Nuclear receptors modulate the interaction of Sp1 and GC-rich DNA via ternary complex formation

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Binding sites for transcription factor Sp1 have been implicated in the transcriptional regulation of several genes by hormones or vitamins, and here we show that a GC-rich element contributes to the retinoic acid response of the interleukin 1 β promoter. To explain such observations, it has been proposed that nuclear receptors can interact with Sp1 bound to GC-rich DNA. However, evidence supporting this model has remained indirect. So far, nuclear receptors have not been detected in a complex with Sp1 and GC-rich DNA, and the expected ternary complexes in non-denaturing gels were not seen. In search for these missing links we found that nuclear receptors [retinoic acid receptor (RAR), thyroid hormone receptor (TR), vitamin D₃ receptor, peroxisome-proliferator-activated receptor and retinoic X receptor] induce an electrophoretic mobility increase of Sp1–GC-rich DNA complexes. Concomitantly, binding of Sp1 to the

GC-box is enhanced. It is proposed that nuclear receptors may partially replace Sp1 in homo-oligomers at the GC-box. RARs and Sp1 can also combine into a complex with a retinoic acid-response element. The presence of RAR and Sp1 in complexes with either cognate site was revealed in supershift experiments. The C-terminus of Sp1 interacts with nuclear receptors. Both the ligand- and DNA-binding domains of the receptor are important for complex formation with Sp1 and GC-rich DNA. In spite of similar capacity to form ternary complexes, RAR but not TR up-regulated an Sp1-driven reporter in a ligand-dependent way. Thus additional factors limit the transcriptional response mediated by nuclear receptors and Sp1.

Key words: interleukin 1 β , mobility shift, reporters, retinoic acid.

INTRODUCTION

Sp1 is a ubiquitous transcription factor in mammals [1–3]. Its activity appears to be modulated by phosphorylation [4], glycosylation [5], levels of expression [3], the specific sequence of the binding sites [6] and by other members of the Sp family [7]. Despite the multiple levels of regulation, Sp1 is generally considered to be a housekeeping factor of rather constitutive activity. In contrast, nuclear receptors (NRs) are principally recognized as ligand-inducible mediators of transcription in metazoans, although for the subset known as orphan receptors cognate ligands have not been identified. The classic mechanism of gene regulation by NRs involves binding of dimers to symmetrical DNA elements. Two major subfamilies of the NR superfamily can be distinguished based on their mode of interaction with DNA: members of the steroid-receptor subfamily (e.g. oestrogen receptor, ER, and progesterone receptor) form homodimers upon interaction with their ligand, and bind preferentially to palindromic sequences [8]. In contrast, members of the thyroid-receptor subfamily [thyroid hormone receptor (TR), vitamin D₃ receptor (VDR), retinoic acid receptor (RAR) and peroxisome-proliferator-activated receptor (PPAR)] bind as heterodimers in complex with the retinoid X receptor (RXR) to direct repeat elements [9–13]. For a review of RAR see [14]. Transactivation by NRs is modulated by cofactors [15].

Retinoids exert profound effects on immune response and wound healing [16,17]. Interleukin 1 β (IL-1 β) [18], a pro-inflammatory protein that is activated in response to infections and many kinds of injury, is transcriptionally up-regulated by all-*trans* retinoic acid (RA) [19–21]. However, no consensus sequence for RAR has been described in its promoter. To elucidate the

mechanism of RA sensitivity of the IL-1 β promoter we analysed the response of promoter CAT (chloramphenicol acetyltransferase) constructs in transfection experiments. These led to the conclusion that an Sp1 site confers RA sensitivity to the IL-1 β promoter, and prompted us to investigate the interaction of RAR, Sp1 and GC-rich DNA. Recently it has been shown in a pull-down assay that a glutathione S-transferase (GST)–RAR fusion protein can bind Sp1 [22]. Similarly, physical interaction of Sp1 with the steroid receptors progesterone receptor [23] and ER [24] and with the orphan receptor COUP-TF (chicken ovalbumin upstream promoter transcription factor) [25,26] had already been demonstrated. That ER and RAR might interact with Sp1 sites via protein–protein interaction was suggested by the observation that Sp1 binding to its cognate DNA in mobility-shift assays is enhanced by ER and also by the RAR fusion protein [22,27]. However, ternary complexes were not detected when receptor was added to binding reactions with Sp1 and GC-rich DNA [27,28] and nor could supershifts of suspected NR–Sp1–GC-rich DNA complexes be induced with anti-receptor-antibodies [22,26]. These results call into question whether NRs can interact with Sp1 when it is bound to GC-rich DNA. The analysis of IL-1 β regulation by RA led us to address this issue. Our data substantiate the hypothesis of a physical NR–Sp1–GC-box interaction, on which the emerging concept of Sp1-mediated hormone and vitamin action is based [22,23,26,29].

