly regulated by the recruitment of CBP.

The three subtypes α, β, and γ of the nuclear receptor RAR have very similar functional profiles, but in a number of tissues, such as epidermis, the predominant subtype is RARγ (32). RARs form heterodimers with the retinoid X receptor (RXR), another nuclear receptor family member, which also contacts DNA (33) with hexameric DNA motifs containing the consensus sequence RGKTCR (R = A or G, K = G or T), arranged as a direct repeat (DR) with five intervening nucleotides, so called DR5-type retinoic acid (RA) REs (34, 35). The nuclear hormone all-trans-RA is the natural RAR ligand and induces a conformational change within the ligand-binding domain of the receptor, resulting in the replacement of co-repressor by co-activator proteins (36). As with activated CREB1, CBP, and other histone acetyltransferases play an essential role in mediating the activating signal of all-trans-RA to the basal transcriptional machinery.

RARs are known to interfere with a variety of other signaling pathways both in a synergistic or an antagonistic way. As we reported previously, retinoids convergence with tumor necrosis factor-α via the use of common co-regulators (15). In addition, RARs are known to cooperate with Sp1/Sp3 transcription factors in transactivation of the CYP26 gene. Similarly, in the presence of cytokines STAT5 cooperates with RARs (37). The interference of retinoids and CREB signaling has been indicated by the observation that retinoids can activate CREB in a non-genomic way through activation of protein kinase C, ERK, and RSK in the absence of the influence of RAR-RXR heterodimers (38).

In this study, we aimed at better understanding of the molecular interactions at the promoter of the HAS2 gene, a central organizer of cell surface and intercellular matrix. This was done by comparing the RE occupation, co-factor recruitment, and transcription activation by two transcription factors (CREB1 and RAR), independently and alone. We found that the first 2250 bp of the promoter contains three CREB binding sites (CREs) and two previously identified functional RAREs. Chromatin immunoprecipitation (ChIP) and re-ChIP assays using overlapping fragments of the promoter showed that forskolin and all-trans-RA modulate the complex formation of CREB1 and RAR with MED1, CBP, and NCoR1 at the predicted sites. Interestingly, there is interdependence between these two signaling pathways, because CREB1 complexes are regulated by all-trans-RA and RAR complexes by forskolin. Reporter gene assays using nested promoter fragments supported these findings. Forskolin and all-trans-RA co-stimulation reduced the binding of CREB1, RAR, and NCoR1, but enhanced the association of MED1 and CBP. RNA interference experiments suggested MED1 and NCoR1 are central for the all-trans-RA induction of the HAS2 gene and CBP for its forskolin response. In general, our findings suggest convergence of CREB1 and RAR signaling and individual properties of each RE in co-regulator preference.

EXPERIMENTAL PROCEDURES

Cell Culture—The human immortalized keratinocyte cell line HaCaT (39) and the human embryonic kidney cell line HEK293 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM l-glutamine. Prior to mRNA or protein extraction, enzyme-linked immunosorbent or ChIP assays, cells were treated at a density of 50 to 60% confluence with solvent (0.1% DMSO), 10 μM forskolin (Calbiochem, Läufelfingen, Switzerland), 0.1 μM all-trans-RA (Sigma) or their combination for time points as indicated under “Results.” The medium was then supplemented with stock solutions of the compounds and was not changed at the moment of cell stimulation.

RNA Extraction and Real-time PCR—Total RNA was extracted using the Mini RNA Isolation II kit (HiSS Diagnostics, Freiburg, Germany) and cDNA synthesis was performed for 1 h at 37 °C using 1 μg of total RNA as a template, 100 pmol of oligo(dT)$_{15}$ primer, and 40 units of reverse transcriptase (Fermentas). Real-time quantitative PCR was performed in an IQ-cycler (Bio-Rad) using the dye SYBR Green I (Molecular Probes, Leiden, The Netherlands). Per reaction, 1 unit of Hot Start Taq polymerase and 3 mM MgCl$_2$ were used and the PCR cycling conditions were: 40 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C. Fold-inductions were calculated using the formula 2$^{\Delta\Delta C_{t}}$, where $\Delta C_{t}$ is $C_{t}$(stimulus) – $C_{t}$(solvent)/ and $C_{t}$ is the cycle at which the threshold is crossed. The gene-specific primer pairs are given in Table S1. PCR product quality was monitored using post-PCR melt curve analysis.

