Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER) α , but not by ER β

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The physiological activities of estrogens are thought to be mediated by specific nuclear receptors, ER α and ER β . However, certain tissues, such as the bone, that are highly responsive to estrogens only express a low level of these receptors. Starting from this apparent contradiction, we have evaluated the potentials of two related receptors ERR α and ERR β to intervene in estrogen signaling. ER α , ERR α and ERR β bind to and activate transcription through both the classical estrogen response element (ERE) and the SF-1 response element (SFRE). In contrast, ER β DNA-binding and transcriptional activity is restricted to the ERE. Accordingly, the osteopontin gene promoter is stimulated through SFRE sequences, by ERR α as well as by ER α , but not by ER β . Analysis of the cross-talk within the ER/ERR subgroup of nuclear receptors thus revealed common targets but also functional differences between the two ERs.

Keywords: estrogen/nuclear receptors/orphan receptors/transcriptional interference

Introduction

Estrogens are lipophilic hormones that regulate a wide variety of physiological processes, such as the reproductive cycle and bone homeostasis (reviewed in Turner *et al.*, 1994). The effects of these hormones are mediated classically by their specific nuclear receptors that act as ligand-dependent transcription factors. The various nuclear receptors form a superfamily of genes that comprises receptors for diverse ligands such as thyroid hormone, retinoic acids, steroids and vitamin D (Gronemeyer and Laudet, 1995; Mangelsdorf *et al.*, 1995). Members of this family display a conserved domain organization. The A/B domain located in the N-terminal part of the protein harbors a ligand-independent transactivation function (AF-1). The C domain is the most conserved part of the molecule and is responsible for the specific DNA-binding activity of the receptors, a property that is also contributed to by parts of the D domain, lying immediately downstream. The E domain contains the ligand-binding hydrophobic pocket and contributes to receptor dimerization. Moreover, the extreme C-terminal part of this domain is necessary for the ligand-dependent transactivation function (AF-2).

Some receptors, such as the estrogen receptor (ER), also comprise an F domain, whose role is poorly understood. In the case of estrogens, two specific receptors have been identified: ER α (Green *et al.*, 1986; NR3A1 according to the Nuclear Receptors Nomenclature Committee, 1999) and, more recently, ER β (Kuiper *et al.*, 1996; NR3A2) that display divergent tissue distributions (Kuiper *et al.*, 1997). Both receptors bind estrogens [and in particular the predominant natural form 17 β -estradiol (E2)], although with different affinities. Both receptors recognize and activate transcription through the same DNA target [referred to as the estrogen response element (ERE)], an inverted repeat of the core element AGGTCA, separated by three nucleotides (Glass, 1994). So far, the individual role of these two receptors has not been well characterized (Giguère *et al.*, 1998), although an opposite interference with the AP1 complex has been reported (Paech *et al.*, 1997).

In apparent contrast to the definition above, the nuclear receptor superfamily also comprises a large number of so-called 'orphan' members, i.e. for which no ligand has been determined to date (Enmark and Gustafsson, 1996). Indeed, it is not known whether these receptors do have ligands that await identification or if they act in a constitutive manner. However, these receptors display the same domain organization as do classical receptors and are conserved through evolution, suggesting that they play critical roles (Laudet, 1997). Two orphan receptors are closely related to the ERs, hence their names: estrogen receptor-related receptor (ERR) α and β (Giguère *et al.*, 1988; NR3B1 and NR3B2, respectively). Sequence identity between these orphan receptors and the ERs is moderate (36%) in the ligand-binding E domain and it is thus no surprise that the ERRs do not bind E2. In contrast, the DNA-binding domain is more conserved within this subfamily (68% identity between the ERs and the ERRs). Accordingly, ERR β has been shown to bind to the ERE, as do the ERs (Pettersson *et al.*, 1996). In contrast, ERR α binds to and activates transcription through a single half-site preceded by a TCA trinucleotide (Bonnelye *et al.*, 1997a; Vanacker *et al.*, 1999), a sequence already described as responding to the SF1 orphan nuclear receptor and hence referred to as the SF1 response element (SFRE; Wilson *et al.*, 1993).

