The hinge region of the human estrogen receptor determines functional synergy between AF-1 and AF-2 in the quantitative response to estradiol and tamoxifen

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Summary

Human estrogen receptors α and β (ERα and ERβ) greatly differ in their target genes, transcriptional potency and cofactor-binding capacity, and are differentially expressed in various tissues. In classical estrogen response element (ERE)-mediated transactivation, ERβ has a markedly reduced activation potential compared with ERα; the mechanism underlying this difference is unclear. Here, we report that the binding of steroid receptor coactivator-1 (SRC-1) to the AF-1 domain of ERα is essential but not sufficient to facilitate synergy between the AF-1 and AF-2 domains, which is required for a full agonistic response to estradiol (E2). Complete synergy is achieved through the distinct hinge domain of ERα, which enables combined action of the AF-1 and AF-2 domains. AF-1 of ERβ lacks the capacity to interact with SRC-1, which prevents hinge-mediated synergy between AF-1 and AF-2, thereby explaining the reduced E2-mediated transactivation of ERβ. Transactivation of ERβ by E2 requires only the AF-2 domain. A weak agonistic response to tamoxifen occurs for ERα, but not for ERβ, and depends on AF-1 and the hinge-region domain of ERα.

Key words: Estrogen receptor alpha, Estrogen receptor beta, Activation domain 1, Activation domain 2, Hinge region, SRC-1, Transcriptional capacity, Tamoxifen agonism

Introduction

Estrogen receptors α and β (ERα and ERβ) are homologous members of the nuclear receptor superfamily and are encoded by two different genes. They control crucial processes in physiology and are prognostic markers in breast cancer; their expression determines whether or not endocrine treatment is given as an adjuvant therapy (Speirs et al., 2004). On binding of agonistic ligands, the DNA-bound receptor recruits cofactors, which enables the receptor to transmit its regulatory information to the cellular transcription complex, including RNA polymerase II (transactivation). ERα and ERβ mediate distinct profiles of gene expression (Chang et al., 2008; Williams et al., 2008). This is due to a combination of differential ligand response (Barkhem et al., 1998; Paech et al., 1997; Paige et al., 1999; Van Den Bemd et al., 1999), distinct interaction with coactivators (Kraichely et al., 2000) and binding to specific estrogen response elements (EREs) (Klinge et al., 2004). Consequently, ERα and ERβ exert different effects on the organization, growth and differentiation of various tissues, including colon, uterus, bone, brain and mammary gland (Forster et al., 2004; Helguero et al., 2005; Kudwa et al., 2005; Wada-Hiraike et al., 2006b; Wada-Hiraike et al., 2006a). Transactivation of ERs can occur in two different ways. One is by classical ER transactivation, in which an ER homodimer binds to a palindromic ERE sequence (Klein-Hitpass et al., 1989). The other way is by non-classical transactivation, in which the ER is tethered to other transcription factors, including the Fos-Jun complex, NF-κB and Sp1, to render transcription of their genes responsive to ER activity (Pearce and Jordan, 2004; Sabbah et al., 1999). ERα and ERβ differ in their classical and non-classical transactivation by estradiol (E2) (Paech et al., 1997; Weatherman et al., 2001). These differences between ERα and ERβ are reflected in differences in their amino acid sequences and/or different interactions between the various domains of the receptor. ERα is composed of 595 amino acids, whereas ERβ is 530 amino acids. The N-terminal A/B domain (also known as the AF-1 domain) has a ligand-independent transactivation function and has 17% amino acid homology between the ERs. The highly conserved central C region, with 96% homology, encompasses the DNA-binding domain (DBD). The flexible hinge, or D region, contains nuclear localization signal (NLS) information and links the C-domain to the multifunctional C-terminal E/F domain. The E/F domain (also known as the AF-2 domain) of the ERs shares 53% amino acid homology and contains the ligand-binding domain (LBD) and the ligand-dependent transactivation domain, including the cofactor-binding groove to which cofactors are recruited when the ER becomes activated. For ERα, it has been shown that interaction between the AF domains is essential for effective transactivation (Metivier et al., 2001; Yi et al., 2002a).

Despite similar in vitro ERE-binding capacities and comparable affinities for E2 (Bowers et al., 2000; Yi et al., 2002b), human ERβ is considerably weaker than ERα with respect to ERE-dependent transactivation following E2 exposure (Yi et al., 2002b). Also, differences between human ERα and ERβ have been reported for

the non-classical pathway (Gustafsson, 2000). The molecular mechanism underlying these differences in transcription by the ER subtypes is still unclear.

The precise process of transactivation of genes by ERs is still unresolved. ER can dimerize and bind to its cofactors even when not bound to DNA, which occurs also in the absence of ligands (Carroll et al., 2006; Padron et al., 2007; Zwart et al., 2007a). For proper recruitment of RNA polymerase II, however, the ER needs to be bound to an ERE (Carroll et al., 2006; Sharp et al., 2006; Zwart et al., 2007a). Effective transactivation of ERα requires interaction between the N-terminal and C-terminal regions of ERα (Merot et al., 2004; Metivier et al., 2001), which is accomplished by the binding of ERα to cofactors (Metivier et al., 2001). The anti-estrogen tamoxifen inhibits ERα-mediated transactivation by arresting the conformation of ERα such that the groove that interacts with the steroid receptor coactivator-1 (SRC-1) cofactor remains covered by the twelfth α helix of the LBD (Shiau et al., 1998). However, tamoxifen also has weak agonistic activity. This mild agonistic behavior can be enhanced by phosphorylation of serine 305 in the hinge region of ERα by protein kinase A (Michalides et al., 2004).

Here, we investigate the differences between human ERα and ERβ with regard to E2-driven transactivation and the agonistic effect of tamoxifen. We show that a specific interaction of the AF-1 domain of ERα with SRC-1 is required, but by itself not sufficient, to induce a maximal response to E2. The maximal response to E2 demands, in addition, the hinge region of ERα. Human ERβ lacks an active AF-1 domain. The weak agonistic response to tamoxifen is dependent on the AF-1 domain of ERα, but also involves the hinge region of ERα for optimal transcriptional activity. The AF-1 domain of ERβ is not involved in tamoxifen-mediated transactivation, explaining the differential response to the anti-estrogen tamoxifen.