Western Blot—24 h after stimulation cells were harvested, proteins extracted with 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate and proteinase inhibitors in phosphate-buffered saline, pH 7.5, and run on 10% SDS-PAGE gels, blotted on nitrocellulose membrane, and immunodetected with primary antibodies against HAS2 (sc-34067, Santa Cruz, Heidelberg, Germany) and actin (A2066, Sigma) with a secondary anti-goat antibody (A-21084, from Invitrogen for HAS2 and sc-1314, from Pierce for actin). Detection was done using an Odyssey infrared imaging system (LI-COR, Lincoln, NE). The amounts of HAS2 were normalized by actin expression.

Hyaluronan Assay—24 h after stimulation the cells were counted and medium was analyzed for hyaluronan content using an enzyme-linked immunosorbent assay as described in detail previously (12).

In Silico Promoter Analysis—The first 2250 bp of the human HAS2 promoter were screened in silico for putative RAR and CREB binding sites. Putative RAREs were identified by screen-
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ing the promoter for two copies of RGKTCA consensus sequence in a DR5 arrangement. The maximal deviation from the consensus sequence was restricted to one position. Putative CREB1 binding sites were screened by using the net-based program ConSite with a transcription factor score cut-off of 85%.

ChIP Assays—Nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% for 7 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 0.15 M and incubating for 5 min at room temperature on a rocking platform. The medium was removed and the cells were washed twice with ice-cold phosphate-buffered saline. The cells were then collected in ice-cold phosphate-buffered saline supplemented with a protease inhibitor mixture (Roche). After centrifugation the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris-HCl, pH 8.1) to result in DNA fragments of 300 to 1000 bp in length. Cellular debris was removed by centrifugation and the lysates were diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors, 16.7 mM Tris-HCl, pH 8.1). Chromatin solutions were incubated with 5 μl of antibody against the antibody against RAR γ, RXRa, and the control empty pSG5-vector with 0.5 μg of DOTAP (Roth, Karlsruhe, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl of phenol red-free DMEM, the liposomes were added to the cells. Phenol red-free DMEM supplemented with 500 μl of 15% charcoal-stripped FBS was added 4 h after transfection. At this time 10 μM forskolin, 0.1 μM all-trans-RA or solvent were added. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche) and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (PerkinElmer Life Sciences). The luciferase activities were normalized with respect to protein concentration and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

siRNA Transfection—Polyethylenimine (Sigma) was used to transfect small inhibitory (si) RNAs into HEK293 cells. siRNA mixtures used were composed of equal amounts of three different siRNAs against Cbp, MEDI, or NcOR1 (see Table S4). 200 pmol of each siRNA mixture were incubated for 15 min with 50 μl of 150 mM NaCl (for 6-well plates). Simultaneously, 10 μg of polyethyleneimine was incubated with 50 μl of 150 mM NaCl. After the 15-min incubation, both solutions were combined and the mixture was incubated for an additional 15 min. Then 900 μl of phenol red-free DMEM supplemented with 5% FBS was added and transfection was continued for 48 h. Ligands were added in phenol red-free DMEM and the silencing effect was determined by real-time PCR.

RESULTS

HAS2 Gene Expression in Response to Forskolin and All-trans-RA in HaCaT and HEK293 Cells—Although the immortalized human keratinocyte cell line HaCaT and the human embryonic kidney cell line HEK293 represent rather different human tissues, both are often used in the field of HAS gene regulation (12–15). The relative mRNA expression of the three HAS genes was first compared by quantitative real-time PCR in relation to the control gene RPLP0 (Fig. S1). The HAS2 gene...
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was found to be the most prominently expressed member of the HAS gene family in both cell types. HAS1 could not be detected at all in HaCaT cells and in HEK293 cells its expression was ~100 times lower than that of HAS2. Also, the expression of HAS3 in HaCaT cells was more than 10 times and in HEK293 cells even nearly 100 times lower than that of HAS2. CREB1 and NCOR1 expressions did not differ significantly in the two cell lines. In HaCaT cells RARγ was the most prominent RAR subtype being 55-fold higher expressed than RARα and nearly 4,000-fold more than RARβ. In HEK293 cells RARα and RARγ were expressed on a similar level nearly 40-fold higher than RARβ. In HEK293 cells the expression of MED1 was nearly 29-fold higher and that of CBP 3.7-fold higher than in HaCaT cells.