The positive effect of estrogens on bone homeostasis can be illustrated by post-menopausal osteoporosis (for a review see Pacifici, 1996). This syndrome can indeed be reversed by administration of natural or synthetic estrogens. However, the precise mechanism by which these compounds act is still poorly understood, as is the nature of their cellular target. Bone is a tissue under constant renewal, a phenomenon (referred to as remodeling) controlled by osteoblasts that accumulate bone mass and osteoclasts that resorb it. In both these cell

types, estrogens induce various responses, ranging from modulation of gene expression to regulation of proliferation. However, the ERs are very poorly expressed in osteoblasts and even less in the osteoclasts (Turner *et al.*, 1994, and references therein). Due to their homology to the ERs, we hypothesized that the ERRs could intervene in the signals induced by E2 in the bone. ERR β expression is restricted to early development and to a few adult tissues (Giguère *et al.*, 1988; Pettersson *et al.*, 1996). In contrast, ERR α has a broader spectrum of expression, including fat, muscle, brain, testis and skin (Bonnelye *et al.*, 1997b). Strikingly, this receptor is also highly expressed in the ossification zones of the mouse embryo (in long bones, vertebrae, ribs and the skull), and is more widely present in osteoblast-like cells than is ER α (Bonnelye *et al.*, 1997a). Moreover, we have shown that ERR α positively regulates the osteopontin gene (Vanacker *et al.*, 1998a), whose product, excreted by osteoblasts in the extracellular matrix, participates in bone remodeling (Denhardt and Guo, 1993).

On this basis, we have questioned the functional relationships between ERs and ERRs. Here, we show that the ERRs bind to and activate transcription through both the ERE and the SFRE. This is also the case for ER α but surprisingly not for ER β , which is unable to recognize the SFRE. The latter sequence thus represents the first differential target site between the ERs. Moreover, the osteopontin gene promoter is an SFRE-dependent, E2-dependent transcriptional target of ER α . The four members of the ER/ERR subgroup of nuclear receptors are thus interconnected in their transactivating properties.

Results

Common DNA-binding properties of ERs and ERRs

The DNA-binding properties of the ERs and the ERRs were first investigated. To this end, *in vitro* synthesized ER α , ER β , ERR α and ERR β proteins were assayed in gel electrophoretic mobility shift assays (EMSAs; Figure 1). ER α , ERR α and ERR β all bound to the SFRE, although with different capacities (Figure 1A). In particular, the ERRs were more efficient than ER α . Most surprisingly, ER β was incapable of such an activity. The same experiment was then performed using the consensus ERE as a probe (Figure 1A). All four proteins, including ER β , bound to this sequence, again with variable efficiencies. It is noteworthy that the ER α -ERE and ER β -ERE signals are within the same range. Since this cannot be reproduced with the SFRE probe, we conclude that the lack of ER β binding to this probe reflected an intrinsic property of this receptor. Control of translation efficiency by SDS-PAGE analysis (Figure 1B) confirmed that ER β was indeed produced. Although no SFRE was embedded in the ERE probe used here (see sequence on Figure 2A), ERR α formed a complex with the latter. To determine whether all SFRE-binding proteins could contact the ERE, we checked the binding capacities of SF1, another SFRE-binding orphan nuclear receptor. This receptor specifically bound to the SFRE sequence but not to the ERE (Figure 1C), indicating that ERE binding is not a general property of SFRE-binding receptors but is ERR specific.

The specificity of the observed bindings was examined by competition EMSAs (Figure 2). *In vitro* synthesized

ER α , ERR α or ERR β were first allowed to bind to radioactively labeled SFRE probe, and unlabeled oligonucleotides were added as competitors (Figure 2B; competitor sequences on Figure 2A). Mutations disrupting the TCA trinucleotide located upstream of the core AGGTCA element [mutants SFRE m1 (lanes 5, 6, 13, 14, 21 and 22) and SFRE m2 (lanes 7, 8, 15, 16, 23 and 24)] impaired the competition ability of the oligonucleotide. Binding of the receptors, and in particular of ER α , is thus SFRE specific. In addition, binding of this receptor to other SFRE-encompassing oligonucleotides differing in their flanking sites was also observed (data not shown), indicating that the latter are not relevant and do not provide any cryptic second half-site. Strikingly, an oligonucleotide comprising only C and T nucleotides in its flanking regions (i.e. outside the consensus SFRE) was also a binding target of ER α (data not shown). However, binding of ERR β to the SFRE probe was competed moderately by SFRE m1 and SFRE m2, suggesting that this receptor has a lower stringency towards the SFRE consensus sequence. Binding to the ERE was also controlled by competitive EMSAs (Figure 2C). Both half-sites were necessary for binding of all three receptors [see mutant ERE m2 (lanes 7, 8, 14, 15, 21 and 22)]. In addition, ERRs, but not ER α , required a particular sequence upstream of these half-sites (mutant ERE m1; compare lanes 5 and 6 with lanes 12, 13, 19 and 20). We next determined the consensus ERE sequence for each of the receptors, using competition EMSAs (Table I). As expected, flanking sites are irrelevant for ER α or ER β binding to the ERE. In contrast, ERRs only recognized a subset of ERE sequences. Thus, all ERs and ERRs share common DNA targets. The SFRE is not only contacted by the ERRs, but also by ER α . This is not the case for ER β , thereby representing the first direct binding difference between the ERs.