MATERIALS AND METHODS

Proteins and cell extracts for electrophoretic mobility-shift assay (EMSA) and binding assays

Affinity-purified Sp1 from HeLa cells infected with recombinant

Abbreviations used: CAT, chloramphenicol acetyltransferase; ER, oestrogen receptor; IL-1 β , interleukin 1 β ; NR, nuclear receptor; PPAR, peroxisome-proliferator-activated receptor; RA, all-*trans* retinoic acid; RARE, RA-response element; RAR, RA receptor; TR, thyroid hormone receptor; VDR, vitamin D₃ receptor; RXR, retinoid X receptor; GST, glutathione S-transferase; EMSA, electrophoretic mobility-shift assay; T₃, 3,3',5-tri-iodo-L-thyronine.

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vaccinia virus was obtained from Promega (Madison, WI, U.S.A.), purified VDR was from PanVera (Madison, WI, U.S.A.) and IgG, albumin and actin were from Sigma, Deisenhofen, Germany. Purified staphylococcal α -toxin was a gift from Dr A. Valeva (University of Mainz, Mainz, Germany). GST-TR α and GST-RAR γ_1 were purchased from Santa Cruz Biotechnology. GST-PPAR γ and GST-RXR α were provided by D. Fenske (University of Mainz, Mainz, Germany) and PPAR γ mammalian expression vector by Dr R. Evans (The Salk Institutes of Biological Research, La Jolla, CA, U.S.A.). Plasmids encoding GST fusions of Sp1 or truncations of Sp1 were provided kindly by Dr H. Rotheneder and Dr E. Wintersberger (University of Vienna, Vienna, Austria). Whole-cell extracts from HaCaT and S2 cells were prepared by lysing cells in high-salt extraction buffer (0.4 M KCl/20 mM Tris/HCl, pH 8.0/2 mM dithiothreitol/20% (v/v) glycerol/protease inhibitors) by three cycles of freeze-thaw. The extracts were centrifuged at 10000 g for 15 min at 4 °C to remove cell debris.

GST-RAR α and deletion constructs

RAR α cDNA was amplified from pECE-RAR α [13] with forward primer (5'-TAGGATCCATGGCCAGCAACAGCAGCTCC-3') and reverse primer (5'-TAGGATCCTCACGGGGAGTGGGTGGCCGG-3') and cloned into the *Bam*HI site of pGEX-2tk (Amersham Pharmacia Biotech) to yield an in-frame fusion of the GST and RAR α cDNAs. Deletion constructs of RAR α -pGEX-2tk were made by using restriction sites in the RAR α cDNA as indicated in Figure 9 (see below). Construct Δ 1 was generated by digesting RAR α -pGEX-2tk with *Kpn*I and *Sac*I and insertion of the linker pair 5'-CCGGTGCCCTCCCTACGAGCT-3'/5'-CGTAGGGAGGCACCGGGTAC-3'; Δ 2 was made by insertion of the linker pair 5'-CATTGAGAAGTGA-3'/5'-TCACTTCTCAATGAGCT-3' into *Sma*I and *Sac*I sites; Δ 3 originated from a digest with *Sma*I cleaving within the C-terminal portion of the RAR α cDNA and also in the multiple cloning site of the parent vector, and re-ligation with the double-stranded linker 5'-GGGCTCCT-3'. The recombinant plasmids were verified by automated sequencing. Expression in *Escherichia coli* BL21 DE3, affinity purification and analysis of fusion proteins were achieved according to the protocols supplied with the GST purification and detection modules (Pharmacia).

Oligonucleotides for EMSA

All oligonucleotides given below and the complementary strands were synthesized by MWG Biotech (Ebersberg, Germany). IL-1 β /WT, 5'-AGCTCAGAGAATTATCTCAGTTTATTAGTCCCCTCCCCTAAGA-3', comprises a sequence of the proximal human wild-type IL-1 β promoter that contains an Sp1-binding site (underlined) [30]. IL-1 β /MUT is a mutated version of IL-1 β /WT with three base changes (in bold) in this Sp1 site (5'-...TCCCCTAAACT...-3'). The GC-box oligonucleotide, 5'-CTTGGTGGGGGCGGGGCTAAGCT-3', corresponds to the core sequence of the oligonucleotide that was used for affinity purification of Sp1 (Promega); the GC-box is underlined. ZIP denotes a promoter region of the IL-2 gene which has been shown to bind Sp1 and other zinc-finger proteins [31], the sequence of the ZIP oligonucleotide used in this study is 5'-TTCTTAAGGGGGTGGGGATACA-3'. The EMSA probes derived from the CD14 and the low-density-lipoprotein receptor genes, 5'-CCAGGAAAGGAAGGGGGCTGGCTCGGAGG-3' and 5'-GCAGGGGGAGGAGTTTGCTAG-3', respectively, have been described earlier [32,33]. The Sp1-binding sites are underlined. The RA-response element (RARE) oligonucleotide with the sequence 5'-AGGGTTCACCGAAAGTTTAC-3' is

derived from the proximal RAR β 2 promoter. The imperfect direct repeat constituting the RARE is underlined [34,35].