Results

Extent of transactivation by ER is dependent on the AF-1 domain

ERα and ERβ differ mostly in their N-terminal AF-1 regions, which share 17% sequence homology and differ in size by 41 amino acids. We therefore examined the contribution of the AF-1 domains to optimal E2-driven transactivation. We generated swap mutants, in which AF-1 of ERα (amino acids 1-185) was replaced by the corresponding region of ERβ (amino acids 1-144) and vice versa, resulting in ERα$^{AF\text{-}1\beta}$ and ERβ$^{AF\text{-}1\alpha}$ mutants (Fig. 1A). A detailed alignment of both AF-1 domains is shown in supplementary material Fig. S1. All four ER variants were analyzed for the extent of E2-induced transcription using an ERE-luciferase reporter construct with various concentrations of ligand (Fig. 1B). Maximal transactivation of ERα was considerably higher than that of ERβ, 47- versus 6-fold induction, respectively. Exchanging the AF-1 region of ERβ for that of ERα in ERβ$^{AF\text{-}1\alpha}$ greatly affected this response and increased transactivation from 6- to 16-fold. Exchanging AF-1 of ERα for that of ERβ in ERα$^{AF\text{-}1\beta}$, however, reduced transactivation from 47- to 6-fold, the level also reached by wild-type ERβ. These differences were not due to differences in RNA or protein levels, as has been determined by quantitative PCR (QPCR) analysis, western blot and luciferase reporter assays (supplementary material Fig. S2). The ERβ AF-1 domain therefore reduces E2-induced transactivation, whereas the AF-1 domain of ERα is responsible for its enhancement, which is in agreement with a previous report (McInerney et al., 1998). Although exchange of AF-1 of ERβ for that of ERα in ERβ$^{AF\text{-}1\alpha}$ did increase maximal transactivation approximately threefold when compared with wild-type ERβ, the extent of transactivation did not match that of ERα. This suggests that additional features are involved in optimal E2-driven transactivation.

We then examined whether ligand-independent and tamoxifen-associated transactivation are also linked to AF-1 of ERα. The AF-1 of ERα appeared responsible for the minor ligand-independent transactivation of ERα, because exchange of the AF-1 region between the ERs completely switched this ligand-independent behavior (see Fig. 1D). These results indicate that the AF-1 domain

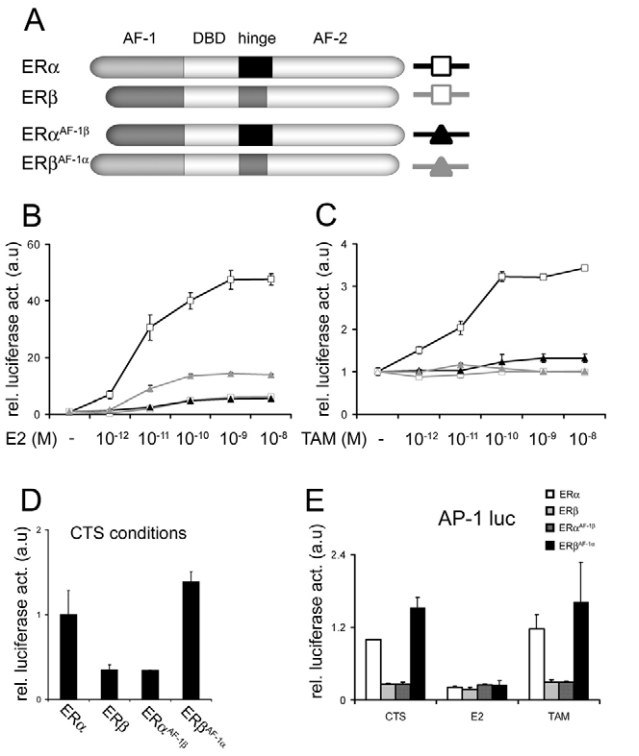

Fig. 1. Extent of transactivation by ER is dependent on the AF-1 domain of ERα. (**A**) Structural overview of ERα, ERβ and AF-1 swap mutants. (**B**) E2-concentration-dependent transcription of an ERE-luciferase reporter. U2OS cells were transfected with ERα, ERβ, ERα$^{AF\text{-}1\beta}$ or ERβ$^{AF\text{-}1\alpha}$ (see key in A), co-transfected with the ERE-luciferase reporter gene and *Renilla* luciferase as the control. Cells were treated with 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M E2 for 36 hours or left untreated, and relative luciferase activity was measured. A representative experiment out of three independent experiments is shown. Error bars indicate standard deviation from duplicate samples. (**C**) The same conditions as in B were applied, but with 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M 4-OH-tamoxifen (TAM). (**D**) Luciferase signal for the tested ER variants under hormone-depleted conditions. Signals for all ER variants are related that of to ERα, which is set at 1. A representative experiment out of two independent experiments is shown. Error bars indicate standard deviation from duplicate samples. (**E**) Non-classical transactivation by ER is influenced by AF-1 of ERα. An AP-1–luciferase reporter assay was performed in U2OS cells, transfected with ER variants, the AP-1–luciferase reporter construct and *Renilla* luciferase as the control. Cells were treated with 10^{-8} M E2, 10^{-7} M tamoxifen or left untreated for 36 hours. Relative luciferase activity was measured and normalized using the *Renilla* signal. The relative luciferase signal for all ER variants was related to the CTS (charcoal-treated serum) value of ERα, which was set at 1. A representative experiment out of three independent tests is shown. Error bars indicate standard deviation from triplicate samples.

of ERα mediates ligand-independent transactivation, whereas such activity is not observed for the AF-1 domain of ERβ.

Tamoxifen exhibits a weak agonistic effect on ERα activity (Michalides et al., 2004; Zwart et al., 2007b). We confirmed these data; transactivation by tamoxifen resulted in a 3.5-fold enhancement over hormone-depleted conditions for ERα (see Fig. 1C). Neither ERβ nor the two swap mutants responded to tamoxifen, indicating that the weak response of ERα to tamoxifen is mediated by AF-1 of ERα. Because the ERβ$^{AF\text{-}1\alpha}$ construct, now containing AF-1 of ERα, did not respond in an agonistic way to tamoxifen, additional features that are not conserved between the two ER isoforms are required for this response (see below).

Our results indicated that AF-1 of ERα determines the extent of E2-mediated transactivation in a classical ERE-mediated transactivation assay. To investigate the role of AF-1 in a non-classical transactivation reaction, we tested ERα, ERβ and the AF-1 swap mutants in an AP-1-driven luciferase assay (Fig. 1E). AP-1-mediated transcription by ERα and ERβ$^{AF\text{-}1\alpha}$ could be inhibited through E2 and not by tamoxifen. These features appeared to be AF-1 mediated, because ERβ and ERα$^{AF\text{-}1\beta}$ failed to respond. These data indicate that the discriminatory role of AF-1 between ERα and ERβ is not restricted to classical ERE-mediated transcription, but also applies to non-ERE, AP-1-dependent transactivation.