Homodimer binding has been demonstrated for ER α on the ERE (for a review, see Glass, 1994) and for ERR α on the SFRE (Vanacker *et al.*, 1999). The ERE contains two half-sites whereas the SFRE encompasses a single site. We thus addressed the question of homodimerization of ER α and ERR α (as typical members of the subfamily) on both probes, using co-translations of wild-type and A/B domain-lacking receptors that were tested in EMSAs (Figure 3). For ER α , as for ERR α , these constructs individually yielded a single complex with either the ERE or the SFRE. In each case, co-translation of a given receptor together with its corresponding deletion mutant generated an intermediately migrating complex that represented heterodimerization between wild-type and the deletion mutant. We thus conclude that ER α homodimerizes not only on the ERE but also on the SFRE probe. The same is also true for ERR α , further delineating DNA-binding activities that are common to ER α and ERR α .

Transcriptional properties of the ERs and the ERRs

The transcriptional behavior of ERs and ERRs was next analyzed by transient co-transfections of ROS 17/2.8 (rat osteosarcoma) cells with reporter constructs containing multimers of either the ERE or the SFRE site (Figure 4). All four receptors activated transcription through the ERE. ERRs and ER α , but not ER β , also enhanced reporter activity driven by the SFRE element. All ER activities were estradiol (E2)-dependent and were inhibited by

Fig. 1. Binding of ERs and ERRs to common DNA targets. (A) Binding of the ERs and ERRs to SFRE and ERE probes. *In vitro* synthesized ERα, ERβ, ERRα or ERRβ proteins were allowed to bind to radiolabeled consensus ERE or SFRE oligonucleotides, the sequences of which are schematized (the arrow represents the AGGTCA core element). A 4 μl aliquot of the translation mixture was used for ERβ, and 2 μl for all the other receptors. Where necessary, reaction mixture were completed with unprogrammed reticulocyte lysate up to 4 μl. Lanes 1 and 6, unprogrammed reticulocyte lysate used as a control (unpr.). Note that the SFRE probe occasionally generates a high background level even with unprogrammed reticulocyte. EMSAs were repeated three times with essentially the same results. (B) Translation efficiency. Translation efficiency was determined by loading the same volume (4 μl for ERβ, 2 μl for all other receptors) of [³⁵S]methionine-labeled translation mixture on an SDS-polyacrylamide gel. (C) Binding of SF-1 on the SFRE, but not on the ERE probe. *In vitro* synthesized SF-1 protein was allowed to bind to the radiolabeled SFRE or ERE probe, as indicated. The arrow indicates the position of the SFRE-SF1 complex. Lanes 1 and 4: the same amount of unprogrammed reticulocyte. Lanes 3 and 6: a 100-fold molar excess of the homologous, unlabeled oligonucleotide was added as a competitor.

addition of anti-estrogen (ICI 164,384) to the culture medium. In contrast, these compounds were inefficient on the ERRs. However, the transcriptional activities of the ERRs required a serum factor removed by charcoal treatment as described in Vanacker *et al.* (1999). As a negative control, the unrelated metallothionein promoter was not regulated by any of the receptors. SF1 orphan nuclear receptor was also examined for its transactivation properties. We used co-transfection in HeLa cells, since no SF1-driven activity could be detected in ROS 17/2.8 cells (Vanacker *et al.*, 1999; data not shown). As expected, this receptor activated transcription through the SFRE but not through the ERE. Our results are thus in agreement with the DNA-binding data presented above and reveal a potential cross-talk between ERs and ERRs at the level of their DNA targets. In addition, experiments using the SFRE demonstrate a clear functional difference between the ERs, ERβ being unable to act through this sequence.

Two activation functions have been identified in nuclear receptors, the activation function 1 (AF-1) embedded in the A/B domain and the AF-2 located in the ligand-binding domain. We analyzed the contribution of these activation functions to stimulation through the SFRE and the ERE by ERα and ERRα (as typical members of the subfamily) using mutated receptor constructs (Figure 5). Both receptors absolutely required their AF-2 domain, as already suggested by their dependence on E2 for ERα and on a unidentified serum compound for ERRα (Figure 5A). Deletion of the AF-1 of ERα resulted in a partial loss of transcriptional activity on SFRE and on ERE, suggesting identical pathways for both activities. In contrast, deletion of the putative AF-1 domain of ERRα

did not reduce its transactivating potential, pointing to mechanistic differences between ERα and ERRα transcription activation. Receptor expression after transfection was examined using EMSA on an ERE probe with proteins extracted under non-denaturing conditions (Figure 5B).

