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# Molecular mechanism of estrogen receptor (ER)α-specific, estradiol-dependent expression of the progesterone receptor (PR) B-isoform

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**Abstract**

The physiological effects of progesterone are mediated by the progesterone receptor (PR) isoforms PRA and PRB, transcribed from a single gene, under control of two distinct promoters. Both the isoforms display different, promoter- and cell line-specific transactivation properties. Upregulation of both isoforms in response to estradiol stimulation has been described, although the two promoters contain no classical estrogen response element (ERE). Therefore, we decided to investigate the regulation of PRB-expression through distinct estrogen receptor (ER)-isoforms: ERα and ERβ We demonstrate, that in HeLa cells treated with E2, PRB promoter activity was enhanced (five-fold) by ERα, but not by ERβ. ERβ was also unable to stimulate activity of the PRB promoter in BT20 and Ishikawa cells, where ERα induced reporter activity by two-fold. Deletion of the AF1—but not AF2 domain from ERα resulted in loss of the transactivation potential in all cell lines tested. Furthermore, in BT20 cells deletion of the AF2 domain of ERα resulted in stronger transcriptional activation than that mediated through wild-type ERα. In SK-BR-3 cells both ERs repressed PRB promoter activity and this repression was enhanced by co-transfection of SRC1. However, strong estrogen-dependent stimulation was observed after deletion of AF2. We conclude that PRB expression is stimulated by ERα but not ERβ in an unique, AF1-dependent but AF2-independent mechanism.
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*Keywords:* Estrogen; Progesterone; Estradiol

## 1. Introduction

The steroid hormones progesterone and estrogen play fundamental roles in the regulation of growth, development and function of female reproductive tissues by stimulating or repressing the expression of specific target genes. The progesterone receptors (PRs) and estrogen receptors (ERs) are ligand-dependent transcription factors that belong to the nuclear receptor superfamily [1]. In absence of hormone these receptors are inactively bound to a large complex of heat shock proteins within the cytoplasma of their target cells. Hormone binding results in dissociation of the heat shock proteins, followed by dimerization and binding to a conserved 13 bp hormone responsive element (HRE) within the promoter region of hormone-regulated target genes [2]. Bound to their HRE the receptors can act as transcription factors by contacting the general transcription machinery [3] or by recruitment of other transcription factors to the promoter (overview in [4]). The up-regulated effector proteins are mediators of cellular actions.

The primary structure of both ER and PR receptor consists of six differentially conserved domains termed A–F. Functional domains are the variable constitutive transactivation function (AF1, located in regions A/B), the highly conserved DNA binding domain (c region) and the low conserved ligand binding domain, that also contains the ligand-dependent transactivation function [5].

The effects of estrogen are mediated by two estrogen receptors termed ERα and ERβ, located on the human chromosomes 6 and 14 [6]. Both receptors are able to bind to the same estrogen response element (ERE) [7] and exhibit similar ligand binding properties [8]. ERα and ERβ are able to function as heterodimers [9], but as a result of their distinct tissue distribution, they may preferentially act as homodimers in most target cells [10]. ERα and ERβ share a high degree of homology in the DNA binding domain (96%) and ligand binding domain (55%), but not in the N-terminal domain. While both receptors seem to contain a functional

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AF2 domain, that works by binding p160 (e.g. SRC1) [11], the activity of AF1 in ERβ is very low compared with that of ERα.

Since estrogen-dependent gene regulation can also be observed for genes that do not contain an ERE, it is reasonable to assume that other alternative pathways of ER-mediated gene regulation exist. The mutated EREs found in the promoters of several estrogen-regulated genes are mostly inactive [12]. Moreover, the ER is involved in regulation of genes containing conserved binding sites for the AP1 protein. AP1, consisting of the proto-oncogenes fos and jun, is a mediator of signals from cell membrane receptors [13,14]. Both potentiating and diminishing effects of ER on AP1 activity have been observed, depending on the cell lines examined [15]. The mechanism of interaction with the AP1 complex has not yet been fully elucidated. Surprisingly, while both ERs interact in an identical fashion at EREs, they exhibit different activities at AP1 sites [15].

However, complex promoters of estrogen regulated genes contain a large variety of binding sites for transcription factors that may interact with the ERs. Thus regulation of complex promoters by estrogen may be different from regulation of minimal promoters containing only a single transcription factor binding site.

To investigate the roles of ERα and ERβ for a classical ER-target gene, we have examined their ability to modulate expression of the human progesterone receptor proximal promoter that contains no consensus ERE. Interestingly, we found that PRB expression is upregulated by estrogen selectively through ERα. For the transactivation of PRB, AF1 but not AF2 of ERα is essential. In addition, the ERα AF2 domain seems to reduce the activity of the ERα AF1 domain in some cell lines. The lack of AF1 activity in ERβ provides an explanation for the inability of ERβ to mediate upregulation of the PRB promoter. To our knowledge, this is the first observation of an estrogen-mediated, AF2-independent upregulation of a classical estrogen responsive promoter.

## 2. Materials and methods

### 2.1. Reporter plasmid construction

The human PRB promoter (−647 to +30 bp, corresponding to the sequence published by Kastner et al. [16]) was PCR amplified (1 min 95 °C, 1 min 60 °C, 1 min 72 °C) using the primers: PP-647: gaagatctctgttcaacatctctactgagg and PP + 30: cccaagcttgcttaccccgattagtgac, thereby generating *Bgl*II and *Hind*III restriction sites, flanking the promoter sequence. The promoter was inserted into the corresponding sites of the pGL2-basic luciferase reporter plasmid (Promega, Madison) to create the PRB promoter reporter plasmid. An AP1 site in the vector backbone was eliminated from the PRB-Luc reporter plasmid by PCR modification. The ERE2-TK-Luc and β-Gal expression plasmids expression plasmids were a gift from Myles Brown (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA) [11]. ERE2-TK-Luc contains two consensus estrogen response elements upstream from the thymidine kinase promoter. In ERE2-TK-Luc no AP1 site was found in the plasmid backbone. ER-expression plasmids are derivatives of the pcDNA expression plasmids (Promega), containing the complete coding sequence of human ERα and human ERβ or truncated ERs (see results for aa deletions).

### 2.2. Cell culture

Ishikawa cells were a gift from Dr. Kluge (University Marburg, Germany). BT20 and HeLa cells were obtained form the American Type Cell Culture Collection (ATCC). Ishikawa cells were maintained in Dulbecos Modified Eagles Medium F12 (DMEM F12) buffered with 15 mM hepes (Life Technologies Inc.) supplemented with 10% FCS (Sigma), penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamin (2 mM). Hela, BT20 and SK-BR-3 cells were cultured in DMEM supplemented with 10% FCS penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamin (2 mM) and 1 × MEM vitamins (Life Technologies Inc.).

Prior to transfection all cell lines were cultured in phenol red-free DMEM for 48 h (supplemented as above), containing 10% charcoal dextran treated and heat inactivated FCS (CFCS). Charcoal dextran treatment and heat inactivation was performed as previously described [17]. Cells were grown to 70–80% confluency prior to transfection.