EMSA

EMSA and the preparation of nuclear extracts were done essentially as described in [30]. Probes were generated by annealing complementary synthetic oligonucleotides and labelling with [γ -³²P]ATP (NEN Life Science Products) using polynucleotide kinase (Roche, Mannheim, Germany). Free [γ -³²P]ATP was removed by non-denaturing PAGE or by two rounds of spin-column chromatography with ChromaspinTM columns (Clontech). EMSA binding reactions contained 10 mM Hepes, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl₂, 10% glycerol and 2 μ g of poly(dI-dC) in a total volume of 30 μ l. After preincubating the various recombinant proteins or cell extracts (see Figure legends) in binding buffer (15 min at 25 °C), probe was added (50000 c.p.m.) and the mixture was incubated for 20 min at 25 °C before electrophoretic separation of protein-bound and free probe by non-denaturing PAGE (5% gel) in 0.09 M Tris/0.09 M borate/2 mM EDTA (pH 8.3) at 8 °C with constant voltage (200 V). Antibodies for supershift experiments and Western blotting (see below) were obtained from Santa Cruz Biotechnology and were used as recommended by the supplier. Some binding reactions contained RA or retinol (both from Sigma) at a final concentration of 1 μ M; all other samples in the respective assays received an equal amount of ethanol which served as a solvent for the ligands.

GST pull-down assay

GST or GST-Sp1 fusion proteins were bound to Sepharose 4B beads (Amersham Pharmacia Biotech). Subsequently, the beads were incubated with purified VDR (100 ng) for 1 h at room temperature, washed five times with NETN buffer (20 mM Tris/HCl, pH 8/50 mM NaCl/1 mM EDTA/0.5% Nonidet P-40), dried, boiled in loading buffer for 5 min and loaded on to SDS/PAGE (10% gel). After electrophoretic separation proteins were blotted on to Hybond-P-membranes (Amersham Pharmacia Biotech). VDR was detected by using an affinity-purified polyclonal antibody (Santa Cruz Biotechnology) and the alkaline phosphatase-based Protoblot detection kit from Promega.

Plasmids for transfection experiments

The construction of hybrid plasmids comprising human IL-1 β promoter segments (−777/+108, −674/+108, −513/+108, 234/+108, −209/+108 and −131/+108) in pBLCAT₃ and the internal deletion construct IL-1 β (−777/−234 U −131/+108)CAT have been described previously [30]. Plasmid IL-1 β (−209/+108)MUT-CAT contained a mutation of the wild-type sequence 5'-TCCCCTCCCCT-3' to 5'-TCCCCTAAACT-3' and was made by PCR-mediated mutagenesis. The reporter construct TRE₂-tk-CAT contained two copies of a perfect palindromic sequence and has been shown to respond to receptors for RA or T₃ (3,3',5-tri-iodo-L-thyronine) [36]. The construction of pECE-RAR and pECE-TR expression vectors has been described previously [13,36]. The sequences of all constructs were verified by dye-terminator cycle sequencing using an ABI 373 automatic sequencer. pPacSp1 for the expression of Sp1 in *Drosophila* cells was a generous gift of Dr R. Tjian (University of California at Berkeley, CA, U.S.A.) [37]. Plasmid preparations for transfections were obtained by two rounds of CsCl density-gradient centrifugation.

Cell lines, culture conditions, transient transfections and CAT assays

CV-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.), Schneider cells (S2) were from Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany) and HaCaT cells [38] were a gift from Dr N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany).

Plasticware for cell cultures was from Nunc (Wiesbaden, Germany) and cell-culture media and fetal bovine serum were from Biochrom (Berlin, Germany). CV-1 cells and HaCaT cells were kept in Dulbecco's modified Eagle's medium containing 10% heat-inactivated (56 °C, 30 min) fetal bovine serum in a humidified incubator with 5% CO₂. S2 cells were cultured at 25 °C in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum (both from Life Technologies). The transfection procedure using calcium phosphate precipitation was essentially as described previously [35]. CV-1 cells were seeded at a density of $\approx 1.5 \times 10^6/100$ mm dish 16–24 h before transfection. Transfection mixtures contained 3 μ g of pCH110 (Amersham Pharmacia Biotech) for β -galactosidase expression to correct for variations of transfection efficiency, 6 μ g of reporter plasmid and between 0.1 and 4.0 μ g of receptor-expression plasmids (see the Figure legends); pBluescript (Stratagene) was added to give a total of 20 μ g of plasmid DNA. Plasmids were diluted in a total volume of 460 μ l of water. Subsequently, 40 μ l of a 2.5 M stock solution of CaCl₂ were added. This mixture was combined with 500 μ l of a 2 $\times$ HEPES buffer (280 mM NaCl/10 mM KCl/1.5 mM Na₂HPO₄·2H₂O/12 mM dextrose/50 mM HEPES), and added to the cultures. Cells were incubated for 16 h with the transfection mixtures. Removal of the media containing the transfection mixtures was followed by a brief rinse with 5 ml of PBS, and fresh media containing charcoal-stripped fetal bovine serum and ligand, or media with solvent alone, were added. Cells were lysed by three cycles of freezing in liquid nitrogen and thawing. Lysates were cleared by centrifugation at 10000 *g* for 5 min.