The osteopontin promoter is regulated by ERα through SFRE sequences

Since our results suggest a new pathway for ERα-induced regulation, we searched for a natural promoter that could be modulated by this receptor through SFRE sequences. The osteopontin (*opn*) gene is expressed in many tissues (Denhardt and Guo, 1993), but preferentially by the bone, where E2 plays a critical role (Turner *et al.*, 1994). Moreover, *opn* expression is enhanced by E2 *in vivo* (Craig and Denhardt, 1991), though its promoter does not contain any ERE sequence. In contrast, seven SFRE-like sequences can be recognized in this promoter (Vanacker *et al.*, 1998a). We first tested the binding capacities of ERα on these sequences by competition EMSAs (Figure 6). The complex formed between ERα and the consensus SFRE can be displaced by addition of unlabeled oligonucleotides corresponding to *opn* SFRE1, 2 and 3 (Figure 6B). Mutations that disrupted the SFRE (see sequences in Figure 6A) impaired the competition ability, indicating that ERα-binding is specific. The other four SFRE-like elements present on the *opn* promoter were unable to inhibit the formation of the SFRE-ERα complex (data not shown).

The ability of ERα to regulate *opn* promoter expression was next tested by transient co-transfection (Figure 7). The *opn* promoter driving the luciferase reporter gene

Fig. 2. Specificity of binding of ER α , ERR α and ERR β to ERE and SFRE probes. (A) Sequence of the oligonucleotides used in this study. The consensus core element is indicated in upper case letters. Underlined letters represent the mutations introduced relative to the consensus sequence. (B and C) EMSAs. The same protocol as above was used for EMSA with the addition of the indicated molar excess of unlabeled oligonucleotides used as competitors. (B) The consensus SFRE was used as a probe; and (C) the consensus ERE was used as a probe. unpr.: unprogrammed reticulocyte lysate.

(plasmid p Δ 777luc) was activated by ER α in an E2-dependent manner. To determine the sequences responsible for this activity, the SFRE-like sequences bound by ER α were then mutated either individually or in combination. The resulting constructs were assayed in transient co-transfections. Mutation in any of the SFRE-like sites resulted in a lower capacity of ER α to activate the *opn* promoter. Disruption of all three ER α -binding SFRE-like sequences was necessary to abrogate the whole ER α -driven transactivation. Consistently, plasmid p Δ 542luc,

which is deleted in the 5' part of the promoter and does not contain the three SFREs, did not respond to ER α . We also tested the effect of ER β on the *opn* promoter. In agreement with our binding data, this receptor was unable to stimulate either the full-length or the deleted construct. Our results show that three SFRE sequences are necessary to ensure maximal ER α stimulation of the *opn* promoter.

We next examined whether these sequences are sufficient to confer this activity. Trimers of the *opn* SFREs were inserted in front of a minimal promoter and the

Fig. 3. ER α and ERR α bind as homodimers. Constructs encoding a wild-type protein (wtER) and a deletion mutant protein (Δ ER) lacking the A/B domain of ER α (left panels) or ERR α (wtERR and Δ ERR; right panels) were co-translated *in vitro*. Reaction products were tested by EMSA on the ERE or SFRE probe, as indicated. The positions and nature of the dimeric complexes are indicated (wt- Δ : heterodimer between the wild-type and deletion mutant). unpr.: unprogrammed reticulocyte lysate used as a control. Wild-type:deletion mutant ratios were 0:100, 25:75, 50:50, 75:25 or 100:0. The asterisk indicates a non-specific band. Note that the ER α experiment was run on a 7.5% acrylamide gel, whereas the ERR α experiment was run on a 5% gel.

Table I. Sequence requirements for ERs and ERRs binding

				ER α	ER β	ERR α	ERR β
ERE	acgggtag	AGGTCA	ctg TGACCT	ctaccgcg	+++	+++	+++
m1	acgggccc	AGGTCA	ctg TGACCT	gggcccgcg	+++	+++	-
m2	acgggtag	AGGTCA	ctg TATGGT	ctaccgcg	-	-	-
m3	acgggtag	AGGTCA	ctg TGACCT	gggcccgcg	+++	+++	+
m4	acgggtca	AGGTCA	ctg TCACCT	ctaccgcg	+++	+++	+++
m5	acgggtac	AGGTCA	ctg TGACCT	gtaccgcg	+++	+++	-
m6	acgggtcg	AGGTCA	ctg TGACCT	cgaccgcg	+++	+++	+++
m7	acgggcag	AGGTCA	ctg TGACCT	ctaccgcg	+++	+++	+++
m8	acgggtcc	AGGTCA	ctg TGACCT	ggaccgcg	+++	+++	-
m9	acgggccg	AGGTCA	ctg TGACCT	cggcccgcg	+++	+++	-
m10	acgggcac	AGGTCA	ctg TGACCT	gtgcccgcg	+++	+++	-
m11	acgggtag	AGGTCA	gacTGACCT	ctaccgcg	+++	+++	+++
Consensus ER α /ER β				AGGTCA nnn TGACCT			
ERR α				ag AGGTCA nnn TGACCT ct			
ERR β				(c/a)g AGGTCA nnn TGACCT g(c/a)			

