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Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor

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Steroid receptor co-activator (SRC1) is one of a number of transcriptional co-activators that are capable of potentiating the activity of nuclear receptors including the oestrogen receptor (ER). Here we report that two isoforms, SRC1a and SRC1e, which diverge at their C-termini, are functionally distinct as they differ in their abilities to enhance the activity of the ER in intact cells. SRC1e enhanced the ability of the ER to stimulate transcription to a greater extent than SRC1a, which had negligible effects on certain promoters. To elucidate the basis of this functional difference, we compared the nuclear receptor-binding properties and mapped the transcriptional activation domains in the two SRC1 isoforms. Both isoforms share a triplet of nuclear receptor-binding motifs (LXXLL motifs) for binding to functional ER dimers, and an activation domain which co-localizes with the CBP-binding domain, while SRC1a contains a unique LXXLL motif in its C-terminus. Although this LXXLL motif increases the affinity for the ER *in vitro*, it does not appear to be responsible for the functional difference between the two isoforms. This difference is due to a second activation domain that is CBP independent and is suppressed in the SRC1a isoform. Thus, SRC1 exists as functionally distinct isoforms which are likely to play different roles in ER-mediated transcription.
Keywords: activation function/co-activator/oestrogen receptor/SRC1

Introduction

The oestrogen receptor (ER) is a member of the nuclear receptor (NR) family of transcription factors that includes receptors for various steroid hormones, retinoids and thyroid hormone (Parker, 1993; Beato *et al.*, 1995; Mangelsdorf *et al.*, 1995; Chambon, 1996). Two transactivation functions (AFs) have been characterized in the ER (Lees *et al.*, 1989; Tora *et al.*, 1989), AF1 in the N-terminal domain, whose activity is regulated by phosphorylation in response to growth factors (Kato *et al.*, 1995; Bunone *et al.*, 1996), and AF2 in the hormone-binding domain (HBD), whose activity is strictly dependent on the presence of ligand. These two activation functions may function independently or synergistically, depending on the cell type and target promoter used (Lees *et al.*, 1989; Tora *et al.*, 1989).

The recent elucidation of the crystal structure of the HBDs of the unliganded retinoic X receptor α (RXRα; Bourguet *et al.*, 1995) and the liganded retinoic acid receptor γ (RARγ; Renaud *et al.*, 1995), thyroid hormone receptor β (TRβ; Wagner *et al.*, 1995) and ER (Brzozowski *et al.*, 1997) has suggested that binding of ligand results in a conformational change of the HBD. This results in the realignment of an amphipathic α-helix, helix 12 (H12), across the ligand-binding pocket which, in the case of the ER, is packed against helices 3, 5/6 and 11 (Brzozowski *et al.*, 1997). The amphipathic character of H12, which is conserved in most NRs, is essential for the function of AF2, since mutations in this helix abolish the transcriptional activity (Danielian *et al.*, 1992; Saatcioglu *et al.*, 1993; Barettino *et al.*, 1994; Durand *et al.*, 1994). The importance of H12 in transcriptional activation by the ER is supported by the observation that it is misaligned in the presence of the oestrogen antagonist raloxifen (Brzozowski *et al.*, 1997).

In common with other transcription factors, the ER stimulates transcription by recruiting a pre-initiation complex. Although direct interactions of the ER with the basal transcription factors TFIIB (Sadovsky *et al.*, 1995) and TATA-binding protein (TBP; Ing *et al.*, 1992) and with a TBP-associated factor (TAF), $TAF_{II}30$ (Jacq *et al.*, 1994), have been demonstrated *in vitro*, these interactions were ligand independent and they were unaffected by mutations in H12 that abolished AF2 activity. In addition, AF2 activity is inhibited by overexpression of receptors, suggesting that other limiting factors are required for hormone-activated transcription (Tasset *et al.*, 1990). A number of candidate proteins have been identified (Cavaillès *et al.*, 1994; Halachmi *et al.*, 1994) and isolated including RIP140 (Cavaillès *et al.*, 1995), TIF1 (Le Douarin *et al.*, 1995), Trip1/SUG1 (Lee *et al.*, 1995; vom Baur *et al.*, 1996), ARA70 (Yeh and Chang, 1996), Trip230 (Chang *et al.*, 1997) three related proteins of 160 kDa (p160) named SRC1 (Oñate *et al.*, 1995; Kamei *et al.*, 1996), TIF2/GRIP1 (Voegel *et al.*, 1996; Hong *et al.*, 1997) and AIB1/ACTR/RAC3/p/CIP (Anzick *et al.*, 1997; Chen *et al.*, 1997; Li *et al.*, 1997; Torchia *et al.*, 1997), and CBP/p300 (Chakravarti *et al.*, 1996; Hanstein *et al.*, 1996; Kamei *et al.*, 1996). The recruitment of these proteins to activated receptors is mediated by α-helical LXXLL motifs (in which L denotes leucine and X denotes any amino acid) which appear to be both necessary and sufficient for the interaction (Heery *et al.*, 1997; Torchia *et al.*, 1997). While the function of some of these proteins remains to be established, the p160 proteins have been shown to potentiate the transcriptional activity of several NRs in transiently transfected cells (Oñate *et al.*, 1995; McInerney *et al.*, 1996; Smith *et al.*, 1996; Voegel *et al.*, 1996; Anzick *et al.*, 1997; Chen *et al.*, 1997; Heery *et al.*, 1997; Hong *et al.*, 1997; Li *et al.*, 1997; Torchia *et al.*, 1997),

and are considered *bona fide* co-activators of NR-mediated transcription. Further evidence for the co-activator function of SRC1 is provided by the observation that a dominant-negative form of SRC1 (Oñate *et al.*, 1995) or micro-injected antibodies against SRC1 (Torchia *et al.*, 1997) block NR function. Similarly, CBP/p300 have also been found to potentiate transcriptional activation by NRs, and microinjection of specific antibodies suppresses ligand-dependent transactivation in intact cells (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996). In addition to their interaction with NRs, SRC1 and CBP/p300 also associate with each other (Hanstein *et al.*, 1996; Kamei *et al.*, 1996; Yao *et al.*, 1996), and SRC1 has been shown to function synergistically with CBP/p300 to enhance NR-mediated transcription (Smith *et al.*, 1996).

It has been shown recently that the two p160 proteins SRC1 and TIF2 are functionally distinct from a third family member p/CIP (Torchia *et al.*, 1997). Here we describe the analysis of two isoforms of human SRC1 and demonstrate that they are also functionally distinct in that they differ in their binding to the ER *in vitro* and, more importantly, in their ability to potentiate transcription by the ER. We therefore propose that the two isoforms may have distinct roles in ER-mediated transcription.

Results

Two major isoforms of SRC1/p160 are widely expressed in different cell lines

In the course of screening cDNA libraries for full-length clones encoding the human SRC1/RIP160 protein, we isolated two groups of clones with homology to the mouse SRC1a and SRC1e clones (Kamei *et al.*, 1996). The two human isoforms are identical up to residue 1385 but differ at their C-termini (Figure 1A) in that SRC1a (1441 amino acids, predicted mol. wt 156.7 kDa) contains 56 unique residues and lacks 14 of the most C-terminal amino acids present in SRC1e (1399 amino acids, predicted mol. wt 152.3 kDa). No clones were isolated that correspond to the N-terminal variant SRC1b or the C-terminal variants SRC1c and SRC1d (Kamei *et al.*, 1996). Both isoforms, overexpressed in COS-1 cells, were shown by far-Western blotting with AF2 of the ER to co-migrate with endogenous receptor-interacting proteins of ~160 kDa (Cavaillès *et al.*, 1994; Halachmi *et al.*, 1994) (Figure 1B). All cell lines examined here (ZR75-1 breast cancer cells, HeLa cells and COS-1 cells) expressed p160 proteins but their relative proportions differ (Figure 1B).

The relative expression of the two SRC1 isoforms was studied by RNase protection analysis in ER-positive (ZR75–1, T47D, MCF7) and ER-negative (BT20, MDA-MB231, MDA-MB468) breast cancer cells, cells derived from endometrium (Ishikawa), placenta (JAR), ovary (JAMA2), cervix (HeLa), kidney (293) and liver (Hep G2), as well as fibroblasts (Butler) and T (Jurkat) and B cells (Bristol-8). A probe was used which hybridizes with both SRC1a and SRC1e transcripts, but results in protected fragments of different size (Figure 1C). Quantification of the protected fragments revealed that the ratio between the mRNAs encoding the two SRC1 isoforms varied between cell lines, but both were detected in all cell lines tested (Figure 1C).