### 2.3. Transfection and luciferase assays

For transfection $5 \times 10^4$ to $1 \times 10^5$ cells were seeded in 12-well plates (3.5 $cm^2$ per well) in triplicat and grown in phenol red-free DMEM, 10% CFCS and cultured for 24 h. For each well 50 ng of ER-expression plasmid (or pcDNA, Promega Madison), 400 ng of reporter plasmid (PRB-Luc or ERE2-TK-Luc) and 50 ng of β-Gal expression plasmid, were transfected using Lipofectamin™ (Life technologies) for Ishikawa cells, or Fugene™ (Roche) for HeLa, BT20 or Hey cells following the manufactures instructions. Twenty-four hours after transfection cells were treated with either vehicle alone, estrogen or anti-estrogens (100 nM each). Forty-eight hours after transfection cells were rinsed twice with PBS and lysed with 60 μl reporter lysis buffer (Promega, Madison). Luciferase assay was performed using 20 μl of lysate by measuring emitted luminescence on an Berthold lumat 9507b luminometer. β-Galactosidase activity was measured using the Tropix β-Gal system including galacto reaction buffer and light emission acceleratorII.

Each luciferase reaction was normalised to β-galactosidase activity to correct for the efficiency of transfection between plates. Experiments were performed in triplicate and repeated at least three times. The results are shown as the average of three individual experiments ± S.D. The unstimulated reaction transfected with pcDNA was set as 1.

### 2.4. Western blot analysis

Cells were pelleted and lysed in lysis buffer (50 mM hepes, 1% Triton X-100, 50 mM NaCl, 0.1 M NaF, 10 mM EDTA, 10 mM Na-orthovanadate, 10 μg/ml aprotinin, 2 mM benzamidine, 0.1% SDS, 2 mM PMSF, pH 7.4) and the lysate was clarified by centrifugation for 15 min at $12{,}000 \times g$. An amount of 50 μg of protein were resolved by SDS-PAGE on 10% polyacrylamide gels and blotted on Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). After blocking with 1× blocking reagent (Roche), membranes were incubated with monoclonal mouse anti-ERα-antibodies (Ab-10, Ab-11, Neo Markers Inc., Fremont, CA, USA), and peroxidase-conjugated anti-mouse IgG (Sigma, Taufkirchen, Germany). Bound antibody was then detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

## 3. Results

### 3.1. Transactivation of PRB through ERα and ERβ

To analyse the transcriptional regulation of the PRB promoter by estradiol, the previously described estrogen-regulatory region of the promoter including $-647 \pm 30$ bp was cloned into a luciferase reporter plasmid [16]. We decided to first analyse estradiol's ability to modulate transcription of the PRB promoter in the endometrial cancer cell line Ishikawa. Although we found low levels of ERα and ERβ expression by RT-PCR (data not shown), after transfection with the PRB-reporter plasmid, neither estradiol nor anti-estrogens exhibited a significant effect on the transcription of the reporter plasmid (Fig. 1A). In contrast, after co-transfection of ERα both estrogens and anti-estrogens were able to stimulate transcriptional activity of the PRB promoter, and the anti-estrogens tamoxifen and raloxifen mediate an even stronger transcriptional activation of the reporter plasmid than estrogen (Fig. 1A).

Interestingly, estrogen was unable to stimulate the reporter when ERβ was co-transfected with the reporter plasmid. However ERβ, like ERα responded to both anti-estrogens to increase luciferase activity in this cell line, with raloxifen being the stronger activator than tamoxifen. Since the estradiol-stimulated, non-classical regulation of promoters, which do not contain an ERE has been shown to strongly depend on the cellular context [15], we decided to analyse estrogen-regulated transcription of the PRB promoter also in HeLa cells. Like for Ishikawa cells, in the absence of co-transfected ER-expression plasmids, the transcription of the PRB-reporter plasmid remained unaltered by estrogens and anti-estrogens. We found low levels of ERα and ERβ expression by RT-PCR (data not shown) (Fig. 1B). Similar to the results obtained in Ishikawa cells, estradiol stimulated transcription of the PRB promoter, when ERα was co-transfected, while co-transfection of an ERβ-expression plasmid was not capable of conferring estradiol sensitivity to this reporter plasmid (Fig. 1B). In addition, tamoxifen and raloxifen were both able to stimulate this promoter via ERα and ERβ. However, unlike in Ishikawa cells, the activation of this promoter was strongest after stimulation with estrogen, and tamoxifen appeared to be a stronger activator than raloxifen when acting through ERα. In summary, in contrast to the differential regulation of AP1-controlled genes through estradiol in these cell lines, regulation of the PRB promoter did not exhibit a differential regulation.

### 3.2. AF1- and AF2 domains synergize to regulate transcription of ERE-dependent promoters

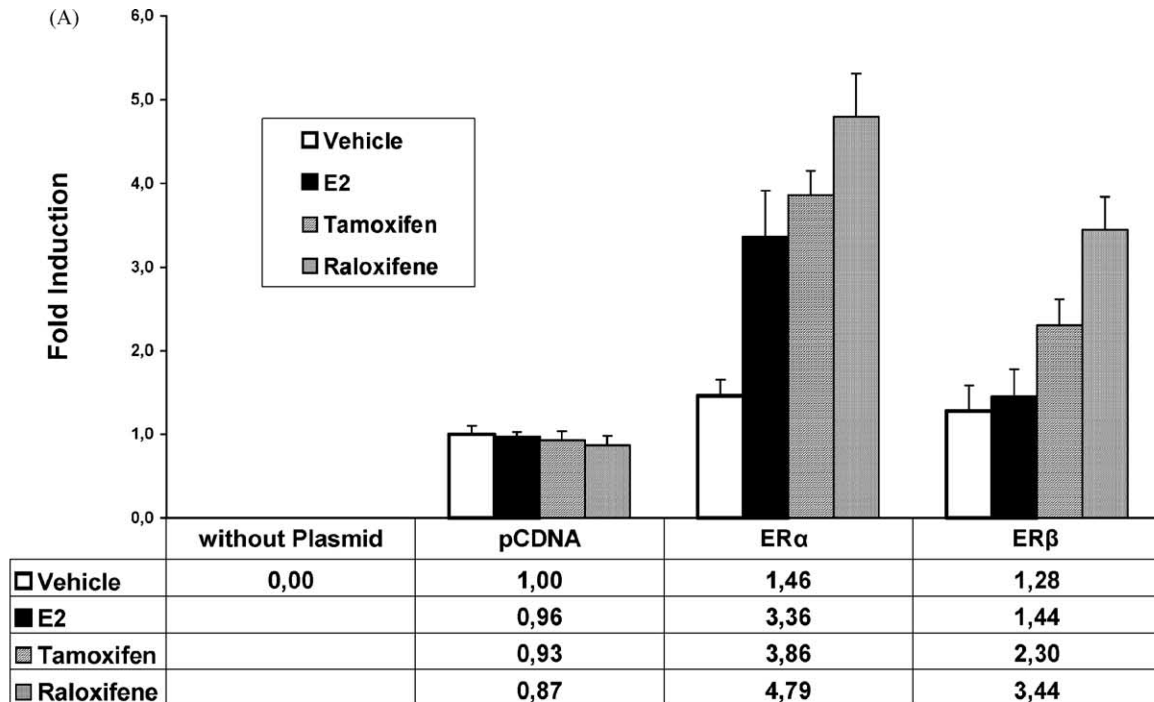
To compare the mechanism of ER-mediated regulation of the PRB promoter with that of ERE-controlled genes, we examined the role of established ER transactivation functions in estrogen and anti-estrogen action on this promoter. Therefore, we investigated the regulation of an ERE-controlled reporter plasmid through wild-type ERα and variants of ERα, lacking AF1 (ERαΔAF1; amino acids: 125–595 aa) or AF2 (ERαΔAF2; 1–534 aa) and an AF2-deficient variant of ERβ (1–439 aa) (Fig. 2A). The ERαΔAF2 variant has been previously described and was shown to exert no affinity to p160 co-activators such as SRC1, while retaining the ability of ligand-induced dimerization [11]. Similar to the results obtained with the PRB-reporter plasmid, no response to estrogen or anti-estrogens was found when ERE2-TK-Luc was transfected in the absence of ER-expression vectors, indicating that both Ishikawa and HeLa cells do not express estrogen receptors high enough to mediate transcriptional activation (Fig. 2A). Both wild-type ERα and ERβ were able to stimulate activity of the ERE2-TK-Luc reporter upon estradiol stimulation (Fig. 2A). Unlike for the PRB promoter (Fig. 1), anti-estrogens showed an antagonistic effect on the ERE-controlled promoter via both receptors (Fig. 2A). For ERα, raloxifen reduced the transcriptional activity of this reporter even beyond the level of the unliganded ERα (Fig. 2A).