EMSAs were performed on *in vitro* synthesized ER α , ER β , ERR α or ERR β proteins bound on the ERE probe. The indicated unlabeled oligonucleotides were added at a 100-fold molar excess. The competition potential of each of these oligonucleotides was evaluated visually and is reported ranging from - (no binding) to +++ (binding identical to the consensus ERE). Core ERE is represented in upper case letters. Mutations relative to the wild-type ERE sequence are underlined. The deduced ERE consensus for each receptor is represented.

resulting constructs were co-transfected together with an ER α -expressing plasmid. A consensus SFRE-containing plasmid was used as a positive control. Only the SFRE2 sequence conferred an ER α response on a minimal promoter in an E2-dependent manner. This indicated out the importance of the promoter context in the case of the *opn* gene. *opn* is thus a target gene of ER α , whose regulation is not exerted through a classical ERE but through SFREs, a property shared with the related orphan receptor ERR α .

Discussion

Three main conclusions can be drawn from the data presented here, and these are schematized in Figure 8. First, the SFRE can substitute specifically for the ERE in

mediating the transcriptional effect exerted by E2, as exemplified both by isolated sites and by the natural *opn* promoter. Secondly, at least certain EREs can be recruited for the regulation of promoter expression in the absence of ERs and even of E2 since ERRs can act through these response elements. Thirdly, our data document the first binding discrimination between ER α and ER β , for which very few functional differences have been demonstrated to date (Paech *et al.*, 1997; Giguère *et al.*, 1998).

A new pathway for E2 action

The results presented in the present report demonstrate that ER α can bind to and activate transcription through the SFRE site. Binding is site specific since the presence of the 5' TCA trinucleotide is required, as judged by our

Fig. 4. Transcriptional activities of ERs and ERRs. Rat osteosarcoma ROS 17.2/8 cells were cultivated in phenol red-free medium supplemented with charcoal-treated serum (strip.) or in normal medium supplemented with untreated serum (norm.). Where stated, ER α , ER β , ERR α or ERR β expression plasmids were transiently co-transfected together with plasmid pBLCAT4 supplemented with three copies of the consensus SFRE and plasmid pGL2pru supplemented with two copies of the consensus ERE. Where indicated, the metallothionein IIa gene promoter driving the Luc reporter sequence (mtIIaLuc) was used as a negative control. The SF-1-expressing plasmid was transfected in HeLa cells cultivated in normal medium. Cells were cultivated further for 48 h in the indicated medium, eventually supplied with 10^{-8} M estradiol (E2). Anti-estrogen ICI 164,384 (10^{-8} M) was added to the stripped medium containing E2 (ERs) or to the normal medium (ERRs). Reporter activities were determined and were plotted as fold activation over the individual CAT or Luc activity without receptor. This represents the average of three independent transfections, with errors bars displaying the variations between the experiments.

competition experiment. It is thus not likely that every core element (AGGTCA)-containing oligonucleotides could mediate ER α transactivation. Indeed, ER α is unable to contact a direct repeat of the core element separated by five nucleotides (DR5; data not shown). Binding of ER α to the classical ERE requires the integrity of both core elements, as exemplified by lack of binding to oligonucleotide ERE m2 (Figure 2B). In contrast, the SFRE only contains one of these half-sites. To exclude the existence of a cryptic second half-site in the SFRE-containing oligonucleotide, experiments were also performed with probes with different flanking sequences (data not shown). ER α binding was observed in all cases, in particular with a probe comprising only three bases outside the consensus SFRE. We conclude that in the absence of a second half-site, trinucleotide TCA is required for ER α binding. Despite the lack of a second half-site as in the ERE, ER α binds as a homodimer on the SFRE. The mechanism of this activity presently is unknown, but it can be suggested that one of the ER α molecules strongly binds to the core half-site, whereas the other weakly contacts the flanking sequences.