For chromosomal localization of SRC1, metaphase spreads were analysed by fluorescence *in situ* hybridization. The SRC1 probe was found to be localized to chromosome band 2p23 (Figure 1D). The gene encoding the related protein TIF2 was localized to chromosome band 8q21.1 (data not shown). No other consistent signal was observed on any other chromosome for either probe.

SRC1 isoforms differ in their binding to AF2 of the oestrogen receptor

To test the SRC1 proteins for their ability to bind the ER, a GST fusion containing AF2 of the ER was incubated with *in vitro* translated [^{35}S]methionine-labelled SRC1 in the absence or presence of 17β-oestradiol (E2) or the anti-oestrogens 4′-hydroxytamoxifen or ICI 182,780. As shown in Figure 2A, both SRC1 isoforms bound to AF2 of the ER in an E2-dependent fashion, while no binding was detected in the absence of ligand or in the presence of the anti-oestrogens. Interaction of SRC1 proteins with three other members of the NR superfamily (human RARβ, TRβ and RXRα) was equally dependent on the presence of ligand (data not shown). In addition, the SRC1 proteins failed to interact with AF1 of the ER or with the basal transcription factors TBP or TFIIB. Next, we tested the SRC1 proteins for their ability to bind to the ER in the presence of DNA. For this, full-length ER expressed in insect cells was pre-bound to an oestrogen response element (ERE) and incubated with ^{35}S-labelled SRC1 proteins in the absence or presence of E2 or anti-oestrogens. Again, the interaction of SRC1 was induced by the presence of E2 and not by anti-oestrogens, but in this assay some binding was detected in the absence of ligand, most clearly in the case of SRC1a (Figure 2B).

To map sequences in SRC1 responsible for ER binding, a series of GST fusion proteins was generated containing non-overlapping regions of the two SRC1 proteins (Figure 2C). These proteins were incubated with ^{35}S-labelled ER in the absence and presence of ligand. Two regions bound to the ER in a ligand-dependent fashion, 570–780 and the SRC1a-specific region 1241–1441, while no binding of these constructs to the transcriptionally inactive L543A/L544A ER mutant could be detected (Figure 2D). Recently, we have identified a short sequence motif in several co-activators that is sufficient for ligand-dependent binding to NRs (Heery *et al.*, 1997). The GST–SRC1 (570–780) construct contains three copies of the LXXLL motif (motifs 1–3), while the SRC1a-specific fusion protein (1241–1441) contains a single copy (motif 4). The C-terminus of SRC1e (1241–1399), which lacks an LXXLL motif, failed to bind the ER, as did the C-terminus of SRC1a in which the leucine doublet of motif 4 was replaced by alanines [(1241–1441)M4]. Two other regions of SRC1 (199–569 and 989–1240), which lack functional LXXLL motifs, were also capable of binding the ER, but the significance of these interactions is unclear, since they bound equally well to the transcriptionally inactive L543A/L544A mutant (see below).

To assess whether the presence of a C-terminal LXXLL motif affected the stability of SRC1a binding to NRs, the two ^{35}S-labelled SRC1 proteins were compared for their binding to AF2 of the ER over a range of salt concentrations. While the binding of SRC1a to the ER was unaffected by increasing the salt concentration from 50 mM to 1.0 M NaCl, binding of SRC1e was reduced when

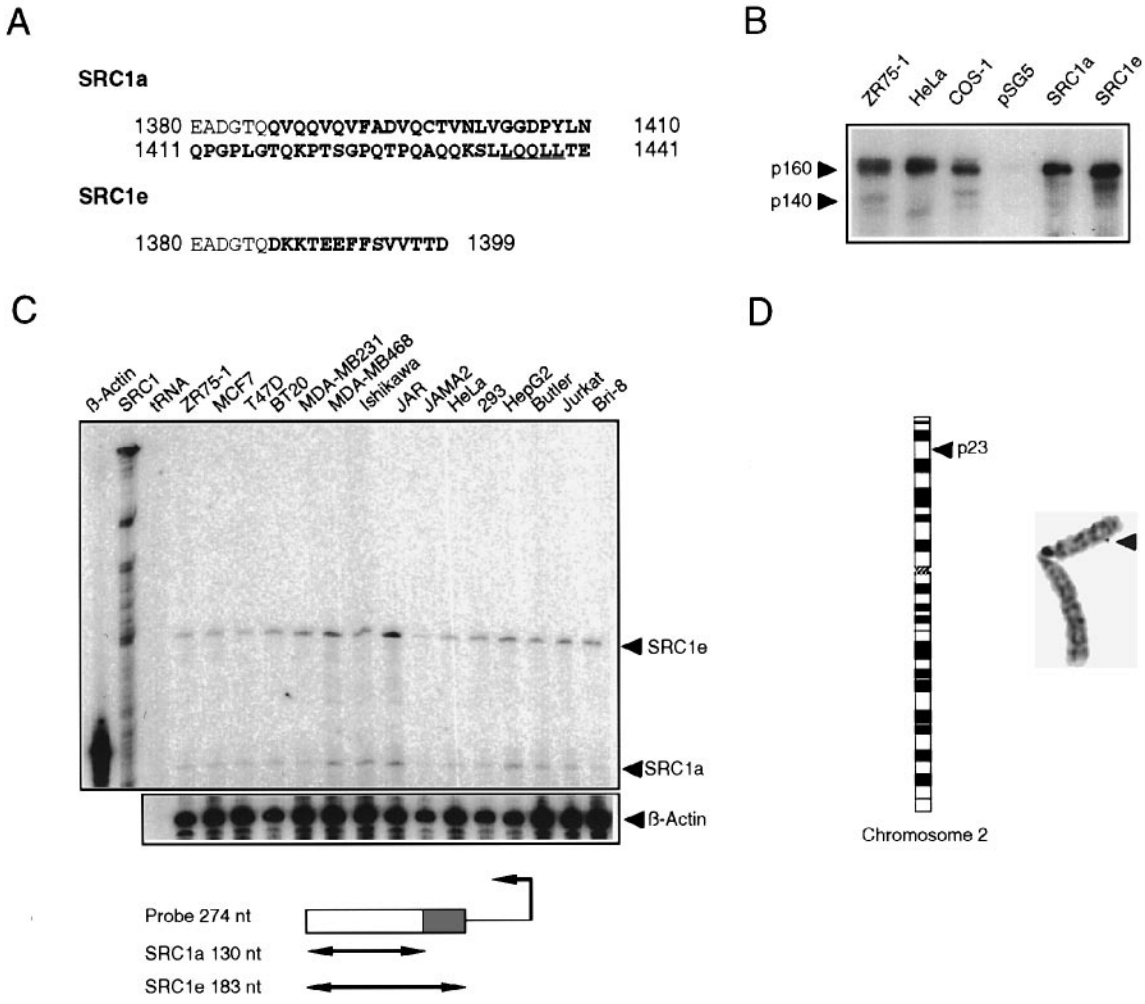

Fig. 1. SRC1/p160 isoforms are widely expressed in human cells. (**A**) Amino acid sequences of the C-termini of the two isoforms of human SRC1. Isoform-specific amino acids are printed in bold, the LXXLL motif in SRC1a is underlined. Numbers refer to amino acids. (**B**) Far-Western blot analysis of the two SRC1 isoforms. Whole cell extracts of ZR75-1, HeLa, COS-1 cells (50 μg of protein) and COS-1 cells overexpressing SRC1a or SRC1e or the empty expression vector (pSG5; 5 μg of protein) were separated by 6% SDS–PAGE and transferred onto filters. After denaturation–renaturation, filters were probed with ^{32}P-labelled GST–AF2 of the ER in the presence of E2 (10^{-6} M), washed and exposed for autoradiography. Arrowheads indicate the positions of the endogenous receptor-interacting proteins of 140 and 160 kD. (**C**) Analysis of SRC1a and SRC1e mRNA expression in human cell lines by RNase protection. Protected fragments resulting from the hybridization of tRNA or total RNA (all 10 μg) with the SRC1 and β-actin probes after digestion with RNase A are indicated on the right, with the undigested probes for SRC1 and β-actin in the first two lanes. The average ratio between SRC1a and SRC1e mRNA from two independent experiments was as follows: ZR75-1, 0.56; MCF7, 1.0; T47D, 0.70; BT20, 1.32; MDA-MB231, 2.0; MDA-MB468, 1.3; Ishikawa, 0.9; JAR, 1.7; JAMA2, 0.6; HeLa, 1.0; 293, 1.56; Hep G2, 0.96; Butler, 0.84; Jurkat, 1.46; Bri-8, 2.7. A schematic representation of the SRC1 probe and the protected fragments is given below. The grey box represents the part of the probe that is specific for the SRC1e isoform. (**D**) Chromosomal localization of the human SRC1 gene. The arrowhead indicates the position of the SRC1 gene on human chromosome 2p23 as detected by fluorescence *in situ* hybridization.

concentrations of 500 mM and higher were used (Figure 2E). An SRC1a protein in which motif 4 was inactivated (SRC1aM4) showed a binding pattern similar to SRC1e (data not shown), indicating that the C-terminal binding domain in SRC1a is responsible for the increased stability of ER binding when compared with SRC1e.

