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The nuclear-receptor interacting protein (RIP) 140 binds to the human glucocorticoid receptor and modulates hormone-dependent transactivation

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Abstract

The glucocorticoid receptor (GR) regulates target gene expression in response to corticosteroid hormones. We have investigated the mechanism of transcriptional activation by the GR by studying the role of the receptor interacting protein RIP140. Both in vivo and in vitro protein–protein interaction assays revealed a ligand-dependent interaction between the GR and RIP140. The ligand binding domain of the GR was sufficient for this interaction, while both the N- and C-terminal regions of RIP140 bound to the receptor. In a yeast transactivation assay RIP140 and SRC-1, a member of the steroid receptor coactivator family of proteins, both enhanced the transactivation activity of a GR protein (GR$\Delta\tau_1$) in which the potent N-terminal τ_1 transactivation domain has been deleted. In contrast, in COS-7 cells increasing amounts of RIP140 significantly inhibited GR$\Delta\tau_1$ function. In cotransfection studies in COS-7 cells, RIP140 also inhibited receptor activity in presence of both SRC-1 and the coactivator protein CBP together. Thus, in yeast cells a stimulation of receptor activity was observed, while in mammalian cells RIP140 repressed GR function. Taken together, these data suggest that, (1) RIP140 is a target protein for the GR and (2) RIP140 can modulate the transactivation activity of the receptor. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

The glucocorticoid receptor (GR) is a ligand activated transcription factor that regulates gene expression in response to corticosteroid hormones (for reviews see [1,2]). The GR in its inactive state is located in the cytoplasm bound to several chaperone proteins, including the heat shock protein hsp90 (see [3] and references therein). Upon binding hormone, hsp90 is released and the hormone-receptor complex translocates to the nucleus and binds as a homodimer to specific palindromic DNA sequences termed glucocorticoid response elements (GRE) associated with target genes (reviewed in [4] and references therein). Depending on the type of GRE and the promoter context, the receptor activates or represses gene transcription. One mechanism whereby the DNA bound GR activates gene expression is thought to involve interactions with components of the general transcription machinery, occurring directly and/or indirectly, via intermediary factors or coactivators (see [5] and references therein).

In keeping with the receptor's role as a transcription factor, discrete domains mediating DNA binding and transcriptional activity have been mapped within the protein. The main transcriptional activity is located in the N-terminus (amino acids 77–262) of the human receptor and termed τ_1 or AF-1 (reviewed in [5]). In addition, a ligand-dependent transactivation function

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(AF-2) has been mapped to the ligand-binding domain (LBD) of the receptor. This includes a region C-terminal of the hinge domain, termed τ_2 (amino acids 526–556) and a highly conserved α-helical region between amino acids 748 and 763 [6]. Significantly, studies with the thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) have shown that the corresponding region in these receptors has intrinsic, albeit weak, transcriptional activity when fused to a heterologous DNA binding domain [7,8].

Initiation of transcription in eucaryotes involves the assembly of a protein complex on the promoter, which includes the RNA polymerase II enzyme and functions to initiate mRNA synthesis. Nuclear receptors, in keeping with other gene regulating proteins, are thought to act by recruiting one or more of the components of the general transcription machinery to the promoter, through binding to basal transcription factors and intermediary cofactors (for recent reviews see [5,9–11]. Thus target proteins for nuclear receptors, within the transcription machinery have been identified that include basal transcription factors (see [12–16]) as well as TATA-binding protein associated factors or TAFs [17–20]. In addition, the use of protein interaction screens has resulted in the identification of an increasing number of putative coactivator proteins for nuclear receptors. These include RIP140, TIF1, RAP 46/Bag-1, ARA70, members of the steroid receptor coactivator (SRC) family SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, ACTR/AIB1/RAC3/pCIP as well as the promiscuous coactivator CREB binding protein (CBP)/p300 (for recent reviews and references see [5,10,11]. Despite considerable efforts in identifying receptor interacting proteins, the role of different putative target proteins for a single receptor is still poorly understood. In order to better understand the mechanisms whereby the ligand-bound GR activates gene transcription, we have studied the binding of the GR with the receptor interacting protein RIP140, originally identified as a protein binding to the estrogen receptor (ER; [21]). Furthermore, the effect of RIP140 on GR-dependent transactivation was investigated using both yeast and mammalian cell culture transcriptional activation assays.