Our transactivation experiments were performed with mouse and human ER α , with identical results (data not shown). The nature of the reporter (CAT or luciferase) and of the minimal promoter used (tk or SV40-derived) was also irrelevant (data not shown). However, promoter context seems to be of importance for ER α -driven activa-

tion through the SFRE. Maximal activation of the *opn* promoter by ER α requires sites S1, S2 and S3. In contrast, ERR α does not bind to site S3, and transactivation of the *opn* promoter by this receptor only requires sites S1 and S2 (Vanacker *et al.*, 1998a), indicating the specificity of the sequence recognition between ER α and ERR α . Only site S2 is capable of transferring an ER α response to a minimal promoter. The reason why sites S1 and S3 are deficient in this activity presently is unknown. Conversely, a perfect SFRE site is present in the promoter driving the thyroid hormone receptor- α gene (*TR α*), and is necessary and sufficient to confer an ERR α -driven transcriptional response (Vanacker *et al.*, 1998b). Although this element confers ER α -responsiveness to a minimal promoter, we have not observed any effect of ER α on *TR α* gene promoter expression (data not shown). Elgort *et al.* (1996) also reported that stimulation of the *RAR α 1* gene promoter by E2 partially depends on an SFRE site. In contrast to the present results, these authors did not report any ER α binding to this sequence. It is thus likely that ER α stimulates transcription through the SFRE in a promoter-specific manner.

opn expression has also been shown to be positively regulated by vitamin D3 (Noda *et al.*, 1990) and tumor-promoting agents (TPA; Craig *et al.*, 1989), through discrete sites mapped on the promoter. Transient transfection experiments have not revealed any cross-talk between activation by vitamin D receptor or TPA on the one hand, and by ER α or ERR α on other (data not shown).

Fig. 5. Domain requirements for transactivation. ROS 17.2/8 cells were co-transfected with the indicated reporter plasmid (0.1 μ g) and 0.5 μ g of the ER α or 0.9 μ g of the ERR α derivative-expressing plasmid. Some of the cells were extracted under non-denaturing conditions and analyzed by in EMSA while the remaining cells were used in reporter tests. mutAF1 mutants harbor a deletion in the A/B domain (ER α , see Pettersson *et al.*, 1997; ERR α , see Vanacker *et al.*, 1999). ER α mutAF2 harbors a point mutation in the E domain (L450Q; Ince *et al.*, 1995) and ERR α mutAF2 is deleted in its C-terminal part (Vanacker *et al.*, 1999). (A) Reporter assays. Reporter activities were determined and were plotted as fold activation over the individual CAT or Luc activity without receptor. This represents the average of three independent transfections, with errors bars displaying the variations between the experiments. (B) Expression assays. The amount of protein in the extracts was quantified using colorimetric tests, and equal amounts were submitted to EMSA using the ERE probe. Arrows represent the specific bands obtained with ER α (left side) or ERR α (right side) derivatives. Lane 1: an extract from pSG5-transfected cells used as a control.

E2-independent stimulation through the ERE

Our results show that ERR α and ERR β stimulate transcription not only through the SFRE but also through the ERE, or at least a subset of these response elements. In the case of ERR α , this was confirmed using cDNAs of human and mouse origin (data not shown). The fact that SF1, though sharing with ERR α the property of binding to the SFRE, does not act through the ERE indicates that transactivation via the latter element is ERR specific. EREs (and the natural promoter containing them) can thus be regulated in an E2-independent manner, i.e. through the ERRs. Consistently, anti-estrogens are also inactive in this pathway. This also indirectly demonstrates that ERR α is

Fig. 6. ER α binds on SFRE-like sites of the *opn* promoter. (A) The *opn* promoter. The schematic structure of the mouse *opn* gene promoter is displayed with the position of the SFRE-like sites relative to the transcription start site. The sequence of the first three SFRE-like sequences (S1, S2 and S3) is shown. Nucleotides diverging from the consensus SFRE site (SFREc) are underlined. Bold characters represent the nucleotides changed in the mutant sequences (S1M, S2M and S3M) used in this study. The arrow represents the promoter transcription start site. The S3 site is in fact in the opposite orientation compared with S1 and S2. (B) Competition EMSA. *In vitro* synthesized ER α protein was allowed to bind to radioactively labeled consensus SFRE. Where indicated, unlabeled oligonucleotides were added as competitors. unpr.: unprogrammed reticulocyte lysate used as a control.

sufficient for this regulation and that it does not require an endogenous ER that would be affected by ICI 164,384. However, ERR α and ERR β require a serum factor that is removed by charcoal treatment, which could suggest the existence of a ligand for these receptors, a hypothesis that we have already proposed for ERR α (Vanacker *et al.*, 1999).

