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Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor

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A conserved region in the hormone-dependent activation domain AF2 of nuclear receptors plays an important role in transcriptional activation. We have characterized a novel nuclear protein, RIP140, that specifically interacts *in vitro* with this domain of the estrogen receptor. This interaction was increased by estrogen, but not by anti-estrogens and the *in vitro* binding capacity of mutant receptors correlates with their ability to stimulate transcription. RIP140 also interacts with estrogen receptor in intact cells and modulates its transcriptional activity in the presence of estrogen, but not the anti-estrogen 4-hydroxytamoxifen. In view of its widespread expression in mammalian cells, RIP140 may interact with other members of the superfamily of nuclear receptors and thereby act as a potential co-activator of hormone-regulated gene transcription.

Keywords: anti-estrogens/co-activator/estrogen receptor/transcriptional activation

Introduction

The estrogen receptor belongs to a superfamily of nuclear receptors that function as ligand-dependent transcription factors (Evans, 1988; Green and Chambon, 1988; Beato, 1989; Parker, 1993). Transcription is mediated by means of two activation regions, AF1 located in the N-terminal domain and AF2 located in the hormone binding domain, whose activities vary depending on the responsive promoter and cell type (Webster *et al.*, 1988; Lees *et al.*, 1989; Tora *et al.*, 1989). These two activation regions appear to function independently on certain promoters but, in some cases, both are required for full transcriptional activity (Tora *et al.*, 1989). Equivalent activation regions have also been identified in other steroid hormone receptors and the receptors for thyroid hormone and retinoids (Webster *et al.*, 1988; Meyer *et al.*, 1990; Zenke *et al.*, 1990; Nagpal *et al.*, 1992; Saatcioglu *et al.*, 1993). One essential element required for the activity of AF2 is a C-terminal region with the characteristics of an amphipathic α-helix (Danielian *et al.*, 1992). This region is highly conserved in the nuclear receptor family and has been shown to be essential for transcriptional activation by estrogen and glucocorticoid receptors (Danielian *et al.*, 1992), thyroid hormone receptors (Saatcioglu *et al.*, 1993; Barettino *et al.*, 1994) and retinoid receptors (Durand *et al.*, 1994). In view of its conservation, we proposed that it might play an important role in ligand-dependent transcriptional activation by all nuclear receptors (Danielian *et al.*, 1992).

The ability of transcriptional activators to stimulate rates of transcriptional initiation by RNA polymerase II is likely to involve the assembly of basal transcription factors into a pre-initiation complex (Ptashne, 1988; Mitchell and Tjian, 1989). A number of activators, including nuclear receptors, have been shown to bind directly to the TATA box binding protein (TBP) and TFIIB *in vitro* (Stringer *et al.*, 1990; Ing *et al.*, 1992; Baniahmad *et al.*, 1993; Choy and Green, 1993; Sadovsky *et al.*, 1995), but recent work has shown that while TBP can support basal transcription, TFIID is required for activated transcription *in vitro* (Dynlacht *et al.*, 1991; Gill and Tjian, 1992; Brou *et al.*, 1993) and *in vivo* (Tansey *et al.*, 1994). TFIID consists of a protein complex containing TBP and sets of TBP-associated factors (TAFs). Tjian and co-workers have proposed that individual TAFs are critical targets that function as co-activators for transcriptional activators (Dynlacht *et al.*, 1991; Chen *et al.*, 1994). It is envisioned that different types of activation domain interact with distinct co-activators and in this way the binding of TFIID is stabilized. Evidence to support this hypothesis was provided by the demonstration that the glutamine-rich activation domain of Sp1 interacts directly and specifically with $TAF_{II}110$ (Hoey *et al.*, 1993), while the acidic activation domain of VP16 interacts specifically with $TAF_{II}40$ (Goodrich *et al.*, 1993). The significance of these interactions was supported by the correlation between binding activity and transcriptional activation (Goodrich *et al.*, 1993; Gill *et al.*, 1994).

Recently $hTAF_{II}30$ has been shown to interact selectively with the hormone binding domain of the estrogen receptor and appears to contribute to transcriptional activity *in vitro*. (Jacq *et al.*, 1994). However, the interaction was unaffected by binding of either 17β-estradiol or anti-estrogens, such as 4-hydroxytamoxifen, and mapped to a region that is inactive in mammalian cells (Pierrat *et al.*, 1994). Therefore, the major targets of AF2 in the hormone binding domain of the receptor have yet to be identified. Candidates include two receptor-interacting proteins of molecular weights 140 (RIP140) and 160 kDa (RIP160) that have been shown to bind directly to the hormone binding domain of the receptor in the presence of estrogen, but not anti-estrogens (Cavaillès *et al.*, 1994; Halachmi *et al.*, 1994). Furthermore, the interaction was abolished when point mutations were

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introduced into the conserved putative amphipathic α-helix required for hormone-dependent transcription (Cavaillès *et al.*, 1994).

In this paper we report the isolation of cDNA clones and characterization of RIP140, which we show is a novel nuclear protein that is widely expressed in mammalian cells. We demonstrate that RIP140 interacts *in vitro* with the wild-type receptor, but not with mutant receptors that are defective in transcriptional activity. RIP140 was also shown to interact with the estrogen receptor in transfected cells and modulated its transcriptional activity in the presence of estrogen, but not the anti-estrogen 4-hydroxytamoxifen. In view of the conservation in the interacting region in receptors, RIP140 is likely to be involved in the regulation of gene expression by other members of the nuclear receptor family.

Results

Isolation and characterization of cDNA clones for RIP140

We have screened an oligo(dT)-primed cDNA expression library, derived from ZR75-1 human breast cancer cells, with a ^{32}P-labeled chimeric protein comprising the hormone-dependent activation domain (GST–AF2), which we used previously to characterize RIP140 and RIP160 (Cavaillès *et al.*, 1994). We isolated five overlapping independent clones corresponding to the same mRNA, the longest of which contained an open reading frame encoding an incomplete protein of ~100 kDa. The shortest cDNA clone (pBRIP3) encoded only the last 407 amino acids, indicating that the receptor interacts with the C-terminal portion of the protein. In order to obtain full-length cDNA clones, the library was rescreened using a ^{32}P-labeled 576 nt fragment from the 5′-end of the longest clone. We obtained an additional cDNA clone containing 1128 extra nucleotides and encompassing the ATG initiation codon. Together these clones spanned 7247 nt and encoded a protein of 1158 amino acids (Figure 1A) with a predicted molecular mass of 127 kDa. In order to determine whether the cloned cDNA corresponded to RIP140 or RIP160, the full-length sequence was inserted into the eukaryotic expression vector pEFBOS (Mizushima and Nagata, 1990) and the recombinant plasmid pEFRIP was transiently expressed in COS-1 cells. Far-Western blot analysis of whole cell extracts using the GST–AF2 probe showed that the band corresponding to the smaller of the two receptor-interacting proteins was dramatically increased, suggesting that the isolated cDNA encoded RIP140 (Figure 1B).