ERαΔAF1 showed a reduced transactivation potential in comparison to the wild-type ERα, demonstrating that AF1 is functional in context of the wild-type ER in this cell line (Fig. 2A). Deletion of the AF2 domain resulted in a complete loss of estrogen-dependent transactivation of the ERE-controlled reporter gene (Fig. 2A). In fact, ERαΔAF2 reduced reporter gene activity upon estrogen-stimulation.

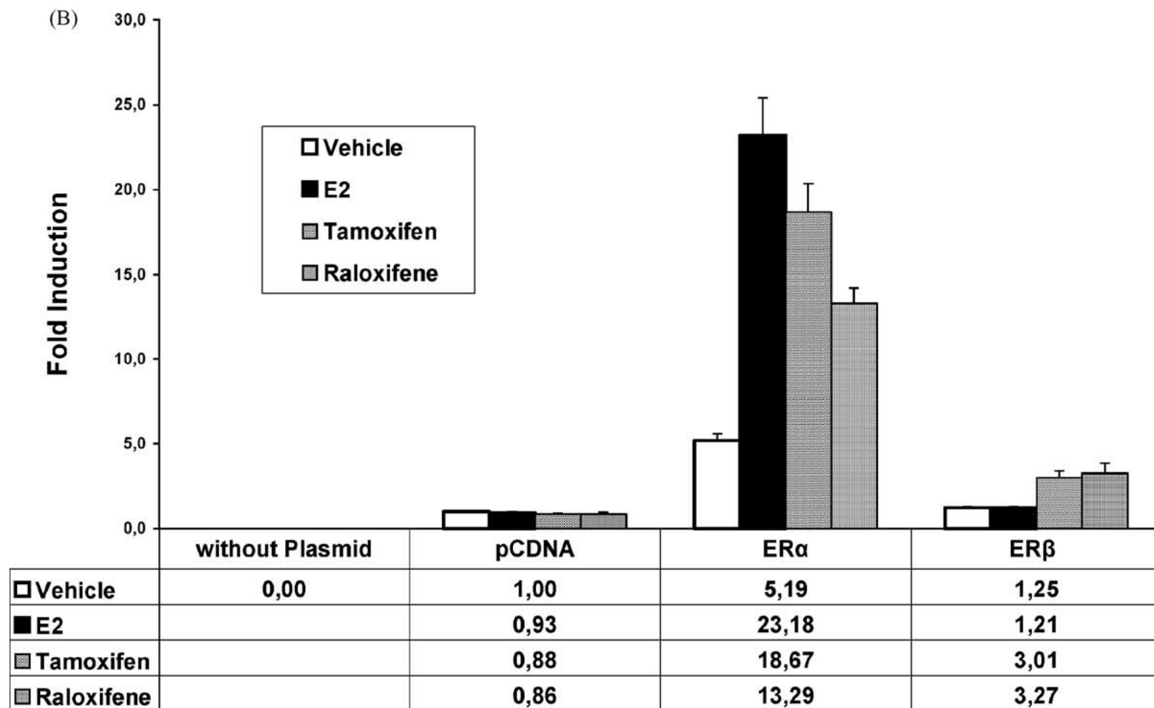
On the other hand, ERβ mediated transcriptional activation of the ERE-controlled reporter gene in response to estradiol stimulation, while the extend was comparable to that mediated by ERαΔAF1, supporting the hypothesis that ERβ has no functional AF1 domain (Fig. 2A).

### 3.3. Estrogen-dependent stimulation of the PRB promoter requires ERα-AF1 but not -AF2

We next determined, whether ERα requires the same transactivation domains for regulation of the PRB promoter

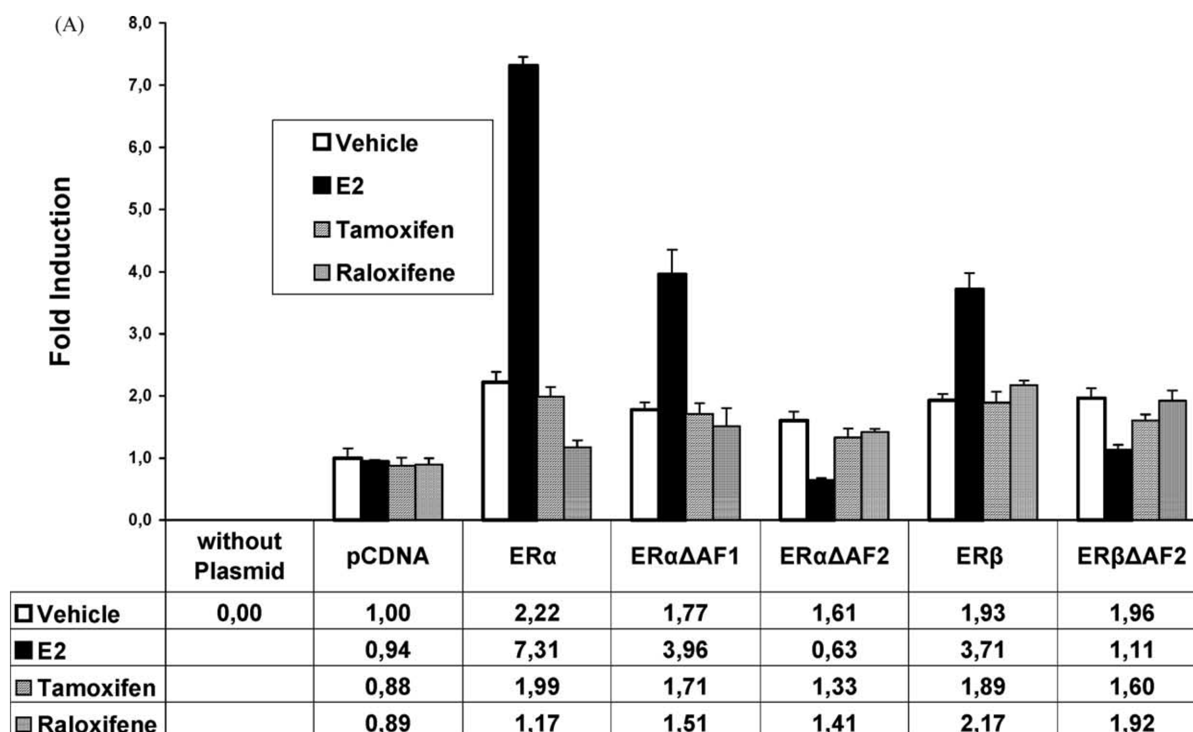



| | without Plasmid | pCDNA | ERα | ERβ |
|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 1,46 | 1,28 |
| E2 | | 0,96 | 3,36 | 1,44 |
| Tamoxifen | | 0,93 | 3,86 | 2,30 |
| Raloxifene | | 0,87 | 4,79 | 3,44 |

