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A comparison of transcriptional activation by ERα and ERβ☆

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Abstract

We have compared the ability of ERα and ERβ to stimulate transcription from a number of reporter genes in different cell lines and demonstrate that the activity of AF1 in ERβ is negligible compared with that of ERα on ERE based reporters. The activity of AF2 in ERα and ERβ is similar and this is likely to reflect their similar ability to bind coactivators. As a consequence, when transcription from a gene depends on both AF1 and AF2 the activity of ERα greatly exceeds that of ERβ but when AF1 is not required ERα and ERβ have similar transcriptional activities.

1. Introduction

The estrogen receptors, ERα and ERβ [1,2], are responsible for mediating the effects of estrogens in target tissues by acting as ligand dependent transcription factors. ERα and ERβ have the potential to function as heterodimers [3,4] but, given that their relative distribution is rather different, the two receptors are more likely to function as homodimers in the majority of target cells. ERα stimulates transcription of target genes by means of two distinct activation functions, AF1 in the N-terminal domain and AF2 in the ligand binding domain, whose activities vary depending upon the target promoter and cell type [5,6]. The activity of AF1 is ligand independent but can be modulated by phosphorylation by the mitogen activated protein kinase (MAPK) pathway [7,8] in response to growth factors [9,10]. The activity of AF2, which depends on the binding of estradiol, is reduced or abolished by mutations in a C-terminal helix which is conserved in most NRs [11,12]. This helix (H12) is packed against helices 3, 5/6 and 11 in the presence of oestrogen [13]. The importance of H12 in transcriptional activity by ERα is supported by the observation that it is misaligned in the presence of the oestrogen antagonist raloxifen [13].

In common with other nuclear receptors, activated ER interacts with a number of target proteins to stimulate transcription. These include a family of proteins with a mol. wt of about 160 kDa encoded by three distinct genes SRC1 [14,15], TIF2/GRIP1 [16,17] and pCIP/ACTR/RAC3/AIB1 [18] and CBP/p300 [15,19,20]. The interaction between the p160 proteins and the receptor appears to be direct while the recruitment of CBP is probably indirect and mediated by the p160 proteins [15,19,20]. Their precise roles in transcriptional activation is unclear but they are likely to be involved in recruiting the basal transcription machinery and in remodelling chromatin.

ERα and ERβ share about 95% homology in the DNA binding domain, both capable of binding to a consensus estrogen response element (ERE) [3,21] and 55% homology in the ligand binding domain, exhibiting similar but not identical ligand binding properties [22]. Both receptors appear to contain a functionally conserved AF2 which is stimulated by binding the coactivator SRC1 [3,21]. Although the two receptors are poorly conserved in the N-terminal domain ERβ, like ERα, appears to contain a MAPK phosphorylation site that results in enhanced transcriptional ac-

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tivity [21]. In this paper we have investigated the ability of ERα and ERβ to stimulate transcription from a number of reporter genes in different cell lines and demonstrate that the activity of AF1 in ERβ is negligible compared with that of ERα on ERE based reporters. As a consequence, when transcription from a gene depends on both AF1 and AF2 the activity of ERα greatly exceeds that of ERβ.

2. Materials and methods

2.1. Plasmid constructions

The isolation and construction of cDNA clones that encode the mouse ERα, and a series of point mutants for analysing transcriptional activation have been described previously [11,23]. The human ERα wild type cDNA from pSG5-HEGO was kindly provided by Pierre Chambon. The 1.6 kb human ERβ cDNA, containing an additional 159 bp of 5′ sequence compared with that published previously [1] (unpublished data), was subcloned into the BamHI site of pSG5. Gal4-AF2β was constructed by inserting the ligand binding domain of ERβ (residues 262–530, generated by PCR) into the BamH1 restriction enzyme site of pSG424. Both wild-type and mutant versions of Gal4-AF2α have been described previously [24]. ERβ helix12 mutants were generated by PCR using oligonucleotides containing the relevant DNA base-pair substitutions (ERβ1: 5′-CAATCCATGCGCCTGGCTACC-3′ in conjunction with either E493A: 5′-TTTCACGTGGGCATTCAGCATCGCCAG-3′, or M494A/L495A: 5′-TTTCACGTGGGCATTCGCCGCCTCCAG-3′). The resulting DNA fragments were cut with restriction enzymes Sac1/Pml1 and ligated into the corresponding sites in pSG5-ERβ and Gal4-AF2β. Gal4-AF1α (residues 1–177) and Gal4-AF1β (residues 1–141) were made by generating AF1 using PCR, cutting the DNA product with restriction enzymes BamH1/Xba1 and then ligating into the corresponding sites of pSG5-Gal (H.Y Mak). The construction and cloning of pSG5-SRC1 and pGEX2TK-SRC1(570–780) have been described previously [25].

2.2. GST pull-down assays

ERα, ERβ and SRC1 protein was synthesised in vitro using the TnT-coupled reticulocyte lysate system (Promega) in the presence of ^{35}S-methionine according to the manufacturer's instructions. GST fusion proteins were expressed and purified as described earlier [26]. [^{35}S]-labelled proteins were incubated with GST-fusion proteins in NETN buffer (20 mM Tris pH 8.0, 1 mM EDTA, 0.5% NP-40) containing 200 mM NaCl, unless stated otherwise, in the absence or presence of E2 (10^{-6} M), 4-hydroxytamoxifen (10^{-6} M) or ICI 182,780 (10^{-6} M) [26]. Samples were subsequently washed and separated on SDS-polyacrylamide gels (8 or 10%). Gels were fixed, dried and the [^{35}S]-labelled proteins were visualised by fluorography.