The AF-1 domain of ERα, but not of ERβ, binds to SRC-1

The AF-1 of ERα appeared to be responsible for efficient transactivation of ERα in a ligand-dependent and -independent manner, unlike AF-1 of ERβ, which lacks this activity. We next investigated whether this difference in transcriptional capacity correlated with the binding of these domains to the p160 coregulator SRC-1. SRC-1 binds to both the AF-1 and AF-2 domains of ERα; an interaction that generates a functional synergy between the two transactivation domains (Metivier et al., 2001; Webb et al., 1998). The Q-rich region of SRC-1 binds AF-1 in ERα, whereas the consensus LxxLL-containing middle region of SRC-1 binds to the ligand-dependent AF-2 domain of ERα (Merot et al., 2004).

We visualized these interactions in intact cells on a genuine ERE promoter sequence – the prolactin promoter-enhancer (PRL) region in HeLa cells. These cells contain a DNA array consisting of ~52 copies of stably transfected modified PRL (Sharp et al., 2006). The PRL array allowed visualization of a defined, ERE-containing DNA structure in the nucleus, to which ER and cofactors can be recruited. In addition, the size of this structure correlates with transcriptional activity of the ER-cofactor complex bound to this region (Hatzis and Talianidis, 2002; Sharp et al., 2006; Zwart et al., 2007a). ERα, ERβ and both AF-1 swap mutants could recruit RNA polymerase II to the array structure under E2 conditions (see Fig. 2A-D). Under these conditions, the array structures were enlarged, which is associated with transcriptional activity. However, whereas ERα was capable of recruiting RNA polymerase II to the array under hormone-depleted conditions, ERβ failed to do so. This difference in RNA polymerase II recruitment was associated with the AF-1 domain of ERα, because swapping this region to ERβ sufficed to enable RNA polymerase II recruitment under hormone-deprived conditions. This suggests that the AF-1 domain of ERα possesses ligand-independent transactivation activity, which is absent in ERβ, supporting the results of the transactivation studies presented in Fig. 1. We confirmed these interactions using truncation mutants of SRC-1 that bind only AF-1 or AF-2. These truncation mutants of SRC-1 act as dominant-negative mutants over the endogenous coactivators (Zwart et al., 2007a). One truncation mutant of SRC-1 (amino acids 1051-1240), which binds to the AF-1 domain of ERα in a yeast two-hybrid assay (Merot et al., 2004), was tested under E2 conditions. In the absence of ER, the SRC-1 truncation mutants were not recruited to the array structure (data not shown). When this SRC-1$_{1051\text{-}1240}$ mutant was co-transfected with ERα, the SRC-1 mutant was specifically recruited to the array structure by ERα, preventing recruitment of RNA polymerase II to the now condensed array structure, which is indicative of an inactive promoter region (Fig. 2E). The SRC-1$_{1051\text{-}1240}$ mutant was not recruited to the array by ERβ and therefore could not prevent RNA polymerase II accumulation on the array structure. Swapping the AF-1 domains of ERα and ERβ resulted in exchange of the effect of the SRC-1 truncation mutant to interfere with accumulation of RNA polymerase II. The results of these inhibition studies indicated that the AF-1 domain of ERα is capable of binding to the Q-rich region of SRC-1, unlike the AF-1 domain of ERβ. These data were verified in a co-immunoprecipitation experiment (supplementary material Fig. S3). Blocking AF-2 using a second SRC-1 truncation mutant (amino acids 623-711), which specifically binds to the AF-2 region of ER (Llopis et al., 2000), prevented RNA polymerase II recruitment for all tested ER constructs (supplementary material Fig. S4), resulting in a condensed array.

The results of these binding studies indicate that the AF-2 domains of both ERα and ERβ directly interact with SRC-1, whereas only AF-1 of ERα is capable of interacting with the AF-1-binding domain of SRC-1.

We next investigated the functional consequences of the difference in SRC-1 binding between the AF-1 domains of ERα and ERβ. We therefore performed an ERE-luciferase reporter assay, in which the function of AF-1, AF-2 or both in each of the ER constructs was inhibited by the different SRC-1 truncation mutants (see Fig. 3). A total of 10 nM E2 was added for all conditions, which resulted in maximal transactivation for each construct (Fig. 1B). The extent of transactivation of ERα was much higher than that of ERβ. The response of ERα was significantly reduced by co-transfection of SRC-1 truncation mutants that inhibit either AF-1 or AF-2. This was not the case for ERβ, for which only transcriptional inhibition by the AF-2-binding, but not by the AF-1-binding, SRC-1 truncation mutant occurred. Exchanging AF-1 of ERβ for that of ERα in ERβ$^{AF\text{-}1\alpha}$ rendered this construct dependent on AF-1 for its transcriptional potency.

These results indicate that the extent of transactivation by E2 is largely determined by both the AF-1 and AF-2 domains of ERα. Because the AF-1 domain of ERβ lacks the ability to bind to SRC-1, this synergism is absent in ERβ. The E2-mediated transactivation of ERβ, which is low in comparison to that of ERα, is due to the transactivation capacity of its AF-2 domain. Replacement of AF-1 of ERβ by the AF-1 domain of ERα in ERβ$^{AF\text{-}1\alpha}$ restored the synergism between both domains, although not to the extent observed with ERα.

Effect of the hinge region on E2-driven transactivation by ER

To investigate the differences in the extent of transactivation by E2 between wild-type ERα and ERβ$^{AF\text{-}1\alpha}$, we exchanged the hinge region of ERα for that of ERβ and vice versa (Fig. 4A), and tested these constructs in an ERE-dependent luciferase reporter assay using various concentrations of E2 (see Fig. 4B). A detailed alignment of the hinge regions of ERα and ERβ, as well as the hinge mutants, is shown in supplementary material Fig. S5. Two different hinge swaps were made for each ER subtype: a small swap in which amino