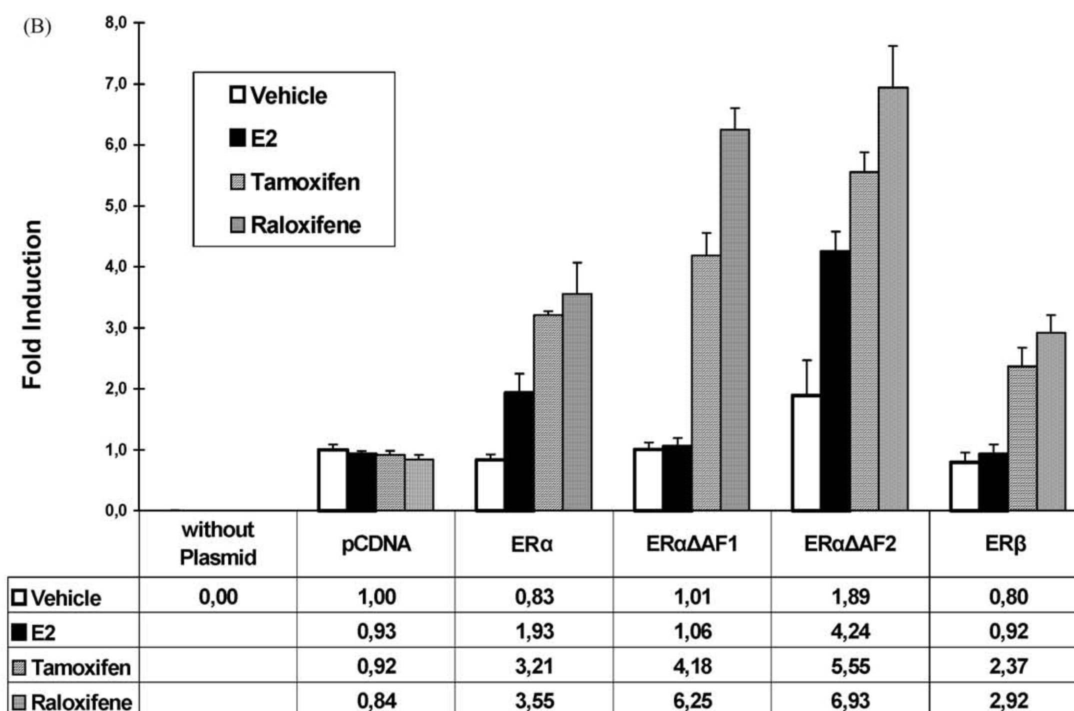



| | without Plasmid | pCDNA | ERα | ERβ |
|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 5,19 | 1,25 |
| E2 | | 0,93 | 23,18 | 1,21 |
| Tamoxifen | | 0,88 | 18,67 | 3,01 |
| Raloxifene | | 0,86 | 13,29 | 3,27 |

Fig. 1. Estrogen induces transcription of an PRB promoter-dependent reporter through ERα but not ERβ in Ishikawa (A) and HeLa cells (B). SERMs (TAM, RLX) exhibit agonistic activity through both ERs. Expression vectors for ERα, ERβ and an empty expression vector (pcDNA) were co-transfected along with the PRB-luciferase reporter plasmid and treated with different ligands (17-βE2, tamoxifen, raloxifen) at 100 nM or vehicle (ethanol). The results are shown as average of three individual experiments ± S.D. The none stimulated reaction transfected with pcDNA was set as 1.

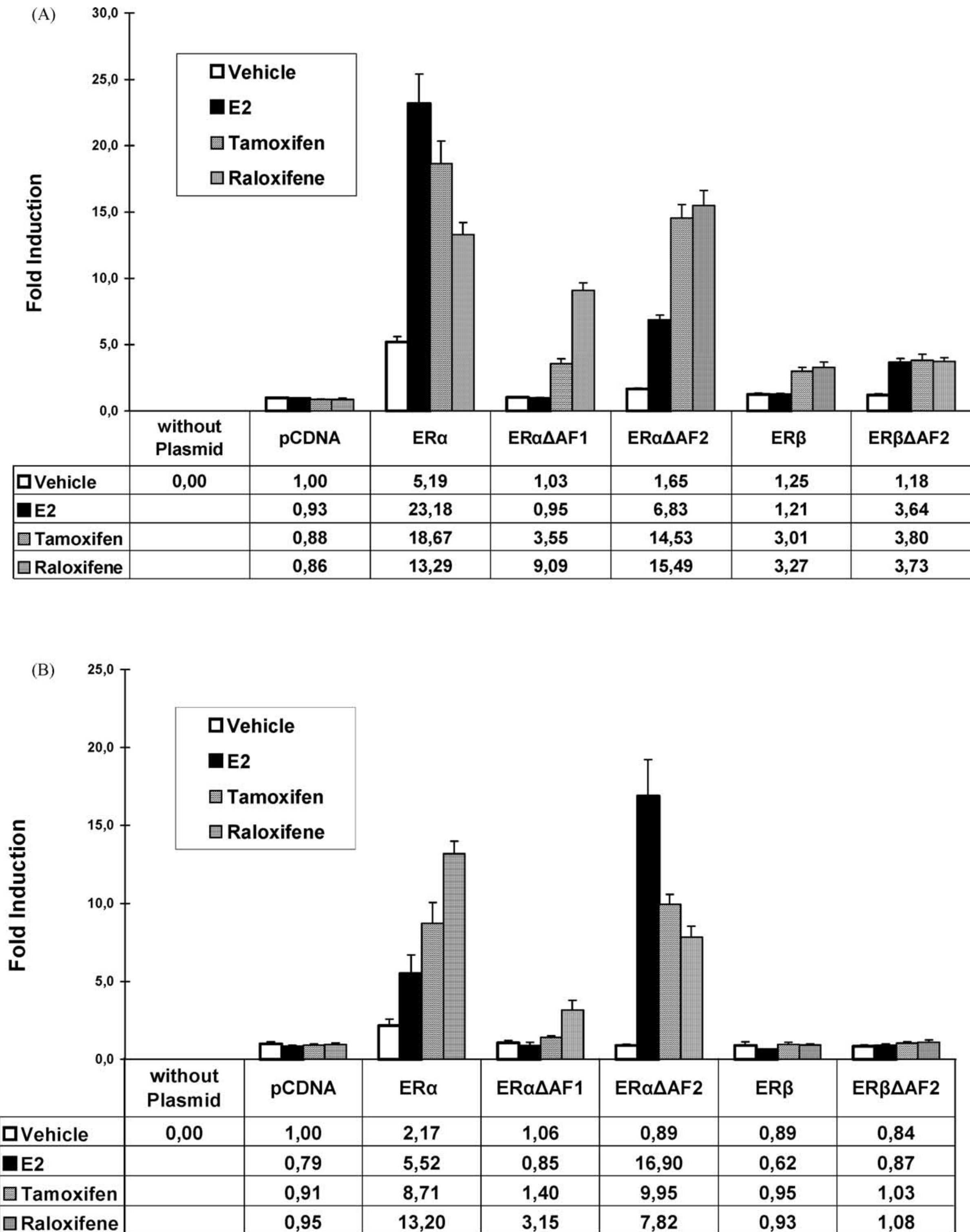



| | without Plasmid | pCDNA | ERα | ERαΔAF1 | ERαΔAF2 | ERβ | ERβΔAF2 |
|---|---|---|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 2,22 | 1,77 | 1,61 | 1,93 | 1,96 |
| E2 | | 0,94 | 7,31 | 3,96 | 0,63 | 3,71 | 1,11 |
| Tamoxifen | | 0,88 | 1,99 | 1,71 | 1,33 | 1,89 | 1,60 |
| Raloxifene | | 0,89 | 1,17 | 1,51 | 1,41 | 2,17 | 1,92 |