2.3. COS-1 cell extract preparation

COS-1 cells were transfected by electroporation using a Bio-Rad gene pulser at 450 V and 250 μF as previously described [11]. Cells were (co)transfected with 20 μg of expression plasmid, as indicated in the Figure legends. Two days after transfection the cells were harvested in chilled PBS, centrifuged for 5 min at 5000 *g* and then snap frozen at −70°C. Whole cell extracts (WCE) were prepared using a high salt extraction buffer (400 mM KCl, 20 mM HEPES pH 7.4, 1 mM DTT, 20% glycerol, plus protease inhibitors), and centrifugation at 50,000 *g* for 20 min at 4°C, the supernatant was stored at −70°C. The protein concentration of the WCE was determined using the Bio-Rad protein assay kit (Bio-Rad).

2.4. DNA binding assay

DNA binding was assayed using an electrophoretic mobility shift or gel shift assay. Aliquots of receptor, translated in vitro or expressed in COS-1 cells, were preincubated for 15 min in 20 μl of binding buffer (20 mM HEPES pH 7.4, 50 mM KCl, 1 mM 2-mercaptoethanol, 10% glycerol) containing 1 μg of poly([dI]-[dC]) and 100 μg of BSA. After the addition of a [^{32}P]-labelled double-stranded oligonucleotide probe containing a consensus ERE sequence (5′CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3′) or Gal4RE (5′-AGCTTCCGGAGGACTGTCCTCCGGT-3′) as indicated in the Figure legends, the samples were incubated for a further 25 min at room temperature. The samples were applied directly onto a prerun nondenaturing 7% polyacrylamide (30% acrylamide, 0.8% bisacrylamide stock solution), 0.5x TBE gels, and electrophoresed in 0.5x TBE for 90 min. Gels were fixed for 15 min in 10% acetic acid, 30% methanol, dried, and autoradiographed.

2.5. Cell culture and transient transfection experiments

Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (Gibco BRL). For transient transfection assays, cells were plated in 24-well microtiter plates (Falcon) in phenol red free medium containing 5% charcoal-dextran stripped foetal bovine serum (CSS). Cells were transfected by calcium phosphate coprecipitation as described earlier [11]. The transfected DNA included a pCMV-βGal control plasmid (150 ng), an