Comparison of the amino acid sequence with those available in databases did not reveal any proteins with extensive homologies. Using basic local search alignment (BLAST) we found some similarity with some serine-rich proteins and several short homologies with transcription factors such as the yeast protein SWI1/ADR6 (52% identity over 23 residues) (O'Hara *et al.*, 1988). When a search was done with the nucleotide sequence in the EMBL database we found two short expressed sequence tags (EST) of <300 nt absolutely identical to parts of the 3′ untranslated region of our cDNA (Adams *et al.*, 1993; Murakawa *et al.*, 1994).

The amino acid sequence revealed little about the function of RIP140. Searches with the ProSite program did not uncover homology to known conserved protein motifs, except potential phosphorylation sites. The protein is highly hydrophilic (Figure 1C) and contains several putative bipartite nuclear localization signals. Overall the predicted sequence is rich in serine (13.9%) and leucine (10.3%) residues that are spread throughout the protein. A serine/threonine-rich domain (34% in 73 residues) was found in the middle of the protein, flanked on each side by acidic and basic domains and by regions rich in charged amino acids (Figure 1D).

Expression and chromosomal location of the RIP140 gene

Northern blot analysis of total RNA from ZR75-1 cells detected a single transcript of ~7.5 kb, indicating that the cloned cDNA was almost full length. RIP140 appeared to be widely expressed, since its mRNA was detected in all human cell lines tested (Figure 2), irrespective of whether the cells were derived from estrogen target tissues. A protein with a similar electrophoretic mobility as RIP140 was also detected by far-Western blotting analysis in extracts from simian, mouse and chicken cells (data not shown). Analysis of 100 metaphase spreads using fluorescence *in situ* hybridization indicated that the RIP140 gene was localized on chromosome 21 in the region q11.2 (not shown).

RIP140 interacts with transcriptionally active estrogen receptors in vitro

We analyzed the interaction between RIP140 and the estrogen receptor by far-Western blotting and by using GST fusion proteins in pull-down experiments. Using the radiolabeled wild-type GST–AF2 probe, we observed a direct interaction between the hormone binding domain and the C-terminal portion of RIP140 (corresponding to residues 752–1158 encoded by pBRIP3 in *Escherichia coli*). The binding was strictly dependent on the presence of estradiol, with no signal observed in the absence of ligand or in the presence of anti-estrogens, such as 4-hydroxytamoxifen (Figure 3A). Interaction between RIP140 and the receptor was also totally abolished when we used a probe containing a mutant AF2, in which conserved hydrophobic residues M547/L548 were replaced with alanines, which binds estradiol but is transcriptionally inactive. Similar results were obtained using longer fragments of RIP140 (data not shown).

The binding of RIP140 to the receptor was also analyzed by testing the ability of GST–AF2 fusion proteins to retain *in vitro* translated RIP140. In addition to the full-length products, *in vitro* translation of RIP140 mRNAs also generated truncated fragments (Figure 3B and C), which were probably derived from initiation at internal ATG codons, since similar fragments were also obtained by translating 5′ deletion mutants of RIP140 cDNA (data not shown). Such internal initiation of translation also occurred in *E.coli*, but not in mammalian cells (Figure 1B). Although a small amount of non-specific binding of ^{35}S-labeled RIP140 fragments to GST alone was detected, specific binding with GST–AF2 was observed with the C-terminal portion of RIP140 (residues 752–1158), as well as with full-length RIP140, and this interaction was strongly enhanced in the presence of estradiol (Figure 3B, tracks