2. Materials and methods

2.1. Plasmid constructs

2.1.1. Two-hybrid vectors

The C-terminal region of human RIP140 (amino acids 431–1158) was excised as a Bgl II fragment from pGAD10–hPIP32 (a two-hybrid clone isolated as a prey for PPAR, see [22]) and cloned into pGBT9 (Clontech) cut with Bam HI, to generate pGDD–RIP140C. The N- and C-terminal parts of hRIP140 (amino acids 1–281 and 431–1158) and the full length hRIP140 (amino acids 1–1158) were cloned into pACT2 to generate pGAD–RIP140N, pGAD–RIP140C and pGAD–RIP140 as described previously [22]. The full length GR (amino acids 1–777) and $GR_{DBD-LBD}$ (amino acids 418–777) were amplified by PCR and the resulting PCR products were cut with Bam HI and cloned into pACT2 (Clontech) to generate pGAD–GR and pGAD–$GR_{DBD-LBD}$. GR LBD (amino acids 485–777) was cut out from pEG202–$GR_{418-777}$ [23] as a Bam HI fragment and cloned into pGBT9 (Clontech) to generate pGDD–GR_{LBD}.

2.1.2. GST-pull down vectors

The expression vector pGEX–RIP140C, encoding RIP140 amino acids 747 to 1158 fused to GST, was constructed as described previously [22].

2.1.3. Vectors for yeast functional assays

The full length GR or GR lacking the τ_1 transactivation domain ($GR\Delta\tau_1$) were cloned as Eco RI fragments into the vector pRS314–NX [24], where the selection marker gene leu2 was exchanged for trp1, to generate pRS–GR and pRS–$GR\Delta\tau_1$ respectively. The *LacZ* reporter pLGZ–TAT has been described previously [25]. The yeast expression vector pYEX–RIP140C (amino acids 431–1158) was constructed by internal deletion of an Asp 718-Xho I fragment (encoding the GAL4 activation domain of pGAD10) from pGAD10–hPIP32. An expression plasmid for steroid receptor coactivator (SRC)-1 (amino acids 1 to 1061; 26) was made by cloning a Bgl II-Sal I fragment into pYEX to generate pYEX–SRC-1.

2.1.4. Vectors for mammalian functional assays

A sequence encoding the hGR lacking the $\tau 1$ transactivation domain ($GR\Delta\tau_1$) [27], was cloned into pCMV4 as a Bgl II-Xba I fragment to generate pCMV–$GR\Delta\tau_1$. Full length SRC-1 and hRIP140 were removed as a Bgl II fragments from pACT–SRC-1 and pACT–RIP140 respectively, and cloned into pSG5 to generate pSG–SRC-1 and pSG–RIP140. pRc/RSV–CBP [28], was a gift from Dr. R. G. Goodman (Vollum Institute, Oregon Health Sciences University, Portland, USA).

2.2. Two-hybrid interactions

The yeast strains HF7c and Y187 were transformed with the vectors pGDD–RIP140C and pGAD–GR or pGAD–$GR_{DBD-LBD}$, respectively, according to manufacturer's instructions (Clontech), and plated out on SD plates lacking tryptophan or leucine respectively. After 2–3 days the yeast were mated according to the manufacturer's instructions (Clontech) and grown on