To determine the relative importance of the three LXXLL motifs common to both SRC1 isoforms (Figure 2C), the motifs were systematically mutated in the full-length protein and the mutant proteins were tested for both their interaction with the ER in GST pull-down experiments and for their effects on ER transactivation in COS-1 cells. The loss of individual motifs had very little effect on the ability of SRC1e to bind to the ER or potentiate its transcriptional activity, although there was a slight reduction in both when motif 2 (M2) was mutated (Figure 3). The effects of mutating motifs 1 and 2 (M12) or 2 and 3 (M23) together were more dramatic and resulted in negligible binding *in vitro* and reduced ability to enhance ER-mediated transcription in transfected cells. However, the combined mutation of motifs 1 and 3 (M13) was less marked (Figure 3). Thus M13 retained partial ER binding in GST pull-down experiments and this was reflected in its ability to potentiate the transcriptional activity of the ER to a greater extent than either M12 or M23. Motif 2 therefore appears to be the preferred site of interaction but both motif 1 and 3 may contribute to optimal binding and activation in intact cells. Finally, mutation of all three motifs completely abolished both the *in vitro* interaction between SRC1e and the ER and its ability to potentiate ER transactivation, indicating that the interactions between the ER and the two fragments of

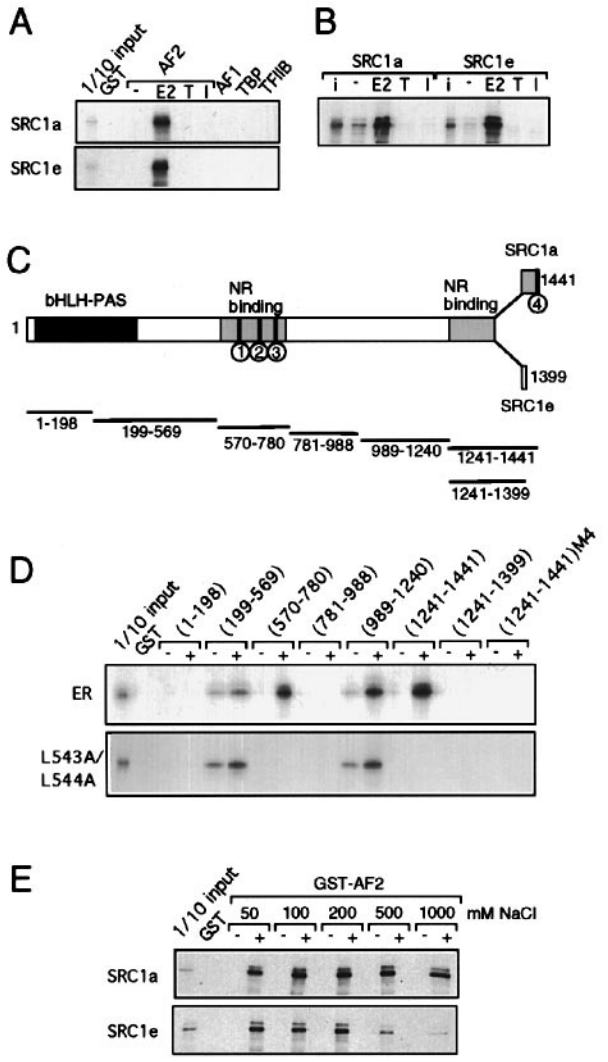

Fig. 2. SRC1 isoforms differ in their binding to AF2 of the ER. (**A**) Binding of GST fusion proteins of AF2 or AF1 of the ER, TBP and TFIIB or GST alone to SRC1 isoforms. GST fusion proteins which previously had been coupled to Sepharose beads were incubated with *in vitro* translated, [^{35}S]methionine-labelled SRC1 proteins, in the absence or presence of 17β-oestradiol (E2), or the anti-oestrogens 4′-hydroxytamoxifen (T) or ICI 182,780 (I; all 10^{-6} M) as indicated. After extensive washing, samples were boiled and separated on 8 or 10% SDS–polyacrylamide gels. Gels were fixed and dried and the labelled proteins were detected by fluorography. The input lane represents 10% of the total volume of the lysate used in each reaction. (**B**) Binding of SRC1 isoforms to DNA-bound ER. Full-length ER expressed in insect cells which had been pre-bound to a consensus ERE was incubated with ^{35}S-labelled SRC1 proteins, in the absence or presence of E2 or anti-oestrogens (T and I, as in A) as indicated. Bound proteins were visualized as in (A). The input lane, representing 10% of the total volume of the lysate used in each reaction, is indicated with i. (**C**) Schematic representation of SRC1 isoforms and the regions that were fused to GST. Numbers refer to amino acids. Indicated are the bHLH-PAS homology region, the binding regions for NRs and the functional α-helical LXXLL motifs (numbered 1–4). The fusion proteins (1241–1441) and (1241–1399) contain amino acid sequences unique to the SRC1a and SRC1e proteins, respectively. (**D**) Binding of GST–SRC1 fusions to the ER. GST fusion proteins were incubated with ^{35}S-labelled wild-type ER or the transcriptionally inactive L543A/L544A mutant in the absence (–) or presence (+) of E2 (10^{-6} M) as described in (A). The fusion protein (1241–1441)M4 contains a mutation of LXXLL motif 4. (**E**) Effect of increasing salt concentration on the interaction between SRC1 isoforms and the ER. The stability of interaction of SRC1a and SRC1e with GST–AF2 of the ER was tested with a range of NaCl concentrations (50 mM–1.0 M) as described in (A).

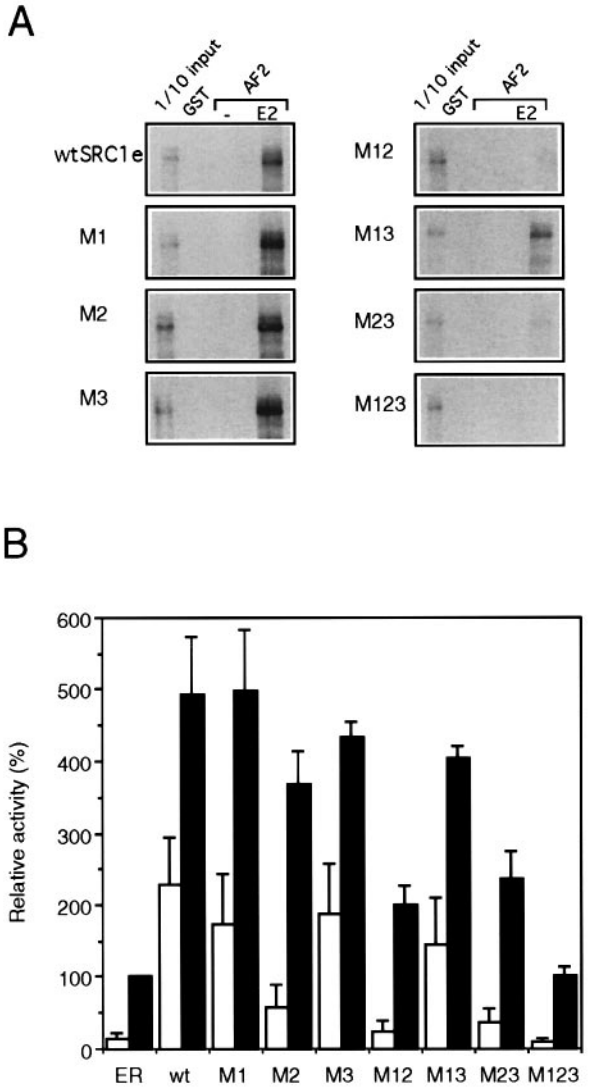

Fig. 3. SRC1 interacts with the ER through LXXLL motifs. (**A**) Binding of wild-type or mutant forms of SRC1e to the ER. ^{35}S-Labelled SRC1e or mutant forms of SRC1e in which the leucine doublet in one or more of its LXXLL motifs (Figure 2C) was mutated to alanines, were incubated with GST fusions of AF2 of the ER, in the absence or presence of E2 (10^{-6} M). Bound proteins were visualized as described in Figure 2A. (**B**) COS-1 cells were transiently transfected with expression vector for the ER, a 3×ERE-TATA-Luc reporter and 100 ng of wild-type or mutant forms of SRC1e. After transfection, cells were washed and incubated with vehicle (white bars) or E2 (10^{-8} M; black bars) for 24 h. Subsequently, cells were assayed for luciferase and β-galactosidase activity. Normalized values are expressed as percentage activity compared with ER alone in the presence of E2 (100%). The results shown represent the average of a minimum of three independent experiments assayed in duplicate ±SEM.