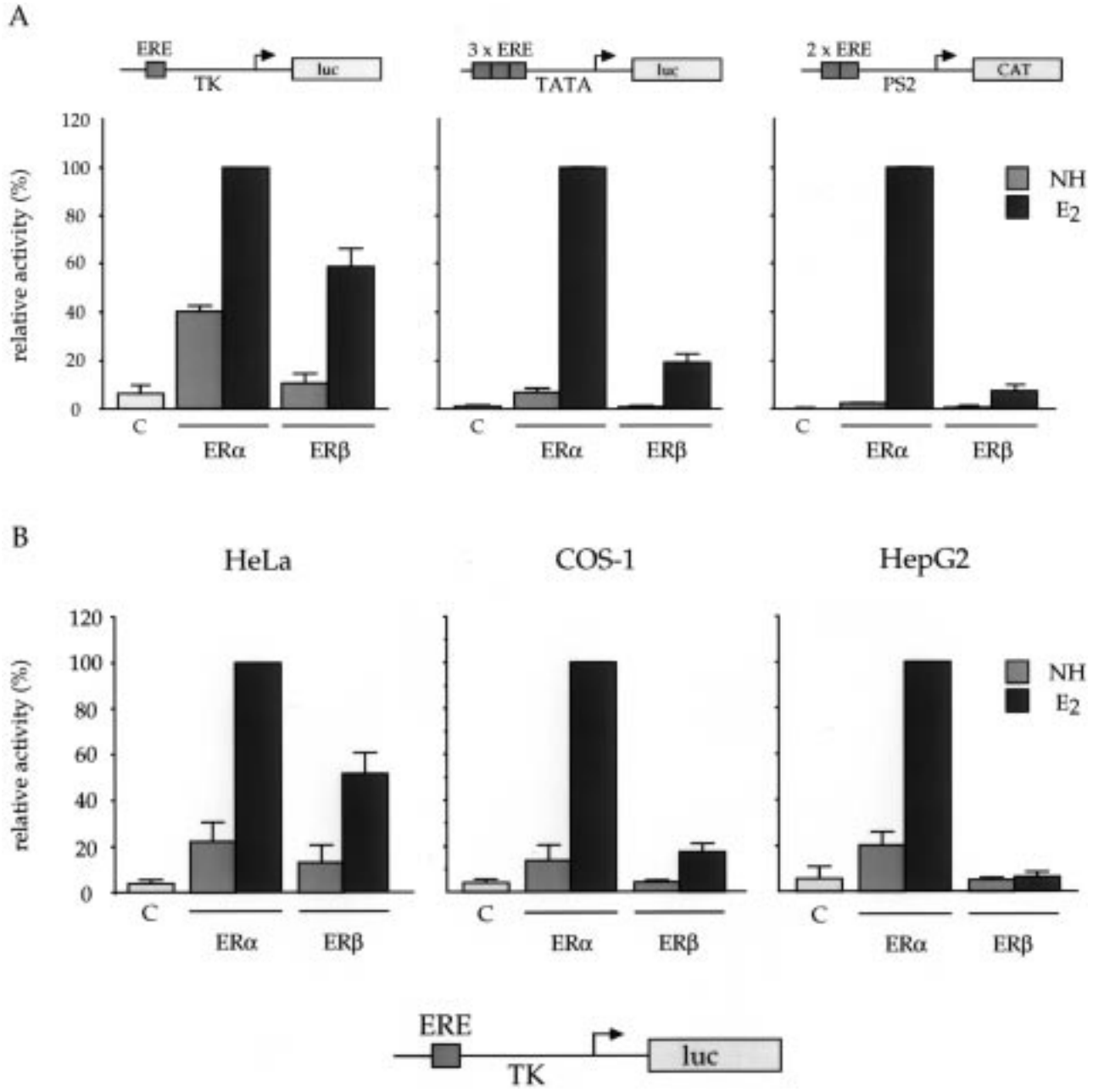

Fig. 1. Transcriptional activation of reporter genes by ERα and ERβ in different cell types. (A) Transactivation by the human ERα and ERβ was tested by transiently transfecting chicken embryo fibroblasts (CEF) with pSG5HEGO or pSG5ERβ respectively and a reporter gene, ERE-TK-Luc, 3ERE-TATA-luc or 2ERE-pS2-CAT as indicated. The internal control was pJ7*lacZ*. Cells were treated for 24 h in the presence or absence of 10^{-8} M 17β-estradiol as indicated, harvested and the extracts tested for reporter activity. After correcting for transfection efficiency using the internal control, transcriptional activity from each of reporters was determined relative to that of ERα in the presence of estradiol. Background activity (without transfected receptor) is denoted by C. The error bars represent the standard deviation of values from at least two separate experiments performed in duplicate. (B) The transcriptional activity of ERα and ERβ from ERE-TK-Luc was determined in COS-1, HeLa and HepG2 cells as described in (A).

ERE containing reporter plasmid, as indicated in the legend (1 μg) and either pSG5-ERα or pSG5-ERβ (20 ng), or varying amounts of the Gal4-AF1 and Gal4-AF2 fusion proteins (5–100 ng). After 16 h, the medium was refreshed and cells were treated with vehicle or E2 (10^{-8} M) for 24 h. Subsequently, cells were harvested and extracts were assayed for luciferase [11] or CAT [27] and β-galactosidase activity, using a

