The AF-2 Region of the Retinoic Acid Receptor α Mediates Retinoic Acid Inhibition of Estrogen Receptor Function in Breast Cancer Cells*

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The growth of estrogen receptor (ER)-positive breast cancer cells is inhibited by all-trans-retinoic acid (RA). In the present study, estrogen (E2) induction of pS2 mRNA levels was significantly reduced within 6 h following cotreatment with RA. In transient transfection experiments, RA repressed transactivation from a vitellogenin E2-responsive element by approximately 50% and wild-type RA receptor α (RARα) or RARβ enhanced this inhibition. Transfection of truncated RARα mutants terminating before or at amino acid 412 markedly decreased RA inhibition of E2-induced reporter gene activity. Expression of RARs with deletions of amino acids 413 and 414 in the transactivation-2 (AF-2) domain also reduced RA inhibition, while deletions and point mutations beyond amino acid 414 behaved like the wild-type RARα. RA-treated MCF-7 cells transfected with an RARα AF-2 region mutant were twice as sensitive to growth inhibition as untransfected and vector-transfected control cells. Thus, the AF-2 domain in the C terminus of the RARα mediates RA inhibition of ER-induced transcription in breast cancer cells. In addition, transcriptional interference between RARs and ERs may contribute to RA inhibition of ER-positive breast cancer cell growth.

Estrogen (E2)1 promotes the growth of E2-dependent breast cancer cells (1, 2). Along with the E2 receptor (ER), many breast cancer cell lines express nuclear receptors for retinoic acid (RA). Retinoic acid has been shown to inhibit the growth of hormone-dependent breast cancer cells both in vivo and in vitro (3–5). Non-additive inhibition has been demonstrated between retinoids and anti-estrogens, wherein the combination of RA and anti-estrogen produced a 75% inhibition of breast cancer cell growth compared with a 50% inhibition when either agent was used alone (3, 6), suggesting that each agent may produce common anti-estrogenic effects. Retinoids have been shown to decrease the production of the mRNA and protein for the progesterone receptor in MCF-7 ER+ breast cancer cells (7, 8), an effect which is in apposition to the positive effects of E2 on the expression of the progesterone receptor (7). A distinct protein of Mr 39,000 has also been identified in breast cancer cells whose synthesis and secretion are inhibited by RA (5).

The retinoic acid receptors (RARs) are members of the steroid/thyroid hormone receptor family which includes the nuclear receptors for vitamin D3, E2, and thyroid hormone (9). Well described properties of retinoic acid (RA) include its activity as a morphogen and a teratogen during development (see Ref. 10 and references therein). This metabolite of vitamin A can also induce the differentiation of embryonal carcinoma cells as well as promyelocytic leukemia cells (11, 12). Most of the biological effects of RA are thought to be mediated through the nuclear RARs, which are ligand-inducible transcription factors. Like other members of the nuclear receptor family, RARs modify the activity of associated promoters by binding to enhancer sequences in the regulatory regions of inducible genes (10, 13). Six functional domains have been described in members of the nuclear receptor superfamily. Regions A and B nearest the N terminus contain a ligand-independent transcription function denoted AF-1, while region C consists of well conserved zinc finger motifs involved in DNA binding and sequence recognition (14). The role of regions D and F are not understood. Region E has several functions including heterodimerization, ligand binding, and ligand-dependent transactivation (AF-2) (15). There are several types of RARs including the α, β, and γ subtypes, which bind the ligands all-trans-RA and 9-cis-RA and the α, β, and γ subtypes of the RXR, which bind only 9-cis-RA. The RXRs have been shown to be heterodimerization partners for RARs, thyroid hormone receptors, and vitamin D3 receptors and their presence enhances by severalfold the binding of these receptors to their preferred response elements (reviewed in Ref. 16).

A compendium of genes responsive to different nuclear receptors and their ligands has been assembled, and the close similarity between these enhancer elements has become clear. The consensus DNA sequence motif to which the retinoid, vitamin D3, and estrogen receptors bind contains the half-site GGTC in the form of a palindrome or as a direct repeat with varying numbers of spacer nucleotides (17, 18). While there is clearly some selectivity with respect to receptor/ enhancer identities, there is also a degree of promiscuity between receptors and enhancers (19).