The fact that the ERRs can activate transcription via the same elements as do the ERs raises interesting new possibilities concerning the mechanisms of action of E2. Indeed, it has been shown that ERR α expression is enhanced *in vivo* by E2, at least in the uterus (Shigeta *et al.*, 1997). This could generate a snowball effect whereby E2 acts not only through its specific receptors but also indirectly through ERR α , both receptors ultimately activating the same ERE and/or SFRE elements. This could prove particularly important in tissues where a massive effect of E2 has been reported together with a low amount of ER. This is the case in bones, where estrogens act as protective agents (Turner *et al.*, 1994; Pacifici, 1996), whereas there are 50- to 100-fold fewer estradiol-binding sites present in osteoblasts than in sexual tissues (Eriksen *et al.*, 1988; Komm *et al.*, 1988). In

Fig. 7. Response of the *opn* promoter to the ERs. (A) ER α stimulates the expression of the *opn* promoter. A schematic representation of the *opn* promoter derivatives used in this study is displayed. Open circles represent wild-type SFRE-like sequences, whereas closed circles symbolize sequences mutated as in Figure 6A. All constructs were verified by sequencing. Reporter plasmids were co-transfected in ROS 17.2/8 cells cultivated in stripped conditions together with ER α or ER β . Estradiol (E2; 10 nM) was eventually added after transfection. Reporter activities were determined 48 h after transfection. The results are plotted as fold activation over the Luc activity without E2 and represent the average of three independent transfections. n.d.: not determined. (B) Response of the isolated *opn* promoter derivatives to ER α . Reporter plasmids containing three copies of the consensus SFRE, S1, S2 or S3 site originating from the *opn* promoter were co-transfected in ROS17.2/8 cells cultivated in stripped conditions together with varying amounts of ER α -expressing plasmids. After transfection, 10 nM E2 was added where indicated. Reporter activities were determined 48 h after transfection. Results are plotted as fold activation over the individual Luc activity without receptor and represent the average of three independent transfections, with errors bars displaying the variations between the experiments.

contrast, our PCR experiments have shown that ERR α is expressed to a greater extent in this cell type than is ER α (Bonnelye *et al.*, 1997a). ERR α expression could also (at least partially) compensate for the absence of ER α in knockout mice, which actually results only in a weak bone phenotype (reviewed in Korach, 1994). The cross-talk hypothesis could also be expanded to other tissues expressing ER and ERR, such as the mammary gland or the uterus (Shigeta *et al.*, 1997; our unpublished results).

Fig. 8. Cross-talk between ERs and ERRs. All ERs and ERRs bind to and activate transcription through the ERE site (at least a subset of these sequences in the case of the ERRs). ERR α , ERR β and ER α , but not ER β , bind to and activate transcription through the SFRE sequence. ERs but not ERRs respond to estradiol (E2). *opn* is an example of a target gene of ER α acting through SFREs. Also schematized is the heterodimerization of ER α and ER β .

A functional difference between ER α and ER β

At present, few functional differences have been described between ER α and ER β . These receptors indeed have distinct, but partially overlapping patterns of expression (Kuiper *et al.*, 1997). Both receptors have been described to contact the same DNA element (the classical ERE) and to bind common ligands, although with different affinities. Paech *et al.* (1997) have shown, however, that these receptors signal in opposite ways on AP1-responsive sites: ER α activates transcription, whereas ER β represses it. Here we show a different behavior at the DNA-binding level, ER β being incapable of binding to the SFRE site. While anti-estrogens reversed the effect of ER α and ER β on AP1 sites (Paech *et al.*, 1997), ICI 164,384, though inhibiting ER α , was unable to induce ER β to bind to or to activate transcription via the SFRE site, whether as an isolated site or on the *opn* promoter (data not shown). Although ER α and ER β heterodimerize on the ERE probe (Cowley *et al.*, 1997; Pettersson *et al.*, 1997; our unpublished results), co-translation of these receptors did not produce any additional complex with the SFRE probe (data not shown). ER α is thus unable to drive ER β to the SFRE, and conversely ER β does not inhibit ER α binding to this probe.

The molecular basis underlying the binding difference between the ERs is unknown at the present time. Wilson *et al.* (1993) pointed out the importance of the T/A box (lying immediately downstream of the DNA-binding domain) in recognition of SFRE elements by nuclear receptors. Interestingly, sequence comparison of the T/A box of ERR α and SF-1 revealed a striking homology (Bonnelye *et al.*, 1997a) that can also be found on ERR β and ER α . On the contrary, the T/A box of ER β is more divergent (data not shown). The molecular relevance of these motifs remains to be determined.