| | without Plasmid | pCDNA | ERα | ERαΔAF1 | ERαΔAF2 | ERβ |
|---|---|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 0,83 | 1,01 | 1,89 | 0,80 |
| E2 | | 0,93 | 1,93 | 1,06 | 4,24 | 0,92 |
| Tamoxifen | | 0,92 | 3,21 | 4,18 | 5,55 | 2,37 |
| Raloxifene | | 0,84 | 3,55 | 6,25 | 6,93 | 2,92 |

Fig. 2. Different transactivation functions are involved in regulation of the PRB promoter and an ERE-dependent promoter. (A) ERα and ERβ stimulate transcription of an ERE-dependent reporter gene in an AF1- and AF2-dependent mechanism in Ishikawa cells. (B) Estrogen-dependent stimulation of the PRB promoter via ERα depends on AF1 but not AF2 in Ishikawa cells. Expression vectors for ERα, ERβ and an empty expression vector (pcDNA) were co-transfected along with ERE2-TK-Luc reporter plasmid (A) or PRB-Luc (B) and treated with different ligands (17-βE2, tamoxifen, raloxifene) at 100 nM or vehicle (ethanol). The results are shown as average of three individual experiments ± S.D. The none stimulated reaction, transfected with pcDNA was set as 1.



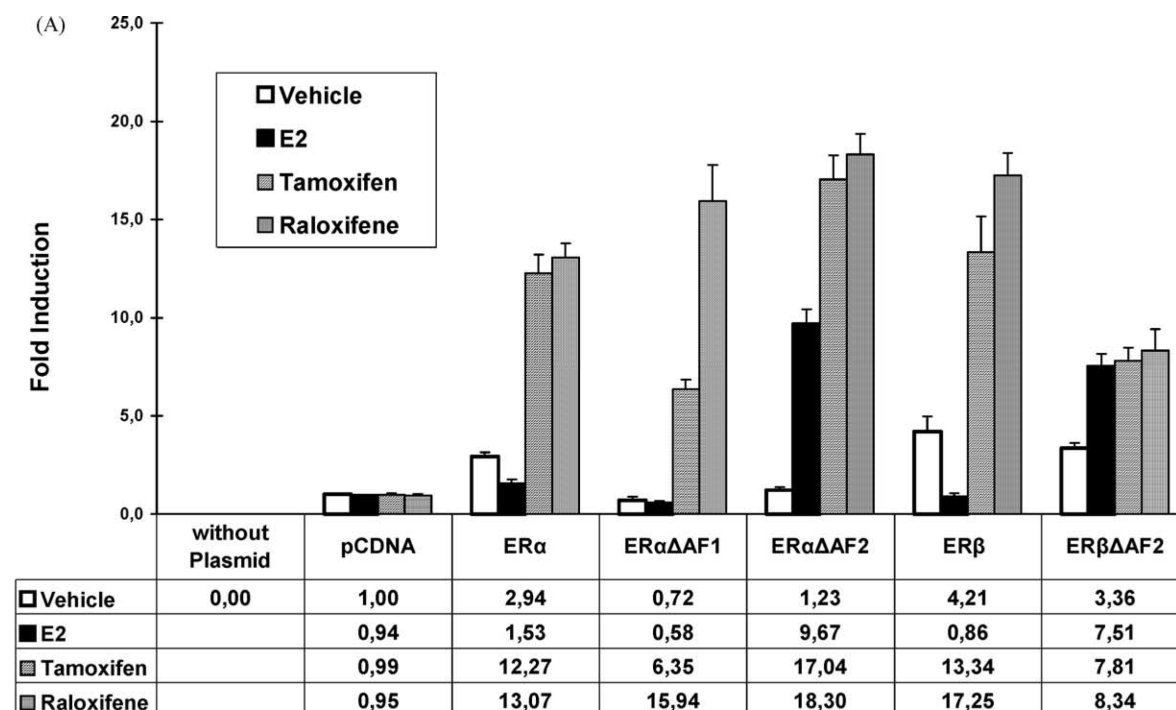

(A)

| | without Plasmid | pCDNA | ERα | ERαΔAF1 | ERαΔAF2 | ERβ | ERβΔAF2 |
|---|---|---|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 5,19 | 1,03 | 1,65 | 1,25 | 1,18 |
| E2 | | 0,93 | 23,18 | 0,95 | 6,83 | 1,21 | 3,64 |
| Tamoxifen | | 0,88 | 18,67 | 3,55 | 14,53 | 3,01 | 3,80 |
| Raloxifene | | 0,86 | 13,29 | 9,09 | 15,49 | 3,27 | 3,73 |

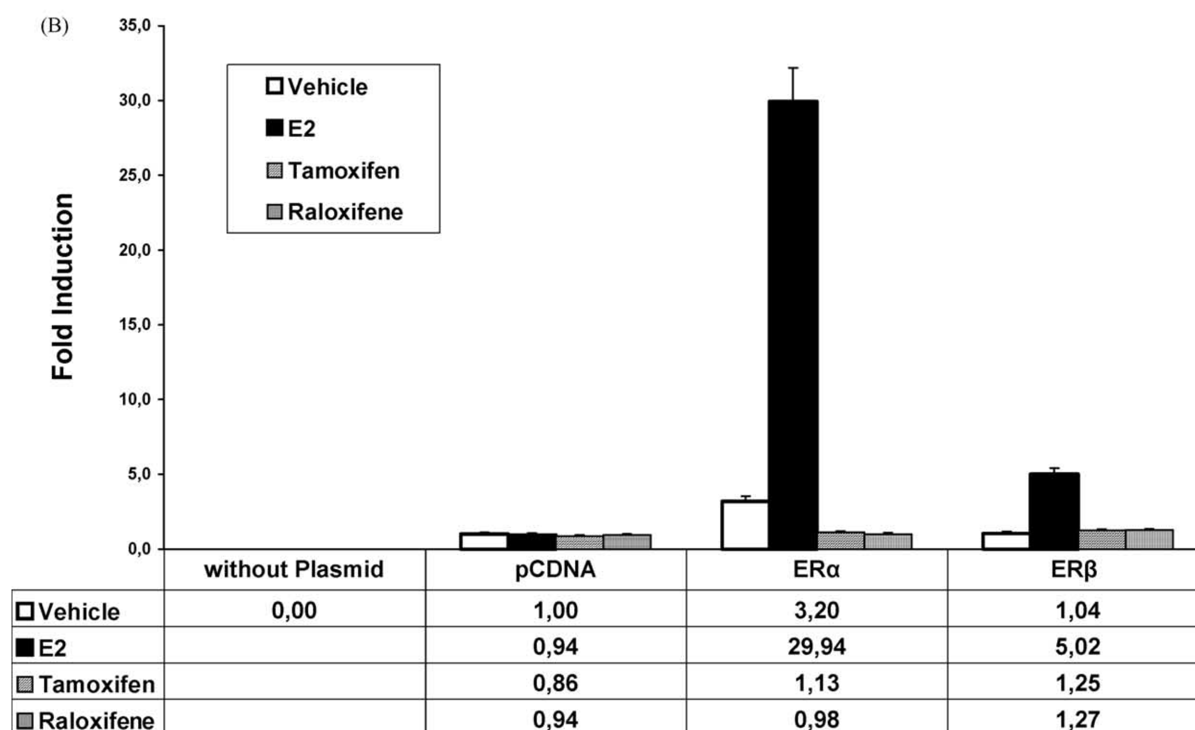
(B)