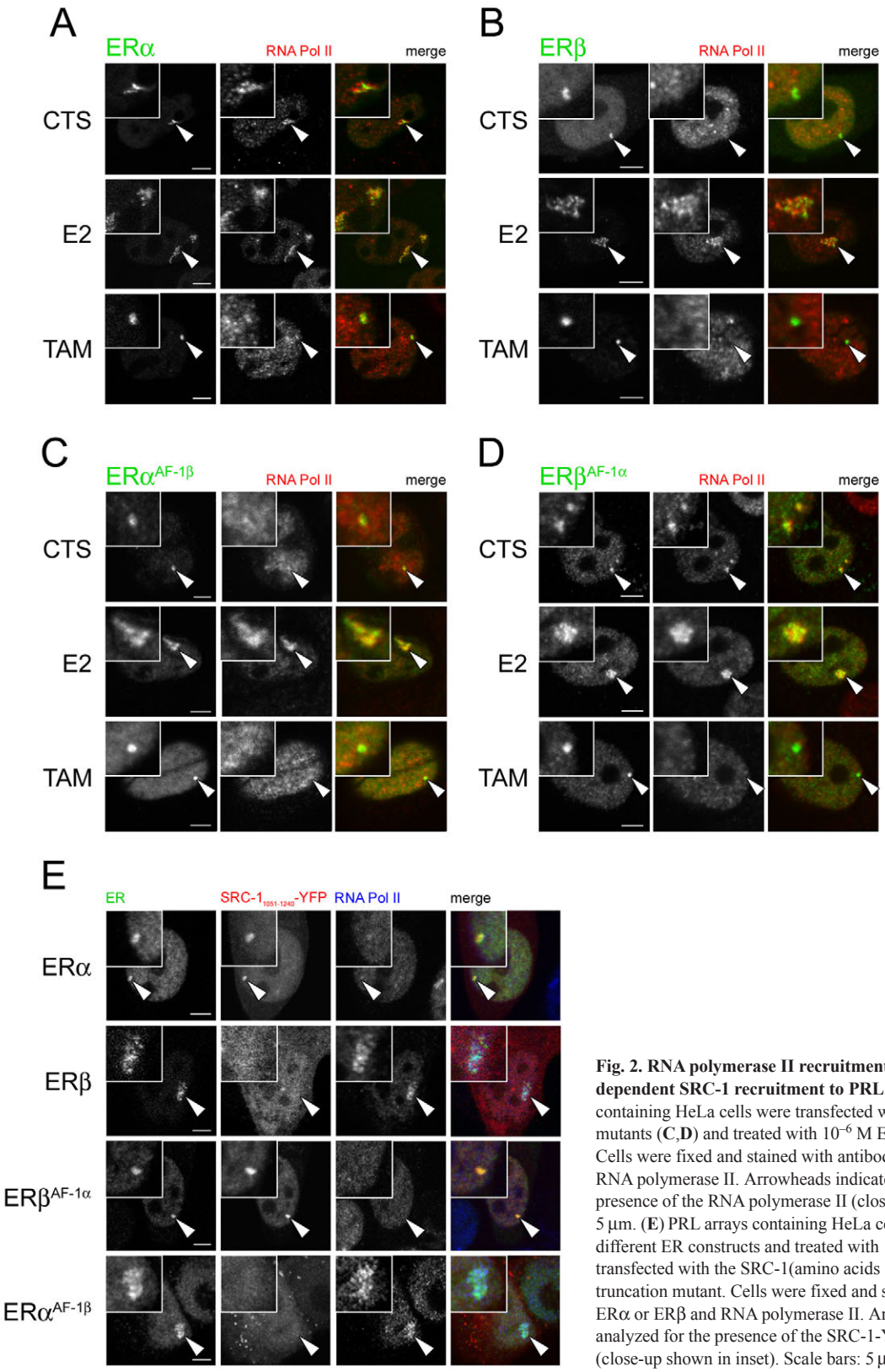

Fig. 2. RNA polymerase II recruitment by ER variants and AF-1-dependent SRC-1 recruitment to PRL array structures. PRL arrays containing HeLa cells were transfected with ERα (**A**), ERβ (**B**) or AF-1 swap mutants (**C**,**D**) and treated with 10^{-6} M E2, tamoxifen or left untreated (CTS). Cells were fixed and stained with antibodies recognizing ERα or ERβ and RNA polymerase II. Arrowheads indicate the array structure, analyzed for the presence of the RNA polymerase II (close-up shown in inset). Scale bars: 5 μm. (**E**) PRL arrays containing HeLa cells were transfected with one of the different ER constructs and treated with 10^{-6} M E2. Cells were then co-transfected with the SRC-1(amino acids 1051-1240)-YFP (AF-1-inhibiting) truncation mutant. Cells were fixed and stained with antibodies recognizing ERα or ERβ and RNA polymerase II. Arrowheads indicate the array structure, analyzed for the presence of the SRC-1-YFP fragment and RNA polymerase II (close-up shown in inset). Scale bars: 5 μm.

acids 294-309 in ERα were replaced with corresponding amino acids 253-262 in ERβ and a larger swap in which amino acids 256-332 in ERα were replaced with corresponding amino acids 225-283 in ERβ. Upon transfection of these constructs, similar mRNA levels were detected for all of the mutants applied (supplementary material Fig. S2). The small hinge swap, $ERα^{Shingeβ}$, showed a similar response to E2 as wild-type ERα, whereas the large hinge swap, $ERα^{Lhingeβ}$, resulted in decreased transactivation, similar to

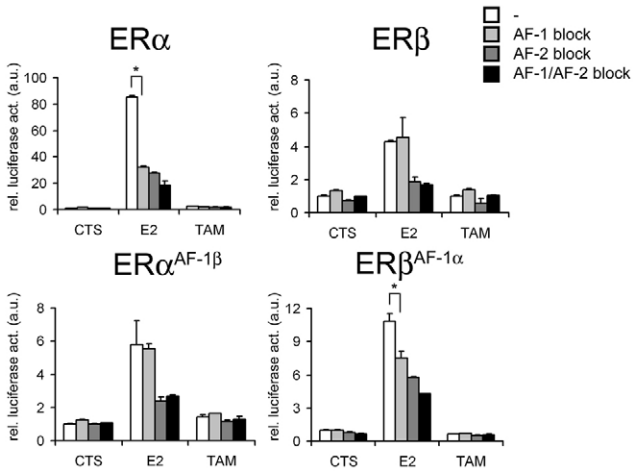

Fig. 3. Functional synergy between AF-1 and AF-2 is ERα and AF-1 specific. An ERE-luciferase reporter assay was performed in U2OS cells transfected with the ER variants, the ERE-luciferase reporter construct and *Renilla* luciferase as a control. Where indicated, cells were co-transfected with SRC-1 amino acids 1051-1240 or SRC-1 amino acids 623-711, inhibiting AF-1 or AF-2, respectively (AF-1 and AF-2 block). Cells were treated with 10 nM E2 or 100 nM tamoxifen or left untreated (CTS) for 36 hours, after which cells were lysed and the luciferase activity was determined. Representative data from two independent experiments is shown. Error bars indicate standard deviation from duplicate samples. The asterisk indicates a statistically significant reduction of luciferase signal by the AF-1 block, $P<0.05$.

that of ERβ$^{AF\text{-}1\alpha}$ (Fig. 4B). This suggests that the reduced response of the ERβ$^{AF\text{-}1\alpha}$ mutant, in which the AF-1 region has been replaced with that of ERα, is due to the composition and/or length of the hinge region. No effect on maximal transactivation of the hinge-swap mutants of ERβ was observed; both mutants behaved similarly to wild-type ERβ or ERα$^{AF\text{-}1\beta}$. This might be because of the absence of any AF-1 activity in ERβ, preventing hinge-mediated functional synergy between AF-1 and AF-2.