Although we concluded that *opn* is not a direct target of ER β , we cannot exclude an indirect effect of this receptor. As pointed out above, E2 has been shown to enhance the expression of ERR α in the uterus (Shigeta *et al.*, 1997). While this regulation remains to be demonstrated in bone cells, ER β , which is the ER subtype preferentially expressed in this tissue (Onoe *et al.*, 1997),

could mediate this phenomenon. Since ERR α overexpression in osteoblast-like cells results in elevating the level of *opn* messengers (Vanacker *et al.*, 1998a), this receptor could be an intermediate between ER β and *opn*. Altogether, the pathways controlled by ERs or by the ERRs, all members of a subfamily of nuclear receptors, are interconnected at various levels. This opens up new perspectives for investigating the mechanisms of action of the signals triggered by these factors.

Materials and methods

EMSA

Proteins were *in vitro* translated (1 h reaction in the presence of RNasin and protease inhibitor) using the TNT reticulocyte lysate kit (Promega) and were allowed to bind to ³²P-end-labeled 30 bp oligonucleotide in a reaction buffer containing 10% glycerol, 10 mM HEPES, 30 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol (DTT) and 1 mM Na₂HPO₄ (pH 7.9). Single-stranded salmon sperm DNA (1 μ g) and poly(dI–dC) (0.4 μ g) were added. Reaction products were run on a 5% native acrylamide gel. Where indicated, a 10- or 100-fold molar excess of 30 bp unlabeled oligonucleotides was added as competitor. [³⁵S]Methionine was added to the translation mixture for protein analyses, which were performed under standard conditions. For the dimerization experiment, ER α and Δ A/BER α (mutant Δ 142 in Pettersson *et al.*, 1997) or ERR α and Δ A/BER α (Vanacker *et al.*, 1999) were co-translated at a 0:100, 25:75, 50:50, 75:25 or 100:0 ratio. EMSAs were performed as above, except that the ER α experiments were run on a 7.5% acrylamide gel.

Cells and transfections

ROS 17.2/8 (rat osteosarcoma) and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) ('normal' conditions). Cells were cleared of steroid hormones by 2 weeks of culture in phenol red-free DMEM supplemented with 10% charcoal-treated FCS ('stripped' conditions). A total of 10⁵ cells seeded in 6-well plates were transfected by 1 μ g of total DNA per assay (comprising 0.1 μ g of reporter plasmid) using 4 μ l of ExGen 500 (Euromedex, Souffelweyersheim, France) under the conditions recommended by the supplier. When necessary, pSG5 plasmid was used as a carrier. The culture medium was changed after transfection, with addition of 10 nM estradiol where indicated. Cells were lysed 48 h after transfection and assayed for luciferase and CAT activity. For a given receptor, the results were essentially identical whether SFRECAT and ERELuc were transfected separately or co-transfected in a single well. Transient transfections performed in HeLa cells yielded the same results.

Plasmid constructions

The ER α AF2 mutation corresponds to mutant 540Q described in Ince *et al.* (1995). The ER α AF1 deletion corresponds to mutant Δ 142 (Pettersson *et al.*, 1997). ERR α deletions have been described in Bonnelye *et al.* (1997) and Vanacker *et al.* (1999).

Deletions of the mouse *opn* promoter (plasmid p Δ 777luc and p Δ 542) have been described elsewhere (Guo *et al.*, 1995). Point mutations were introduced into the p Δ 777luc *opn* promoter derivative by a two-step PCR using standard methods. Mutated fragments were transferred into pGL2-basic reporter plasmid (Promega) and the resulting constructs were sequenced entirely. Mutations were also introduced in combination. Oligonucleotides encompassing the consensus SFRE or the SFRE-like sites were cloned as trimers in plasmid pGL2-PRM (Promega). Individual clones were sequenced to ensure the correct number of SFRE derivatives and the appropriate head-to-tail orientation with respect to the transcription sense.

The cloned oligonucleotides had the following sequences (SFRE is underlined): pSFREx3-luc, 5'-GATCCGGCGATTGTCAAGGTCAACACAGTA-3'; pS1x3-luc, 5'-GATCCACGTCTCTAAAGGTCAAGTGGAGGCA-3'; pS2x3-luc, 5'-GATCCGAGGAATTCAGGGTCACTGTGTGGA-3'; and pS3x3-luc, 5'-GATCCCCCAACTGACCTGGAACACAGTAA-3'. Note that the SFRE-like sequence present in plasmid pS3x3-luc is in the reverse orientation relative to pS1x3-luc and pS2x3-luc plasmids, with respect to its orientation in the original *opn* promoter.

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