Following our hypothesis that the signaling of the adenylyl cyclase activator forskolin and that of the RAR ligand all-trans-RA converge in the up-regulation of hyaluronan synthesis, we stimulated both cell types with the compounds alone and in combination (Fig. 1). In the combined treatment both stimuli were added at the same time.

Has2 mRNA Levels—We performed in both cell lines a time course stimulation over 1, 2, 4, 6, and 24 h and measured HAS2 mRNA by using quantitative real-time PCR (Fig. 1, A and B). Forskolin-induced HAS2 mRNA in HaCaT cells 2.7-fold after 1–2 h, 1.4–1.5-fold after 4–6 h and, surprisingly, even 4.6-fold after 24 h. The all-trans-RA response of the gene was already 6.5-fold after 1 h, reached a peak of 8.9-fold after 4 h and gradually declined to 2.2-fold after 24 h. The combination of forskolin and all-trans-RA induced the HAS2 gene 6.2-fold after 1–2 h, decreasing to only 2.7-fold after 24 h, suggesting that, at 4 h forskolin inhibited the all-trans-RA response of the gene, whereas at 24 h all-trans-RA disturbed the forskolin response. In HEK293 cells the HAS2 gene showed a broad response to forskolin with an ~3-fold induction after 1 to 6 h and an increase to 7.8-fold after 24 h. All-trans-RA induced the gene ~2-fold after 1 to 4 h treatment, showed a peak with 3.1-fold induction after 6 h, which decreased to 1.7-fold after 24 h. The combination of forskolin with all-trans-RA resulted in a steady increase of HAS2 gene expression over time from 3.6-fold after 1 h to 10.7-fold after 24 h.

Western Blotting of Has2—Next we tested, whether the up-regulation of HAS2 mRNA is also reflected at the protein level. Western blotting analysis of both cell lines stimulated for 24 h showed that the effect of forskolin was more prominent than that of all-trans-RA in both HaCaT and HEK293 cells (Fig. 1, C and D). The combination of all-trans-RA and forskolin increased the level of HAS2 protein to the same extent as forskolin alone in HaCaTs but slightly more in HEK293 cells. The responses of HAS2 protein to forskolin and all-trans-RA thus roughly corresponded to those of the HAS2 mRNA.

Synthesis of Hyaluronan—Finally, we estimated HAS enzyme activity in both cell lines by assaying hyaluronan content in the medium after a 24-h treatment with forskolin and all-trans-RA, alone and together (Fig. 1, E and F). In HaCaT cells forskolin alone did not increase hyaluronan content, whereas in HEK293 cells the levels increased 2.7-fold. All-trans-RA induced the hyaluronan content 5.2-fold in HaCaT cells and 1.4-fold in HEK293 cells. By a combined treatment with forskolin and all-trans-RA the amount of hyaluronan was increased 15.9-fold in HaCaT cells, and 3.2-fold in HEK293 cells. The increases of HAS2 mRNA, HAS2 protein, and hyaluronan synthesis were therefore roughly parallel in HEK293 cells (Fig. 1F). On the other hand, forskolin-treated HaCaT cells showed hyaluronan synthesis lower than expected based to the HAS2 mRNA.
**CREB and RAR Convergence on the HAS2 Promoter**

**FIGURE 2. Overview of the human HAS2 promoter**. The first 2250 bp of the HAS2 promoter were screened in silico for putative RAREs (DR5-type) and putative CREs by using our own software for the modified hexamer binding site search (44) and the net-based program ConSite (applying the transcription factor binding search cut-off 85%), respectively. The sequence and location of the putative REs, the locations of the genomic fragments used in ChIP assays, the borders of the promoter fragments used in reporter gene assays, and repetitive sequences are indicated.

Data, and higher than expected when added together with all-trans-RA (Fig. 1F). This suggests that forskolin had a post-transcriptional influence on hyaluronan synthesis in HaCaT cells.

Taken together, in HaCaT and HEK293 cells HAS2 mRNA and protein levels were induced by each forskolin and all-trans-RA alone. However, only in HEK293 cells did we observe under all conditions an additive or even synergistic effect when both compounds were applied together. Moreover, forskolin alone was unable to increase the hyaluronan content in HaCaT medium. Therefore, we selected HEK293 cells to further investigate the mechanisms of the responses to the two different stimuli.