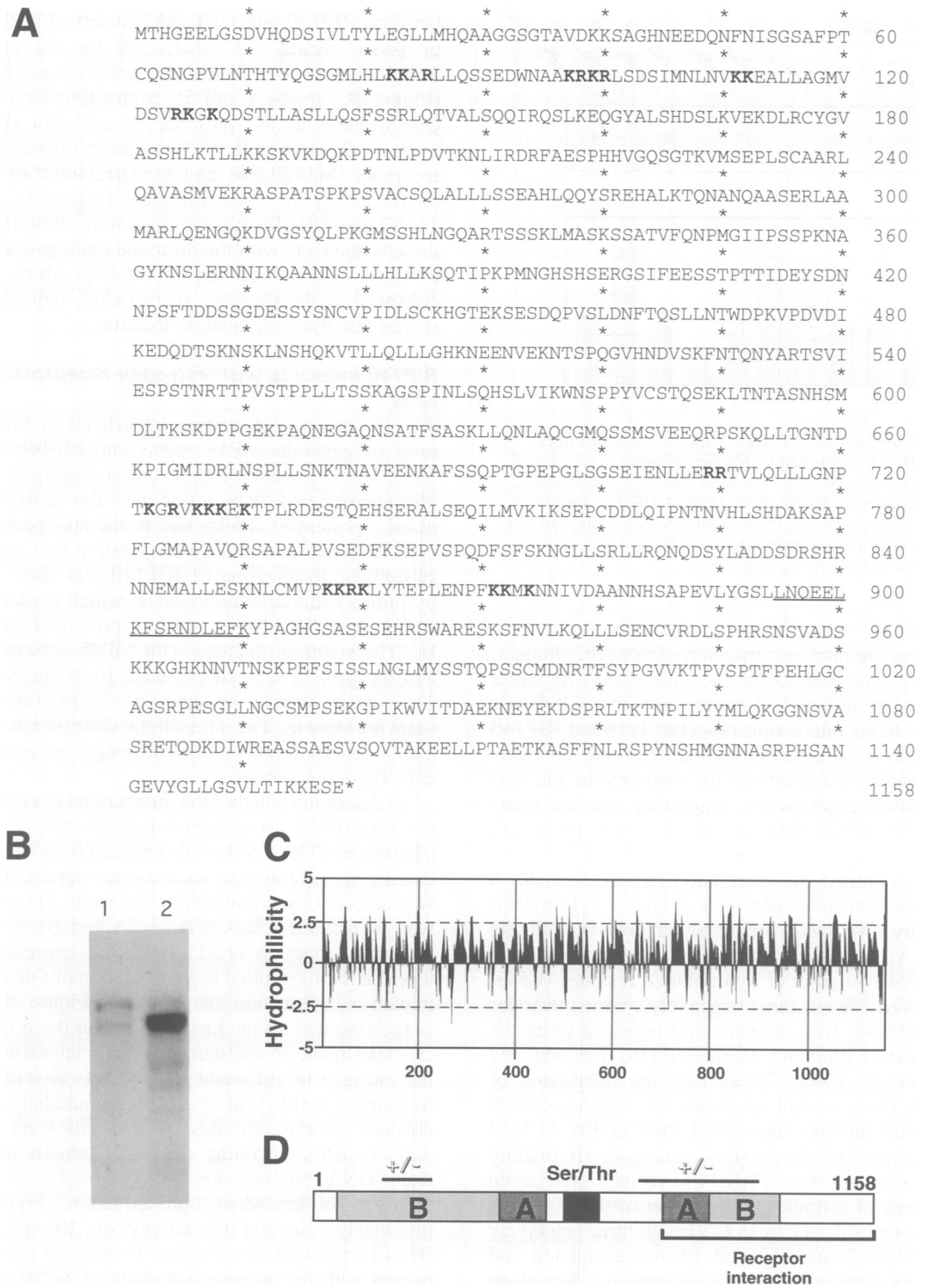

Fig. 1. Characterization of cDNA clones for RIP140. (**A**) The RIP140 protein sequence predicted from composite pBRIP140 cDNA clones is shown in single-letter code. A number of basic residues that might serve as a nuclear localization signal are highlighted. In addition, the position of a 16 amino acid peptide used to generate rabbit polyclonal antiserum is underlined. (**B**) Far-Western blotting was used to demonstrate that the recombinant cDNA clones encoded RIP140. Using a ^{32}P-labeled GST–AF2 probe, endogenous RIP140 and RIP160 were detected in COS-1 cell extracts (track 1). After transfection with the eukaryotic expression vector pEFRIP, only the lower band of the doublet corresponding to RIP140 was overproduced (track 2). (**C**) Kyte and Doolittle hydrophilicity plot of RIP140 (window = 7) using IBI MacVector software. (**D**) Schematic representation of the RIP140 molecule showing the central 73 amino acid domain, which contains 34% of serine or threonine residues (black box) flanked by basic (B) and acidic (A) domains. In addition, the C-terminal receptor-interacting domain and the two regions of the protein enriched in charged residues (>31%) are also shown (+/–). The schematic is aligned with the hydrophilicity plot (C).

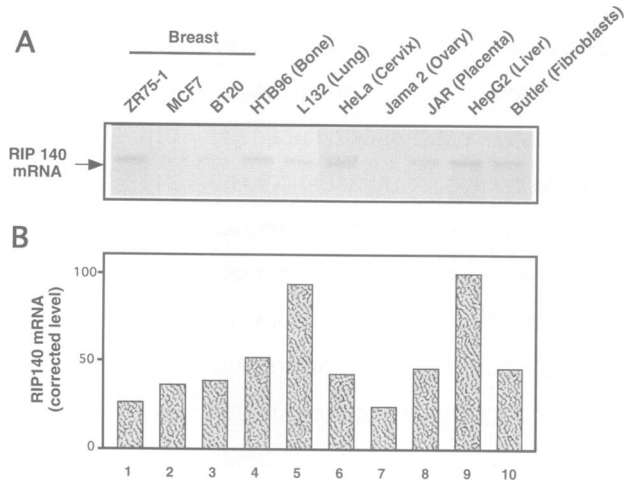

Fig. 2. Expression of RIP140 mRNA in various human cell lines. (**A**) RIP140 mRNA was detected by Northern blotting in samples of total RNA (20 μg) isolated from human cell lines. The name and origin of the cell lines are shown above the blot. (**B**) The amount of RIP140 mRNA relative to that of γ-actin mRNA was determined using a Molecular Dynamics PhosphorImager and expressed as percent of the level obtained in the Hep G2 cells.

4 and 5, and C, tracks 2 and 3). Similar results were obtained using the human estrogen receptor AF2 domain, whereas no specific interactions were observed with either the mouse or the human AF1 domains fused to GST (not shown). The basal interaction observed between RIP140 and GST–AF2 in the absence of added hormone contrasts with the absolute requirement for estrogen in the far-Western blotting experiments, suggesting that the interaction may be stabilized by additional factors present in cell extracts.

We then examined the interaction of RIP140 with a series of mutant receptors that were defective in transcriptional activity. We have previously shown that in the absence of AF1, transactivation by AF2 was negligible when either of two pairs of hydrophobic residues (L543/L544 or M547/L548) or three negatively charged residues (D542/E546/D549) were mutated and impaired when the highly conserved glutamic residue (E546) was mutated (Danielian *et al.*, 1992). To analyze the interaction of RIP140 with these mutant receptors, we expressed them as GST fusion proteins and tested their ability to bind *in vitro* translated RIP140. As shown in Figure 3B, binding of the C-terminal portion of RIP140 was negligible, even in the presence of estradiol, when either of the two pairs of hydrophobic residues (tracks 6–9) or all three negatively charged residues (tracks 12 and 13) were mutated and was markedly reduced when the conserved E546 alone was mutated (tracks 10 and 11). When full-length RIP140 protein was used similar results were obtained for hydrophobic mutants (Figure 3C, tracks 11 and 12), whereas a slight increase was observed with the triple acidic mutant (Figure 3C, tracks 15 and 16). Thus the ability of a number of mutations in the conserved region of the receptor to disrupt the estrogen-induced interaction with RIP140 correlates with their effects on hormone-dependent transcriptional activity of the receptor (Danielian *et al.*, 1992).

We also examined the effect of estrogen antagonists on the binding properties of RIP140. The basal interaction between RIP140 and GST–AF2 observed in the absence of added ligand was unaffected by the presence of 4-hydroxytamoxifen or the pure anti-estrogen ICI 182780 (Figure 3C, tracks 4 and 5), contrasting with the strong enhancement in the presence of estradiol (Figure 3C, track 3). Binding of RIP140 to transcriptionally defective receptors (M547/L548 and D542/E546/D549) was also unaffected by these anti-estrogens (Figure 3C, tracks 13, 14, 17 and 18). Together these results show that RIP140 directly interacts with the hormone binding domain of the estrogen receptor and that the binding, which is strongly enhanced in the presence of estradiol, requires the conserved putative amphipathic α-helix.

RIP140 interacts with estrogen receptors in intact cells

The 16 amino acid peptide underlined in Figure 1 was used to generate MP45 rabbit anti-RIP140 antibodies, which were purified by affinity chromatography. In Western blot experiments (data not shown) this antiserum recognized a protein of similar size to the endogenous RIP140 detected in cell extracts by far-Western blot analysis. The subcellular localization of RIP140 was then determined by indirect immunofluorescence, which revealed that the endogenous RIP140 is a nuclear protein (Figure 4A and B). The staining obtained with MP45 appeared granular and the protein seemed excluded from nucleoli. Control experiments showed no staining with pre-immune serum (data not shown). The intracellular distribution was similar after overexpression of the full-length protein in COS-1 cells (data not shown).

To determine whether the interaction between the receptor and RIP140 also occurred in intact cells, we transiently transfected COS-1 cells with a mutant form of the receptor lacking specific nuclear localization signals. This mutant was detected by immunofluorescence in the cytoplasm as well as in the nucleus (Figure 5A and B), as previously described (Dauvois *et al.*, 1993). Co-expression of full-length RIP140 resulted in translocation of this cytoplasmic mutant receptor into the nucleus (Figure 5C and D), suggesting that an interaction between these two proteins can take place in cultured cells. An interaction between the estrogen receptor and RIP140 was also demonstrated by using a two-hybrid system in mammalian cells. When chicken embryo fibroblast (CEF) cells were transfected with a vector encoding the AF2 domain fused to the Gal4 DNA binding domain (Gal4–AF2) we observed an estradiol-dependent transactivation (Figure 5E), as previously described (Cavaillès *et al.*, 1994). Co-transfection of a plasmid encoding the C-terminal part of RIP140 tagged with the activation domain of VP16 (RIP–VP16) produced a significant increase in CAT activity, resulting probably from the recruitment of RIP–VP16 by Gal4–AF2 in the presence of estradiol. In the presence of 4-hydroxytamoxifen, Gal4–AF2 transactivation was negligible and not significantly enhanced by co-transfection with RIP–VP16. As expected, no transcriptional activity was observed when RIP–VP16 was transfected without Gal4–AF2. We also expressed the VP16 domain alone together with Gal4–AF2 and observed a decrease in AF2 transactivation, probably due to 'squelching', in which VP16 might interact with one or more target factors required for the action of the estrogen receptor (Tasset