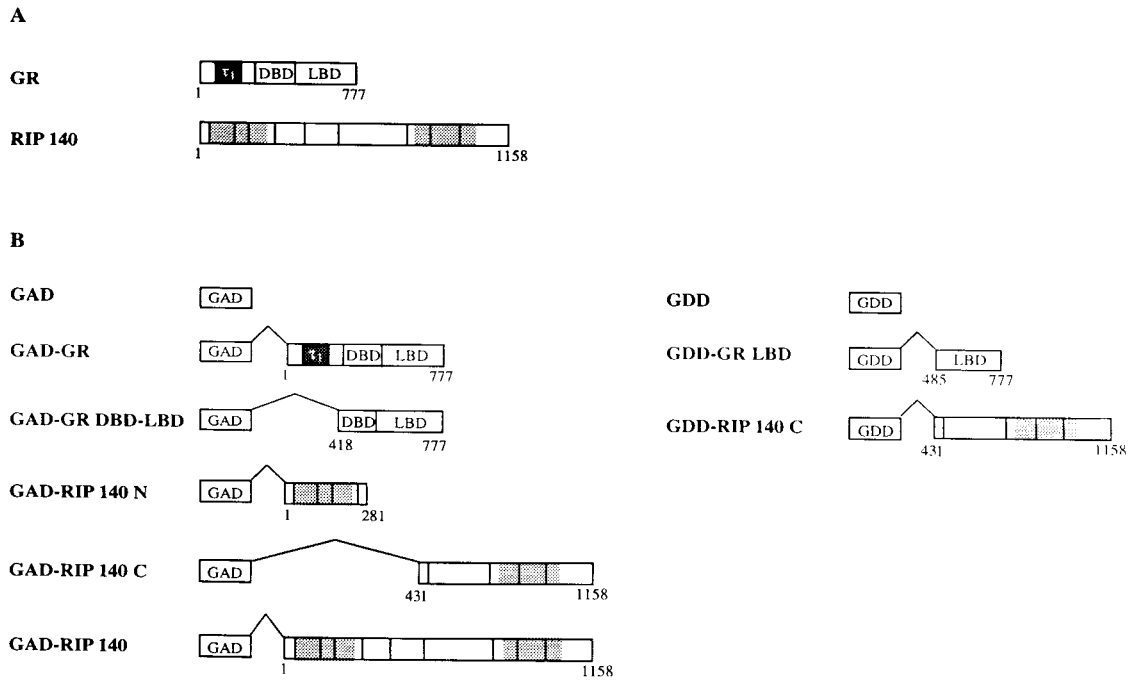

Fig. 1. Glucocorticoid receptor and RIP140 constructs used in two-hybrid analysis. A, Schematic representations of the human GR and the receptor interacting protein RIP140. The DNA (DBD) and ligand (LBD) binding domains of the receptor and the N-terminal transactivation domain (τ_1, amino acids 77–262) are all indicated. The nuclear receptor binding sites, amino acids 27–241 and 753–981, including the LXXLL motifs are indicated by the shaded areas and vertical lines respectively (see [52]). B, Schematic representations of the fusion proteins used in the yeast two-hybrid studies. GAD and GDD represent the Gal4 transactivation domain (amino acids 768–881) and DNA binding domain (amino acids 1–147) respectively.

plates lacking tryptophan and leucine. After a further 2–3 days the colonies were streaked out on plates lacking tryptophan and leucine or plates lacking tryptophan, leucine and histidine, in the presence or absence of 50 μM triamcinolone acetonide. Possible interactions were assayed for by blue color formation in a filter assay (Clontech) or by quantitating β-galactosidase activity in liquid culture assay [25].

2.3. In vitro protein–protein interactions

2.3.1. Expression and purification of recombinant receptor proteins

Bacterial cells expressing GST or GST–RIP140C were harvested by centrifugation and the cell pellets resuspended in buffer A (10 mM Tris–HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) and stored at −70°C. Soluble protein lysates were prepared and incubated with GST–Sepharose beads (Pharmacia) for 30 min (GST alone) or overnight (GST–RIP140C), and the unbound proteins subsequently removed by washing with PBS.

2.3.2. GST-pull down

The full-length GR from vector pGEM–7-GRα was synthesized in vitro in the presence or absence of 1 μM dexamethasone, using T7 polymerase and the TNT coupled rabbit reticulocyte lysate system (Promega) supplemented with [^{35}S]-Methionine (Amersham). The GST–Sepharose beads with the coupled GST or GST–RIP140C were washed with 1 × 10 ml PBS and 2 × 1 ml PBS and transferred into pull down buffer (PDB: 20 mM Hepes–KOH, pH 7.9, 10% glycerol, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 0.1% Tween 20) + 0.02 mg/ml BSA to give a 50% slurry of beads. Samples (20–100 μl) of the beads were then incubated with ^{35}S–GR in the absence or presence of 1 μM dexamethasone for 2.5 h at 4°C in a total volume of 230 μl PDB containing 0.02 mg/ml BSA. Subsequently the beads were washed with 5 × 200 μl PDB and resuspended in 20 μl SDS-sample buffer. The bound proteins were resolved on a 12.5% polyacrylamide SDS-gel and visualized by autoradiography. The gels were scanned using an Image Densitometer (Model GS-690, Bio Rad Laboratories) and the bound GR quantitated using the Multi-Analyst program (Bio Rad Laboratories).