**In Silico Screening of the HAS2 Promoter**—Because the HAS2 gene showed a primary response to forskolin, we screened the first 2250 bp of the promoter in silico for putative CREs by using the net-based program ConSite with a transcription factor score cut-off of 85% (Fig. 2). This led to the identification of three putative CREs at positions −656 to −663, −1731 to −1751, and −1878 to −1889 on the HAS2 promoter. Previously we had already identified and confirmed a cluster of two overlapping DR5-type RAREs (position −1208 to −1237) (12, 15). To investigate the accessibility of the putative REs by ChIP assays, we designed in total six genomic PCR primer pairs specific for genomic fragments containing the putative REs on the first 2250 bp of the HAS2 promoter (Fig. 2 and Table S2). Similarly, the borders of 12 HAS2 promoter fragments (Table S3) covering approximately the same respective chromatin fragments as the ChIP primers, are indicated.

In summary, the first 2250 bp of both the HAS2 promoter contain three CREs and two RAREs. The genomic fragments containing these binding sites may be sufficient for a mechanistic understanding of the response of the HAS2 gene to forskolin and all-trans-RA.

**Forskolin- and All-trans-RA-modulated Association of Nuclear Proteins to the HAS2 Promoter**—To assess the basal and stimulation-dependent association of transcription factors CREB1 and RARγ, pPol II, and co-regulators MED1, CBP, and NCoR1 with the HAS2 promoter, we performed ChIP assays with chromatin extracts from HEK293 cells, which had been treated for 60 min with solvent, forskolin, or all-trans-RA (Fig. 3). Using genomic PCR we determined for six fragments of the HAS2 promoter (containing the TSS and the putative CREs and RAREs, see Fig. 2) their relative amount in the immunoprecipitated chromatin fraction. The resulting PCR products were quantified, the individual amount of unspecified binding of chromatin to IgG was subtracted, and the result was expressed as a percentage (%) of input control (the amount of PCR products obtained from non-immunoprecipitated chromatin templates).

In the absence of forskolin stimulation, CREB1 associated, as expected, with all those fragments of the HAS2 promoter that contained putative CREs, i.e. fragments 2 and 3 (CRE1), 5 (CRE2), and 6 (CRE3) (Fig. 3A). In addition, binding to fragment 1 (TSS) was observed, which reflects DNA looping from the upstream CREs. This binding profile was stable in the presence of forskolin or all-trans-RA with one exception. Stimulation with all-trans-RA significantly increased the CREB1 association with the TSS (fragment 1).

Ligand-independent binding of RARγ was found on the TSS (fragment 1) and on fragment 2 (CRE1) of the HAS2 promoter (Fig. 3B). Stimulation with all-trans-RA increased RARγ association with the TSS (fragment 1) and fragment 2 (CRE1) and induced binding to fragments 3 and 4 (RAR1 and 2 with flanking effects) and 6 (flanking effect of a previously described upstream RARE (12)). Interestingly, forskolin induced RARγ binding to the RARE containing fragment 4 and RARE flanking fragments 3 and 6 suggesting that all-trans-RA and forskolin might interfere in their mechanisms of HAS2 regulation.

Moreover, we observed basal association of pPol II with the TSS (fragment 1) and fragments 2 and 3 (CRE1) and 4 (RAREs) of the HAS2 promoter (Fig. 3C). This indicates stimulation-independent looping of the TSS to fragments 2, 3, and 4, which may be caused either by basal binding of CREB1 and RAR binding (see Fig. 3, A and B) or by other unrelated transcription factors. Forskolin treatment increased pPol II binding to the TSS (fragment 1), fragments 3 (CRE1), and 6 (CRE3), but also with fragment 4 (RAREs). All-trans-RA stimulated the association of pPol II with fragment 4 (RAREs), but interestingly also with fragment 5, which contained CRE2. The latter is another example of the possible interference of CREB and RAR signaling.

MED1 is part of the mediator complex that forms a bridge between transcription factors and basal transcriptional machinery. Thus association of MED1 with a RE containing fragment is one sign of the functionality of the RE. Basal MED1 association was observed with fragments 1 (TSS), 2 and 3 (CRE1), and 5 (CRE2) of the HAS2 promoter (Fig. 3D). Forskolin induced the binding of MED1 to fragments 2 (CRE1) and 6 (CRE3), but reduced it with fragment 5 (CRE2). Treatment with
all-trans-RA reduced association of MED1 with fragments 2 (CRE1) and 5 (CRE2).