Synergy between AF-1 and AF-2 is also essential for the agonistic behavior of tamoxifen (Zwart et al., 2007a). We therefore studied the role of the hinge region in the synergy involved in tamoxifen response. The tamoxifen response of the ERα large hinge-swap mutant was impaired, whereas that of the ERα small hinge-swap mutant resembled ERα (Fig. 4C). Tamoxifen did not increase the response of any of the ERβ hinge-swap mutants. Interestingly, exchanging the hinge region of ERα for that of ERβ in the ERα large hinge-swap mutant, resulted in a tenfold shift in EC_{50} (effector concentration for half-maximum response) values in the response curves for E2 (Fig. 4B), as well as for tamoxifen (Fig. 4C), indicating that the ligand efficacy is influenced by the hinge region. The EC_{50} of the ERβ hinge mutants was also affected (Fig. 4B). This does not involve AF-1 and AF-2 functional synergy, but is determined by the hinge region alone.

Taken together, these results indicate that full E2-driven transactivation of ERα involves cooperation of the AF-1 and AF-2 domains that is dependent on the length and/or composition of the hinge region. For ERβ, E2-mediated transactivation is associated with only its AF-2 domain.

The hinge domain determines tamoxifen- and PKA-induced conformational alterations in ERα and ERβ

After tamoxifen binding to ERα, a conformational change is induced in the receptor, which is indicative of ERα inactivation (Michalides et al., 2004; Zwart et al., 2007b; Zwart et al., 2009). This conformational alteration can be monitored by intramolecular

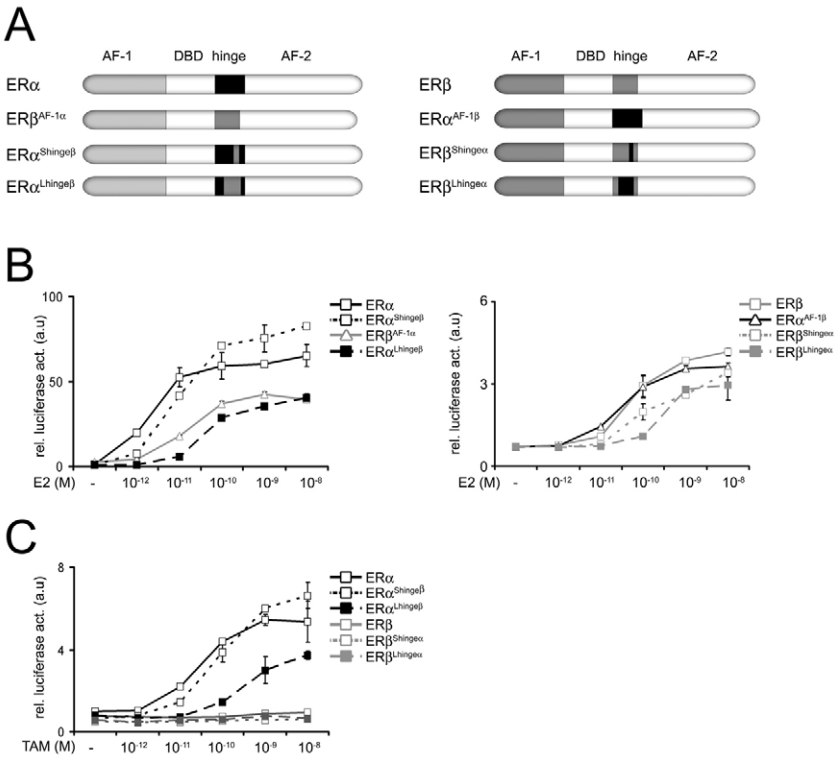

Fig. 4. The hinge region enables complete AF1 and AF2 synergy in ERα. (**A**) Structural overview of ERα, ERβ and its hinge-swap mutants used in this experiment. (**B**) The ERE-luciferase reporter assay was performed in U2OS cells transfected with ER variants, the ERE-luciferase reporter construct and *Renilla* luciferase as the control. Cells were treated with 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M E2 for 36 hours or left untreated (CTS), and relative luciferase activity was measured. A representative experiment out of three independent tests is shown. Error bars indicate standard deviation from duplicate samples. (**C**) The same conditions as in B are applied, but with 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M 4-OH-tamoxifen (TAM).

fluorescence resonance energy transfer (FRET), in which two fluorophores (typically CFP and YFP) are fused to both termini of the receptor. FRET is the radiationless energy transfer from a donor fluorophore to a suitable acceptor fluorophore, and is highly dependent on the distance between them and their orientations (Förster, 1948), enabling a probe to monitor even subtle conformational changes. In the case of tamoxifen resistance, as induced by protein kinase A (PKA)-mediated phosphorylation of serine 305 close to the hinge region of ERα, this tamoxifen-induced conformational change does not occur (Michalides et al., 2004). To monitor the possible influence of the hinge region on the conformational changes induced by ligand binding, we tested the effect of tamoxifen and PKA activation on the FRET responses of the swap mutants (Fig. 5). We monitored FRET by fluorescent lifetime imaging microscopy (FLIM), in which the lifetime of the donor fluorophore emission is measured. The lifetime of the donor CFP is typically 2.7 ns (Vermeer et al., 2004) and is reduced when the energy of the donor is transferred to the acceptor fluorophore in the case of FRET (Bastiaens and Squire, 1999). Cells expressing YFP-ER-CFP were co-cultured with cells only expressing CFP to provide an internal control for the FLIM measurements (Zwart et al., 2009). As we reported previously for ERα, the FRET signal was increased by treating the cells with tamoxifen, which could be prevented by pre-incubation with the PKA activator forskolin (Michalides et al., 2004). In the case of ERβ, no increase in FRET signal was detected after tamoxifen treatment and after forskolin treatment. Combining both treatments, however, did induce a significant increase in FRET efficiency. When the hinge regions of both receptors were swapped, the characteristic responses to tamoxifen and PKA activation were also exchanged between receptors; $ERα^{Lhingeβ}$ did not show a large increase in FRET efficiency after tamoxifen or forskolin exposure, whereas combination of these treatments did induce a conformational change. These FRET characteristics of $ERα^{Lhingeβ}$ are therefore comparable to those of ERβ. $ERβ^{Lhingeα}$ showed a response comparable to ERα and tamoxifen now induced a FRET change. The combination of tamoxifen and forskolin treatment did not induce an increase in FRET for the $ERβ^{Lhingeα}$ mutant, whereas this was observed for wild-type ERβ. Therefore, introducing the hinge of ERα into ERβ sufficed to swap the ligand-induced conformational characteristics. The small hinge swaps showed intermediate effects. There were no differences in transcriptional potency, as induced by tamoxifen and forskolin, between the ER hinge-swap mutants (supplementary material Fig. S6). This indicated that the N- and C-terminal orientations within the ER variants are determined by the non-conserved hinge regions in ERα and ERβ. More importantly, this hinge-specific feature appeared to be responsible for the different conformational responses induced by ligand treatment and kinase activities.