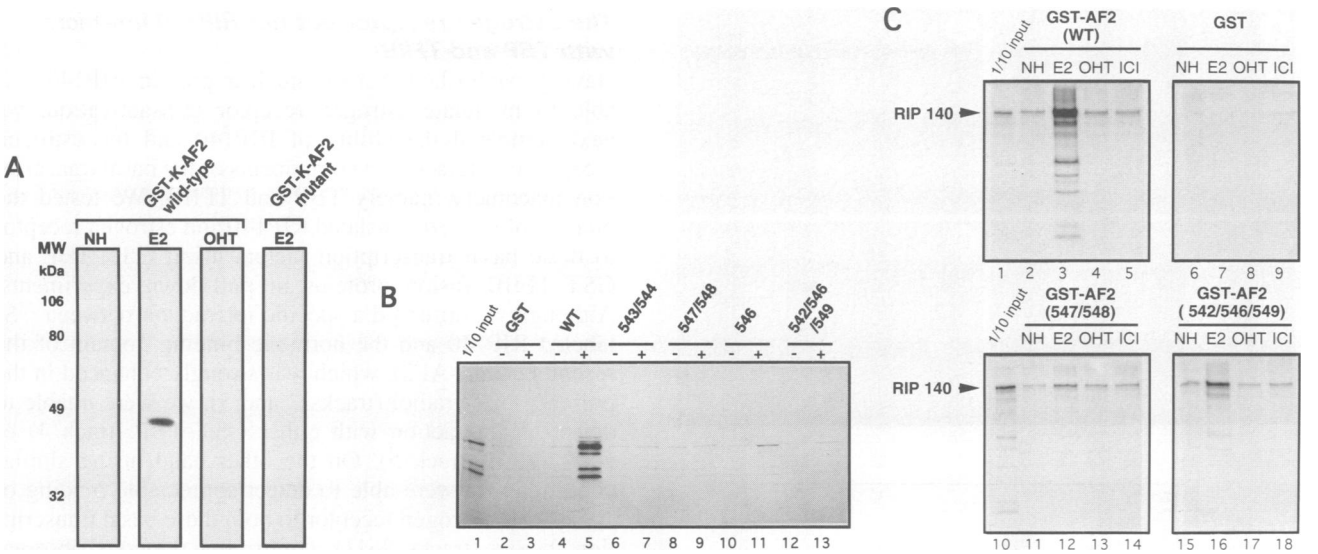

Fig. 3. *In vitro* interaction of RIP140 with the estrogen receptor. (**A**) Direct interaction between the C-terminal portion of RIP140 (amino acids 752–1158 encoded by pBRIP3) and the estrogen receptor was analyzed by far-Western blotting using ^{32}P-labeled wild-type or (M-547A/L-548A) mutant GST–AF2 probe (Cavaillès *et al.*, 1994). Probes were incubated with the blots with no added hormone (NH) or in the presence of 100 nM estradiol (E2) or 100 nM 4-hydroxytamoxifen (OHT). (**B**) Interaction of RIP140 with wild-type and mutant AF2 domains of the mouse estrogen receptor was analyzed by testing the ability of GST–AF2 fusion proteins linked to glutathione–agarose beads to bind the ^{35}S-labeled C-terminal portion of RIP140 (residues 752–1158 encoded by pBRIP3). The AF2 domains tested were the wild-type (WT, tracks 4 and 5), L543A/L544A mutant (543/544, tracks 6 and 7), M547A/L548A mutant (547/548, tracks 8 and 9), E546A mutant (546, tracks 10 and 11) and D542N/E546Q/D549N mutant (542/546/549, tracks 12 and 13). Tracks 2 and 3 show binding to GST alone. The interaction was analyzed in the absence (–) or presence (+) of 100 nM estradiol. Track 1 shows 1/10 the amount of input C-terminal RIP140 fragments. (**C**) Effect of estrogen antagonists on the interaction between RIP140 and the estrogen receptor AF2 domain. GST–AF2 fusion proteins comprising either the wild-type AF2 (WT, tracks 2–5), M547A/L548A mutant (547/548, tracks 11–14), D542N/E546Q/D549N mutant (542/546/549, tracks 15–18) or GST alone (tracks 6–9) were tested for their ability to bind ^{35}S-labeled RIP140. The analysis was done with no added hormone (NH) and/or in the presence of 100 nM estradiol (E2), 100 nM 4-hydroxytamoxifen (OHT) or 100 nM ICI 182780 (ICI). Tracks 1 and 10 show 1/10 the amount of input ^{35}S-labelled RIP140, whose position is indicated on the left.

et al., 1990). Together our results indicate that the nuclear factor RIP140 is able to interact with the hormone binding domain of the estrogen receptor in intact nuclei.

RIP140 modulates transcriptional activity of the estrogen receptor

To assess the consequences of an association between the receptor and RIP140, we analyzed the effects of RIP140 overexpression on the transcription of an estrogen-sensitive reporter gene in transiently transfected CEF cells, which are devoid of endogenous estrogen receptor. Both activation domains of the transfected receptor are transcriptionally active in these cells and this allowed us to monitor the effects of tamoxifen, which has been shown to act through AF1 (Webster *et al.*, 1988; Lees *et al.*, 1989), as well as estrogen. Co-transfection of increasing amounts of the expression plasmid for RIP140 produced a biphasic effect on estrogen receptor-mediated transcription of the reporter gene (Figure 6A). At low levels of expression plasmid pEFRIP (1 ng) the estrogen-stimulated transactivation was increased 2-fold, but as the amount of RIP140 expression plasmid was increased (>100 ng) transcription in the presence of estrogen was almost totally abolished. The inhibition was slightly reduced by increasing the levels of the estrogen receptor expression plasmid (data not shown), suggesting that the estrogen receptor was partially squelched by high levels of RIP140 and the remaining inhibition was probably due to its ability to sequester one or more target proteins required for transcriptional activity of the receptor. A similar decrease in CAT activity after RIP140 overexpression was

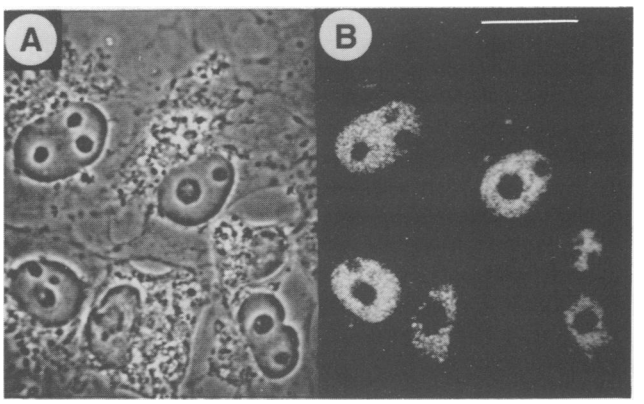

Fig. 4. Nuclear localization of RIP140 in COS-1 cells. (**A**) Phase contrast image of the COS-1 cells shown in (B). (**B**) Endogenous RIP140 was detected by indirect immunofluorescence using the MP45 affinity-purified rabbit antiserum raised against a synthetic peptide corresponding to residues 895–910 of RIP140. The picture shows the granular staining of RIP140 only in the nucleus and excluded from the nucleoli. The white bar corresponds to 20 μm.