The protein complex on a functional RE should also contain co-activators. Constitutive association of the co-activator CBP was found on fragments 2 and 3 (CRE1), 4 (RAREs), and 5 (CRE2) of the HAS2 promoter (Fig. 3E), forskolin-stimulated CBP binding to the TSS (fragment 1) and fragment 6 (CRE3), but reduced co-activator binding to fragment 4 (RAREs). All-trans-RA induced CBP association with fragments 2 (CRE1) and 6 (CRE3), but reduced association with fragments 4 (RAREs) and 5 (CRE2).

In the absence of stimuli co-repressor proteins often bind to promoter regions, but usually dissociate from REs after gene activation. In the absence of stimulation NCoR1 associated with fragments 2 and 3 (CRE1), 4 (RAREs), 5 (CRE2), and 6 (CRE3) of the HAS2 promoter (Fig. 3F). Both forskolin and all-trans-RA reduced NCoR1 association on fragments 3 (CRE1), 4 (RAREs), 5 (CRE2), and 6 (CRE3). However, on fragment 2 (CRE1) all-trans-RA had no effect and on the TSS (fragment 1), it even increased NCoR1 association.

Taken together, CRE-containing fragments 2, 3, 5, and 6 as well as RARE-containing fragment 4 of the HAS2 promoter associate in part constitutively and as expected after ligand stimulation with CREB1, RARγ, and their co-regulators. However, a number of cross-reactions suggested marked interplay between CREB and RAR signaling on this promoter.

Complexes of CREB1 and RARγ with Co-regulators on the HAS2 Promoter—Re-ChIP assays monitor the simultaneous association of two nuclear proteins to the same genomic frag-

ment in living cells. After stimulation of HEK293 cells with forskolin and all-trans-RA a first round of ChIP was performed with antibodies against CREB1 (Fig. 4, A–C) or RARγ (Fig. 4, D–F) and then a second precipitation with antibodies against MED1 (Fig. 4, A and D), CBP (Fig. 4, B and E), and NCoR1 (Fig. 4, C and F).

Both forskolin and all-trans-RA stimulated CREB1-MED1 binding to fragments 3 (CRE1), 5 (CRE2), and 6 (CRE3) of the HAS2 promoter indicating that all-trans-RA interferes with CREB signaling (Fig. 4A). Moreover, forskolin stimulated CREB1-CBP complex binding to the TSS (fragment 1), whereas all-trans-RA induced the complex on fragment 4 (RAREs). A CREB1-CBP complex was observed only on fragment 5 of the HAS2 promoter and only after stimulation with forskolin (Fig. 4B).

Basal CREB1-NCoR1 complexes were found on fragments 2 and 3 (CRE1) of the HAS2 promoter (Fig. 4C). Forskolin and in particular all-trans-RA stimulated CREB1-NCoR1 binding to CRE1 (fragment 3). Moreover, forskolin induced CREB1-NCoR1 association with fragment 5 (CRE2).

Significant association of RARγ-MED1 complexes were observed only on the TSS (fragment 1), and fragments 2 (CRE1) and 4 (RAREs) of the HAS2 promoter (Fig. 4D). Forskolin treatment reduced this complex binding to the TSS (fragment 1) and all-trans-RA to the association with fragments 2 (CRE1) and 4 (RAREs).

Basal association of RARγ-CBP complexes was found only on the TSS (fragment 1) of the HAS2 promoter (Fig. 4E). Interestingly, forskolin treatment stimulated the association of the complex on fragments 2 (CRE1), 4 (RAREs), 5 (CRE2), and 6 (CRE3), whereas all-trans-RA induced it only on fragments 4 (RAREs) and 5 (CRE2 or possible flanking effect from the RAREs).

Finally, in the absence of stimulation RARγ-NCoR1 complexes were found only on the TSS (fragment 1) of the HAS2 promoter (Fig. 4F). Interestingly, forskolin treatment stimulated binding of complexes to fragments 2 (CRE1) and 4 (RAREs), whereas all-trans-RA had no effect.

In summary, on the HAS2 promoter both CREB1 and RARγ form complexes with the co-regulators MED1, CBP, and NCoR1. The observation that (i) these complexes are not only observed on fragments with respective CREs and RAREs but also on unexpected promoter fragments and (ii) the response of CREB1-containing complexes to all-trans-RA and that of RARγ-containing complexes to forskolin confirmed that there...
is an interaction between these two signaling pathways on the HAS2 promoter.