SRC1 which lacked LXXLL motifs (amino acids 199–569 and 989–1240; Figure 2D) do not occur in the full-length SRC1 protein in intact cells. As described before, SRC1 proteins can be tested for their binding to GST–CBP (Heery *et al.*, 1997), and this interaction was found to be unaltered in all mutants compared with wild-type SRC1e (data not shown).

Taken together, these data show that the binding of both SRC1a and SRC1e to the HBD of the ER depends on the presence of ligand. The SRC1 isoforms differ, however, in their *in vitro* binding characteristics, which

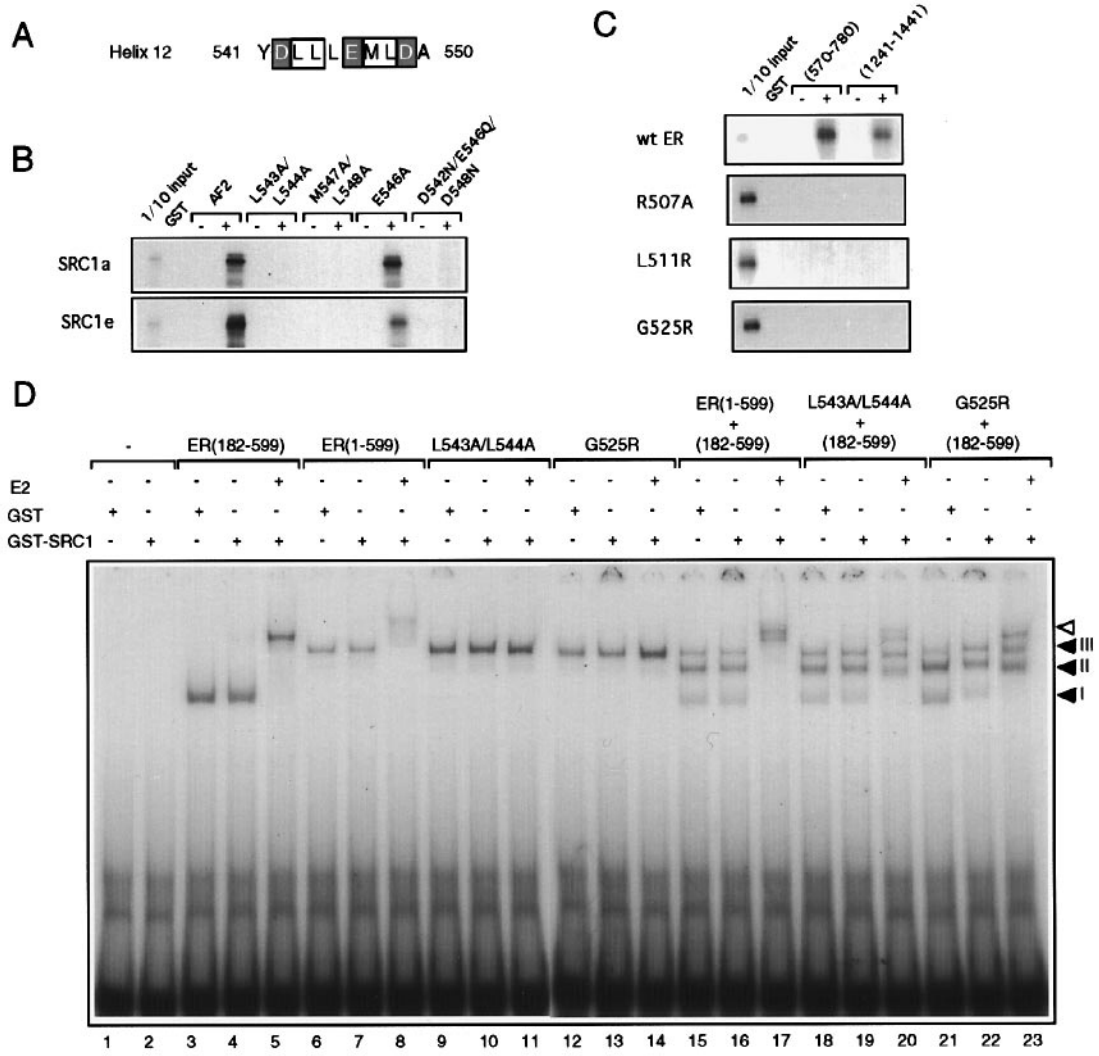

Fig. 4. SRC1 requires two functional AF2 domains in an ER dimer for binding. (**A**) Amino acid sequence of helix 12 of the mouse ER. Hydrophobic and charged residues are indicated by white and grey boxes, respectively. Numbers refer to amino acids. (**B**) Binding of GST fusions of AF2 of the ER containing mutations in helix 12 to SRC1 isoforms. GST fusions of AF2 of the ER containing the indicated point mutations were incubated with ^{35}S-labelled SRC1 proteins as described in Figure 2A in the absence (–) or presence (+) of E2 (10^{-6} M). (**C**) GST fusions containing amino acids 570–780 or 1241–1441 of SRC1 were incubated with ^{35}S-labelled ER or the mutants R507A and L511R, which are defective for dimerization, or the G525R mutant, which is defective for E2 binding, in the absence (–) or presence of E2 (+) as described in Figure 2. (**D**) Wild-type ER (1–599) and the mutants L543A/L544A and G525R were translated *in vitro*, either separately or in combination with the truncated wild-type ER (182–599), and analysed for DNA-binding capacity in a gel retardation assay. Proteins were incubated with a ^{32}P-labelled oligonucleotide containing a consensus ERE in the presence of GST or GST–SRC1(570–780) and in the absence or presence of E2 (10^{-6} M). Protein–DNA complexes were separated from unbound DNA on 7% non-denaturing SDS–polyacrylamide gels in 0.5× TBE and visualized by autoradiography. Black arrowheads indicate homodimers of truncated receptors (complex I), heterodimers between truncated and full-length receptors (II) or homodimers of full-length receptors (III), while the open arrowhead indicates the position of the complexes retarded due to E2-dependent GST–SRC1 binding.

reflects the presence of an additional NR-binding motif in the C-terminus of the SRC1a. Of the three LXXLL motifs present in the central region of both SRC1 proteins, motif 2 is sufficient for *in vitro* binding to the ER and for the potentiation of ER-mediated transcription in transiently transfected cells.

Dimers of ER require two functional AF2 domains for SRC1 binding

Having identified regions in the SRC1 proteins that are important for SRC1–ER interaction, we next investigated the requirements in the ER protein for SRC1 binding. For this, GST fusions of the ER were used in which mutations had been introduced into the H12 of AF2 that result in complete or partial loss of transcriptional activity (Figure 4A). Substitution of the hydrophobic residues at amino acid positions 543/544 or 547/548 or the charged residues at positions 542/546/549 abolishes both transcriptional activity in the full-length receptor (Danielian *et al.*, 1992) and the *in vitro* binding to SRC1 isoforms (Figure 4B). Therefore, a good correlation exists between transcriptional activity and SRC1 binding. Mutation of residue 546 from glutamic acid to alanine, which results in only a partial loss of transcriptional activity (Danielian *et al.*, 1992), had no appreciable effect on the binding of SRC1a to the ER, while SRC1e binding was reduced, indicating that subtle differences between the receptor-binding characteristics of the two SRC1 isoforms exist.