Since RA inhibits the growth of E2-dependent breast cancer cells, we set out to determine if RA could inhibit E2-induced transcription and the mechanism by which this occurs. In addition, we wished to investigate the contribution of RA-induced transcriptional inhibition of the ER to the growth inhibitory activity of RA. The pS2 gene is expressed in hormone-dependent MCF-7 breast cancer cells in an estrogen-responsive manner, and an estrogen response element (ERE) has been identi-
RARE AF-2 Region Interference with ER Activation

MATERIALS AND METHODS

Northern Blot Analysis—RNA was extracted using the LiCl/urea procedure (21). RNA was separated on 1% agarose 1.1 M formaldehyde gels then transferred and cross-linked to Hybond N (Amersham Corp.). Hybridization was carried out with multi-prime labeled cDNA probes for pS2 (22) and tubulin to control for loading equivalency.

Cell Culture and Transfection—MCF-7 cells were maintained in α-minimal essential medium (Life Technologies, Inc.) supplemented with nonessential amino acids, 0.3% glucose, and 5% fetal bovine serum. For experiments involving E2 induction, MCF-7 cells were grown for 7 days to 80% confluence in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% 2-mercaptoethanol/charcoal-stripped fetal bovine serum. The medium was changed 12 h before the addition of ligands. For transfection experiments, MCF-7 cells were grown in α-minimal essential medium, then washed with phosphate-buffered saline and the medium changed to phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% 2-mercaptoethanol/charcoal-stripped fetal bovine serum. The following day the cells were split and allowed to grow for 48 h in E2-free medium as described above before transfection.

Cells were transfected using the calcium phosphate precipitate method (24). Routinely, 5 μg each of either the kinase-dead kinase-dead galactosidase-phosphorylated thyroid hormone receptor-α2 (Vit-ERE) or vector denoted by the C-terminal (hatched region).

RESULTS

Retinoic Acid Inhibits Induction of mRNA for pS2 by Estrogen in MCF-7 Cells—The pS2 gene encodes a secreted polypeptide with homology to a pancreatic protein, which inhibits gastrointestinal motility and acid secretion (22) and is expressed under the control of E2 in MCF-7 breast cancer cells. To study the effect of RA on an endogenous E2-inducible gene, Northern blot analysis of pS2 gene expression was performed on MCF-7 cells treated for up to 8 h with 10−8 M E2 or with 10−7 or 10−6 M RA. The results in Fig. 2 show that, as expected, cells grown in phenol red-free medium and 5% charcoal-stripped fetal calf serum for 7 days did not produce pS2 mRNA, while cells treated for 24 h with E2 expressed the pS2 transcript. After 3 h of exposure to E2, there was little difference between cells treated with either concentration of RA. However, by 6 h of E2/RA exposure, there was markedly less pS2 transcript level in cells treated with 10−6 M RA compared with untreated cells, and by 8 h this decrease was more pronounced and also evident in cells treated with 10−7 M RA.

The RARα/RXR heterodimer binds with low efficiency to the ERE—To determine if RA inhibition of E2-induced transcription might be due to inhibition of ER binding to the ERE, we performed in vitro binding experiments. The result in Fig. 3 shows the gel mobility shift analysis obtained when equimolar amounts of the RARα, mutant RARαγ, and RXRβ were incubated individually or together with the ERE oligonucleotide. As expected, only the ER bound with high efficiency to the ERE as a homodimer. The RARα and RARαγ both bound the ERE as heterodimers with the RXRβ, although with much reduced efficiency compared with the ER, and did not compete with the ER when present in equimolar amounts. In addition, we have not observed RA-mediated decreases in the expression of the ER (data not shown). Under the conditions used for binding and electrophoresis, we did not observe binding of the RXRβ to the ERE. In contrast, the RARα/RXRβ heterodimer bound with high efficiency to the RARE oligonucleotide derived from the direct repeat RARβ-2 enhancer (RAREβ), while the ER alone or in combination with RXRβ did not.
Inhibition of E2-responsive Transcription—