Figure 1 An Sp1-binding site contributes to the RA response of the IL-1 β promoter

IL-1 β -CAT reporter constructs comprising varying segments of the IL-1 β promoter were generated. The positions of restriction-enzyme-recognition sites and the Sp1 site are indicated; the mutated site is crossed out. CV-1 cells were co-transfected by calcium phosphate precipitation (see the Materials and methods section for details) with 6 μ g of the respective reporter construct, and expression vectors for RAR (1 μ g) and β -galactosidase (3 μ g). The data represent the ratios of β -galactosidase-corrected CAT activities from parallel cultures treated with RA (1 μ M) or solvent only. Deletion or mutation of the Sp1 site reduces induction by RA. The number of independent experiments (*n*) is indicated; S.E.M. are given for data sets with *n* > 4.

The activity of CAT in lysates was determined using a liquid scintillation/extraction procedure employing [³H]acetyl-CoA (NEN Life Science Products) as a substrate [39]. The activity of β -galactosidase in the lysates was assessed with *o*-nitrophenyl β -D-galactopyranoside (Sigma) as a substrate, as described previously [40,41]. Transfection of S2 *Drosophila* cells was performed using a calcium phosphate precipitation procedure [42], and CAT activities were determined 72 h after transfection with equal amounts of protein from whole-cell lysates. Protein was measured using Bradford reagent (Bio-Rad, Richmond, CA, U.S.A.).

RESULTS

An Sp1 site renders the IL-1 β promoter sensitive to RA

IL-1 β is transcriptionally up-regulated by RA [19–21]. However, a classic RARE has not been described in its gene. To identify

Figure 2 Influence of NR, ligand and Sp1 on the activation of the IL-1 β promoter

(A) CV-1 cells were co-transfected with IL-1 β (-777/+108)CAT (6 μ g) and RAR α (0.5 μ g). Parallel cultures were treated with various concentrations of RA (□) or retinol (○); data points represent the means from experiments performed in triplicate. (B) On the left-hand side, data from CV-1 cells co-transfected with IL-1 β (-234/+108)CAT (6 μ g) and either RAR α (1 μ g) or TR α (1 μ g) are shown. The fold induction of CAT activity by 1 μ M RA (dark-grey columns) or 0.1 μ M T₃ (light-grey columns) was determined in parallel cultures; data are means from experiments performed in triplicate. As a control (right-hand side) for functional receptor expression, TRE₂-tk-CAT (4 μ g; an RAR- and TR-responsive reporter) was co-transfected with either receptor. (C) *Drosophila* cells were co-transfected in six-well plates. Each well received 7 μ g of IL-1 β (-209/+108)CAT together with either 1.5 μ g of empty expression vector (—) or the same amount of Sp1 expression plasmid (Sp1). Parallel cultures of both transfection mixes were treated with 10 μ M ponasterone (dark-grey columns) or solvent (ethanol, light-grey columns).

Figure 3 EMSA with GST-RAR α , Sp1 and GC-rich DNA

(Top-left panel) EMSA were carried out with IL-1 β WT (left-hand panel) or IL-1 β MUT probes (right-hand panel), comprising the wild-type or mutated Sp1 sites of the IL-1 β promoter respectively. Binding reactions contained 100 ng of GST-RAR α or GST, 25 ng of Sp1 or combinations of these proteins. Some binding reactions received 1 μ M RA. The right panel was exposed for a longer period in order to reveal weaker signals. The arrowheads indicate Sp1 complexes of different mobilities, depending on the absence or presence of receptor. (Bottom-left panel) EMSA with Sp1 probes from the IL-2, CD14 and low-density-lipoprotein receptor (LDLR) promoters were performed with sub-saturating amounts of Sp1 (5 ng), and 100 ng of GST-RAR α . The arrowheads indicate Sp1 complexes forming in the absence and presence of receptor with the LDLR Sp1 probe. (Top-right panel) EMSA with the GC-box oligonucleotide and varying amounts of Sp1 in the presence or absence of 100 ng of GST-RAR α . Arrowheads point to Sp1 complexes of low and high mobility. (Bottom-right panel) Titration of GST-RAR (0.8, 4, 20 and 100 ng) with a fixed sub-saturating amount of Sp1 (5 ng) and an Sp1 probe (CD14 oligonucleotide). Controls shown in the first three lanes from the left contained no protein, 5 ng of Sp1 and 100 ng of GST-RAR α . Arrowheads indicate Sp1 complexes produced as the concentration of GST-RAR is increased.