The binding of SRC1 by the ER is dependent on ligand and the integrity of H12, but it is unclear whether the interaction is dependent on the presence of two functional AF2 domains in a receptor dimer. To test this, we initially analysed dimerization-defective receptors, R507A and L511A, for their ability to bind SRC1. We found that these mutants, both of which are capable of binding hormone (Fawell *et al.*, 1990), were unable to bind to either of the two receptor-binding regions of SRC1 (Figure 4C), indicating that dimerization of receptors is a requirement for SRC1 binding in this assay. The G525R mutant of ER, which can still dimerize but has lost its ability to bind E2 (Fawell *et al.*, 1990; Danielian *et al.*, 1993), was unable to interact with either receptor-binding domain of SRC1. We next tested whether both AF2 domains in an ER dimer have to be functional to allow SRC1 binding. Therefore, ER heterodimers comprising one functional AF2 domain were compared with homodimers with two intact AF2 domains for their ability to bind GST–SRC1(570–780), which contains the common receptor-binding domain. The interaction was analysed on DNA using gel retardation assays with full-length wild-type ER (1–599), an N-terminally truncated ER (182–599), the transcriptionally defective L543A/L544A mutant or the oestrogen-binding G525R mutant, all of which bind to a consensus ERE (Fawell *et al.*, 1990). These receptors were translated *in vitro* either alone to form homodimers or in various combinations to form heterodimers. As shown in Figure 4D, the mobility of ER homodimers, both truncated (complex I, lane 5) and full length (complex III, lane 8), and of heterodimers between the two (complex II, lane 17) was retarded by GST–SRC1 in the presence of E2, reflecting the ability of SRC1 to interact with receptor dimers with two intact AF2 domains on DNA. In contrast, GST–SRC1 had no effect on the mobility of the L543A/L544A mutant or the G525R mutant, either as a homodimer (complex III, lanes 11 and 14, respectively) or as heterodimers with the ER (182–599), containing one functional AF2 domain (complex II, lanes 20 and 23). Similar results were obtained in a GST pull-down assay (data not shown). These results demonstrate that SRC1 requires the presence of two functional AF2 domains in the ER dimer for binding.

SRC1 isoforms differ in their ability to potentiate transcription by the ER

To investigate whether the two SRC1 isoforms also differed in their ability to potentiate ER-mediated transcription, different amounts of the respective expression vectors were co-transfected in COS-1 cells with an expression plasmid encoding the ER and different reporter plasmids. As shown in Figure 5, both isoforms stimulated the activity evoked by the ER in the presence of E2 on a reporter containing a single ERE in front of the herpes simplex virus thymidine kinase promoter (ERE-tk-Luc), with SRC1e being more potent in its action than SRC1a. It is noteworthy that both SRC1 isoforms enhanced the transcriptional activity of the ER in the absence of ligand, which is probably due to the overexpression of SRC1, since it is more apparent as the levels of expression vector are increased. When the two SRC1 isoforms were tested on a simple reporter (3×ERE-TATA-Luc), SRC1a had no effect, with higher concentrations being inhibitory (data not shown), while SRC1e stimulated ER-mediated transcription (Figure 5; middle panel). Similar results were obtained using a 1×ERE-TATA-Luc reporter (data not shown). Finally, both isoforms were tested on a reporter construct containing the –363/+16 upstream region of the rat oxytocin gene (RO-Luc), which contains a natural ERE (Adan *et al.*, 1992). On this reporter even low concentrations of SRC1a inhibited ER-mediated transcription, while co-transfection of SRC1e again increased reporter activity (Figure 5; lower panel). Similar results were obtained in HeLa cells although the maximal enhancement of ER activity by SRC1e was lower than

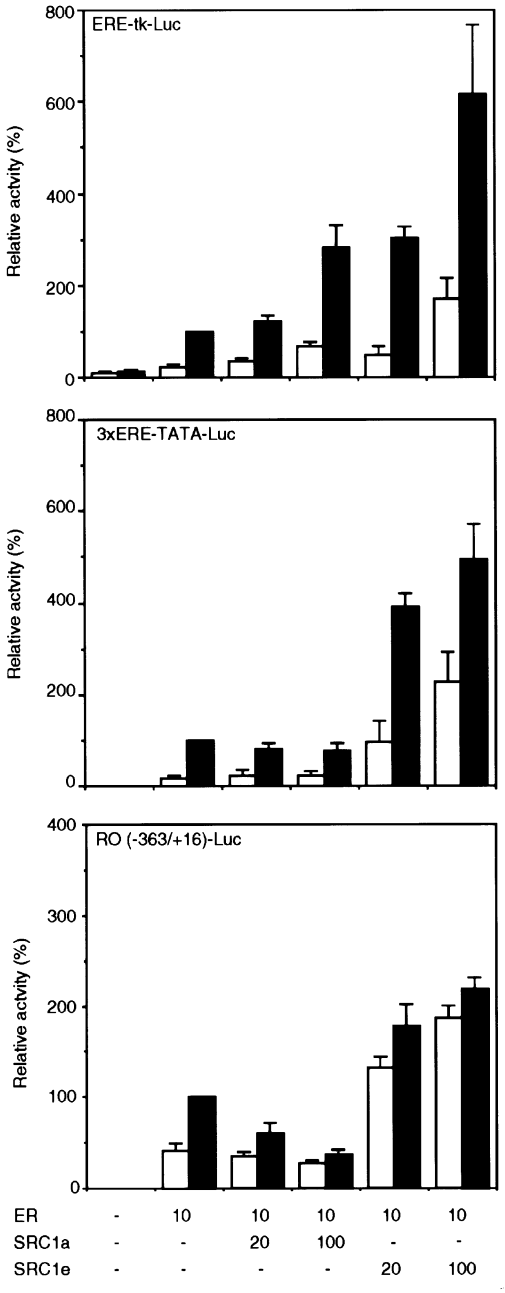

Fig. 5. SRC1 isoforms differ in their ability to stimulate ER-mediated transcription. COS-1 cells were transiently transfected with an expression vector for the ER and the reporter plasmids ERE-tk-Luc (upper panel), 3×ERE-TATA-Luc (middle panel) or (–363/+16)RO-Luc (lower panel) and different amounts of SRC1a or SRC1e (20 or 100 ng), as indicated. Data are presented as described in Figure 3B.

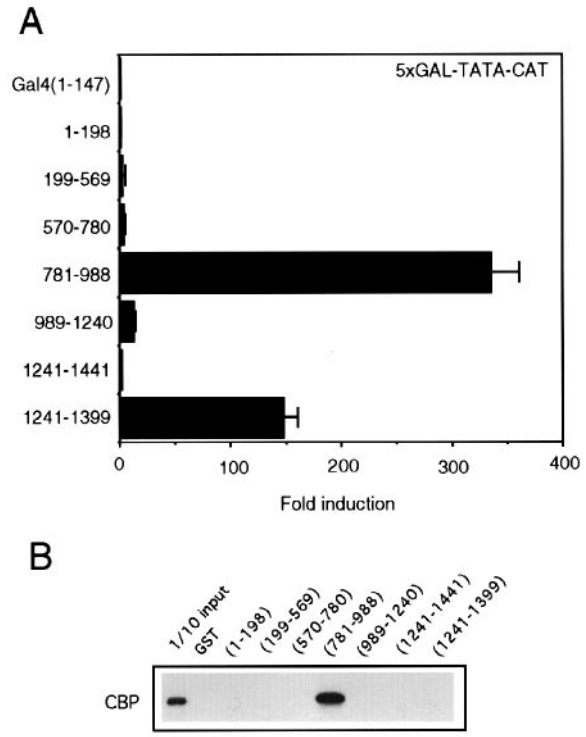

Fig. 6. SRC1 contains two activation functions. (**A**) Regions of SRC1 (Figure 2C) were fused to the DNA-binding domain of the yeast transcription factor Gal4 (amino acids 1–147) and tested for their ability to stimulate a Gal4 reporter in HeLa cells. The fusion proteins (1241–1441) and (1241–1399) contain amino acid sequences unique to the SRC1a and SRC1e proteins, respectively. Fold induction indicates the activity of the Gal4 fusions over the empty Gal4 vector. (**B**) Binding of CBP to GST fusions of SRC1. GST fusions of SRC1 (Figure 2C) were incubated with ^{35}S-labelled CBP as described in Figure 2.

that in COS-1 cells (data not shown). Taken together, these findings indicate that on a range of reporter constructs SRC1e is a more potent co-activator for the ER than SRC1a, whose ability to stimulate transcription depends on the promoter context.