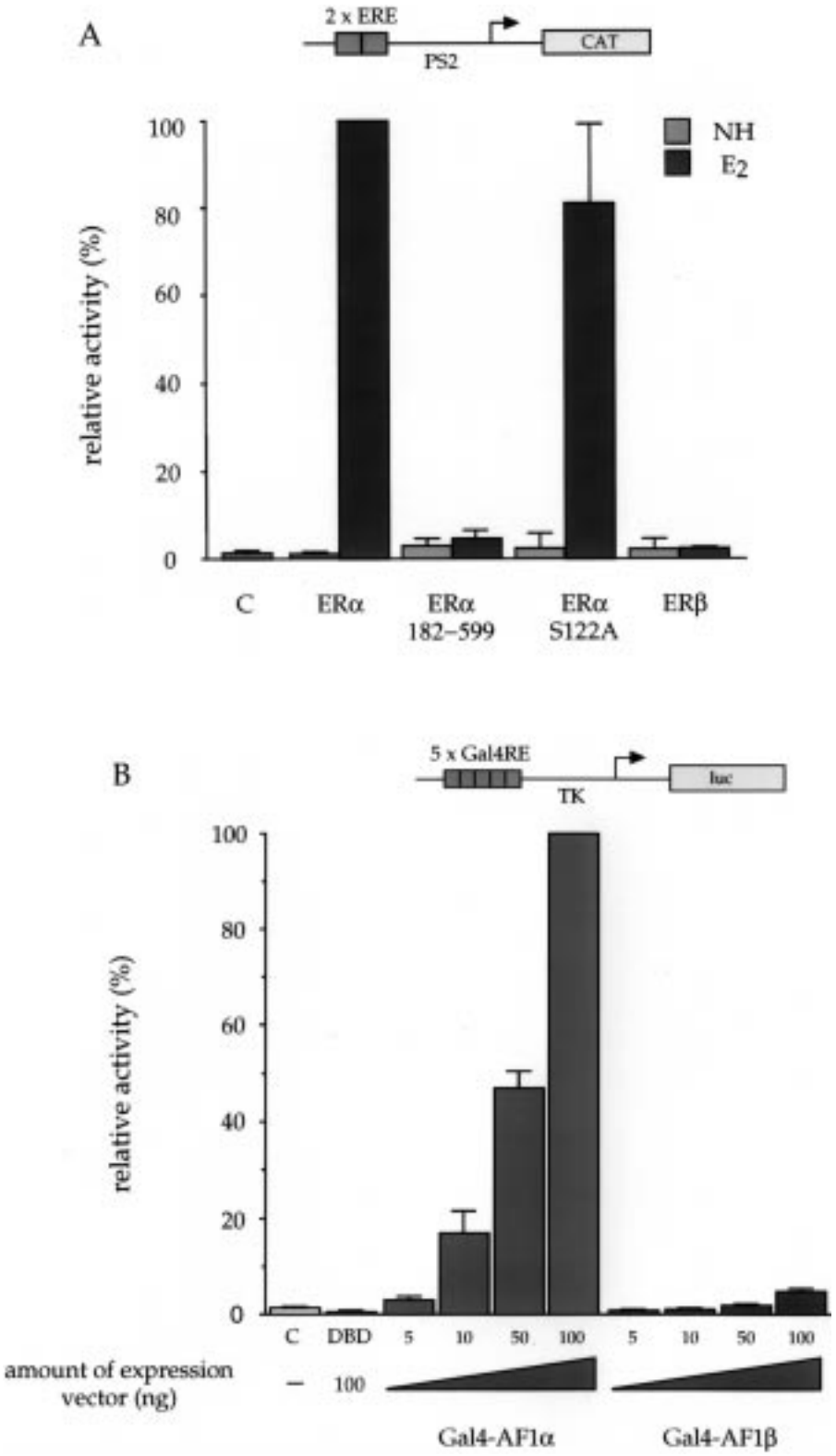

Fig. 2. ERα and ERβ differ in their AF1 activities. (A) The ability of ERα, ERβ, or mutant versions thereof, to stimulate transcription from 2ERE-PS2-CAT was determined in HeLa cells. Cells were treated for 24 h in the absence or presence of 10^{-8} M 17β-oestradiol as indicated and then harvested and analysed as described in Fig. 1. Transcriptional activity relative to that of ERα in the presence of estradiol is presented. (B) AF1 activity in ERα and ERβ cells was analysed by determining the ability of Gal4AF1α or Gal4AF1β to stimulate transcription from Gal4RE-TK-luc in HepG2 cells. Cells were harvested 48 h after transfection and reporter activity measured in the extracts. After correcting for transfection efficiency using the internal control the relative reporter activity was determined. Background activity (without transfected receptor) is denoted by C and error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

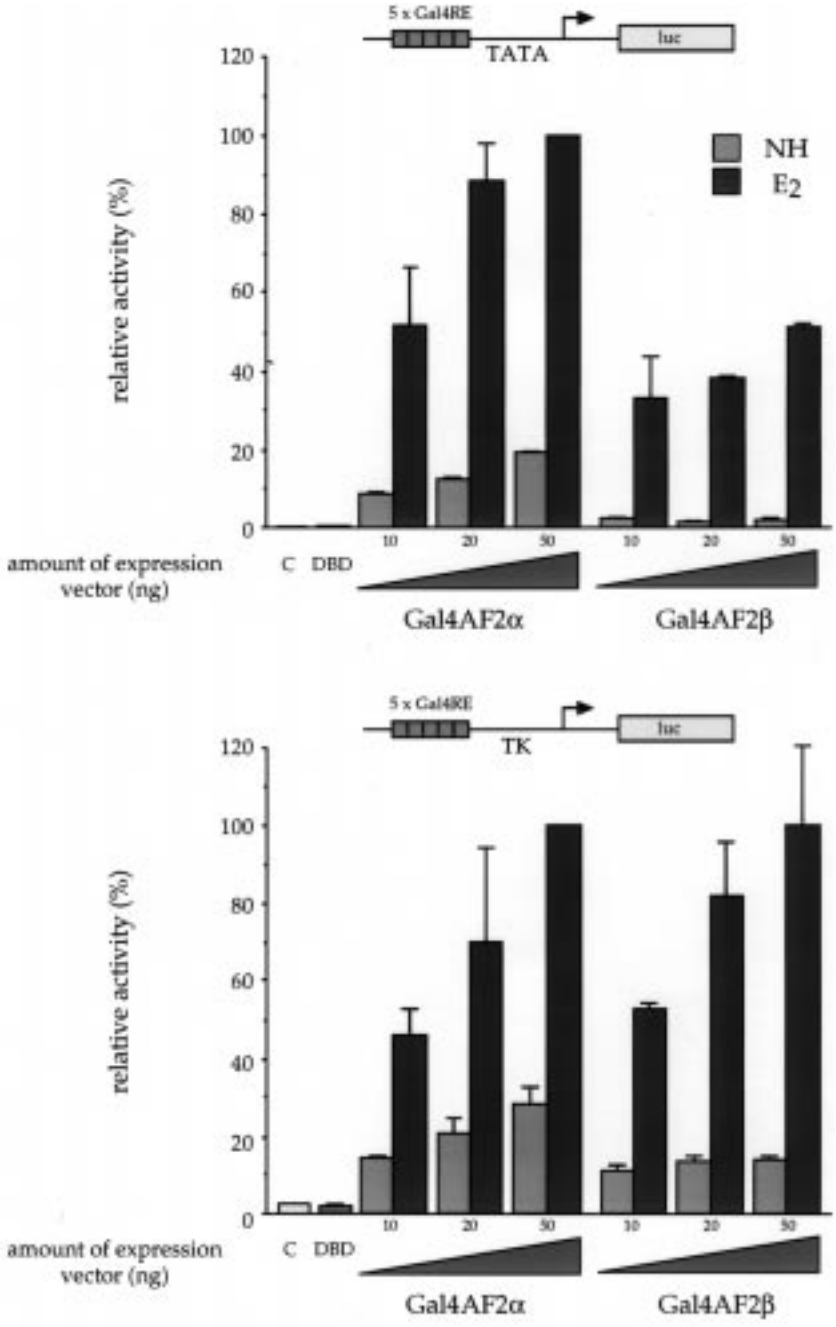

Fig. 3. ERα and ERβ have similar AF2 activities. AF2 activity was analysed by testing the ability of Gal4-AF2α or Gal4-AF2β to stimulate transcription from either 5Gal4RE-TATA-luc or 5Gal4RE-TK-luc as indicated in HeLa cells. Cells were treated for 24 h with or without 1×10^{-8} M 17β-estradiol, harvested and the extracts tested for reporter activity. Values for reporter activity were corrected for transfection efficiency using the internal control. Background activity (without transfected receptor) is denoted by C and error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

Galacto-Light chemiluminescent assay (Tropix). β-Galactosidase activity was used to correct for differences in transfection efficiency.