The AF-2 Region of the RARα Is Required for RA-mediated Inhibition of E2-responsive Transcription—The results above indicated that RA can inhibit the E2 induction of an endogenous E2-responsive mRNA. In order to test whether or not this inhibition occurred at the transcriptional level, a reporter gene construct containing the Xenopus vitellogenin ERE linked to CAT was transfected into MCF-7 cells and the cells were treated with E2 or E2 and RA. Fig. 4 shows the results of a typical experiment. Addition of E2 to the cells resulted in an approximate 6-fold induction of CAT activity, while the simultaneous addition of RA decreased the activation of this promoter by about 50% (Fig. 4 and Table I). To evaluate the role of the RARα on RA-mediated inhibition of E2-dependent transcription, an RA receptor expression plasmid was cotransfected into the cells with the Vit-CAT reporter gene construct. Transfection of wild-type RARα into MCF-7 cells resulted in an approximate 30% increase in the fold induction of CAT activity as a result of a decrease in control background levels of transcription (Fig. 4). The presence of transfected RARα in these cells also resulted in significantly greater inhibition of E2-induced transcription following treatment with RA. As our results showed that the liganded RARα could function to inhibit E2-induced transcription, we set out to determine which part of the receptor mediates this effect. Since the C terminus of the RARα has been shown to be important for ligand-dependent transcriptional activation (29, 30), we tested the effect of introduction of a C-terminally truncated mutant of the RARα on RA inhibition of the E2 response. MCF-7 cells were transfected with a truncated mutant of the RARα called RARα', which is missing 70 amino acids from the C terminus such that all of the F domain and a small fraction of the ligand binding domain are deleted. This receptor has been shown previously to be a dominant repressor of RA-induced transcription from RA response elements (11). Table I shows that, in contrast with the wild-type RARα, E2 induction of gene transcription was only weakly inhibited by addition of RA. To further delineate the region of the RARα necessary for interference with the ER, we cotransfected mutants of the RAR downstream of the RARα truncation into MCF-7 cells along with the Vit-CAT reporter gene (Table I and Fig. 4). Slightly better RA inhibition was obtained following transfection with the RARα C-terminal deletion mutants 404Δ and 412Δ, although it still remained below 30%. Transfection of RARα deletion mutants 414Δ and 419Δ resulted in recovery of RA-induced inhibition of the E2-induced Vit-CAT activity to wild-type levels. To define this region more precisely, we transfected the RARα mutants, ML413,414Δ and E415A/E418A, both of which terminate at amino acid 419, into MCF-7 cells along with the Vit-CAT reporter gene. The RARα ML413,414Δ prevented RA inhibition of E2-induced CAT activity to the same extent as 412Δ and the other larger deletions while RARα E415A/E418A produced wild-type levels of inhibition. Taken together, these results demonstrate that either one or both of the AF-2 region amino
acid residues 413 and 414 are essential for mediating RA-dependent inhibition of ER transactivation.

Interference with E2-Induced Transcription by RA Is a Dominant Effect—In order to determine whether RA inhibition of E2-responsive transcription is RARα concentration-dependent, we transfected various concentrations of the RARα mutant Δ412 into MCF-7 cells. The results in Fig. 5 show that, as expected, RA treatment of MCF-7 cells transfected with the empty expression pcDNA3 vector reduced E2-induced CAT activity by approximately 50%. Transfection of as little as 0.25 µg of 412A significantly reduced RA-mediated inhibition of E2-induced ERE activation, an effect that remained almost constant for all transfected expression plasmid concentrations up to 2.5 µg. As noted for all RARα expression plasmid transfections, the control background levels of CAT activity decreased in an RARα concentration-dependent manner, resulting in a net increase in overall fold induction in the presence of E2.

Stable Expression of the RARα in MCF-7 Cells Prevents RA-Mediated Growth Inhibition—In order to assign a role for the RARα in RA antagonism of E2-induced growth, we have stably transfected MCF-7 cells with RARα expression plasmids. Four high expressing clones were obtained, two transfected with pcDNA3-RARα6, and two with CMX-RARα2 (Fig. 6, A and B, respectively) as assessed by Northern blot analysis. These clones were then assessed for growth inhibition by RA. The results shown in Fig. 7 (A and B) indicate that treatment of with 10−6 M RA over a 5-day period resulted in a 70% growth inhibition of untransfected MCF-7 cells and a clone of transfected MCF-7 cells, which did not express the CMX-RARα2 expression vector. In contrast, both clones of MCF-7 (pcDNA3-RARα2) cells were less than 30% growth inhibited at the end of the treatment period (Fig. 7, C and D) and the MCF-7 (CMX-RARα2) cells were completely refractory to growth inhibition (Fig. 7, E and F). In addition, while growth-inhibited clones underwent a characteristic change in morphology consisting of increases in intercellular spaces and the formation of processes, no such changes were observed in the MCF-7 (CMX-RARα2) clones (data not shown).