2.4. Functional assays

2.4.1. Yeast

The yeast strain W303-1A was transformed with the

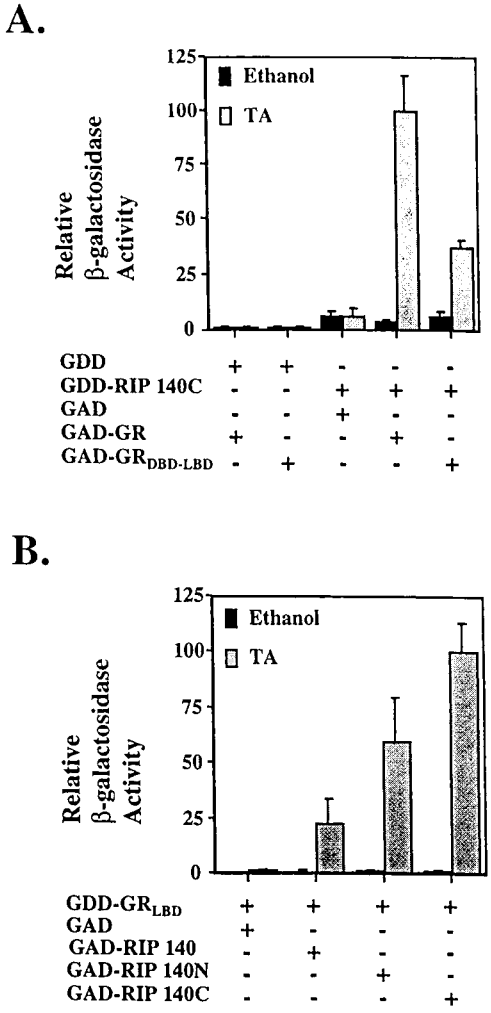

Fig. 2. Ligand-dependent interaction between the glucocorticoid receptor ligand binding domain and RIP140 in a yeast two-hybrid assay. A, The yeast strains HF7c and Y187 were transformed with the vector expressing GDD-fused to a C-terminal fragment of RIP140 (GDD–RIP140C) and the vectors expressing GR or $GR_{DBD–LBD}$ fused to the GAD (GAD–GR, GAD–$GR_{DBD–LBD}$), respectively, or the empty vectors expressing only GDD and GAD. The two yeast strains were mated and positive clones grown in selective, minimal medium in the presence of 50 μM triamcinolone acetonide (TA) or ethanol vehicle and β-galactosidase activity measured in a liquid culture assay. The graph shows the mean and standard deviation of four independent experiments measured in quadruplicates and the activity in the presence of both RIP140C and full length GR is set at 100%. B, The yeast strain HF7c was transformed as in (A) with the vector expressing GAD-fused to the full length, or N- or C-terminal fragments of RIP140 (GAD–RIP140, GAD–RIP140N or GAD–RIP140C, respectively), or the empty vector (GAD), while the yeast strain Y187 was transformed with the vector expressing GR_{LBD} bound to the GDD (GDD–GR_{LBD}). The assay was performed as in (A). The graph shows the mean and standard error of two independent experiments measured in duplicates and the activity in presence of both RIP140C and GR_{LBD} is set at 100%.

GR expression plasmid pRS–GRΔτ_1 together with pLGZ–TAT and pYEX, pYEX–RIP140C or pYEX–SRC-1 using lithium acetate [29] and the cells grown on selective plates in the absence of tryptophan, leucine and uracil. Colonies were grown overnight in selective minimal medium and the cultures diluted to an $OD_{600nm} = 0.05$ and ligand added to a final concentration of 0, 10 or 25 μM triamcinolone acetonide (TA). The cells were grown to $OD_{600nm} = 0.3$, and harvested and stored at −20°C, before they were assayed for β-galactosidase activity and protein concentration [25].

2.4.2. Mammalian cells

COS-7 cells were split onto 5 cm dishes (3×10^4 cells per dish) and incubated at 37°C overnight. The cells were transiently cotransfected with 0.3 μg pCMV–GRΔτ_1 and 5 μg p19LucTK, containing two GREs [27] in the absence or presence of increasing amounts of pSG–RIP140, pSG–SRC-1 or pRc–CBP using DOTAP, according to the manufacturer's instructions (Boehringer Mannheim). After 24 h incubation, dexamethasone or ethanol vehicle was added to a final concentration of 1 μM and the cells incubated for a further 24 h before being harvested and assayed for luciferase activity. Total DNA was kept constant by addition of the corresponding parental vector.