3. Results

We have compared the ability of ERα and ERβ to stimulate the transcriptional activity of a number of reporter genes containing consensus oestrogen response elements (ERE) initially in chicken embryo fibroblasts. ERα and ERβ stimulated transcription from reporters containing complex promoters such as ERE-TK-luc to a similar extent, but on reporters with simple promoters such as 3ERE-TATA-luc and 2ERE-PS2-CAT, ERα had much more activity than ERβ

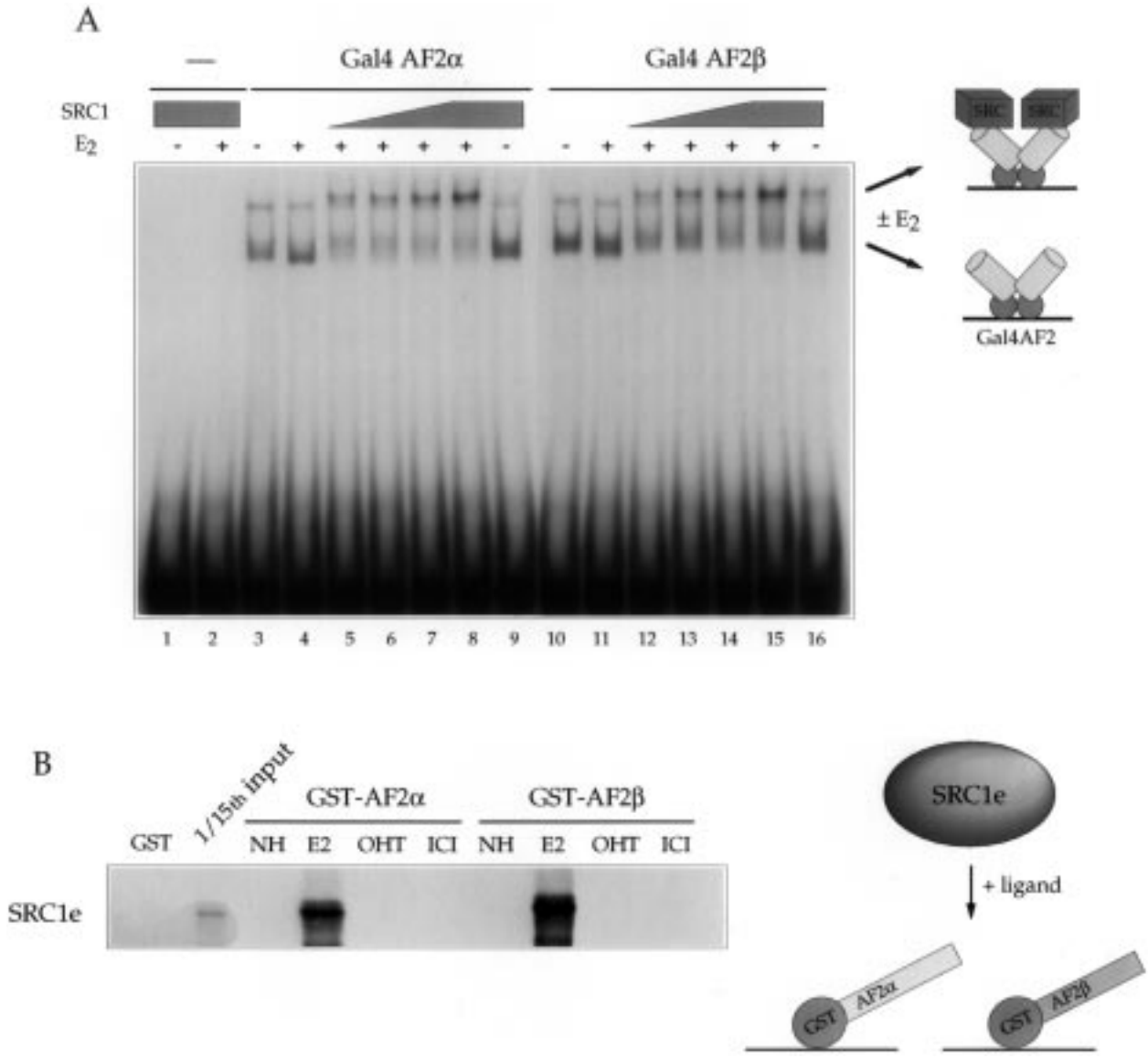

Fig. 4. The ligand binding domains of ERα and ERβ bind SRC1 similarly. (A) COS cell extracts expressing either Gal4AF2α or Gal4AF2β were prebound to a [^{32}P]-labelled Gal4RE and incubated with increasing amounts of GST-SRC1(570–780) in the presence or absence of 2.5×10^{-7} M 17β-estradiol as indicated. The predicted positions of Gal4AF2-DNA and SRC1 bound complexes are shown on the right hand side. (B) Binding of [^{35}S]-methionine labelled SRC1 with GST, GST-AF2α or GST-AF2β was analysed in the presence or absence of 1×10^{-6} M 17β-estradiol, 1×10^{-6} M 4OH-Tamoxifen or 1×10^{-6} M ICI 182780 as indicated. Bound SRC1 was eluted and analysed by SDS-PAGE followed by autoradiography.

(Fig. 1A). We then analysed the response of ERE-TK-luc in other cell types and found that the relative ERβ activity was similar in Hela cells but only 20% in COS-1 cells and negligible in HepG2 cells (Fig. 1B). Thus, the transcriptional activity of ERα is greater than that of ERβ on ERE-containing reporter genes, the extent of which varies in different cell types.