**DISCUSSION**

The growth response of hormone-dependent breast cancer cells to estrogen has been shown to be inhibited by retinoids (3–5), however the molecular basis of inhibition is yet to be defined. One way in which retinoids could antagonize the effects of E2 on growth is by preventing E2-induced transcription. Previous studies have reported conflicting results regarding the ability of RA to inhibit E2-induced transcription in MCF-7 cells; however, it has been suggested that the discrepancies may be due to donor variations in the expression of the RA binding site.

| Expression plasmid | Inhibition (%) | std dev |
|--------------------|----------------|---------|
| pcDNA3             | 50.1 ± 2.4     |         |
| RARα               | 18.4 ± 6.8     |         |
| RARα 404Δ'         | 29.0 ± 4.2     |         |
| RARα 414Δ'         | 61.4 ± 9.4     |         |
| RARα               | 65.4 ± 2.0     |         |
| RARβ               | 74.3 ± 4.5     |         |
| pcDNA3-T(47D)      | 28.6 ± 1.8     |         |

**Fig. 5. RARα AF-2 mutant dose independent repression of RA inhibition.** MCF-7 cells were transiently transfected with empty expression vector (0) or the indicated amounts in micrograms of the RARα 412Δ expression plasmid construct along with the Vit-CAT reporter gene. The total amount of transfected expression vector was constant for all experiments. The bars represent relative CAT activity ± the standard deviation for a duplicate experiment. A, vehicle; B, E2; C, E2 + RA.

**Fig. 6. Northern blot analysis of RARα′ expressing MCF-7 cells.** MCF-7 cells were transfected with either pcDNA3-RARα′ or CMX-RARα′ expression plasmids selected in G418 as described under "Materials and Methods." Clones were expanded and assayed for expression by Northern analysis using the RARα′ as a probe. Arrows point to the RARα′ transcript. A, wild-type MCF-7 RNA (C) and MCF-7 (pcDNA3-RARα′) clones (2 and 3). B, MCF-7 (CMX-RARα′) clones (3 and 4) and a non-expressing CMX-RARα′ clone (C).

RARs and ERS (31). In this study we have shown that RA inhibits E2 induction of the endogenous p52 gene as well as a transfected E2-responsive promoter reporter gene construct in MCF-7 ER-positive breast cancer cells. Fontana et al. (32) and Demirencse (33, 34) have both shown that retinoids antagonize the E2 induction of p52 mRNA expression in MCF-7 cells after 24 and 12 h of RA treatment, respectively. In the present study, we have shown that RA significantly inhibits E2 induction of p52 mRNA within 6 h of treatment with both ligands. The reason for this delay in inhibition is not clear but may be due to a lower affinity of the RAR-RXR complex for an ER coactivator molecule(s) (see discussion below) and/or the requirement for the expression of additional RARs such as the RARβ to achieve transcriptional inhibition. Potential mechanisms of RA-induced transcriptional inhibition include the possibility that RA bound to transcriptionally inactive RARα-RXRβ heterodimers on the ERE might directly block transactivation by the ER. In this scenario the RARα′-RXR heterodimers would be deficient in binding to the ERE when compared with wild-type RARα: RXR heterodimers. However, the results of the gel shift analysis suggests that this is not the case since both wild-type RARα-RXRβ and RARα′:RXRβ heterodimers bind equally weakly to the ERE. This weak binding may be responsible for the detected ligand independent inhibition of E2-induced transcription upon transfection of both the wild-type RARα and mutant RARs, which decreased the relative levels of both back-
Demirpence et al. (34) used chimeric receptors containing a GAL4 DNA binding domain and ER C-terminal domain to show that the DEF region of the ER is insufficient to confer RA-sensitivity to E2-induced transactivation and suggested that RA inhibits ER transactivation by direct interference at the level of the ERE in MCF-7 cells. They did not, however, show that RA increases the binding of RAR complexes to the ERE, which would be a requirement in a model of ligand-induced transcriptional inhibition.