also observed in the absence of added estrogens, suggesting that the 'stripped' serum probably contained residual estradiol. In contrast, the agonistic activity of 4-hydroxytamoxifen was unaffected, irrespective of the concentration of pEFRIP vector used. This result suggests that the *in vivo* interaction between the receptor and RIP140 is weaker in the presence of an anti-estrogen than that in the presence of estrogens, confirming the observations made *in vitro* (Figure 3). A similar biphasic effect of RIP140 titration

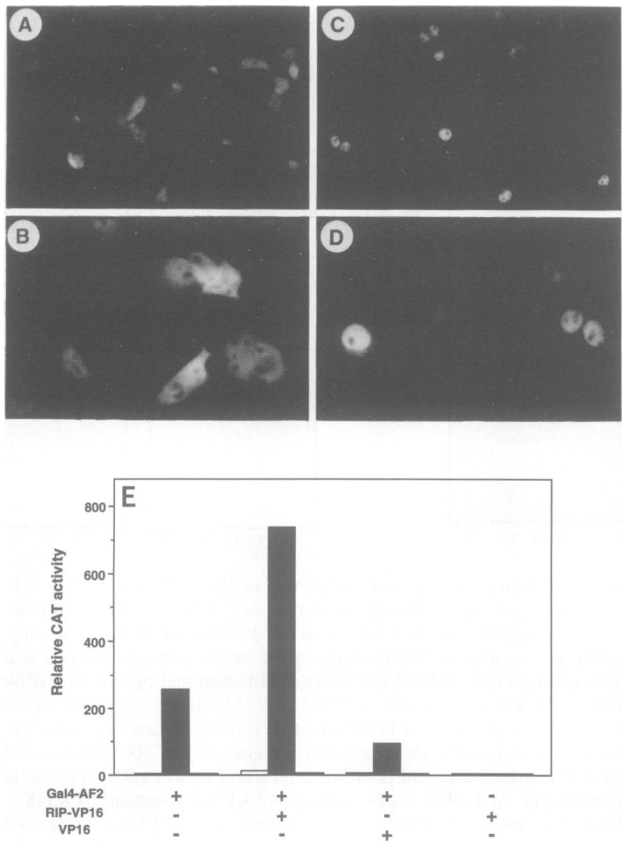

Fig. 5. Interaction of the estrogen receptor with RIP140 in intact cells. (**A–D**) Cytoplasmic forms of the estrogen receptor are translocated into the cell nucleus by RIP140. The nuclear localization-deficient mutant (MOR Δ252–307) was expressed alone (A and B) or with RIP140 (C and D) in transiently transfected COS-1 cells as described in Materials and methods. The subcellular localization of MOR Δ252–307 was monitored with the estrogen receptor monoclonal antibody H222. The magnification is ×100 in (A) and (C) and ×400 in (B) and (D). (**E**) Two-hybrid system in mammalian cells. The ability of a fusion protein comprising the hormone binding domain of the estrogen receptor and the DNA binding domain of Gal4 (Gal4–AF2) to interact with either the C-terminal portion of RIP140 fused to the activation domain of VP16 (RIP–VP16) or VP16 alone was examined in CEF cells by determining the level of CAT activity from the reporter plasmid G5E1BCAT. As a control, the effect of RIP–VP16 alone was also determined. Transcriptional activation after treatment of the cells without added ligand (open bars), with 10 nM estradiol (solid bars) or 100 nM 4-hydroxytamoxifen (hatched bars) is expressed as relative CAT activity corrected for transfection efficiency.

on estradiol-dependent transactivation of the estrogen receptor was obtained using the MCF-7 human breast cancer cell line (data not shown).

We also monitored the inhibitory effect of RIP140 overexpression on deletion mutants of the receptor lacking either AF1 (MOR182–599) or AF2 (MOR1–339). Transcriptional activation mediated by AF1 was decreased by <2-fold, as expected, since it lacks the interacting region of the receptor, whereas transactivation by AF2 was reduced by >90%, slightly more than that for the full-length receptor (Figure 6B). Together these results suggest that RIP140 is a transcriptional modulator of the hormone-dependent activation function AF2 of the estrogen receptor.

The estrogen receptor but not RIP140 interacts with TBP and TFIIB

Having established that the nuclear protein RIP140 was able to modulate estrogen receptor transactivation, we next compared the ability of RIP140 and the estrogen receptor to interact with components of the basal transcription machinery, namely TBP and TFIIB. We tested the binding of *in vitro* translated RIP140 and estrogen receptor to these basal transcription factors using GST–TBP and GST–TFIIB fusion proteins in pull-down experiments. Although we observed a specific interaction between ^{35}S-labeled RIP140 and the hormone binding domain of the receptor (GST–AF2), which was strongly enhanced in the presence of estradiol (tracks 2 and 3), we were unable to detect an interaction with either GST–TBP (track 4) or GST–TFIIB (track 5). On the other hand, using similar conditions we were able to detect appreciable binding of ^{35}S-labeled estrogen receptor to both these basal transcription factors (tracks 8–11), confirming previous observations (Ing *et al.*, 1992; Sadovsky *et al.*, 1994). However, their interaction with the receptor was unaffected by estrogen binding, in contrast with that of RIP140.

Discussion

A conserved region with the characteristics of an amphipathic α-helix (Danielian *et al.*, 1992) plays an important role in ligand-dependent stimulation of transcription by nuclear receptors (Zenke *et al.*, 1990; Danielian *et al.*, 1992; Saatcioglu *et al.*, 1993; Barettino *et al.*, 1994; Durand *et al.*, 1994). In this paper we have isolated cDNA clones corresponding to a human protein, RIP140, whose direct interaction with the estrogen receptor requires this conserved region. Using two distinct approaches, namely far-Western blotting and GST pull-down experiments, the interaction was found to be enhanced in the presence of estrogen, but not anti-estrogens, consistent with the effects of these ligands on the transcriptional activity mediated by the hormone binding domain. Furthermore, the *in vitro* binding activity of RIP140 to estrogen receptor mutants correlates with the effects of the mutations on transcriptional activity (Danielian *et al.*, 1992). Thus both hormone-dependent RIP140 binding and transcriptional activation were abolished when either pair of hydrophobic residues were substituted with alanines and reduced when the charged residues were replaced with their corresponding amides. In all cases the mutations had no significant effect on the steroid and DNA binding activity of the receptors, indicating that these residues are specifically involved in transcriptional activation (Danielian *et al.*, 1992).