Previous work demonstrated that the affinity of ERα for a consensus ERE was slightly greater than an ERβ [3] but this difference is unlikely to account for the 10-fold variations in relative ERα/ERβ activity observed with certain promoters and cell types. An alternative explanation is that the relative activities of AF1 and AF2 in ERα and ERβ differ. Previous work has shown that their activities in ERα vary depending on the responsive promoter and cell type [5]. Deletion of AF1 from ERα almost completely abolished transcription from the 2ERE-pS2-CAT reporter gene (Fig. 2A) which contrasts with the observation that it is not required for transcription from ERE-TK-luc (data not shown, [11]). We also tested the effect of replacing Ser 122 with Ala, given that this residue can be phosphorylated by the MAP kinase pathway in response to growth factors, but transcription from this reporter

was essentially unchanged. Thus, AF1 is required to stimulate transcription from 2ERE-pS2-CAT irrespective of whether it is phosphorylated.

To analyse the relative AF1 activities in ERα and ERβ directly we fused their respective N-terminal domains with the DNA binding domain of Gal4. Increasing concentrations of Gal4-AF1α and Gal4-AF1β were tested for their ability to stimulate transcription from the Gal4 reporter gene, 5GalRE-TK-luc. The relative activity of AF1β was negligible compared with that of AF1α irrespective of the concentration of expression vector used (Fig. 2B). Thus ERβ appears to lack AF1 activity found in ERα. Therefore the meagre ERβ activity observed using the 2ERE-pS2-CAT reporter gene may reflect the lack of an AF1 activity similar to that found in ERα. To compare AF2 activities in ERα and ERβ we fused their ligand binding domains with the DNA binding domain of Gal4 to generate Gal4AF2α and Gal4AF2β fusion proteins. We tested their ability to stimulate transcription from two Gal4 reporter genes, one containing a simple TATA promoter, 5Gal4RE-TATA-luc, and one containing the TK promoter, 5Gal4RE-TK-luc, in Hela cells. Gal4AF2α stimulated transcription from 5Gal4RE-TATA-luc about 2-fold more than Gal4AF2β (Fig. 3A) while their ability to stimulate 5Gal4RE-TK-luc transcription was similar (Fig. 3B). Thus, when both AF1 and AF2 are required for optimum receptor activity we would predict that ERβ would be much less active than ERα in view of the

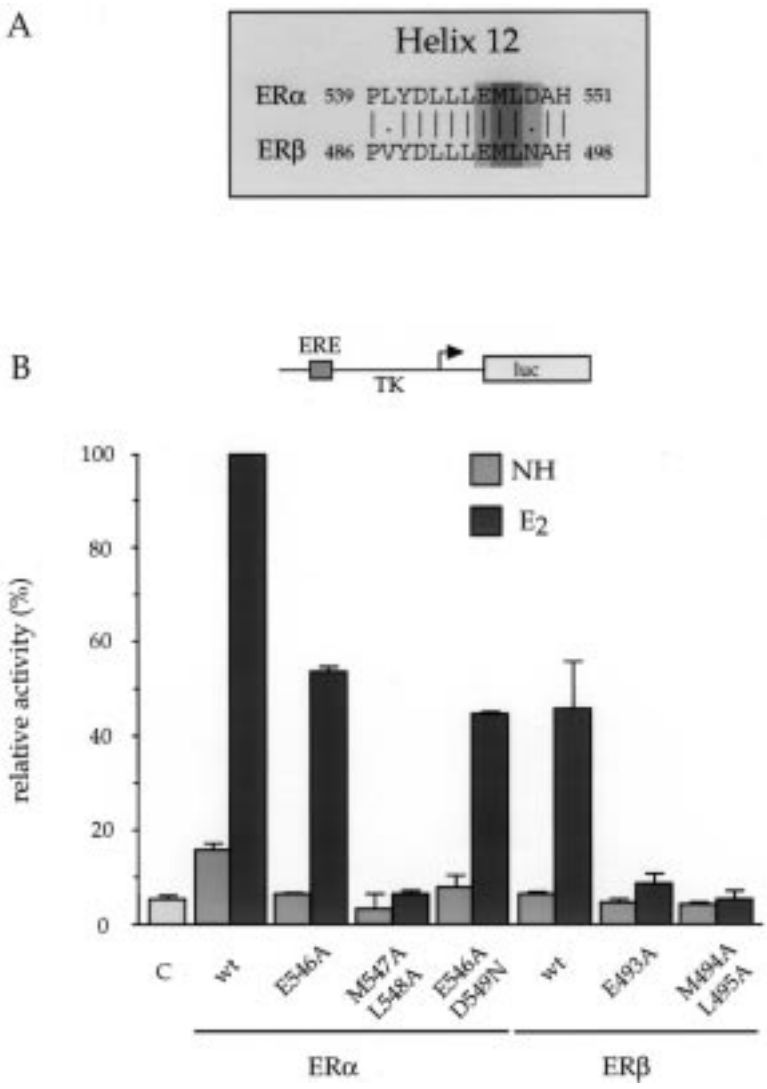

Fig. 5. Mutations in helix 12 of ERα and ERβ differentially effect their ability to activate transcription. (A) sequence comparison of helix 12 from ERα and ERβ, mutated residues are shaded. HeLa cells were transiently transfected with ERα, ERβ or mutant versions thereof (B) or with Gal4-AF2 wild-type or mutant versions thereof (C). Cells were treated as shown in Fig. 1 and after correction for transfection efficiency using the internal control relative reporter activities are presented. Background activity (without transfected receptor) is denoted by C and error bars represent the standard deviation of values from at least two separate experiments performed in duplicate.