| | without Plasmid | pCDNA | ERα | ERαΔAF1 | ERαΔAF2 | ERβ | ERβΔAF2 |
|---|---|---|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 2,17 | 1,06 | 0,89 | 0,89 | 0,84 |
| E2 | | 0,79 | 5,52 | 0,85 | 16,90 | 0,62 | 0,87 |
| Tamoxifen | | 0,91 | 8,71 | 1,40 | 9,95 | 0,95 | 1,03 |
| Raloxifene | | 0,95 | 13,20 | 3,15 | 7,82 | 0,93 | 1,08 |

Fig. 3. Estrogen-dependent stimulation of the PRB promoter via ERα depends on AF1 but not AF2 in HeLa (A) and BT20 cells (B). Expression vectors for ERα, ERβ and an empty expression vector (pcDNA) were co-transfected along with PRB-luciferase reporter plasmid and treated with different ligands (17-βE2, tamoxifen, raloxifene) at 100 nM or vehicle (ethanol). The results are shown as average of three individual experiments ± S.D. The none stimulated reaction transfected with pcDNA was set as 1.




| | without Plasmid | pCDNA | ERα | ERαΔAF1 | ERαΔAF2 | ERβ | ERβΔAF2 |
|---|---|---|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 2,94 | 0,72 | 1,23 | 4,21 | 3,36 |
| E2 | | 0,94 | 1,53 | 0,58 | 9,67 | 0,86 | 7,51 |
| Tamoxifen | | 0,99 | 12,27 | 6,35 | 17,04 | 13,34 | 7,81 |
| Raloxifene | | 0,95 | 13,07 | 15,94 | 18,30 | 17,25 | 8,34 |




| | without Plasmid | pCDNA | ERα | ERβ |
|---|---|---|---|---|
| Vehicle | 0,00 | 1,00 | 3,20 | 1,04 |
| E2 | | 0,94 | 29,94 | 5,02 |
| Tamoxifen | | 0,86 | 1,13 | 1,25 |
| Raloxifene | | 0,94 | 0,98 | 1,27 |

Fig. 4. Estrogen stimulates the expression of an ERE-dependent promoter (B) but not the PRB promoter (A) in SKBR3 cells. Expression vectors for ERα, ERβ and an empty expression vector (pcDNA) were co-transfected along with ERE2-TK-Luc reporter plasmid (B) or PRB-Luc (A) and treated with different ligands (17-βE2, tamoxifen, raloxifene) at 100 nM or vehicle (ethanol). The results are shown as average of three individual experiments ± S.D. The none stimulated reaction transfected with pcDNA was set as 1.

as it does for an ERE-controlled promoter. Therefore, we performed co-transfection experiments of the PRB-reporter plasmid with wild type and mutant ERα expression plasmids (Fig. 2B). In contrast to the results obtained with the ERE-controlled promoter, deletion of the AF1 domain in the ERαΔAF1 plasmid resulted in a complete loss of estradiol-stimulated PRB transcription, while the transcriptional activation in response to anti-estrogens remained unaltered (Fig. 2B).

Unlike for the ERE2-TK-Luc promoter, deletion of the ERαΔAF2 domain did not affect estradiol's ability to stimulate transcription of the PRB promoter in response to estrogen or anti-estrogens (Fig. 2B). Thus, estrogen-dependent regulation of the PRB promoter in Ishikawa cells was strictly AF1 but not AF2-dependent. To investigate, whether AF2-independent regulation of the PRB promoter is a cell type specific phenomenon, we performed similar experiments in cancer cell lines derived from different tissues. Therefore, the cancer cell lines HeLa (cervix) and BT20 (breast) were analysed. As shown in Fig. 3, estradiol-mediated stimulation of the PRB promoter was AF2 domain-independent in all cell lines tested. Moreover, in BT20 cells, deletion of the AF2 domain resulted in an enhanced estrogen response, which was increased from 2- to 18-fold (Fig. 3). A tendency for an enhanced activity of ERαΔAF2 was also detected in Ishikawa cells but not in HeLa cells.

Like for Ishikawa cells, the estrogen-mediated upregulation of the PRB promoter was completely abolished, when the AF1 domain of ERα was deleted. Similarly, co-transfection of ERβ failed to stimulate transcription of the PRB promoter in response to estradiol. However, in all three cell lines, both basal and anti-estrogen-mediated reporter gene activity was largely reduced with both variants (Fig. 3). In BT20 cells, anti-estrogens were completely unable to stimulate the PRB-reporter plasmid through ERβ, while raloxifen had some activity via ERαΔAF1.

### 3.4. Estrogen-dependent stimulation of the PRB promoter is inhibited by AF2 in human SK-BR3 breast cancer cells

In Ishikawa (endometrium), HeLa (cervix), Hey (ovary) and BT20 (breast) cells estrogen stimulated the activity of the PRB promoter via ERα. In contrast, both ERα and ERβ are unable to mediate estradiol-dependent stimulation of the human PRB promoter in human SK-BR-3 breast cancer cells (Fig. 4A), although both ERs were able to stimulate an ERE-regulated promoter in the same cell line (Fig. 4B). Instead, both ERα and ERβ could repress PR promoter activity when estradiol was used as ligand. Interestingly, this repression appeared to be mediated by the AF2 domains of these receptors as demonstrated by the AF2 deficient variants of ERα and ERβ. Unlike the ERαWT receptor ERαΔAF2 and ERβΔAF2 did not show an estrogen-dependent repression of the PRB promoter (Fig. 4A). ERαΔAF2 could effectively stimulate the activity of the human PRB promoter and an estradiol mediated stimulation is also found for