A second possibility is that the ligand bound RAR-RXR complex titrates out a common auxiliary factor, which is necessary for transactivation by both retinoid receptors and ERs. Transcriptional interference between the steroid receptors for E2, progesterone, and glucocorticoid has been shown to involve both the N terminus and the hormone binding domain of these receptors (35). Danielian et al. (36) have suggested that the C terminus of the hormone binding domain in steroid hormone receptors contains sequences necessary for ligand dependent transactivation. Supported by earlier structure/function studies of the ER and RAR (37, 39), both Tate et al. (40) and Durand et al. (30) have found that the region between residues 404 and 419 in the RARα contains the C-terminal transactivation function. Notably, three of the residues in this region (Glu-412, Met-413, and Leu-414) are conserved in the AF-2 region of the ER (36).

Fig. 7. RA-mediated growth inhibition of MCF-7 cells. Cultures of MCF-7 cells were treated with vehicle or 10^{-6} M RA. A, untransfected MCF-7 cells; B, a non-expressing CMX-RARα clone; C and D, clone 1 and clone 2, respectively; E and F, clone 3 and clone 4, respectively. On day 3 of each experiment, the medium was changed and fresh drug added. Cells were enumerated on a hemocytometer after vital staining with trypan blue. Points represent the mean of triplicate cultures, and bars represent the standard deviation.
The observation that mutant RARα receptors in which amino acids 413 (Met) and 414 (Leu) are deleted effectively prevent the RA inhibition of E2-stimulated gene transcription demonstrates the participation of the AF-2 in ligand-dependent transcriptional interference with the ER. This observation supports that of Barettonio (41), who showed that the glucocorticoid receptor can interfere with the activity of the RAR, while the glucocorticoid receptor mutant M770AL771A cannot. Recent evidence has suggested that the C terminus of the ER binds several factors (42), which may include the proteins ERAP160 (43) and RIP140 (44), both of which modulate ER activity. Notably, the RAR can also bind ERAP 160 (43). Another candidate for such a factor is the mammalian homologue of S. cerevisiae SW12/SNF2 and Drosophila brahma (45) called BRG-1, which has been shown to be a coactivator for both the ER and RAR (46). The yeast SNF protein, SPT6, also enhances ER activity and binds to the AF-2 region of the ER (47).

The ability of mutant receptors to repress RA inhibition of E2-induced gene transcription is independent of ligand binding, since while 412Δ, ML413,414Δ and 414Δ can all bind trans-RA and 9-cis-RA, the RARα is predicted to be incapable of binding either all-trans- or 9-cis-RA (28). Since the inhibition of RA-mediated repression of E2-induced transcription by AF-2 region mutants was a dominant effect, we suggest that RA inhibition requires heterodimerization with limiting amounts of RXR in MCF-7 cells. To this end, we and others (48, 49) have shown that MCF-7 cells express low levels of all-RAR and RAR (46). The yeast SNF protein, SPT6, also enhances ER activity and binds to the AF-2 region of the ER (47).

Inhibition of RA-mediated repression of E2-induced transcription or inhibition also contributes to the AF-2 region of the RAR. This has been shown to be a common transcriptional accessory factor by retinoid receptors (48, 49) which may also harbor a different complement of ER coactivator(s), which do not bind with high affinity to the RAR.

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ERα cells transfected with the RAR acquire sensitivity to RA-mediated inhibition of the E2 response in T-47D cells, which may reflect differences in relative levels of RARα mutants in which amino acids 413 (Met) and 414 (Leu) are deleted effectively prevent the RA inhibition of E2-stimulated gene transcription demonstrates the participation of the AF-2 in ligand-dependent transcriptional interference with the ER. This observation supports that of Barettonio (41), who showed that the glucocorticoid receptor can interfere with the activity of the RAR, while the glucocorticoid receptor mutant M770AL771A cannot. Recent evidence has suggested that the C terminus of the ER binds several factors (42), which may include the proteins ERAP160 (43) and RIP140 (44), both of which modulate ER activity. Notably, the RAR can also bind ERAP 160 (43). Another candidate for such a factor is the mammalian homologue of S. cerevisiae SW12/SNF2 and Drosophila brahma (45) called BRG-1, which has been shown to be a coactivator for both the ER and RAR (46). The yeast SNF protein, SPT6, also enhances ER activity and binds to the AF-2 region of the ER (47).

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