In accordance with the proposal that transcriptional activators are involved in the assembly of transcription pre-initiation complexes (Ptashne, 1988; Mitchell and Tjian, 1989), estrogen receptors have been demonstrated to stabilize the formation of such a complex in a cell-free transcription system (Elliston *et al.*, 1990). The estrogen receptor has been shown to interact *in vitro* with TFIIB (Ing *et al.*, 1992) and with the TATA box binding protein TBP (Sadovsky *et al.*, 1994), but these interactions were unaffected by mutations in the conserved amphipathic α-helix which abolish transcriptional activity of the receptor in mammalian cells (Sadovsky *et al.*, 1994; G.Lopez and P.J.Kushner, unpublished results) and were similar

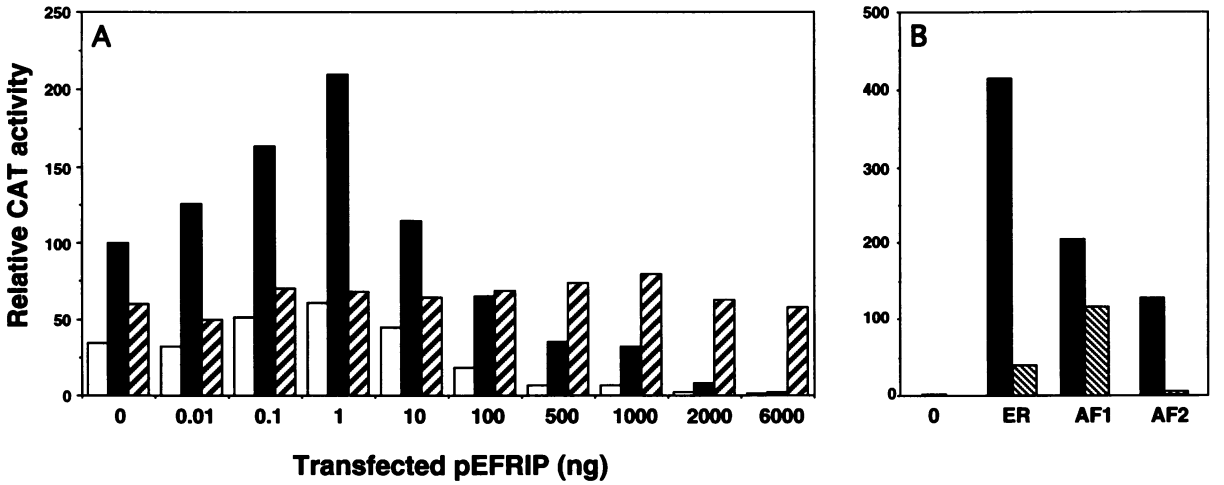

Fig. 6. Effect of RIP140 on transcriptional activation by the estrogen receptor. (**A**) The ability of increasing amounts of RIP140 (up to 6 μg transfected pEFRIP vector) to modulate the activity of the estrogen receptor (expressed from 1 μg pJ3MOR1–599) was examined by co-transfection with the reporter plasmid pEREBLCAT (5 μg) in CEF cells treated with no added hormone (open bars), 10 nM estradiol (solid bars) or 100 nM 4-hydroxytamoxifen (hatched bars). Transcriptional activation is expressed as relative CAT activity corrected for transfection efficiency. (**B**) Effect of RIP140 overexpression on the activity of AF1 and AF2. CEF cells were co-transfected, as described in Materials and methods, with the reporter plasmid pEREBLCAT alone (0) or with pJ3MOR1–599 (ER), pJ3MOR1–339 (AF1) or pMT2MOR182–599 (AF2) in the absence (solid bars) or presence (hatched bars) of 1 μg pEFRIP. The transcriptional activation was expressed after correction for transfection efficiency.

in the presence and absence of estrogen (Figure 7). Furthermore, 'squelching' experiments (Tasset *et al.*, 1990) and *in vitro* transcription assays (Brou *et al.*, 1993a,b) suggest that the hormone binding domain was likely to interact with additional targets distinct from basal transcription factors. Recently the receptor was shown to interact with $hTAF_{II}30$ (Jacq *et al.*, 1994), but this too was unaffected by mutations in the conserved amphipathic α-helix and was retained in the presence of estrogen antagonists, suggesting that this factor might not be the main target of the AF2 domain. In contrast, RIP140 is a nuclear protein whose *in vitro* binding properties to the estrogen receptor make it a good candidate for a role in transcription.

Indeed, we show that RIP140 interacts with the estrogen receptor in intact cells and modulates its transcriptional activity in transient transfection experiments. The effects were ligand-specific, in that the stimulation upon introduction of small amounts of RIP140 expression vector was observed in the presence of estrogen but not 4-hydroxytamoxifen, consistent with the *in vitro* binding properties of RIP140. The modest stimulation of estrogen receptor transactivation by RIP140 might be accounted for by the presence of endogenous protein in mammalian cells, giving rise to a high basal receptor activity, but cells devoid of RIP140 have yet to be identified. In a similar type of experiment it has been found that overexpression of the CREB binding protein CBP did not potentiate CREB activity in all cell lines (Arias *et al.*, 1994) and the effects of hbrm on glucocorticoid receptor transcriptional activity were only apparent in cells devoid of the endogenous protein (Muchardt and Yaniv, 1993). Alternatively, it is conceivable that RIP140 is part of a multisubunit complex, in which case its increased expression might not lead to transcriptional stimulation if the other components of the complex are in limiting concentrations. The possibility that RIP140 is part of the SWI–SNF complex was examined, since this complex appears to be required in yeast for optimum activity of a number of activators,

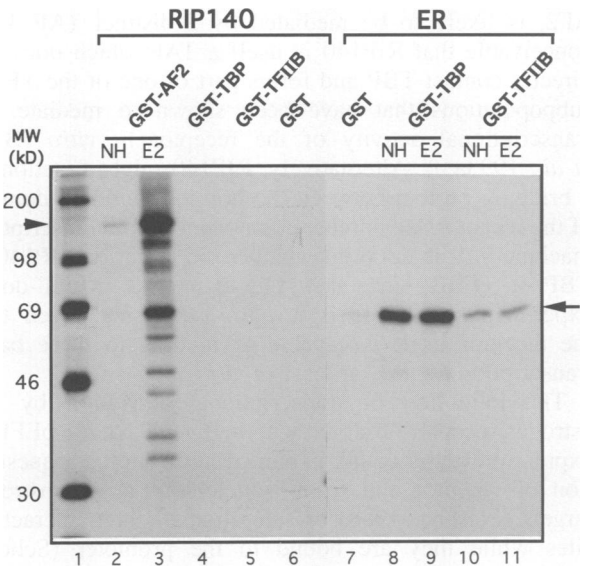

Fig. 7. *In vitro* interaction of RIP140 and the estrogen receptor with TBP and TFIIB. Interaction of ^{35}S-labeled RIP140 (tracks 2–6) and ^{35}S-labeled estrogen receptor (tracks 7–11) with GST alone (tracks 6 and 7) and GST fusion proteins comprising GST–AF2 (tracks 2 and 3), GST–TBP (tracks 4, 8 and 9) and GST–TFIIB (tracks 5, 10 and 11) was tested using the pull-down assay in the absence (NH) or presence of 10 nM estradiol (E2). The position of molecular weight markers is shown in track 1 and those of full-length RIP140 and the estrogen receptor are shown by an arrowhead and arrow respectively.

including steroid hormone receptors (Yoshinaga *et al.*, 1992). Moreover, human homologs of yeast SWI2 stimulated the transcriptional activity of the glucocorticoid, retinoid and estrogen receptors in mammalian cells (Muchardt and Yaniv, 1993; Chiba *et al.*, 1994). However, although the human SWI–SNF complex contains proteins of a similar size to RIP140 (Kwon *et al.*, 1994), rabbit polyclonal antisera MP45 specific for RIP140 failed to cross-react with purified SWI–SNF complex preparations in Western blotting experiments (unpublished data).