promoter regions that may be involved in RA-mediated transcriptional regulation we transiently co-transfected CV-1 cells with IL-1 β -promoter-CAT constructs and RAR α . As shown in Figure 1, full induction by RA was dependent on a promoter segment between positions -234 and -131. Because Sp1 sites have been implicated in vitamin or hormone action we suspected that an atypical Sp1 site, which we had identified previously in this promoter region [30], might be involved in the regulation of the IL-1 β promoter by RA. This idea was then supported by the finding that a mutation of the Sp1 site virtually abolished the response to RA.

The RA response of the IL-1 β promoter is ligand-specific and depends on RAR

Dose-response curves for RA and retinol revealed the ligand-specificity of IL-1 β activation by RA (Figure 2A). A slight inhibition of basal levels was observed in retinol-treated cultures. Retinol might compete with minute amounts of RA present in the culture system, and act as an inhibitory ligand of regulatory proteins. While RAR α induced the reporter in the presence of RA, TR α was not capable of activating IL-1 β (-234/+108)CAT in the presence or absence of its cognate ligand. A reporter

containing two copies of a perfect palindromic sequence was employed as a control for functional expression and ligand activation of RAR and TR. The promiscuous response element was activated strongly by either combination of receptor and cognate ligand (Figure 2B). Thus differential activation of the IL-1 β promoter by RAR and TR was not due to differential expression of functional receptors. RA-dependent activation of IL-1 β (-209/+108)CAT increased with the amount of transfected RAR cDNA in a range between 0.1 and 2.0 μ g. Of the RAR subtypes, RAR α proved to be the most efficient ($\approx$ 4–5-fold increase with 1 μ M RA), whereas RAR β yielded 2–3-fold activation and RAR γ virtually no induction with RA (results not shown). This resembled the receptor-typic activation pattern observed previously with some classic RAREs [43,44].

Sp1 and ponasterone activate the IL-1 β promoter in *Drosophila* cells

Sp1 sites may bind a variety of regulatory proteins. Before embarking on studies of molecular interactions, we therefore wished to establish that Sp1 can functionally interact with the IL-1 β promoter. For this purpose we performed transfection experiments with insect cells, which do not naturally express Sp1. Co-transfection of Sp1 expression vector increased the activity of the IL-1 β -promoter reporter 5-fold ($P < 0.02$ for Sp1 versus empty expression-vector control; Figure 2C). When ponasterone, a ligand for the nuclear ecdysone receptor of insect cells, was added to these cultures an additional 5-fold activation was observed, while a 2-fold induction was seen in control cells without Sp1. Although this difference did not reach statistical significance ($P < 0.1$ for Sp1- versus control-transfected cells), it may reflect a functional interaction of ecdysone receptor with Sp1.

GST-RAR, Sp1 and the GC-box form a ternary complex

To study potential interactions of Sp1, RAR and the Sp1-binding site of the IL-1 β promoter we labelled an oligonucleotide probe comprising this element and employed it in EMSAs with bacterially expressed RAR fusion protein and purified Sp1. As expected, Sp1 retarded the labelled probe and GST-RAR α did not. However, addition of GST-RAR α to Sp1 increased both the mobility and intensity of Sp1 bands (Figure 3, top-left panel). GST exerted no effect. RA did not influence complex formation in the absence or presence of receptor. Complexes did not form when oligonucleotides containing mutations in the Sp1 site were used. A number of TC-rich sites from other promoters gave results similar to the IL-1 β Sp1-site oligonucleotide (Figure 3, bottom-left panel), and we reasoned that enhancement of Sp1 binding by receptor may be limited to a sub-population of Sp1 sites, possibly to sites of low affinity for Sp1 [6]. However, the enhancing effect of GST-RAR α was also evident when a consensus GC-box oligonucleotide was used as a probe (Figure 3, top-right panel). An unexpected but consistent finding in these EMSA experiments was that Sp1 complexes migrated faster in the presence of receptor. Addition of sub-saturating amounts of receptor led to Sp1 complexes of intermediate intensity and intermediate electrophoretic mobility (Figure 3, bottom-right panel).