SRC1 contains two distinct transactivation domains

To examine whether the different abilities of the two SRC1 isoforms to stimulate ER-mediated transcription could be explained by differences in intrinsic transactivation domains, the regions of the two SRC1 isoforms analysed for receptor binding (Figure 2C) were fused to the DNA-binding domain (DBD) of the yeast transcription factor Gal4. When these fusion proteins were tested for their ability to activate a Gal4 reporter, strong activity was observed with the Gal4 DBD fusion containing amino acids 781–988 (Figure 6A). This region, which is present in both SRC1 isoforms and binds to p300 (Yao *et al.*, 1996), showed strong *in vitro* interaction with the C-terminus of CBP in a GST pull-down assay (Figure 6B). The intrinsic transcriptional activity of this domain when tethered to DNA, which we termed activation domain 1 (AD1), is therefore likely to reflect its ability to recruit CBP/p300. A second Gal4–SRC1 fusion (amino acid 1241–1399), which contains SRC1e-specific amino acids, also had considerable transcriptional activity, while the corresponding SRC1a-specific fusion protein (1241–1441) was inactive. This SRC1e-specific transactivation domain (1241–1399), termed activation domain 2 (AD2), failed to interact with CBP (Figure 6B). It seems likely, therefore, that the difference between the two SRC1 isoforms in potentiating ER-mediated transcription (Figure 5) is not due to AD1, which is present in both isoforms, but to the CBP-independent activation domain AD2 in the C-terminus of SRC1e.

Activation domain 2 is suppressed in the SRC1a isoform

Next, we investigated the difference in transcriptional activity between the C-termini of the two SRC1 isoforms in more detail. As shown in Figure 1A, a major difference between the two SRC1 isoforms is the presence of an NR-binding motif in the C-terminus of the SRC1a protein. Inactivation of this LXXLL motif in the SRC1a-specific Gal4 DBD fusion [1241–1441(M4)] did not change its transcriptional activity (Figure 7A). Similarly, when this motif was altered in the full-length SRC1a protein [SRC1a(M4)], this protein was unable to potentiate ER-mediated transcription on a 3×ERE-TATA-Luc reporter (Figure 7B), as was also true for wild-type SRC1a. From these results, we conclude that the functional difference between the two SRC1 isoforms observed here is not related to the presence of an isoform-specific LXXLL motif in the C-terminus of SRC1a. Therefore, the C-terminal amino acids common to both SRC1 isoforms (1241–1385), as well as the regions specific for SRC1a (1386–1441) and SRC1e (1386–1399), were fused to the Gal4 DBD and tested as described above (Figure 7A). No transcriptional activity was detected with the Gal4 fusions containing the SRC1a- or SRC1e-specific amino acids, while the region common to both isoforms activated transcription (Figure 7A), similarly to the C-terminus of SRC1e (1241–1399; Figure 6A). These data show that AD2 is located between amino acids 1241 and 1385, and suggest that the 56 C-terminal amino acids specific to SRC1a (Figure 1A) suppress the function of this activation domain. To confirm this isoform-specific difference in the context of the full-length protein, cells were transiently transfected with a truncated form of SRC1 (amino acids 1–1385), which lacks the isoform-specific amino acids (Figure 7C). As shown in Figure 7B, this protein stimulated ER-mediated transcription similarly to full-length SRC1e, indicating that in the context of the full-length protein the SRC1a-specific amino acids also function as a suppressor of transcriptional activity. When SRC1a(M4) and SRC1(1–1385) were tested on other reporters (ERE-tk-Luc and RO-Luc), their effect on ER-mediated transcription was also found to be similar to SRC1a and SRC1e, respectively (data not shown).

From these findings, we conclude that the C-terminal transactivation domain AD2 in SRC1 is suppressed in the SRC1a isoform, thereby resulting in reduced ability to potentiate ER-mediated transcription.

Discussion

In this study, we have characterized two isoforms of human SRC1 for their ability to interact with nuclear receptors and to potentiate transcription by the ER in intact cells. As a consequence of different C-terminal

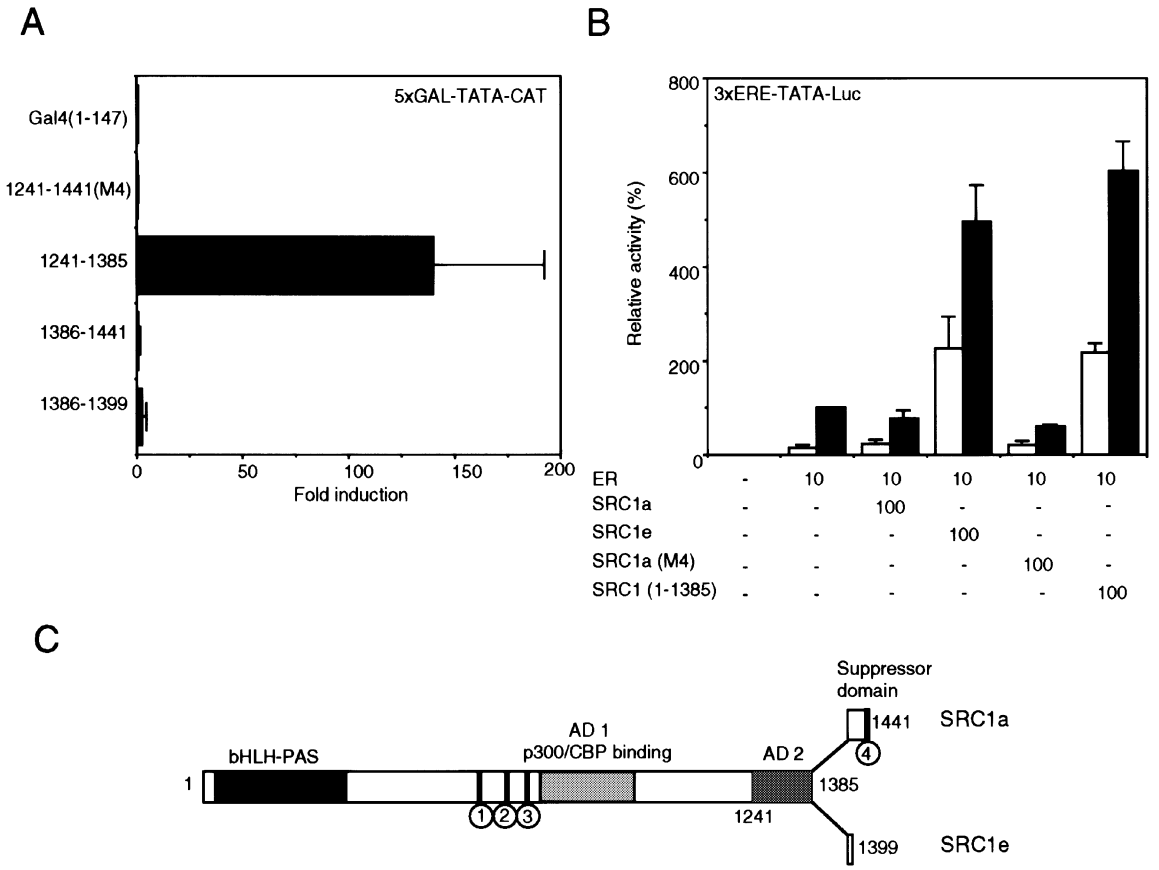

Fig. 7. Activation domain 2 is suppressed in SRC1a. (**A**) Regions of SRC1 (shown in C) were fused to the DNA-binding domain of the yeast transcription factor Gal4 and tested for their ability to stimulate a Gal4 reporter in HeLa cells. The fusion protein (1241–1441)M4 contains a mutation of LXXLL motif 4. Data are presented as described in Figure 6A. (**B**) COS-1 cells were transiently transfected with an expression vector for the ER and a 3×ERE-TATA-Luc reporter and 100 ng of SRC1a, SRC1e, SRC1a(M4) or SRC1(1–1385). Data are presented as described in Figure 3B. (**C**) Schematic representation of SRC1 isoforms. Numbers refer to amino acids. Indicated are the bHLH-PAS homology region, the nuclear receptor-binding motifs (LXXLL motifs, numbered 1–4), the CBP/p300-dependent activation domain AD1, the CBP/p300-independent activation domain AD2 (light and dark grey, respectively) and the suppressor domain in SRC1a.