Functional Activities of the HAS2 in Response to Forskolin and All-trans-RA—We next aimed to confirm the functionality of the HAS2 promoter as suggested by ChIP assays (Figs. 3 and 4) by reporter gene assays (Fig. 5). For this purpose a nested set of 12 fragments of the HAS2 promoter (covering the genomic fragment from +43 to −2118, see Fig. 2 and Table S3) (14, 15) were subcloned into the pGL3 vector. Without overexpression of RARγ and RXRs, or CRE1, we observed in transiently transfected HEK293 cells a linear increase of basal gene activity of the HAS2 promoter fragment (number 12, containing the two RAREs and all three CREs, Fig. 5C). Surprisingly, fragment 11, which contains two RAREs, did not respond to the retinoid receptor overexpression.

The combined overexpression of CRE1 and retinoid receptors (Fig. 5D) resulted in a mixed response of the single overexpression series (Fig. 5, B and C). The basal activity of the 12 HAS2 promoter fragments (Fig. 5D) was comparable with that of the endogenous conditions (Fig. 5A), with the exception of fragment 7, which showed a lower basal activity than the neighboring fragments. Forskolin inducibility was observed in CRE-containing fragments 8, 11, and 12, but also in CRE-free fragments 2 and 5. Similarly, an all-trans-RA response was observed in RARE-containing fragments 11 and 12, but also with RARE-free fragments 6 and 8. Significant inductions after treatment with both ligands were found with fragments 2, 5, 8, 10, 11, and 12, but compared with the single treatments no synergistic effects were observed.

Taken together, under CRE1 overexpressing conditions the reporter gene assays with the HAS2 promoter fragments support the findings of in silico screening and ChIP assays concerning the location and functionality of CREs and RAREs on the HAS2 promoter. Also in this experimental system a number of unexpected cross-coupling effects between CREB and RAR signaling supported the notion of convergence between both signal transduction pathways, and often interference of their respective stimulatory effects.

Combined Effects of Forskolin and All-trans-RA on the Recruitment of CREB1, RARγ, and Their Co-regulators—To analyze a possible synergistic effect of forskolin and all-trans-RA in modulating the binding of CRE1, RARγ, pPol II, and co-regulators to the TSS, CRE- and RARE-containing fragments of the HAS2 promoter, ChIP assays were performed on chromatin from HEK293 cells that had been stimulated by both compounds (Fig. 6). Constitutive CREB1 association was found on fragment 1 (TSS), fragments 2 and 3 (CRE1), and fragments 5 (CRE2) and 6 (CRE3) of the HAS2 promoter (Fig. 6A). The
combined application of forskolin and all-trans-RA was neutral on most fragments but reduced CREB1 binding to fragment 3 (CRE1) and induced it on fragment 4 (RAREs).

Ligand-independent binding of RARγ was observed on the TSS (fragment 1), on fragment 2 (CRE1), and to much lower extent also on fragments 3 (CRE1), 4 (RAREs), and 5 (CRE2) of the HAS2 promoter (Fig. 6B). Combined ligand application increased RARγ association only on fragments 2 and 3 (CRE1).

In the absence of stimulation pPol II association was found on the TSS (fragment 1), on fragment 2 (CRE1), and to a much lower extent also on fragments 3 (CRE1), 4 (RAREs), and 5 (CRE2) of the HAS2 promoter (Fig. 6C). Stimulation with forskolin and all-trans-RA resulted in increased pPol II binding on the TSS (fragment 1), and fragments 5 (CRE2) and 6 (CRE3).

Constitutive MED1 binding was found on fragment 1 (TSS), fragments 2 and 3 (CRE1), and 4 (RAREs) and 5 (CRE2) of the HAS2 promoter (Fig. 6D). Co-treatment with forskolin and all-trans-RA strongly increased the binding of MED1 to fragments 2 (CRE1), 4 (RAREs), and 6 (CRE3), but reduced it on fragment 5 (CRE2).

Ligand-independent CBP binding was observed on fragments 2 and 3 (CRE1), 4 (RAREs), and 5 (CRE2) of the HAS2 promoter (Fig. 6E). Combined ligand treatment enhanced CBP binding to all four fragments and in addition also to the TSS (fragment 1).