3. Results

3.1. GR_{LBD} interacts with RIP140 in a yeast two-hybrid assay

Most, if not all, the receptor interacting proteins that have been identified and cloned to date appear to interact with more than one member of the nuclear receptor superfamily (see [5] and references therein). RIP140 was originally identified as an ER-binding protein [21] and has more recently been shown to interact with the TR, RAR, retinoid X receptor (RXR) [30] and peroxisome proliferator-activated receptor (PPAR; [22]).

In order to test whether RIP140 could also interact with the GR, a two-hybrid interaction assay was utilized: the GR was fused to the Gal4 transactivation domain (GAD) and RIP140, lacking the first 430 amino acids (RIP140C), was fused to the Gal4 DNA binding domain (GDD) (Fig. 1). Coexpression of GDD–RIP140C and GAD–GR resulted in a strong ligand-dependent (triamcinolone acetonide, TA) activation of the *LacZ* reporter gene (34-fold; Fig. 2A). Thus, indicating a ligand-dependent interaction between the human GR and RIP140. In order to delineate the region of the receptor involved in this interaction, a GR construct lacking the N-terminal transactivation domain ($GR_{DBD–LBD}$) was fused to the Gal4 transactivation domain and tested for binding to

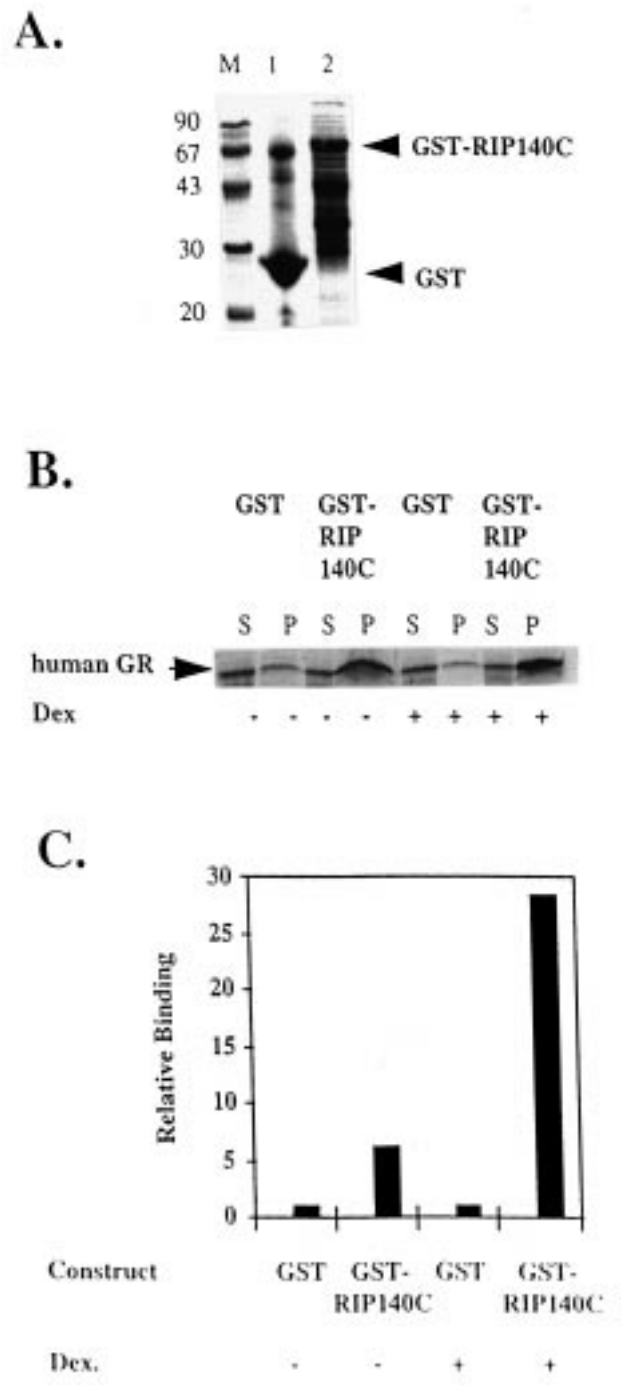

Fig. 3. Direct binding of RIP140 by the human glucocorticoid receptor. A, GST and GST–RIP140C were expressed in bacteria and bound to glutathione–agarose beads as described in the Materials and methods and analysed by SDS-PAGE. The lane M represents the molecular weight markers and lanes 1 and 2 represent GST and GST–RIP140 respectively. B, Equal amounts of GST or GST–RIP140C were incubated with radiolabelled GR in the absence (–) or presence (+) of 1 μM dexamethasone (dex). After centrifugation and washing the material bound to the beads (P) and remaining in the supernatant (S) was analysed by SDS-PAGE and autoradiography. C, The autoradiography of the gel in part (B) was scanned and the material bound to GST–RIP140 quantitated relative to GST alone using the Multi-Analyst program (Bio Rad Laboratories). Note for the gel shown, in the presence of 1 μM dexamethsene less than 1% of the input labelled receptor bound to GST, while 10% bound to GST–RIP140.