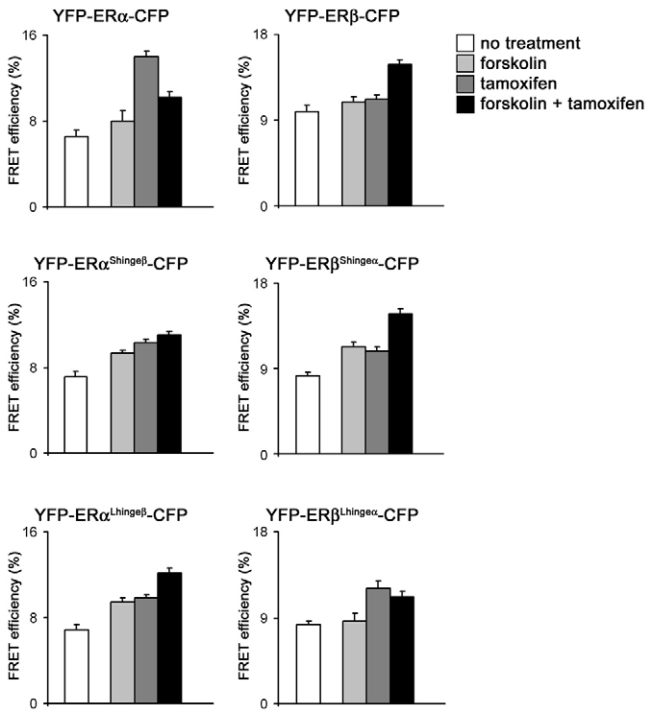

Fig. 5. Tamoxifen and PKA-induced conformational changes of ER and hinge mutants. Intramolecular FRET, as determined by FLIM, was measured in U2OS cells expressing YFP-ERα-CFP, YFP-ERβ-CFP or their hinge-swap mutants. FRET was determined prior to ligand addition, after 1 μM tamoxifen addition or PKA activation by 10 μM forskolin, or a combination of both treatments in CTS. Donor FRET efficiency (E_D) was calculated as E_D=1–(lifetime cell of interest/lifetime reference cell). For each condition, *n*>25 cells.

Discussion

ERβ arose from ERα by a duplication event to different chromosomes approximately 450 million years ago (Kelley and Thackray, 1999). The homology between the receptors is largely maintained in the DNA-binding domains (96%) and ligand-binding domains (53%), which indicates a functional selection pressure. In the present study, we confirmed previous work (Metivier et al., 2001) showing that the AF-1 domain of ERα binds to SRC-1. This binding, in combination with the hinge region of ERα, is responsible for the extent of transactivation by E2 and for ligand-independent transactivation. Both of these functions are lost in ERβ, which reflects the largely deviating sequence of AF-1 and the hinge region of ERβ compared with ERα. The expression of ERα and ERβ is different in various human tissues, ranging from exclusive expression of ERβ in the colon and brain to variations in the relative expression of ERα and ERβ in other tissues. This variation influences the extent of response to E2. Indeed, ERβ has been reported to interfere with E2-driven proliferation of breast cancer cells (Strom et al., 2004), either as homodimers or as heterodimers of ERα and ERβ (Li et al., 2004).

Our study confirms a previous report (McInerney et al., 1998) and illustrates that the AF-1 domain of ERα is crucial for enhanced transactivation by E2. This is mediated by a unique feature of AF-1 of ERα, because it directly interacts with the Q-rich region of the SRC-1 cofactor (Metivier et al., 2001), whereas AF-1 of ERβ does not. As a consequence, the small E2-driven transactivation and RNA polymerase II recruitment by ERβ is solely mediated through its AF-2 activity. We also showed that the orientation between the AF-1 and AF-2 domains, determined by the composition and/or length of the hinge region, affects the final extent of E2-driven transactivation.

The results of the luciferase reporter studies (Fig. 3) confirmed the co-immunoprecipitation experiments (supplementary material Fig. S3) and in situ localization studies (Fig. 2). These results showed that non-liganded ERα binds to SRC-1 through its AF-1 domain, which is sufficient for RNA polymerase II recruitment. The composition of the AF-1 domain is crucial for binding to SRC-1, whereas the spacing and/or orientation between the AF-1 and

AF-2 domains are essential for transactivation of ERα. When the large hinge region of ERα was replaced with that of ERβ, the resulting ERα^{Lhingeβ} mutant was equally active as the ERβ^{AF-1A} mutant (Fig. 4). This suggests that a combination of AF-1–AF-2 interactions, together with the hinge region, determines the prominent E2-driven transactivation of ERα. This spatial positioning of the AF domains might also be relevant for resistance to particular antagonists, because phosphorylation of serine 305 of ERα within the hinge region by protein kinase A or PAK-1 (Michalides et al., 2004; Rayala et al., 2006) results in resistance to tamoxifen.

Both the length and the composition of the hinge region might affect the capacity for transactivation, because the hinge region of ERα is a target of extensive post-translational modifications that affect the stability and/or activity of the receptor. In particular, the residues in the hinge between K299 and S305 are targeted for acetylation, ubiquitylation, methylation and phosphorylation. These modifications might interfere with one another and affect final activation of ERα (Eakin et al., 2007; Kim et al., 2006; Michalides et al., 2004; Subramanian et al., 2008; Wang et al., 2001). These sequences and corresponding opportunities for modification and modulation of the receptor are lacking in the hinge region of ERβ. Indeed, exchanging the hinge regions between ERα and ERβ altered the conformation of ER, as induced by tamoxifen and the PKA-activating compound forskolin (Fig. 5).

The antagonist tamoxifen also has weak agonistic activity (32). We showed that the AF-1 domain of ERα enhanced this tamoxifen-mediated transactivation, but only on an ERα background (Fig. 1C). This background involves the proper hinge region, which facilitates interaction between AF-1 and AF-2 and thereby the extent of transactivation for ERα, but not for ERβ (Fig. 4C).