Constitutive NCoR1 binding was detected on all six fragments of the HAS2 promoter (Fig. 6F). On fragments 1 (TSS), 3 (CRE1), 4 (RAREs), 5 (CRE2), and 6 (CRE3) forskolin and all-trans-RA treatment significantly reduced the NCoR1 association, whereas it increased it on fragment 2 (CRE1).

In summary, with a few exceptions the constitutive binding of CREB1, RARγ, pPol II, and co-regulators to selected fragments of the

**FIGURE 5. Functionality of the HAS2 promoter.** Reporter gene assays were performed with extracts from HEK293 cells that were transiently transfected with luciferase reporter gene constructs carrying the indicated 12 different HAS2 promoter fragments in addition to empty expression vectors (pSG5 or pFN2K, A) or with expression vectors for human CREB1 (B), RARγ and RXRα (C), or their combination (D). Cells were treated for 16 h with solvent (0.1% DMSO), forskolin (10 μM), all-trans-RA (0.1 μM) alone or in combination. Relative luciferase activity was determined. Bars represent the mean ± S.D. of at least three experiments. Two-tailed paired Student’s t tests were performed to determine the significance of the effects of the overexpression reference to the endogenous control (A) (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
When comparing individual treatment with forskolin and all-trans-RA with their combined application, the latter was rather neutral on the association of transcription factors CREB1 and RAR/H9253 with promoter fragments but significantly modulated the association of the co-regulators, i.e. it increased complex formation of co-activators MED1 and CBP and enhanced dissociation of the co-repressor NCoR1.

**Co-regulator Silencing Modulates Ligand Response of HAS2 Expression**—The co-regulators MED1, CBP, and NCoR1 seem to be critical for ligand response of the HAS2 gene expression. For a more detailed analysis of their role, they were individually knocked-down by siRNA mixtures against their respective genes (Fig. 7). HEK293 cells were first transfected with siRNAs, then stimulated with forskolin and all-trans-RA (0.1 μM) alone or in combination for 2 h. HAS2 mRNA levels in relation to the control gene RPLP0 were measured by real-time quantitative PCR. The silencing effect was measured by comparing the value of a specific siRNA to that of the unspecific control. Bars represent the mean ± S.D. of at least three experiments. Two-tailed paired Student’s t tests were performed to determine the significance of the effects of the ligand treatment in reference to control siRNA (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**DISCUSSION**

In this study two important transcription factors, CREB1 and RARγ, were studied for their effects on human HAS2 gene expression and for a possible convergence in their signaling pathways. HAS2 was already known to be a primary target of transcription factors RAR, STAT3, SP1, and NF-kB (12–15). The retinoid responsiveness of the HAS2 gene, which we had previously characterized in HaCaT cells (12, 15), was confirmed here in HEK293 cells. Moreover, we demonstrated for the first time that HAS2 displays a rapid response to forskolin suggest-
ing that it is a primary target of the bZIP transcription factor CREB1. The in silico identification of three putative CREs within the first 2250 bp of the HAS2 promoter as well two RAREs each within the same sequence, suggested that the investigated genomic fragments are sufficient for a mechanistic understanding of CREB and RAR signaling.

ChIP assays confirmed the association of CREB1 and RARγ with those HAS2 promoter fragments that contain the predicted CREs and RAREs, respectively. The RARE cluster could be confirmed as the mediator of retinoid response also in HEK293 cells. In addition, three CREs were functional CREB1 binding sites. Moreover, re-ChIP assays indicated at the expected fragments the stimulation-dependent dissociation of NCoR1 and the association of MED1 and CBP from fragments that carry at the same time CREB1 or RARγ. This suggests that the respective chromatin fragments form functional complexes that mediate activation of the basal transcriptional machinery. Reporter gene assays support these assumptions.

The average size of chromatin fragments of 300 to 1000 bp, as used in the ChIP assays, limits the resolution of promoter scanning. For this reason we also observed some effects in fragments flanking the CREs and RAREs. In addition, we cannot exclude that CREB1 and RAR also associate with other than the predicted CREs and RAREs, maybe even without direct DNA contact but in conjunction with other transcription factors. Alternatively, these transcription factors may associate with more distant REs. The puzzling reporter gene assay result that the fragment between positions −432 and −320 mediated all-trans-RA responsiveness supports these possibilities. Despite such alternative explanations for some of the unexpected observations, it is obvious that CREB1 and RARγ converge in many ways in their signaling. For example, the binding of CREB1 to CREs results in local opening of chromatin and facilitates access of RARγ to neighboring RAREs and vice versa RARγ seems to help CREB1 to its sites.