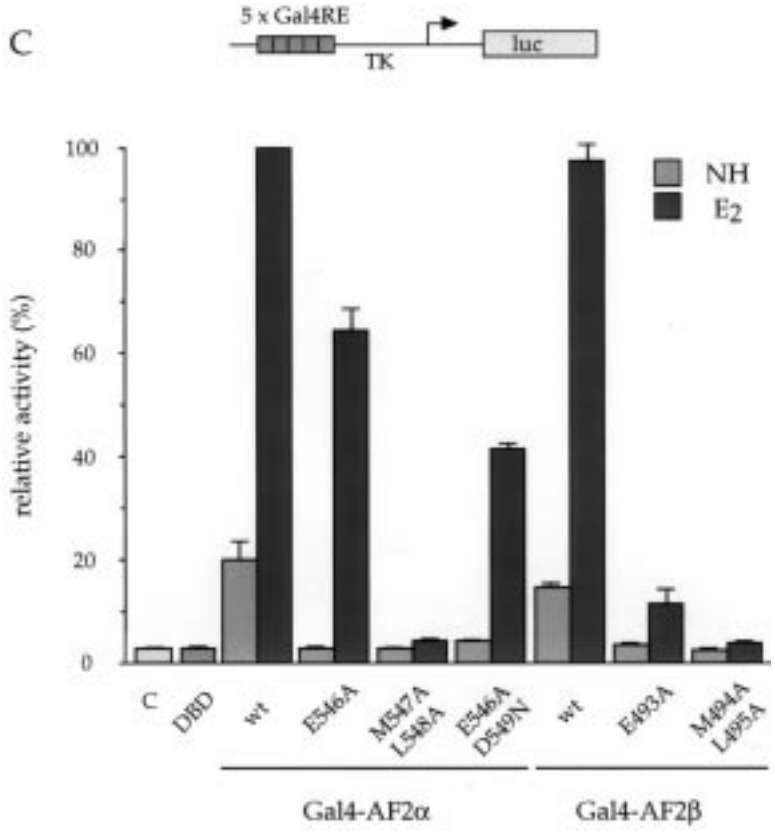

Fig. 5 (*continued*)

difference in their AF1 activities, but when AF1 is not required we predict that the activities of the two receptors would be similar. This is essentially what we observed in Fig 1. Thus on promoters where AF1 is not required, such as ERE-TK-luc [11], ERα and ERβ have similar activities whereas on TATA- and pS2 based promoters, which depend on both AF1 and AF2 [5], ERα is much more active than ERβ.

Given that AF2α and AF2β have similar transcriptional activities we investigated whether they also interacted similarly with the coactivator SRC1. The ability of the receptor interacting domain of SRC1 to interact with AF2α and AF2β was analysed when the receptors were prebound to DNA. Gal4AF2α and Gal4AF2β, expressed in COS 1 cells, were bound to a [^{32}P]-labelled consensus Gal4RE and analysed by gel shift analysis. The amounts of the two complexes were similar and their mobility, as expected, was slightly increased in the presence of oestrogen (Fig. 4A tracks 4 and 11). When the two chimaeric receptors were mixed with increasing amounts of GST-SRC1(570–780) we detected a similar dose-dependent increase in ternary complex formation for both AF2α and AF2β. Similar results were obtained when we tested the ability of GST-AF2α and GST-AF2β fusion proteins to bind full-length SRC1e (Fig 4B), SRC1a and TIF2 (data not shown). The interaction was dependent on the addition of estradiol and there was no interaction in the presence of the antiestrogens 4-hydroxytamoxifen or ICI 182780. Thus the interaction between SRC1 isoforms or TIF2 with ERα and ERβ is similar.

Given the degree of sequence conservation between the ligand binding domains of ERα and ERβ and their similar coactivator binding properties we presumed that a similar surface of the two receptors would be involved in the recruitment of coactivators. To investigate this, we analysed the effect of introducing a number of mutations in helix 12, which is required for oestrogen dependent transactivation by ERα [11]. We introduced mutations into both the full-length receptor and a Gal-AF2 chimaeric receptor and found that mutation of the hydrophobic residues Met 547/Leu 548 in ERα and the corresponding residues Met 494/Leu 495 in ERβ essentially abolished oestrogen dependent transactivation (Fig. 5B, C), consistent with our assumption. However, the effect of replacing the highly conserved Glu residue with Ala was much more marked in ERβ than in ERα. Thus the activity of E546A, in both full-length ERα and Gal-AF2α was 50–60% that of the wild-type receptor whereas the activity of E493A in ERβ and Gal-AF2β was markedly reduced (Fig. 5B, C). One possible explanation for this