TR, VDR, PPAR and RXR, but not several unrelated proteins, enhance binding of Sp1 to the GC-box

We wished to know whether other members of the thyroid-receptor subfamily could also serve as auxiliary factors for Sp1.

Figure 4 Comparison of the effect of GST-TR, native VDR and other proteins on Sp1 binding

Controls with no protein and 5 ng of Sp1 alone are shown in lanes 1 and 2 of each panel (reading from the left). EMSA binding reactions in the left-hand panel contained Sp1 (5 ng) and/or GST-TR α (100 ng) together with Sp1 probe (ZIP oligonucleotide). EMSAs shown in the right-hand panel were performed with GC-box probe, 5 ng of Sp1 ($\approx$ 0.05 pmol) and 3 pmol of purified VDR, actin, albumin, *Staphylococcus aureus* α -toxin (α Tox) or IgG.

Figure 5 Stability of VDR-Sp1-GC-box and Sp1-GC-box complexes

Binding reactions with GC-box probe and 20 ng of Sp1 (left-hand half of the gel), or 2 ng of Sp1 plus 10 ng of VDR (right-hand half) were performed in the presence of a 200-fold excess of unlabelled probe oligonucleotide and incubated for the indicated times (t_{comp} : ', min; '', s) before electrophoretic separation of complexes and free probe.

Figure 6 Comparison of Sp1 complexes from extracts or purified protein

(A) Binding reactions contained GC-box probe, and either 10 μ g of protein from extracts of HaCaT cells (H) [38] or 50 ng of purified Sp1. Arrowheads on the left indicate Sp1 complexes; $\blacklozenge$, a complex that is supershifted by antibodies against the zinc-finger protein Sp3 (results not shown); $\blacktriangleleft$ (left- and right-hand panels) uncharacterized complexes. The antibody-mediated shift identifies the Sp1 complex from cell extract (H); no shift was induced by a number of other antibodies (results not shown). (B) Extracts (10 μ g of protein) from S2 *Drosophila* cells (D) and/or 5 ng of purified Sp1 were incubated with GC-box probe and analysed by EMSA. The arrowhead indicates a complex only obtained in the presence of both Sp1 and S2 cell extract.

In fact, GST-TR α enhanced Sp1 binding to the GC-box (Figure 4). Virtually identical results were also obtained with GST-PPAR γ and GST-RXR α (results not shown). In order to investigate the effect of a native class-II receptor on Sp1 binding to GC-rich DNA, we extended our study to a VDR preparation from a baculovirus expression system. Like the fusion proteins of RAR, TR, RXR and PPAR, native VDR produced marked complex formation with Sp1 at concentrations where neither protein alone generated visible bands. In contrast, a panel of various proteins unrelated to NR did not cause enhanced Sp1 binding. This ruled out the possibility of an unspecific effect being responsible for the increased band intensity.

VDR stabilizes binding of Sp1 to its target site

Competition experiments with unlabelled oligonucleotides were performed to compare the stability of Sp1–GC-rich DNA and Sp1–VDR–GC-rich DNA complexes. These experiments revealed that the ternary complexes are far more stable. While Sp1 complexes disassembled after brief exposure (30 s) to a 200-fold excess of unlabelled oligonucleotide, ternary Sp1–VDR complexes remained stable for at least 16 min under these conditions (Figure 5).

Comparison of Sp1 complexes from cell extracts and purified Sp1

If Sp1 associates with NRs or other proteins in the cellular milieu, this would be expected to result in an increased mobility of Sp1 complexes from cellular extracts as compared with complexes from purified Sp1. In fact, Sp1 bands obtained with

Figure 7 Antibody-induced mobility shift of RAR–Sp1–GC-box complexes

EMSA with the GC-box probe was performed with mixtures of RAR fusion protein and Sp1 in various ratios (the numbers given shown represent pre-diluted protein preparations in μ l: 1 and 4 correspond to 25 and 100 ng of GST-RAR γ_1 , and 3 and 6 to 5 and 10 ng of Sp1). Antibodies (2 μ g per lane) were added to the protein mixtures 2 h prior to the addition of probe. α -Sp1 and RAR α both induced a mobility shift of the complexes while α -CEBP (CCAAT-enhancer-binding protein) had no effect.