RIP140C. As with the full-length GR there was a ligand-dependent increase in *LacZ* transcription when GDD–RIP140C and GAD–$GR_{DBD-LBD}$ were coexpressed (6-fold), but not with either construct alone (data not shown). In control experiments, the parental vector expressing GDD alone, together with GAD–GR or GAD–$GR_{DBD-LBD}$ failed to activate the reporter gene; similarly, GDD–RIP140C together with the parental vector expressing GAD alone showed negligible activity (Fig. 2A) thus indicating the specificity of the interaction.

To further characterize the regions of the GR and RIP140 involved in the interaction, the next series of experiments analyzed the interactions in the reverse orientation. Thus, the GR_{LBD} alone (amino acids 485 to 777) was fused to GDD and tested with full-length or the C- or N-terminal regions of RIP140 fused to GAD (Figs. 1 and 2). As expected, under these conditions a ligand-dependent interaction between the GR_{LBD} and full length RIP140 (80-fold) was seen (Fig. 2B). Significantly, both the N- and C-regions of RIP140 interacted with the GR_{LBD} in a ligand-dependent manner, resulting in 160- and 240-fold activation of the reporter gene respectively. Therefore, as with previously described RIP140-receptor interactions (see above), the binding to GR was ligand-dependent and involved the ligand-binding domain (LBD) of the receptor and either the N- and/or C-terminal regions of RIP140.

SRC-1 and CBP are two other receptor interacting proteins that have been implicated in GR function [26,31,32], and it was of interest to test if they interacted with the receptor protein under similar conditions. In the two-hybrid assay the GR_{LBD} was found to interact with SRC-1 but, interestingly not with the N-terminus of CBP (data not shown). Thus, in vivo experiments in yeast cells show that the GR_{LBD} interacted in a ligand-dependent manner with both RIP140 and SRC-1.

3.2. The GR binds to RIP140 in vitro

To confirm that the interactions seen in yeast represent a direct binding of the GR to RIP140, these interactions were tested in vitro using a GST–RIP140 fusion protein and a [^{35}S]-methionine-labelled receptor. GST and GST–RIP140 were expressed in bacteria and bound to glutathione–Sepharose beads (Fig. 3A). The full-length GR was synthesised in vitro (in the presence or absence of ligand) and incubated with GST or GST–RIP140 in the presence or absence of 1 μM dexamethasone. The beads were recovered and washed extensively with buffer and analysed by SDS-PAGE. Figures 3B and C show a representative gel and quantitative data respectively, for GR recovered in the supernatant (S) and pellet fractions (P). While some