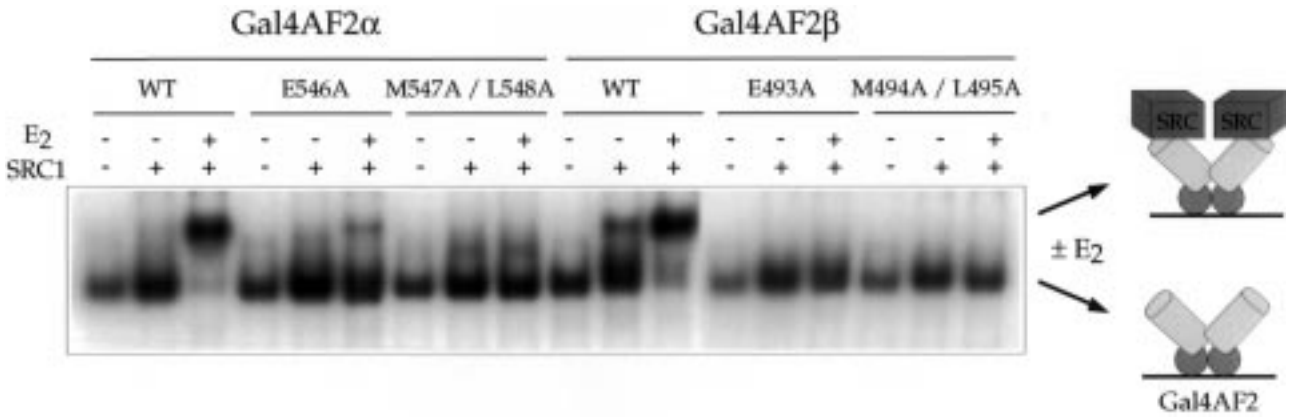

Fig. 6. Mutations in helix 12 of ERα and ERβ differentially effect their ability to bind SRC1. COS cell extracts, expressing either Gal4AF2α or Gal4AF2β, or mutant versions thereof, were prebound to a consensus Gal4RE and incubated with GST-SRC1(570–780) in the presence or absence of 2.5×10^{-7} M 17β-estradiol. The predicted position of DNA-bound complexes is shown on the right hand side.

observation is that ERα contains three acidic residues, whilst ERβ contains only two, Asp 549 in ERα is an Asn in ERβ (Fig. 5A). However, the ERα double mutant E546A/D549N had a similar activity to ERα E546A (Fig. 5B, C), it is therefore unlikely that the lack of the third acidic residue in helix 12 of ERβ is responsible for the greater deleterious effect of ERβ E493A compared to ERα E546A. We conclude that the surface required to interact with coactivators may be similar but is not identical in the two receptors.

Finally we investigated whether the requirement for a Glu residue in helix 12 of ERβ but not ERα could be explained by differences in coactivator binding. We examined this by performing the type of experiment described in Fig. 4A, in which we tested the ability of GST-SRC1(570–780) to interact with mutant Gal4AF2α and Gal4AF2β prebound to DNA. As previously observed, SRC1 bound to the wild-type α and β receptors in an oestrogen dependent manner but not with the hydrophobic mutants (Fig 6). The effect of replacing the conserved Glu residue was different, however, since SRC1 was able to interact with E546A in GalAF2α, albeit less well than with the wild-type receptor but not with E493A in GalAF2β. Thus the Glu residue seems to play a crucial role for coactivator recruitment by ERβ but not ERα.

4. Discussion

Previous work has established that the activity of AF1 and AF2 in ERα varies depending on the target gene and cell type [5,28]. Transcription from certain promoters, such as the pS2 promoter depends on both activation domains whereas on others, such as the vitellogenin or C3 promoters, AF1 or AF2 was sufficient. In the latter case, the ability of AF1 and AF2 to function independently was cell type dependent. Differential transcriptional activity from ERα and ERβ has been observed on AP1 response elements [42], suggesting a difference in their respective transcriptional activation domains. In this study we demonstrate that the intrinsic activity of AF1 in ERβ, fused to the Gal4 DNA binding domain, is negligible compared with that in ERα. Since AF1 is required for ERα to stimulate transcription from some but not all promoters, we would expect ERβ to stimulate transcription from promoters where AF1 was not required but not from those where it is essential. This is precisely what we observed, namely, transcription from ERE-TK-luc is stimulated similarly by both ERα and ERβ whereas ERα is much more active than ERβ on TATA- and pS2 based promoters, which require both AF1 and AF2 [5], As a consequence, we propose that when AF1 is required for transcription from ERE-dependent promoters ERβ will be a poor transcriptional activator. The absence of an AF1 activity can also explain the lack of agonist activity exhibited by oestrogen antagonists, such as 4-hydroxytamoxifen [21], which is mediated by AF1 in ERα [29,30].

While AF1 is less well defined than AF2, analysis of ERα suggests that it comprises at least three distinct features. Firstly, a region between residues 41 and 150 is required for AF1 activity [29,31,32]. Secondly, a region between residues 91 and 121 are required for synergy with the ligand binding domain to generate optimum transcriptional activity [11] that may reflect their interaction [33]. Thirdly, AF1 is a target for phosphorylation by the MAPK pathway in response to growth factors [7,8,34]. Although ERβ lacks a functional AF1 similar to that found in ERα, consistent with the lack of sequence homology in this region of the two receptors it does, however, contain a MAPK phosphorylation site. That this is functional is supported by the observation that expression of H-Ras^{V12} potentiates the transcriptional activity of ERβ and this

effect is abolished by mutation of the site [21,35]. Whether there is any synergy or functional interaction between the N-terminal domain and the ligand binding domain in ERβ remains to be determined.

The transcriptional activity of AF2 in ERα and ERβ, tested in the context of a heterologous DNA binding domain, and their ability to bind the coactivator SRC1 are very similar. This is consistent with the observation that residues required for AF2 in ERα, namely, a lys residue in helix 3 [36] and hydrophobic residues in helices 3, 5 and 12 ([11] and Ho Yi Mak, data not shown) are conserved in ERβ. It seems likely that these residues are induced to form a surface upon ligand binding [13] which contacts the conserved LXXLL helices that mediate the binding of coactivators to nuclear receptors [18,37]. One potential difference in the surface of the two receptors is the contribution of the highly conserved Glu residue in helix 12. It seems to play a more important role in coactivator binding and AF2 activity in ERβ than in ERα, which also seems to be the case in other nuclear receptors [38–41].

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