sequences, the two isoforms SRC1a and SRC1e contain both common and distinct functional domains. We and others recently have identified short α-helical peptide sequences in NR-interacting proteins that are both necessary and sufficient to bind to NRs (Heery *et al.*, 1997; Torchia *et al.*, 1997). Both SRC1 isoforms contain three copies of the LXXLL motif clustered in the central region of the protein, but SRC1a contains an additional copy of the motif at its C-terminus. This C-terminal binding domain was originally identified as a PR-binding domain (Oñate *et al.*, 1995), while only the central region was found to bind to RAR (Yao *et al.*, 1996). These results are unlikely to reflect receptor specificity but are rather the consequence of the SRC1 isoforms used (SRC1a and SRC1e, respectively). Our results showing that both regions bind the ER and that the three LXXLL motifs in the central region as well as the C-terminal motif all interact with the ER and RAR in a yeast two-hybrid assay (Heery *et al.*, 1997) support this view. Although the C-terminal motif in SRC1a stabilizes the *in vitro* binding to the ER, its role in NR signalling is unclear since mutation of this motif had no effect on the activity of SRC1a in potentiating transcriptional activity of the ER in transiently transfected cells. In this respect, other p160 family members, TIF2/GRIP1 (Voegel *et al.*, 1996; Hong *et al.*, 1997) and AIB1/ACTR/RAC3/p/CIP (Anzick *et al.*, 1997; Chen *et al.*, 1997; Li *et al.*, 1997; Torchia *et al.*, 1997) resemble SRC1e in so far as they also contain three LXXLL motifs, which are highly conserved in both sequence and spacing, but lack a motif at the C-terminus. The only NR interaction protein identified so far that shares an LXXLL motif at its very C-terminus with SRC1a is Trip230 (Chang *et al.*, 1997), but this protein does not share extensive sequence homology with p160 proteins.

The recruitment of SRC1 to the ER following hormone binding depends on the presence of two functional AF2 domains in a receptor dimer since mutations which inhibit receptor dimerization or destroy the integrity of one H12 in a heterodimer abolish the binding of SRC1 *in vitro*. Thus the realignment of H12 in monomers of the HBD, which is proposed to occur upon ligand binding (Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995; Wurtz *et al.*, 1996; Brzozowski *et al.*, 1997), is insufficient for the interaction with SRC1. In ER homodimers, it seems likely that two AF2 surfaces, each comprising H12 (Danielian *et al.*, 1992) and H3 (Henttu *et al.*, 1997), are required to recruit SRC1 (DiRenzo *et al.*, 1997; Henttu *et al.*, 1997; present study). SRC1 is capable of binding to the ER through LXXLL motif 2 but, since additional motifs appear to result in optimal binding, a single molecule of SRC1 may interact with both receptor molecules in an ER dimer. Alternatively, it is conceivable

that the two AF2 surfaces may contact distinct SRC1 molecules, with motif 2 being the preferred site of interaction, and the requirement for functional receptor dimers might then indicate that SRC1 binds as a dimer.

The SRC1 isoforms differ not only in their ER-binding properties but also in their ability to potentiate the transcriptional activity of the ER in transiently transfected cells. While SRC1e stimulated ER-mediated transcription on various reporters, SRC1a only enhanced activity on an ERE-tk-Luc reporter. Both SRC1 isoforms contain a CBP interaction domain, and CBP has been shown to be essential for NR signalling (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996). However, the inability of SRC1a to potentiate the transcriptional activity of the ER on simple reporters and the oxytocin reporter suggests that on some promoters the recruitment of CBP/p300 is not sufficient for maximal potentiation of ER-mediated transcription in transiently transfected cells. The presence of a CBP-independent transactivation domain in the C-terminus of the SRC1e isoform indicates that it interacts with another target protein, which is probably important for the maximal potentiation of ER-mediated transcription. Our results indicate that this interacting protein is not TBP or TFIIB, so its identity remains to be established. Interestingly, the region encompassing the C-terminal activation domain is also present in the SRC1a isoform, but its activity is apparently repressed in the context of this protein. Whether the 56 amino acids that are unique to this isoform (Figure 1A) suppress the activity of this domain by masking it directly or indirectly, through interaction with another protein, remains to be established. Since the maximal stimulation by SRC1e was greater in COS-1 cells than in HeLa cells, the expression of the SRC1e-interacting protein possibly differs between cell lines. Similarly, TIF2 was shown previously to be a more potent co-activator for NRs in COS-1 cells compared with HeLa cells (Voegel *et al.*, 1996), suggesting that these two p160 proteins may interact with a common target important for NR signalling.

In addition to its NR- and CBP/p300-binding domains, SRC1 recently has been shown to contain an intrinsic histone acetyltransferase (HAT) domain (Spencer *et al.*, 1997). CBP/p300 has also been shown to contain a HAT domain (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996), and both SRC1 (Spencer *et al.*, 1997) and CBP/p300 (Yang *et al.*, 1996) have been demonstrated to interact with the HAT-containing protein p/CAF. Proteins with intrinsic HAT activity acetylate lysine residues in the N-terminal tails of histones, resulting in chromatin remodelling, and recruitment of HAT activity to a promoter region is thought to be an essential step in transcriptional regulation (Wade and Wolffe, 1997). The HAT domain in SRC1 was mapped to the C-terminus of the protein, between amino acids 1107 and 1441 (Spencer *et al.*, 1997), while only marginal activity was observed when this region was truncated on its N-terminus (1216–1441). Since amino acids 1107–1216 are important for HAT activity, and since the HAT activity was observed in the C-terminus of the SRC1a isoform (Spencer *et al.*, 1997), which we have shown to be transcriptionally suppressed, it seems unlikely that HAT activity is responsible for the transcriptional activity of the C-terminal activation domain AD2 (amino acids 1241–1385). p/CAF is also unlikely to be involved in AD2 function, since the two p/CAF interaction regions in SRC1 (amino acids 1027–1139 and 1139–1250) only marginally overlap with AD2 and since p/CAF does not function as a transcriptional activator when fused to the Gal4 DBD in transient transfection assays (Spencer *et al.*, 1997). To appreciate the relative importance of the intrinsic HAT activity and the activation domains of SRC1 in NR signalling, it will be necessary to use chromatin-based templates.

A recent comparison between SRC1, TIF2 and p/CIP by Rosenfeld and co-workers revealed that p160 proteins are functionally more distinct than first expected. Firstly, the role of SRC1 appears to be limited to NRs, while p/CIP is also required for other CBP/p300-dependent transcription factors like STATs (Torchia *et al.*, 1997). In addition, SRC1 was shown to be essential for RAR signalling, but TIF2/GRIP1 is dispensable (Torchia *et al.*, 1997). These differences, combined with the functionally distinct SRC1 isoforms described here, indicate that p160 proteins may play different roles in NR-mediated transcription. An important challenge for the future will therefore be the dissection of the complexes that are associated with activated NRs when bound to the promoter regions of target genes.

Materials and methods

Isolation of SRC1 cDNA clones

Two human cDNA libraries, a B-cell pCDNA3 plasmid library (Glynne *et al.*, 1993) and a ZR75- breast cancer cell λgt11 phage library (Cavaillès *et al.*, 1995), were screened with two DNA fragments corresponding to nucleotides 1–560 and 1202–3182 of the original SRC1 cDNA clone (Oñate *et al.*, 1995). Five individual clones were isolated from the B-cell library containing an open reading frame (ORF) of 1399 amino acids, which is preceded by stop codons in all three frames, and were named human SRC1e. In addition, shorter clones were isolated from both libraries which were identical to the SRC1e clones except for their 3′ end, resulting in a different C-terminus. These clones were named human SRC1a. The existence of SRC1a mRNA encoding a protein of 1441 amino acids was confirmed by RT–PCR using specific SRC1a primers. Clones were sequenced on both strands by automated sequencing. Several differences were observed with the human SRC1 cDNA sequences reported by O'Malley and co-workers (DDBJ/EMBL/GenBank accession Nos U40396 and U90661), some of which result in amino acid changes (A214G, with the second letter representing the amino acid deduced from the sequences reported in this study, K457Q, K466N, P474S, T591I, A685E, A794P, F999S and T1154M).