It is becoming clear that transcriptional activators are able to interact with multiple targets, for example the transcriptional activator VP16 has been shown to interact not only with TFIIB (Lin and Green, 1991; Lin *et al.*, 1991) and, to some extent, with TBP (Ingles *et al.*, 1991), but also with $dTAF_{II}40$ (Goodrich *et al.*, 1993) and the PC4 adapter that contacts TFIIA (Ge and Roeder, 1994). VP16 contains two distinct activation domains (Regier *et al.*, 1993), of which only one, VP16C, contacts TAFII40, so that Tjian and co-workers have suggested that the other domain, VP16N, might interact with a distinct TAF (Goodrich *et al.*, 1993). The estrogen receptor also interacts with multiple target proteins which appear to contact different regions of the hormone binding domain. Thus its interaction with RIP140 (and RIP160) depends on the functional integrity of the conserved putative amphipathic α-helix required for hormone-dependent transcription, while those of TFIIB, TBP and $TAF_{II}30$ are unaffected by disruptive mutations in this region (Jacq *et al.*, 1994; Sadovsky *et al.*, 1994; G.Lopez and P.J.Kushner, unpublished results). The site of interaction of $TAF_{II}30$ has been mapped to the N-terminal portion of the hormone binding domain, referred to as AF2a (Pierrat *et al.*, 1994), but the transcriptional activity of the major activation domain, AF2, is likely to be mediated by a distinct TAF. It is conceivable that RIP140 is itself a TAF which does not directly contact TBP and forms part of one of the TFIID subpopulations that have been shown to mediate the transcriptional activity of the receptor *in vitro* (Brou *et al.*, 1993a,b). Alternatively, RIP140 might function as a bridging protein between the hormone binding domain of the receptor and another component of the transcription machinery. It is doubtful whether the target of RIP140 is TBP or TFIIB, since they fail to interact in pull-down experiments, but this is not too surprising given that the receptor itself is capable of binding to these basal transcription factors, at least *in vitro*.

The inhibition of transcriptional activation by the estrogen receptor observed with high levels of the pEFRIP expression vector could, in part, result from the sequestration of receptor and from 'squelching' of downstream targets (Ptashne, 1988) or saturation of their interaction sites while they are bound to the promoter (Scholer *et al.*, 1991), thereby preventing them from mediating the transcriptional activity of the estrogen receptor. While appreciable inhibition is unlikely to occur at the levels of RIP140 normally found in mammalian cells, it is conceivable that an increase in the ratio of RIP140 to receptor might reduce the activity of the receptor in the presence of estrogen without affecting its tamoxifen sensitivity. In view of this, it would be worth while determining the relative expression of the estrogen receptor and RIP140 in breast tumors from patients that differ in their response to tamoxifen. In any event, our results support the view that the agonistic effect of anti-estrogens involves distinct targets, since RIP140 has no effect on the transcriptional activity of the estrogen receptor in the presence of 4-hydroxytamoxifen, in contrast to that observed when estrogen is bound to the receptor.

RIP140 is clearly not restricted to target cells for estrogens, but its widespread expression in many types of mammalian cell was not entirely unexpected, given that the interaction of RIP140 with the estrogen receptor appears to require a region that is conserved in other nuclear receptors (Zenke *et al.*, 1990; Danielian *et al.*, 1992; Saatcioglu *et al.*, 1993; Barettino *et al.*, 1994; Durand *et al.*, 1994). It is doubtful whether any mammalian cell is completely devoid of one or more nuclear receptors and preliminary experiments confirm that RIP140 is able to interact with a number of nuclear receptors *in vitro*. It is therefore possible that the role of RIP140 is to function as a bridging protein between components of the basal transcription machinery and members of the nuclear receptor family and thereby modulate their transcriptional activity.

Materials and methods

Isolation of RIP140 cDNA clones

Using poly(A)$^+$ RNA from ZR75-1 human breast cancer cells, a cDNA expression library was constructed in the λZAPII vector using the ZAP cDNA synthesis kit (Stratagene) following the manufacturer's protocol. The first strand cDNA was synthesized using a primer containing a poly(dT) sequence preceded by a *Xho*I site. After second strand replacement, *Eco*RI adapters were ligated to blunt-ended DNA fragments, allowing their directional insertion as *Eco*RI–*Xho*I inserts into the Uni-ZAPXR vector. The library screening was performed using the *in vitro* ^{32}P-labeled GST–AF2 probe, as previously described for far-Western blotting (Cavaillès *et al.*, 1994). Five positive clones (pBRIP1–5) were purified, screened again and pBluescript phagemids containing the inserts of interest were then excised *in vivo* by co-infection with the R408 helper phage. The library was rescreened using a ^{32}P-labeled 576 nt fragment from the 5′-end of the longest clone isolated in the first screening (pBRIP4). An additional cDNA clone containing 1128 extra bp was isolated and the 7247 bp full-length cDNA (pBRIP140) was generated in pBluescript IISK by inserting the insert from pBRIP16 as a *Bam*HI fragment into pBRIP4. Inserts were sequenced by the dideoxy chain termination method using a Sequenase Version 2.0 DNA Sequencing kit (US Biochemicals).

Construction of recombinant vectors

To construct the eukaryotic expression vector for RIP140 (pEFRIP), a 5569 bp *Spe*I fragment containing the 288 bp 5′ UTR, the RIP140 coding sequence and 1.8 kb of 3′ UTR from pBRIP140 was inserted into the *Xba*I site of vector pEF-BOS downstream of the polypeptide chain elongation factor 1α (EF-1α) promoter (Mizushima and Nagata, 1990). In order to generate the RIP–VP16 expression vector, we first mutated the UAA stop codon to GAA in pBRIP3 and incorporated a downstream *Bam*HI site using PCR amplification. We then transferred, in the correct reading frame, a *Bam*HI fragment containing the last 1432 bp of the RIP140 coding sequence, upstream of the region encoding residues 410–490 of the herpes simplex virus (HSV) transcriptional activator VP16, into the SD06 yeast vector (Dalton and Treisman, 1992). An *Eco*RI fragment containing sequences coding for RIP–VP16 was then subcloned downstream of the EF-1α promoter into the pEFLINK vector (constructed by R.Marais). This vector then allowed expression of the C-terminal portion of RIP140 (residues 683–1157) preceded by the peptide MAGSEFGTR and followed by the VP16 activation domain. The same domain of VP16 was also introduced by A.Butler into the pSG5 plasmid (Green *et al.*, 1988). Construction of recombinant vectors allowing expression of full-length (pJ3MOR1–599), AF2 deletion mutant (pJ3MOR1–339), AF1 deletion mutant (pMT2MOR182–599) and nuclear localization deficient mutant (pJ3MORΔ252–307) mouse estrogen receptor has been described elsewhere (Lees *et al.*, 1989; Dauvois *et al.*, 1993; Lahooti *et al.*, 1994). GST–AF2 (residues 313–599) expression vectors were generated by inserting a *Sal*I fragment encoding either the wild-type or mutant proteins into pGEX-2X (Sadovsky *et al.*, 1994). The Gal4–AF2 construct encoding the AF2 domain of the mouse estrogen receptor (residues 313–599) fused to the Gal4 DNA binding domain has been described previously (Cavaillès *et al.*, 1994). The reporter vectors pEREBLCAT and G5E1BCAT contain respectively an estrogen response element (ERE) upstream of the HSV thymidine kinase promoter or five GAL4 DNA binding sites upstream of the E1b TATA box, linked to the reporter gene chloramphenicol acetyl transferase (CAT) (Danielian *et al.*, 1992). The pGL2 control vector (Promega Corp.) was used to monitor transfection efficiency.