Figure 8 Sp1–RAR interaction on a RARE

EMSA was performed with a RARE as the probe. Binding reactions contained 25 ng of Sp1 and/or 500 ng of GST-RAR α , and antibodies (Ab) as indicated. $\blacklozenge$, RAR–RARE complex; arrowheads mark supershifted bands; $\blacksquare$, undefined complex; $\bullet$, free probe.

mammalian cell extracts migrated faster than Sp1 complexes obtained with the purified protein (Figure 6). As expected, insect-cell extracts did not yield the typical Sp1 band. However, when combined with low amounts of Sp1 that were insufficient to give

Figure 9 RAR α domains involved in ternary complex formation with Sp1 and GC-rich DNA

(A) Deletions ($\Delta 1$ – 3) of GST-RAR α (WT) used in the experiment shown in (B). Construction of the plasmids is described in detail in the Materials and methods section. The restriction-enzyme-recognition sites used for construction are indicated. The DNA-binding domain (DBD) and the ligand-binding domain (LBD) are indicated by grey shadows. (B) EMSA binding reactions contained either 100 ng of wild-type GST-RAR α fusion protein (wt) or an aliquot of deletion mutant, or no fusion protein (0); amounts of fusion proteins were equalized for GST enzyme activity. All reactions contained Sp1 probe (ZIP) and 10 ng of Sp1.

an appreciable signal in the absence of helper factors, insect-cell extracts supported the formation of a strong Sp1 complex. Notably, Sp1 complexes from extracts migrate like those of purified Sp1 plus receptor. Thus cell extracts contain helper factors, which potentially enhance and modulate Sp1 interaction with the GC-box.

Detection of RAR and Sp1 in a complex with the GC-box by antibody-mediated mobility shift

To demonstrate the presence of Sp1 and receptor in the presumptive ternary complexes, EMSAs with specific antibodies were performed. While anti-Sp1 readily induced a supershift, initial experiments using antibodies against peptide sequences of the C-terminus of RAR α or anti-GST antibodies failed (results not shown). In contrast, when we performed an experiment with RAR $\gamma 1$ and an affinity-purified antibody raised against the whole molecule a shift of the ternary complex was elicited; no shift was observed with anti-CEBP (CCAAT-enhancer-binding

Figure 10 Delineation of Sp1 domains interacting with receptor

Pull-down assays were performed with GST-Sp1 or GST fusions of truncated Sp1 bound to Sepharose. Binding of VDR to the immobilized Sp1 fusion proteins was detected by Western blotting as described in the Materials and methods section. The positions of the molecular-mass markers are given on the left and the left-most lane contained 20% of VDR input.

protein; see Figure 7). Additional negative controls not shown in Figure 7 included a series of other irrelevant antibodies, and binding reactions where larger amounts of Sp1 were used and RAR was omitted.

Sp1 and RAR can interact on a RARE

Up to this point, all EMSAs had been performed with Sp1-binding sites. We wished to know whether complexes can also form with RAR, Sp1 and consensus sites for the receptor. Accordingly we employed a response element for RAR as a probe. As expected, no binding of Sp1 alone was seen. In contrast, GST-RAR yielded a retarded complex, and the intensity of the band was enhanced when Sp1 was added to the binding reaction. Antibody against Sp1 completely abolished this signal and antibody to RAR α yielded two species of supershifted bands. From these data we conclude that in principle Sp1 can interact with RAR bound to a RARE (Figure 8).

RAR domains in the N- and C-terminal halves contribute to ternary complex formation with Sp1 and GC-rich DNA

In order to delineate receptor domains required for ternary complex formation with Sp1 and GC-rich DNA we constructed GST-RAR α fusion proteins containing partial deletions of the receptor, and employed them in EMSAs (Figure 9). A deletion of the extreme C-terminal end had only a minimal effect, and removal of the ligand binding domain almost abolished ternary complex formation. A construct with a deletion of the DNA-binding domain had a reduced capacity to enhance Sp1 binding.

The C-terminus of Sp1 interacts with VDR

To determine domains of Sp1 that interact with NRs we performed pull-down assays with VDR and GST fusion proteins containing Sp1 or parts of the molecule. Immobilized GST fusion proteins comprising either the C-terminus of Sp1 (622–788)

or the whole Sp1 molecule bound VDR (Figure 10). Upon extended development, binding to the Sp1(1–622) fusion protein was also evident (results not shown). In contrast, VDR did not interact with the N-terminus of Sp1 (1–293).

DISCUSSION

The primary finding of this work is that NRs can form ternary complexes with Sp1 and GC-rich DNA. Recently, this interaction has been postulated to explain Sp1-site-dependent hormone or vitamin responses of several target genes in the absence of classic response elements. Our data suggest that such a mechanism may also be involved in the activation of the IL-1 β promoter by RA. The identification of NR–Sp1–GC-box complexes was accomplished by showing that the mobility of Sp1–GC-box complexes changes in the presence of NRs, and by inducing a shift of the ensuing complexes with antibody to receptor (RAR) and also with antibody to Sp1. Unexpectedly, NR, Sp1 and the GC-box assembled into complexes of increased electrophoretic mobility compared with Sp1–GC-box complexes. This phenomenon could be due to an increased relative charge of the ternary complex [45]. Alternatively, RAR–Sp1–GC-box complexes might be of lower molecular mass than Sp1–GC-box complexes. This is possible because purified Sp1 binds as a homo-oligomer (principally as a tetramer) to the GC-box [46]. Partial replacement of Sp1 in tetramers by the smaller NR molecules would yield ternary complexes of lower mass (Figure 11). When Sp1 bound to RAR/RARE, it did not cause a lower mobility of the ensuing complex. Therefore, if charge shift is the cause of the mobility increase after addition of RAR to Sp1–GC-box complexes, it must result specifically from this interaction. Titration of receptor revealed at least two forms of ternary complex, and the receptor-induced mobility increase of Sp1 complexes was accompanied by a dose-dependent enhancement of band intensity. Recently,