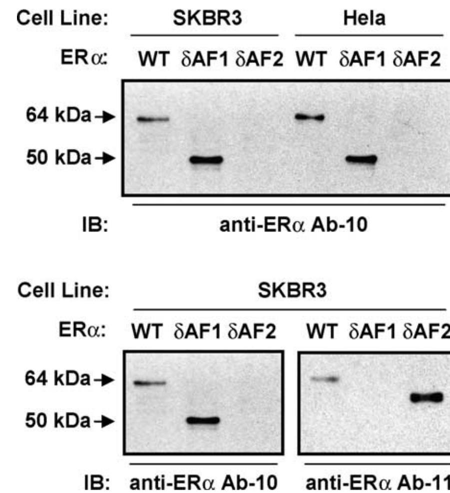
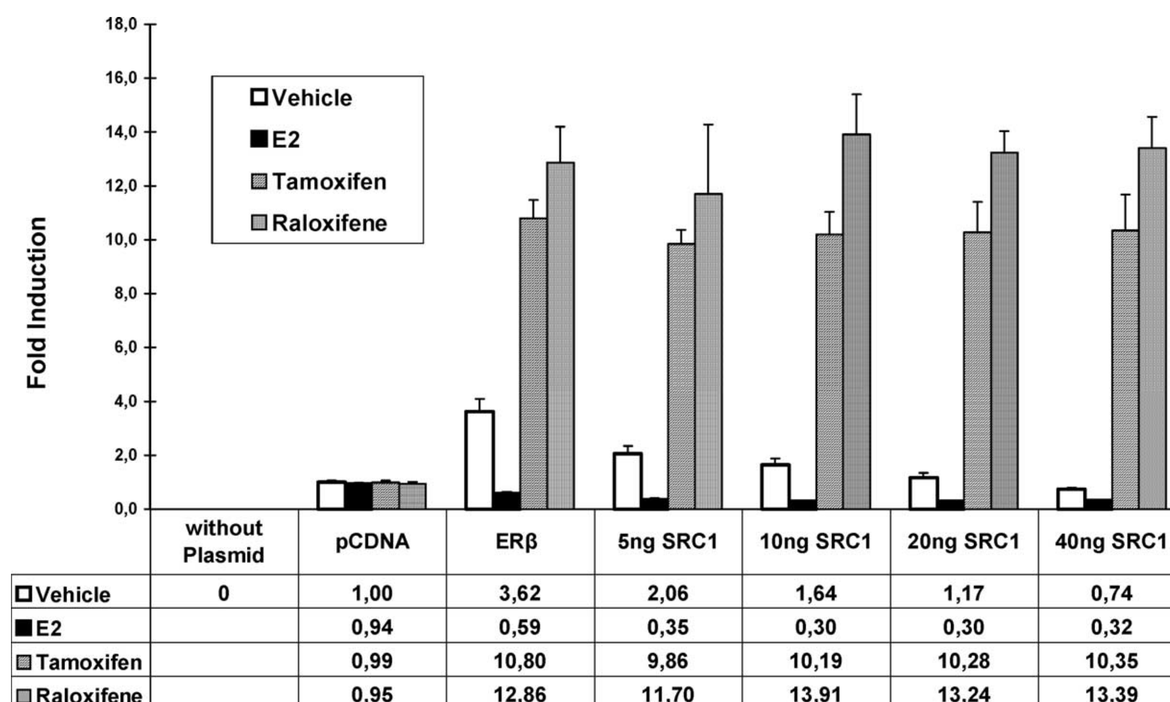



Fig. 5. Estrogen receptor variants are expressed to similar degree in various cell lines. Expression vectors for ERα, ERαΔAF1 and ERαΔAF2 were transfected in SKBR3 and HeLa cells. Protein extracts were analysed for ER expression with an antibody directed against the C-terminus of ERα (upper panel). Expression vectors for ERα, ERαΔAF1 and ERαΔAF2 were transfected in SKBR3 cells. Protein extracts were analysed for ER expression with an antibody directed against the N-terminus and with antibody directed against the C-terminus of ERα (lower panel).

ERβΔAF2 (Fig. 4A). Raloxifene and tamoxifen stimulated the human PRB promoter via ERα and ERβ, and deletion of either AF1 or AF2 domains showed only minor effects on anti-estrogen-mediated stimulation of the PRB promoter.

To exclude the possibility, that differential effects observed for different ER-expression plasmids resulted from differential transfection efficiency of these cell lines or from altered expression of the different constructs, we next determined the expression of ERα and the described mutant variants ERαΔAF1 and ERαΔAF2, by Western blot analysis with anti-ER-specific antisera to control for protein expression of these receptors. First, we tested the expression of ERα in parallel in two cell lines, where ER-variants exhibited opposite effects in terms of transcriptional regulation, i.e. SKBR-3 and Hela cells. As shown in Fig. 5, both ERαWT and ERαΔAF2 were expressed in comparable amount in these cell lines, ruling out the possibility, that different effects observed for transcriptional regulation in these cell lines resulted from different degrees of ER expression. Moreover, we analysed the relative expression of the ERαWT, ERαΔAF1 and ERαΔAF2 in different cell lines. Since there are no antibodies available, which recognize all these different variants, we analysed expression of ERαΔAF2 relative to ERαWT by Western blot analysis with an antibody directed to the N-terminus of ERα and expression of ERαΔAF1 relative to ERαWT by Western blot analysis with an antibody directed to the C-terminus of ERα. This analysis revealed, that relative to ERαWT both variants were expressed to the same extend, again ruling out, that different effects of these mutants were caused by variations in expression efficiency (Fig. 5).




| | without Plasmid | pCDNA | ERβ | 5ng SRC1 | 10ng SRC1 | 20ng SRC1 | 40ng SRC1 |
|---|---|---|---|---|---|---|---|
| Vehicle | 0 | 1,00 | 3,62 | 2,06 | 1,64 | 1,17 | 0,74 |
| E2 | | 0,94 | 0,59 | 0,35 | 0,30 | 0,30 | 0,32 |
| Tamoxifen | | 0,99 | 10,80 | 9,86 | 10,19 | 10,28 | 10,35 |
| Raloxifene | | 0,95 | 12,86 | 11,70 | 13,91 | 13,24 | 13,39 |

Fig. 6. Co-transfection of SRC1 with ERβ does not abolish the estrogen-mediated repression of the PRB promoter in SK-BR-3 cells. Expression vectors for ERα, ERβ and an empty expression vector (pcDNA) were co-transfected along with PRB-Luc and increasing amounts of SRC1 expression plasmid. Transfected cells were treated with different ligands (17-βE2, tamoxifen, raloxifene) at 100 nM or vehicle (ethanol). The results are shown as average of three individual experiments ± S.D. The none stimulated reaction transfected with pcDNA was set as 1.

Since transcriptional activation through AF2 domains occurs through recruitment of p160 co-activators, we next investigated, whether AF2-dependent repression of the PRB promoter in SKBR-3 cells not only depends on ER AF2 domains, but also on p160 co-activators. Therefore, we investigated the effect of increasing amounts of co-transfected SRC1 on PRB repression (Fig. 6). Since ERβ (unlike ERα) binds SRC1 only via the AF2 domain but not through an AF1 domain, we chose ERβ to perform this experiments. Co-transfection of increasing amounts of SRC-1 resulted in an enhanced ability of estradiol to inhibit transcription of the PRB promoter (Fig. 6), indicating that indeed not only the AF2 domain but also its recruitment of p160 co-activators are required for estradiol-stimulated inhibition of PRB transcription.

## 4. Discussion

The phyiological effects of progesterone are mediated by two progesterone receptors designated PRA and PRB. PRA is a truncated isoform of PRB lacking the N-terminal 164 amino acids [16]. The two isoforms originate from a single gene by transcription from distinct promoters and alternate translation initiation from two different AUG codons. Both isoforms are able to mediate progesterone-activated transcription. However, their transactivation properties differ dependent on cell type and promoter context [18–20]. A recent study on PRA knockout mice clearly showed that both PRs are functionally distinct mediators of progesterone action and that PRA regulates a subset of reproductive functions [21].