We have performed these studies in U2OS osteosarcoma cells; this is a model cell line for testing the direct effects of transactivation of ERα and ERβ (Michalides et al., 2004; Stossi et al., 2004), because these cells do not express endogenous ER and allow reconstitution of the ER-cofactor complex. We have focused in this study on the interaction between ER and SRC-1 as a most relevant cofactor of ER. There are three different SRC cofactors, which all belong to the p160 cofactor family (Xu and Li, 2003). They interact with the AF-2 domain of ERα and ERβ through their LxxLL motifs (Heery et al., 1997). However, when AF-1 of ERα was blocked using an AF-1-inhibiting fragment of SRC-1 (Fig. 2), no recruitment of RNA polymerase II is observed in the presence of E2. These data showed that this SRC-1 truncation functions in a dominant-negative manner, implying no residual interaction between AF-1 of ERα and SRC-2 or SRC-3 that would otherwise have resulted in recruitment of RNA polymerase II.

It has been reported that AF-1 of ERβ binds to SRC-1 when the complex is bound to the SP-1-directed promoter of the TIEG gene in osteoblast cells, enhancing its E2-mediated expression (Hawse et al., 2008). This study and our results indicate that the effects of ERα and ERβ are dependent on the promoter region to which they (in)directly bind, as was also suggested by others (Klinge et al., 2004). The results of our study emphasize that, with the 'classical' palindromic ERE-containing promoters, E2- and ligand-independent transactivation are determined by the binding of AF-1 of ERα and SRC-1, which does not occur in ERβ. AF-1 of human ERβ does not bind to SRC-1, unlike mouse ERβ (Tremblay et al., 1999). This corresponds to a difference in transactivation by E2 between human ERα and ERβ, whereas there is no such difference between mouse ERα and ERβ (Picard et al., 2008). In the mouse, specific phosphorylation sites in the AF-1 domain of mouse ERβ can alter its activity (Tremblay et al., 1999) and stability (Picard et al., 2008).

This difference between human ERα and ERβ also emphasizes the importance of domain interactions in ERα. The orientation between the AF-1 and AF-2 domains in ERα is affected by selective anti-estrogens and can be subtly modified by the structure of these anti-estrogens. How the various domains of ERα and cofactors collaborate in breast-tumor growth and in the response to anti-estrogens is gradually becoming clear.

Materials and Methods

Cell culture and antibodies

Human osteosarcoma U2OS, MCF-7 and HeLa PRL array cells (Sharp et al., 2006) were cultured in DMEM medium in the presence of 10% FCS and standard antibiotics. Cells containing ER constructs were cultured in phenol-red-free DMEM containing 5% charcoal-treated serum (CTS; HyClone).

Antibodies used were raised against ERα (NovoCastra/Cell Signaling Technology), tubulin (Sigma), ERβ (Santa Cruz Biotechnology), RNA polymerase II (8WG16; Covance) and GFP (van Ham et al., 1997).

Cloning procedures and oligomers

ERα, ERβ, SRC-1 (amino acids 623-711)-YFP and SRC-1 (amino acids 1051-1240)-YFP constructs were generated as described previously (Michalides et al., 2004; Zwart et al., 2007a). The ERα$^{AF-1β}$ and ERβ$^{AF-1α}$ swap constructs were made by pairwise ligation of four separate PCR fragments in a second PCR reaction. The AF-1α (amino acids 1-180) and AF-1β (amino acids 1-144) fragments were made using forward primer (A) 5′AATTGGATCCACCACCATGGCATACCCATACGACGTCCCAGACTACGCTATGACCATGACCCTCCACACC and reverse primer (B) 5′GCGCAGAAGTGAGCATCCTTGGCAGATTCCATAGCC, and forward primer (C) 5′AATTGGATCCACCACCATGGCATACCCATACGACGTCCCAGACTACGCTATGGATATAAAAAACTCACC and reverse primer (D) 5′GCACAGTAGCGAGTCTCCCTCTTTGAACCTGGACC, respectively, introducing a *Bam*HI restriction site and a hemagglutinin tag at the 5′ ends, and a ERβ or ERα overhang at the 3′ ends of the fragments. The ERα$^{ΔAF-1}$ and ERβ$^{ΔAF-1}$ fragments, lacking the AF-1 regions, were made using forward primer (E) 5′GGTCCAGGTTCAAAGAGGGAGACTCGCTACTGTGC and reverse primer (F) 5′TGGGGGATCCTTATCAGACTGTGGCAGGGAAACC, and forward primer (G) 5′GGCTATGGAATCTGCCAAGGATGCTCACTTCTGCGC and reverse primer (H) 5′AATTGGATCCTCACTGAGACTGTGGGTTCTGG, respectively, introducing a ERβ or ERα overhang at the 5′ ends, and a stop codon and *Bam*HI restriction site at the 3′ ends of the fragments. The AF-1α and ERβ$^{ΔAF-1}$ and AF-1β and ERα$^{ΔAF-1}$ fragments were then ligated to each other in a second PCR reaction using forward primer (A) and reverse primer (H), and forward primer (C) and reverse primer (F). These swap constructs were cloned in the dephosphorylated *Bam*HI site of the pcDNA3 vector.

ERα^{Shingeβ}, ERα^{Lhingeβ}, ERβ^{Shingeα} and ERβ^{Lhingeα} constructs were made by sequential PCR reactions, ligating ten separate smaller PCR fragments into four different larger fragments containing the chimeric hinge regions.

The two PCR fragments used for the construction of ERα$^{Shingeβ\ (a.a.\ 253\text{-}262)}$ were made using forward primer (1) 5′AATTCCGCGGCCGCCGCCAACGCGCAGG and reverse primer (2) 5′GGCGTCCAGCAGCAGCTCCCGCACTCGGGGTGGCCAAAGGTTGGC, and forward primer (3) 5′CCCCGAGTGCGGGAGCTGCTGCTGGACGCCCTGACGGCCGACCAG and reverse primer (4) 5′TGGTCTAGAAGGTGGACCTGATCATGGAG. The fragments were then ligated in a second PCR reaction using forward primer (1) and reverse primer (4), introducing a *Sac*II restriction site at the 5′ end and an *Xba*I site at the 3′ end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ERα vector. The two PCR fragments used for the construction of ERβ$^{Shingeα\ (a.a.\ 294\text{-}309)}$ were made using forward primer (5) 5′CCAGATATCACTATGGAGTCTGGTCGTGTG and reverse primer (6) 5′GGACAAGGCCAGGCTGTTCTTCTTAGAGCGTTTGATCATGAGCGGGCTCGCGTGGCCGCCACTTCTCTTGGCC, and forward primer (7) 5′AGCCCGCTCATGATCAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGAGCCCCGAGCAGCTAGTGCTCACC and reverse primer (8) 5′TGGGAATTCCTTCTACGCATTTCCCCTCATCC. The fragments were then ligated in a second PCR reaction using forward primer (5) and reverse primer (8), and introducing an *Eco*RV restriction site at the 5′ end and an *Eco*RI site at the 3′ end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ERβ vector.