GST probes

The expression and purification of GST fusion proteins were carried out as previously described (Cavaillès *et al.*, 1994; Sadovsky *et al.*, 1994) using a modification of the method of Kaelin *et al.* (1991). Fusion proteins were then purified on glutathione–Sepharose beads (Pharmacia) and then either radiolabeled with ^{32}P for screening purposes and far-Western blotting (Cavaillès *et al.*, 1994) or used directly for GST pull-down experiments (Sadovsky *et al.*, 1994).

In vitro protein–protein interaction assays

Far-Western blotting. Cell extracts from *E.coli* expressing pBRIP3 were obtained as described for GST fusion protein production and 150 μg protein were subjected to SDS–PAGE and electroblotted onto nitrocellulose in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.01% SDS). After denaturation/renaturation in 6–0.187 M guanidine–HCl (Calbiochem) in HB buffer [25 mM HEPES, pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)] filters were saturated at 4°C in blocking buffer (5 then 1% milk in HB buffer plus 0.05% NP40) before incubation with ^{32}P-labeled GST–AF2 probes. This was performed overnight at 4°C in H buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP40, 1% milk, 1 mM DTT) using 200 000 c.p.m./ml probe and cold GST to block non-specific binding. After three washes with H buffer, filters were dried and exposed for autoradiography at –70°C.

GST pull-down. Recombinant pBRIP3 or pBRIP140 cDNA in pBluescript was transcribed and translated in rabbit reticulocyte lysates (Promega, Madison, WI) following the manufacturer's instructions. The fusion proteins loaded on glutathione–Sepharose beads were incubated with ligands (100 nM estradiol, 100 nM 4-hydroxytamoxifen or 100 nM ICI182780) for 30 min at 4°C and then incubated with the ^{35}S-labeled proteins, *in vitro* expressed from pBRIP140 or pBRIP3, for 1.5 h at 4°C in a total volume of 150 μl IPAB buffer [150 mM KCl, 0.02 mg/ml bovine serum albumin (BSA), 0.1% Triton, 0.1% NP40, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.9, protease inhibitors]. Beads were washed five times with IPAB without BSA. The beads were then dried under vacuum, resuspended in 20 μl loading buffer and analyzed by SDS–PAGE. Signals were amplified by fluorography for ^{35}S extracts (Amplify; Amersham) and gels exposed at –70°C.

Northern blotting

Total RNA was isolated as described using phenol:chloroform (50:50) extraction (Ham *et al.*, 1988). The RNA (20 μg) was then fractionated on a 1% formaldehyde–agarose gel, transferred onto a nylon membrane (Pall Biodyne) and prehybridized as previously described (Dauvois *et al.*, 1992). Probes corresponding to pBRIP3 and γ-actin (Enoch *et al.*, 1986) were ^{32}P-labeled *in vitro* (sp. act. 1.5×10^8 c.p.m./μg DNA) using the Ready-To-Go DNA labeling kit (Pharmacia) according to the manufacturer's conditions and hybridized overnight to the blots. After washing (Dauvois *et al.*, 1992) blots were exposed for autoradiography and quantification was done using a Molecular Dynamics PhosphorImager. RIP140 mRNA levels were corrected for the γ-actin signals and expressed as percent of the highest level (Hep G2).

Fluorescence in situ hybridization

Chromosomal localization of the RIP140 cDNA was by fluorescence *in situ* hybridization using standard procedures (Senger *et al.*, 1993). A 500 μg aliquot of the pBRIP140 probe was mixed with 4 μg Cot1 DNA (BRL) and hybridized to reversed banded metaphase spreads, together with probe pZ21A (donated by A.Baldini), which is specific for the centromeres of chromosomes 13 and 21.

Cell culture and transient transfection experiments

Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 (T47D, JAR, Butler) containing 10% fetal calf serum and, in the case of CEF cells, 1% chicken serum (Gibco, Paisley, UK). CEF cells were transfected using calcium phosphate co-precipitation as previously described (Danielian *et al.*, 1992). The transfected DNA included a reporter plasmid pEREBLCAT (5 μg), the pGL2 internal control plasmid (1 μg), pJ3MOR (0.5 μg) in the presence of variable amounts of vectors pEF-RIP or pEF-BOS to a total of 10 μg DNA/dish. In the two-hybrid assay the reporter was G5E1BCAT (1 μg) and GAL4–AF2 (1 μg) was transfected in the absence or presence of RIP–VP16 (3 μg) or VP16 (3 μg). Following transfection, cells were maintained in the absence or presence of 10 nM estradiol or 100 nM 4-hydroxytamoxifen as indicated. After 48 h the cells were harvested and extracts assayed for luciferase (De Wet *et al.*, 1987) and CAT activity (Sleigh, 1986). Luciferase activity was used to correct for differences in transfection efficiency in all experiments. COS-1 cells were transfected by electroporation using a Bio-Rad Gene pulser at 450 V and 250 μF as previously described (Danielian *et al.*, 1992). Cells were transfected with 12.5 μg pEFRIP in the absence or presence of 12.5 μg nuclear localization-deficient mutant pJ3MOR Δ259–307.

Antibody production

The peptide corresponding to residues 895–910 of RIP140 (see Figure 1A) was synthesized on a model 430A Applied Biosystems Solid Phase Synthesiser and analyzed by reverse phase HPLC and mass spectroscopy. After coupling with glutaraldehyde to keyhole limpet hemocyanin (KLH), this peptide was used to immunize rabbits. The resultant antiserum (MP45) was affinity-purified on a Sepharose column coupled to the immunizing peptide (Hancock and Evan, 1992).

Indirect immunofluorescence studies

The expression of endogenous RIP140 and mutant estrogen receptor following transient transfection was analyzed in COS-1 cells. The rabbit polyclonal antibody MP45 was used to detect RIP140, while the estrogen receptor was detected using the rat monoclonal antibody H222 (Greene *et al.*, 1984). The cells were plated on 22 mm poly-L-lysine precoated coverslips and stained as previously described (Dauvois *et al.*, 1993). In all cases untransfected or transfected cells incubated without first antibody were included as controls. Slides were examined using a Zeiss laser confocal microscope or a Zeiss Axiophot fluorescence microscope.

Accession number

The EMBL accession no. for the sequence reported in this paper is X84373.

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