Upregulation of PR mRNA expression by estrogen has been shown for both isoforms in studies on cultured cells [22]. However the ratio of the two isoforms varies among target tissues [23]. Importantly, PR has been defined as an estrogen-regulated gene in breast cancer and has gained importance as a standard prognostic value for hormone responsiveness of these tumours, although the exact mechanism of estradiol regulation remains largely unclear.

We have compared the regulation of an ERE-dependent promoter and the regulation of the human PRB promoter by ERα and ERβ in response to different ligands using reporter gene technology. Endogenous ER seems to have no effect on the reporter gene, since we could not detect any estrogen-dependent stimulation in the absence of co-transfected ERs. Co-transfection of ERs revealed striking differences in the regulation of both promoters through ERα and ERβ. On an ERE-dependent promoter, both ERα and ERβ are able to mediate estrogen responsiveness. In our experiments both transactivation functions of ERα contribute to the estrogen response of the ERE-dependent promoter, as previously demonstrated [24]. Deletion of ERαΔAF1 results in reduction of estrogen response in this cell line, demonstrating that AF1 has a slight but detectable effect in this cell line. We did not generate an N-terminal deficient

variant of ERβ, because ERβ has been shown to have no AF1 activity on ERE-dependent promoters [25]. Therefore, it appears conceivable that ERβ shows an estrogen response similar to the estrogen response of ERαΔAF1.

Deletion of ERαΔAF2 resulted in a complete loss of the transactivation potential, although the used variant (Δ534) retained an intact dimerization domain and binds estradiol with wild-type affinity (Kd $< 1$ nM) [11]. This mutant has been shown not to bind p160 co-activators and therefore fails to transactivate on ERE-dependent promoters [11]. However, in some cell lines, an AF2-deficient ERα variant can still activate transcription via AF1, as demonstrated by other authors [26]. We did not find such AF1-mediated transactivation via ERαΔAF2, although AF1 seems to be active in Ishikawa cells, as demonstrated by deletion of AF1. Instead, we found a negative response to estrogen in a variety of different cell lines (data not shown). We speculate that ERαΔAF1 may be overridden by recruitment of repressive factors to the promoter via the defective AF2 domain. Expectedly, an AF2-deficient variant of ERβ shows no estrogen-dependent transactivation.

While ERα and ERβ exhibited similar transcriptional activity on the ERE-dependent promoter upon estradiol-stimulation, we detected profound differences between the two ER-isoforms in the regulation of the human PRB promoter ($-647 \pm 30$ bp [16]). Whereas both ERs were efficient in transactivation from an ERE element in response to estrogen, only ERα was capable of mediating estradiol-stimulated transcription of the PRB promoter. It has been previously demonstrated that estrogen stimulates transcription from the human PRB promoter through ERα [16]. We demonstrate for the first time, that estrogen was unable to stimulate transcription of this promoter via ERβ (Fig. 1). However, tamoxifen and raloxifen were potent agonists of ERβ at this promoter. A similar regulation has been observed for other promoters, that do not contain an ERE site. For example promoters that contain an SF response or an AP1 site have been shown to be upregulated by estrogen through ERα but not ERβ [15,27]. A similar regulation has also been observed for promoters containing SP1 sites [28]. In contrast to our findings, the estrogen-mediated regulation of these promoters has been shown to depend on both transcriptional activation functions of ERα. In contrast, in our experiments, upregulation of the PRB promoter depends on ERαΔAF1 but is independent of ERαΔAF2 in all cell lines tested. Furthermore, AF2 seems to block the estradiol-dependent stimulation of the PRB promoter in some cell lines. This is especially evident in human BT20 breast cancer cell lines, where deletion of AF2 results in a largely enhanced estrogen response from 2- to 18-fold. In addition, in human SK-BR-3 breast cancer cells ERα cannot stimulate the activity of the PRB promoter when AF2 is intact (Fig 6B), although ERα can stimulate an ERE-regulated promoter in this cell line (Fig. 6A). Instead, estradiol-dependent repression of the PRB promoter activity is found with WT ERα and ERβ and the repression is abolished when AF2 is removed. Furthermore, PRB repression via ERβ is enhanced by SRC1 in this cell line, confirming that the repression is in fact mediated by AF2, since AF2 works by binding p160 co-activators. However, this effect is cell line specific and cannot be found in HeLa cells, where deletion of AF2 results in a slightly decreased estrogen-dependent activity. It is interesting to speculate that two activation functions within the same receptor may antagonize.14 each other in regulation of the same promoter. In face of the cell line specificity of PRB repression by AF2, we believe that the degree of repression is mediated by cell specific cofactors.

We cannot directly address the mechanism of action of AF1 and AF2 on the PRB promoter. We found no consensus ERE or AP1 element in the cloned promoter fragment. Furthermore, regulation via AP1 or SP1 has been demonstrated to depend on AF2 [29,30]. A variant of ERα with an additional viral transactivation function showed enhanced transactivation properties on ERE and AP1-dependent promoters but not on the PRB promoter (data not shown). The ER may bind to the promoter in conjugation with another transcription factor like described for the PRA promoter [29,30]. Alternatively, the ER may be tethered to the promoter via interaction with another protein. Transcriptional repression may be mediated by sequestration of other transcription factors from the promoter. Clearly, promoter mapping studies are needed to address that issue.

To our knowledge, this is the first description of a promoter activated by estrogen solely via AF1 of ERα. Moreover, the strictly AF1-dependent regulation of the PRB promoter explains ERβ's inability to activate the PRB promoter in response to estrogen. This finding offers a molecular explanation for PR regulation in endometrium. However, regulation of the endogenous promoter is likely more complex than indicated by our simplified studies on a small promoter fragment. Although the cloned promoter fragment is inducible by ERα, we cannot exclude the existence of other important regulatory sites upstream or downstream of the cloned promoter fragment. For example, the expression of PRA is modified by a cluster of two SP1 sites and an ERE half side at position +571 bp and an AP1 site is also involved in its regulation [29,30]. Although it is known that PRB and PRA are regulated separately [23], we cannot exclude mutual interaction between both promoters. Interestingly, estradiol-mediated regulation via AP1 or SP1 is mediated by ERα but not ERβ, indicating that expression of both PR isoforms may be primarily controlled by ERα [15,28]. In support of this hypothesis, overexpression of an ERβ splice variant, termed ERβCX, that represses ERα activity but not ERβ activity, resulted in a significant decrease of PR expression without affecting ER expression [31]. In addition, expression of ERβCX showed an inverse correlation with PR expression in human tumour samples.

Our current understanding of PR regulation derives largely from detailed studies in mammalian uterus and

cancer cell lines, which led to the general view, that estrogen increases PR expression. However, in the mouse endometrium, both the stroma and epithelium express PR, and estrogen induces PR in the stromal cells, whereas it reduces PR in the luminal epithelial cells [32]. In the ERβ knockout mouse, estradiol-dependent stimulation of PR expression in stromal cells remains unmodified, while transcriptional down regulation in the luminal epithelium is abolished [33]. These findings are in good agreement with our finding that ERα but not ERβ is responsible for the upregulation of the PRB promoter.

In summary, our results demonstrate for the first time a strictly AF1-dependent transcriptional regulation of an ER-target gene, thereby explaining the functional difference between ERα and ERβ in the regulation of the PRB promoter. The identification of cellular factors interacting with the ERα-AF1 domain responsible for this regulation will largely increase our understanding of ER-regulated transcriptional activation and will potentially define novel targets in the pharmacological modification of ER action.

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