Plasmids

A double-stranded oligonucleotide containing several restriction sites followed by stop codons in all three reading frames (sense strand 5′-GGAATTCCCCGGGATGCTCGAGGGATCCAGATCTGACTGACTG-ATC-3′) was cloned into *Eco*RI–*Bgl*II sites of the mammalian expression vector pSG5 (Green *et al.*, 1988), and named pSG5(MCS). The complete ORF of SRC1e was amplified by PCR using Elongase (Gibco BRL) and cloned into the *Sma*I–*Bgl*II sites of this vector. To generate pSG5-SRC1a, a short region at the 3′ end of the SRC1e cDNA in the pSG5-SRC1e construct was exchanged for the 3′ end of the SRC1a clone. pSG5-SRC1a(M4) and pSG5-SRC1(1–1385) were made in a similar fashion. pSG5-SRC1e constructs containing leucine to alanine mutations at positions 636/637, 693/694 and 752/753 were created by recombinant PCR. Different regions of SRC1 were fused to GST and the DBD of the yeast transcription factor Gal4 by cloning PCR fragments, or a double-stranded oligonucleotide in the case of the 1386–1399 construct, into the *Sma*I site of the pGEX-2TK vector (Pharmacia) and pSG424 vector (Sadowski and Ptashne, 1989), respectively. All PCR-created constructs were verified by automated sequencing. A *Sma*I–*Bam*HI fragment of mouse CBP encoding amino acids 1917–2440 was cloned into the respective sites of pSG5(MCS) and used for *in vitro* translation. The GST–AF1 fusion containing amino acids 1–182 of the mouse ER was made by ligating an *Eco*RI–*Bcl*I fragment into *Eco*RI–*Bgl*II-cut pGEX2TK in the presence of annealed oligonucleotides encoding amino

acids 179–182 (constructed by F.L'Horset). The 1×ERE- and 3×ERE-TATA-Luc reporters, made by cloning oligonucleotides encoding the E1b TATA box and one or three copies of a consensus ERE into the pLuc vector (originally provided by Dr M.Schratl), were kind gifts of Dr B.van der Burg. The following plasmids have been described before: GST fusions of AF2 of the mouse ER and its mutants (Cavaillès *et al.*, 1994, 1995), GST–AF2 fusions of RARβ (Folkers and van der Saag, 1995), TRβ (Tone *et al*., 1994) RXRα (Mangelsdorf *et al*., 1991), GST–TBP and GST–TFIIB (Cavaillès *et al.*, 1995), GST–CBP (Heery *et al.*, 1997), pMT2 MOR (Lahooti *et al.*, 1994), the reporter plasmids $p(Gal)_5$CAT (Lillie and Green, 1989), ERE-tk-Luc (White *et al.*, 1994) and (–336/+16)RO-Luc (Adan *et al.*, 1992), the pSP65 plasmids containing the wild-type mouse ER and its mutants which were used for *in vitro* translation (Fawell *et al.*, 1990; Danielian *et al.*, 1992; Lahooti *et al.*, 1994).

Cell culture and transient transfection experiments

Cells routinely were maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 (T47D, JAR, Butler), containing 10% fetal bovine serum (Gibco BRL). For transient transfection assays, COS-1 or HeLa cells were plated in 24-well microtitre plates (Falcon) in phenol red-free medium containing 5% charcoal–dextran-stripped fetal bovine serum (CSS). Cells were transfected by calcium phosphate co-precipitation as described earlier (Danielian *et al.*, 1992). The transfected DNA included a pCMV-βGal control plasmid (150 ng), 3×ERE-TATA-Luc or $p(GAL)_5$CAT reporters (1 μg) and either pMT2-MOR (10 ng) and varying amounts of pSG5-SRC1a or pSG5-SRC1e, or the Gal4 fusions of SRC1 (200 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 (Danielian *et al.*, 1992) or CAT (Sleigh, 1986) and β-galactosidase activity, using a Galacto-Light chemiluminescent assay (Tropix). β-Galactosidase activity was used to correct for differences in transfection efficiency.

For far-Western analysis, SRC1a and SRC1e were overexpressed in COS-1 cells. After electroporation of the cells at 450 V and 250 μF in the presence of 20 μg of pSG5-SRC1a, pSG5-SRC1e or the empty expression vector, cells were plated out in DMEM containing 5% CSS and grown for 48 h. Whole cell extracts were prepared in buffer containing 0.4 M KCl, 20 mM HEPES (pH 7.4), 1 mM dithiothreitol and 20% glycerol, and the protein content was determined using a colorimetric method (Bio-Rad).

RNA isolation and RNase protection assay

Total RNA was isolated by the acid–phenol method (Chomczynski and Sacchi, 1987). RNase protection probes were generated by *in vitro* transcription of the plasmids described below, using Sp6 RNA polymerase. A 183 bp PCR fragment from the human SRC1e cDNA was cloned into the pGEM-T vector (Promega), which upon linearization with *Nco*I results in a 274 bp template. The β-actin probe has been described before (Kalkhoven *et al.*, 1995). Total RNA (10 μg) was hybridized for 16 h at 45°C with 10^5 c.p.m. of SRC1 probe and 10^4 c.p.m. of β-actin probe in a final volume of 25 μl containing 80% formamide, 40 mM PIPES, 400 mM NaCl and 1 mM EDTA. Subsequently, samples were digested with RNase A (20 μg/ml) for 30 min at room temperature and digestion was terminated with 0.125 mg/ml proteinase K and 0.5% SDS. Finally, protected fragments were phenol/chloroform-extracted, ethanol-precipitated and separated on a 6% polyacrylamide sequencing gel as described before (Kalkhoven *et al.*, 1995). Protected fragments were quantified on a PhosphoImager (Molecular Dynamics) using ImageQuant software, using the amount of β-actin as an internal standard, and values were corrected for probe length and distribution of labelled nucleotides along the probes.

Far-Western blotting

Whole cell extracts from ZR75-1, HeLa and COS-1 cells (50 μg) or COS-1 cells overexpressing SRC1a or SRC1e (5 μg) were separated on 6% SDS–polyacrylamide gels and blotted onto nitrocellulose. Proteins were denatured–renatured and probed with ^{32}P-labelled GST–AF2 of the ER as described (Cavaillès *et al.*, 1994) in the presence of E2 (10^{-6} M). Subsequently, filters were washed and exposed for autoradiography.

GST pull-down assays

Recombinant cDNAs in the pSG5 expression vector or the pSP65 vector were transcribed and translated *in vitro* in reticulocyte lysate (Promega) in the presence of [^{35}S]methionine according to the manufacturer's description. GST fusion proteins were induced and purified as described earlier (Cavaillès *et al.*, 1995). ^{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 100 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) (Cavaillès *et al.*, 1995). Samples subsequently were washed and separated on SDS–polyacrylamide gels (8 or 10%). Gels were fixed and dried and the ^{35}S-labelled proteins were visualized by fluorography.

Protein–protein interaction assays in the presence of DNA

The ability of SRC1 to interact with DNA-bound ER was assessed with a modified version of the assay employed by Kurokawa *et al.* (1995), as described (L'Horset *et al.*, 1996). ER (2 pmol), expressed in insect cells by using a baculovirus expression vector, was bound to a double-stranded biotinylated oligonucleotide (1 μg) containing one copy of the ERE from the vitellogenin A2 promoter in the absence or presence of E2 or anti-oestrogens as described above, and then immobilized on streptavidin MagneSphere Paramagnetic beads (Promega). The beads were washed and subsequently incubated with ^{35}S-labelled SRC1. After washing, samples were separated by SDS–PAGE and visualized as described above for GST pull-downs.

Gel retardation assays

In vitro translated wild-type ER and ER mutants were incubated with ^{32}P-labelled annealed oligonucleotides consisting of a 32 bp fragment of the vitellogenin A2 gene promoter encoding a consensus ERE (upper strand 5′-CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3′) as described (Danielian *et al.*, 1992). GST fusions proteins were eluted off GST beads (20 mM glutathione in 100 mM Tris pH 8.0, 120 mM NaCl) and added to the binding reactions in the absence or presence of E2 (10^{-6} M). Receptor–DNA complexes were separated from unbound DNA on non-denaturing 7% polyacrylamide gels and visualized by autoradiography.

Fluorescence in situ hybridization

Metaphase spreads were prepared from phytohaemagglutinin-stimulated normal human lymphocytes by use of standard techniques. Probe DNA was labelled and hybridized using standard techniques (Jones *et al.*, 1997). Briefly, 400 ng of labelled probe was mixed with 5 μg of Cot-1 DNA (Gibco BRL), precipitated, denatured, allowed to pre-anneal and then applied to a denatured slide and hybridized overnight. Slides were washed and probe signal detected using avidin–fluorescein isothiocyanate. Slides were counterstained with 4,6-diamidino-2-phenylindole and then mounted in Citifluor. Chromosome images were captured as described (Jones *et al*., 1997).

Accession numbers

The human SRC1a and SRC1e sequences have been submitted to DDBJ/EMBL/GenBank under accession Nos AJ000881and AJ000882, respectively.

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