The general features of recruitment of transcriptional regulators to the HAS2 promoter in response to different stimulations are depicted in a model (Fig. 8). Forskolin and all-trans-RA both recruit the same co-regulators indicating functional convergence but also show individual differences. Without any stimuli the different fragments containing CRE1, the RARE cluster, or both CRE2 and CRE3 show a unique pattern of transcription factor and co-regulator binding. All those fragments associate with both co-repressors and co-activators indicating the presence of large protein complexes on the HAS2 promoter. All-trans-RA seems to regulate mainly NCoR1 association, whereas forskolin affects association with NCoR1 and also to some extent that with MED1 and CBP. Unknown bridging factor(s) (named X) are postulated to mediate the contact between co-activators and co-repressors. Both stimuli showed also some transcription enhancing effects on fragment(s) not containing any putative RE for their corresponding transcriptional machinery.
CREB and RAR Convergence on the HAS2 Promoter

According to ChIP results, CRE1 MED1 and NCoR1 seem to be the main regulators, whereas on CRE2 and CRE3 CBP together with NCoR1 may be central.

In cases of co-treatment with forskolin and all-trans-RA there seems to be competition between both transcription factors resulting in a reduction of their individual binding, as observed by ChIP. However, it can be assumed that the remaining association of CREB1 and RARγ with the respective promoter fragments is more specific and selective, because it results in increased steady state mRNA levels of HAS2. Similar effects are also observed with more selective pPol II associations, which represent looping of the respective chromatin fragments to the TSS. At this stage, we do not know the causes of this “background.” Indeed it might not be background and may reflect additional transcription factors that utilize the examined promoter fragments (including un-induced CREB1 and RARs). In contrast, co-treatment with forskolin and all-trans-RA results in increased binding of activating co-regulators, such as MED1 and CBP, whereas the association of repressing co-regulators, such as NCoR1, decreased. A number of co-regulators are rather promiscuous in their interaction with transcription factors, for example, MED1, CBP, and NCoR1 interact both with CREB1 and RARγ. Therefore it is possible that the chromatin made more accessible to transcription factors by the two stimulating transcription factors may allow for other constitutively active transcription factors to bind their target sequences and associate with these promiscuous factors. Alternatively, the short receptor interaction domains, composed of the sequence LXXLL in case of co-activators (40) and LXXXLXXX(I/L) in case of co-repressors (41), do not require very specific target recognition. Therefore, these co-regulators are well suited to integrate the actions of basically all transcription factors in which they interact. In addition, it has been suggested that all-trans-RA may activate CREB1 in a non-classical, RAR-independent way via protein kinase C or ERK (38).

All putative RE-containing fragments showed a unique pattern of transcription factor and co-regulator binding in response to different treatment conditions. This indicates that the surrounding chromatin contributes to the function of the transcription factor binding site RE. Moreover, the surprising finding that both co-activators and co-repressors can associate to the same fragment further complicates understanding of the regulation on the chromatin level. DNA looping may combine different complexes containing either co-activators or co-repressors via bridging proteins as suggested in a recent study on GATA transcription factors (42). The relative abundance and position of the corresponding factor then may finally determine the direction of the gene regulation.

In general, HAS2 mRNA levels were well correlated with similar changes in HAS2 protein, and further corresponded also to the synthesis of hyaluronan, stressing the importance of the molecular interactions that occur on the HAS2 promoter. The extensive cross-talk of different signaling pathways on the HAS2 promoter, in some cases competing with each other, sometimes additive, is quite understandable considering the sensitivity of hyaluronan synthesis toward numerous kinds of external stimuli and cellular differentiation and developmental programs (1, 2, 4–8, 43). Indeed, there is a continuous fine balance in the regulation of hyaluronan production due to its key function in the pericellular glyocalyx and as an organizer of the extracellular matrix.

In conclusion, we showed that forskolin and all-trans-RA regulate the human HAS2 gene and that the promoter immediately adjacent to the TSSs of this gene contains both functional CREs and RAREs. These transcription factors share common co-regulators that demonstrate more idiosyncratic association patterns. These observations demonstrate the general as well as individual mechanisms for the convergence of CREB and RAR signaling on the HAS2 gene that have a critical impact on fine-tuning hyaluronan production.

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