The three PCR fragments used for the construction of ERα$^{Lhingeβ\ (a.a.\ 220\text{-}283)}$ were made using forward primer (1) and reverse primer (9) 5′CACATCTCTCTCTCCGTATCCCACCTTTCATCATTCCC, forward primer (10) 5′CGGAGAGAGAGATGTGGGTACC and reverse primer (11) 5′GCGGCTGATCAGCACATGGGGC, and forward primer (12) 5′TGTGCTGATCAGCCGCCCTACCAGACCCTTCAGTGAAGCTTCG and reverse primer (4). The fragments were then sequentially ligated in a second and third PCR reaction using forward primer (1) and reverse primer (11), and forward primer (1) and reverse primer (4), respectively, introducing a *Sac*II

restriction site at the 5′ end and an *Xba*I site at the 3′ end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ERα vector.

The three PCR fragments used for the construction of ERβ$^{Lhingeα\ (a.a.\ 256\text{-}332)}$ were made using forward primer (5) and reverse primer (13) 5′CTCCTCTTCGGTCTTTTCGGGAGCCACACTTCACCATTCCC, forward primer (14) 5′CGAAAAGACCGAAGAGGAGGGAG and reverse primer (15) 5′ATCATACTCGGAATAGAGTATGGG, and forward primer (16) 5′CTCTATTCCGAGTATGATCCCAGTGCGCCCTTCACCGAGG and reverse primer (8). The fragments were then ligated in a sequential second and third PCR reaction using forward primer (5) and reverse primer (15), and forward primer (5) and reverse primer (8), respectively, introducing an *Eco*RV restriction site at the 5′ end and an *Eco*RI site at the 3′ end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ERβ vector.

For QPCR analysis of ER expression levels, hybrid primers were applied based on the two DNA-binding domains, with forward primer 5′GAGAAGCATTCAAGGACATAACGAT and reverse primer 5′CCACATTTCACCATTCCCAC. As a control for equal loading, the observed ER signals were related to β-actin RNA levels, using a forward primer 5′CCTGGCACCCAGCACAAT and reverse primer 5′GGGCCGGACTCGTCATACT.

Immunoprecipitation

U2OS cells were transfected with ERα, ERβ or ERβ$^{AF\text{-}1α}$ in the presence or absence of SRC-1 (amino acids 1051-1240)-YFP using polyethylenimine (PEI). Twenty-four hours after transfection, cells were lysed in 125 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% Nonidet P-40, 10 mM $MgCl_2$ and protease inhibitor cocktail (Roche) on ice, sonificated and debris removed by centrifugation. The supernatant was used in immunoprecipitation using anti-GFP antibody immobilized on protein A-sepharose beads (Invitrogen) during incubation overnight. Samples were taken from the supernatant for analysis of the total lysate. Beads were extensively washed, boiled and samples were analyzed by western blotting. For detection, antibodies identifying ERα, ERβ and GFP were used, and the signal was detected using an ECL detection kit (Amersham).

ERE- and AP-1-dependent luciferase assays

Luciferase assays were performed as described previously (Bindels et al., 2002), transfecting 2 ng of ER and 0.2 μg ERE-tk-firefly luciferase, or 0.2 μg of ER and 0.2 μg AP-1-tk-firefly luciferase using PEI (25 kDa; Polysciences) (Boussif et al., 1995). As a control, 2 ng Simian virus (SV40) *Renilla* luciferase was used. For specific inhibition of the AF-1 or AF-2 activity of ERα, SRC-1 truncation mutants comprising amino acids 1051-1240-YFP or amino acids 623-711-YFP (Zwart et al., 2007a) were co-transfected at 0.5 and 0.2 μg per well, respectively, and supplemented with pcDNA3 empty vector to equalize the total amount of DNA per well.

Microscopy

RNA polymerase II recruitment was assayed as described before (Zwart et al., 2007a). Where indicated, cells were co-transfected with YFP-tagged SRC-1 fragments comprising amino acids 623-711 or amino acids 1051-1240. Two hours before fixation, cells were treated with 1 μM estradiol, tamoxifen or left untreated. Cells were fixed with 3.7% formaldehyde in PBS, permeabilized for 5 minutes with 0.05% Triton X-100 in PBS at room temperature, and subsequently stained with antibodies detecting ERα, ERβ and RNA polymerase II, and secondary antibodies conjugated to AlexaFluor405 and AlexaFluor647 (Molecular Probes). After staining, cells were mounted in Vectashield mounting medium (Vector Laboratories). The specimens were analyzed with confocal laser-scanning microscopes (TCS-SP1, TCS-SP2 or AOBS; Leica) equipped with HCX Plan-Apochromat 63× NA 1.32 and HCX Plan-Apochromat lbd.bl 63× NA 1.4 oil-corrected objective lenses (Leica). The acquisition software used was LCS (Leica).

Fluorescence lifetime imaging microscopy

Prior to FLIM experiments, cells on cover slips were mounted in bicarbonate-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 23 mM $NaHCO_3$, 10 mM glucose and 10 mM HEPES at pH 7.3) in a heated tissue-culture chamber at 37°C under 5% CO_2. FLIM experiments were performed on a Leica inverted DM-IRE2 microscope equipped with a Lambert Instruments frequency domain lifetime attachment (Leutingewolde), controlled by the vendor's LI FLIM software. CFP was excited at 430 nm with ~4 mW power using an LED modulated at 40 MHz. Emission was collected at 450-490 nm using an intensified CCD camera. FLIM measurements were performed in U2OS cells, transfected with YFP-ERα-CFP, YFP-ERβ-CFP or one of the hinge-swap mutants. Calculated CFP lifetimes were referenced to a 1 μM solution of rhodamine-G6 in medium that was set at a 4.11 ns lifetime, and internally calibrated using co-cultured CFP containing MelJuSo reference cells, for which the lifetime was set to 2.7 ns (Vermeer et al., 2004). Donor FRET efficiency (E_D) was calculated as E_D=1–(lifetime cell of interest/lifetime reference cell).

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