enhancement of Sp1 complexes by ER [24,27,47] or by GST–RAR [22] has been reported; however, no concomitant mobility increase has been observed in these studies. The reason for the discrepancy with our results is unclear. Possibly, the differential mobility of NR–Sp1 compared with Sp1 complexes depends on the degree of Sp1 glycosylation and on the NRs studied. Differences between Sp1 preparations might play a role. The mobility change represents valuable information because it indicates a change of the composition, and not only of the quantity, of the ensuing complex.

What is the mechanism of enhanced Sp1 binding in the presence of receptor? First, conformational changes may increase the stability of Sp1–GC-rich DNA interaction. The experiment shown in Figure 5 documents that ternary complexes are in fact more stable than binary Sp1–GC-box complexes. A second mechanism could be the displacement of Sp1 molecules from homo-oligomers because it could make more Sp1 molecules available for binding to oligonucleotide.

The selectivity of Sp1–NR interactions was demonstrated with a panel of proteins unrelated to transcription factors; they had no effect on Sp1 binding in EMSA. The differential binding of receptor to N-terminal and C-terminal domains of Sp1 underscore the specificity of the interaction. That the Sp1 C-terminus serves as an interaction domain for VDR parallels recently published data with ER and with transcription factor E2F (adenovirus E2 promoter-binding factor) [29,48]. The ligand-binding domain of NR turned out to be most critical for the interaction with Sp1–GC-box complexes, but deletion of the DNA-binding domain significantly diminished ternary complex formation as well. This suggests that relatively large portions of the receptor may be important for binding to Sp1 and the GC-box, and could explain the failure to induce supershifts with anti-peptide antibodies, while an antibody raised against the whole RAR molecule shifted the ternary complex.

All receptors tested here enhanced Sp1 binding to DNA, which implies that they may compete for Sp1. The large number of potential interaction partners of Sp1 and the stability of receptor–Sp1 complexes also raise the question of whether binding of Sp1 to GC-rich DNA regularly involves auxiliary factors. Cross-talk between Sp1 and NRs is suggested by the recent finding that both utilize a protein that is part of a co-activator complex required for transcriptional activation by Sp1 [49]. It is tempting to speculate that NR can serve to bridge this protein to other components of the co-activator complex. Functional synergy between Sp1 and VDR or RAR γ may also be reflected by their genomic organization in a synteny group on chromosome 12q in humans and on chromosome 7 of the rat [50].

Despite the common theme of Sp1–RAR interaction in the present study and the previous work of Suzuki et al. [22], different mechanisms may be involved in these two cases. Thus in contrast to the IL-1 β -CAT constructs used here, the urokinase-reporter hybrids in the work of Suzuki et al. [22] did not respond to ligand. Possibly, various pathways can lead to ligand-induced transcription via Sp1–NR. These may include post-translational modifications, enhanced Sp1 binding as a consequence of receptor upregulation and recruitment of cofactors. Which pathway prevails may depend on the specific tissue and other conditions. The different results obtained with the urokinase and the IL-1 β promoter may be due to differential tissue expression of cofactors, since different cell types were used in these studies. A requirement for restricted cofactors may also explain the finding that TR α failed to induce the IL-1 β reporter in CV-1 cells, although it enhanced binding of Sp1 to the GC-box in EMSA. This is in line with the recent finding with ER subtypes α and β . ER α activates

Figure 11 Hypothetical model of ternary complex formation between Sp1, GC-rich DNA and NRs

NRs can form ternary complexes with Sp1 and the GC-box. Sp1 molecules may be displaced from homo-oligomers by receptor and might become available for binding to additional GC sites. Size and stoichiometry of NR–Sp1–GC-rich DNA complexes may be variable.

transcription via Sp1 sites in a tissue- and ligand-dependent fashion while ER β is inactive, although both ER subtypes can interact with Sp1 [29].

To summarize, the analysis of an atypical RARE led to the identification of a ternary complex composed of NR, Sp1 and GC-rich DNA. The observations can be accommodated in a model according to which NR would partially replace Sp1 in homo-oligomers. It appears that many, if not all, NRs may bind to Sp1; consequently it will be important to analyse the constraints that control Sp1-dependent activation by a given NR.

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