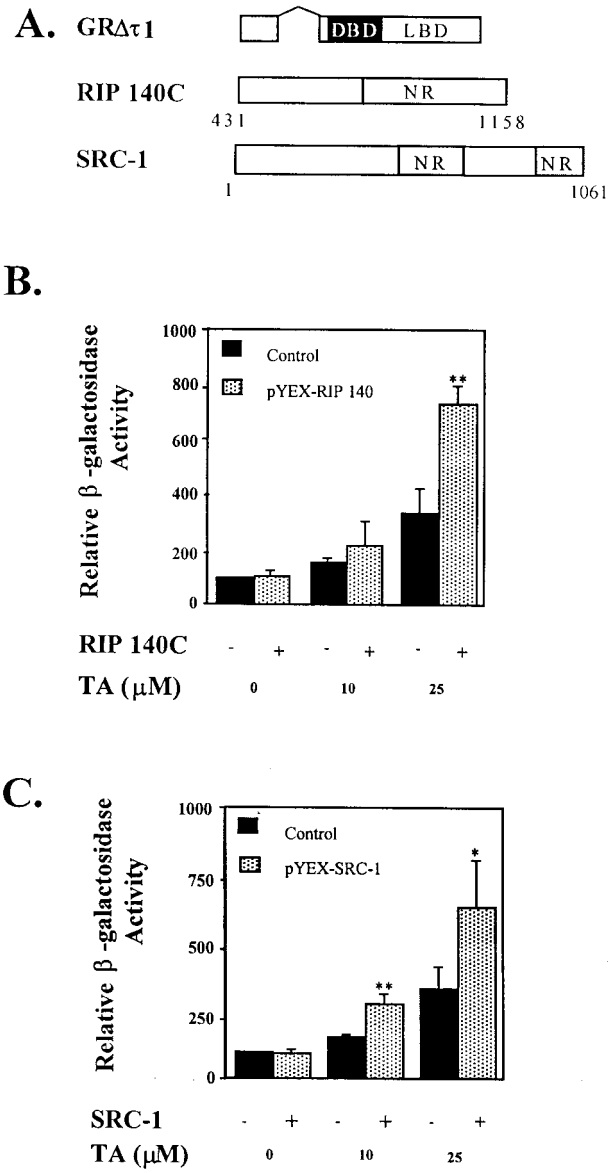

Fig. 4. RIP140 and SRC-1 potentiate GR$\Delta\tau_1$ activity in yeast. A, Schematic drawing of the GR$\Delta\tau_1$, C-terminal 728 amino acids of RIP140 and full length SRC-1 constructs. Nuclear receptor interaction regions are indicated by 'NR' in shaded boxes. B, The expression vector containing the C-terminal region of RIP140 (pYEX–RIP140C) or the control empty vector (pYEX) was cotransformed into the yeast strain W303-1A together with the vector containing the GR with the N-terminal transactivation domain (τ_1) deleted (pRS–GR$\Delta\tau_1$) and the *lacZ* reporter plasmid (pLGZ–TAT). The yeast cells were grown in selective, minimal medium in the presence of increasing amounts of triamcinolone acetonide (TA) or ethanol vehicle and assayed for β-galactosidase activity. The graph shows the mean and standard deviation of three independent experiments performed with three different colonies measured in quadruplicates. C, As in part B except the expression plasmid for full length SRC-1 (pYEX–SRC-1) was used. Transcriptional activity in the presence of RIP140 or SRC-1 relative to vector only control were compared using a student *t*-test: $^{*}p < 0.05$ and $^{**}p < 0.01$. Note, at 25 μM of TA RIP140 and SRC-1 enhanced GR$\Delta\tau_1$ activation by 2.2-fold and 1.9-fold respectively.

binding of the GR to RIP140 was observed in the absence of ligand there was a clear enhancement of binding in the presence of 1 μM dexamethasone (4.5-fold). Under identical conditions there was no significant binding of the receptor to GST alone (Fig. 3B and C). Thus the results of the in vitro binding studies demonstrate that the GR can bind directly to the receptor interacting protein RIP140 and that this interaction is stimulated by the presence of ligand.

3.3. RIP140 and SRC-1 enhance GRΔτ$_1$ activity in yeast

The above binding studies (in vivo and in vitro) clearly showed that GR could bind to RIP140 in a ligand-dependent manner. It was therefore important to determine whether such an interaction can influence the transactivation activity of the receptor. Yeast is a useful model system for these studies as yeast cells do not contain either nuclear receptors or the putative coactivator proteins. Previously, it was shown that the full-length GR, in the absence of coexpression of a coactivator protein, strongly activated a GRE driven reporter gene in yeast cells [25]. Therefore, for the present studies a derivative of the GR lacking the strong N-terminal τ_1 transactivation domain was used (GR$\Delta\tau_1$), and cells were cotransformed with a reporter gene driven by two GREs and expression plasmids for either RIP140C, SRC-1 or parental vector. The levels of receptor-dependent transactivation were then measured in the presence of increasing amounts of TA (0 to 25 μM). Figure 4 shows that GR$\Delta\tau_1$ activated transcription poorly at low ligand concentration (10 μM) but resulted in about a 3-fold activation (solid bars) at the highest concentration of TA used. This contrasts with the activity of the full-length GR which resulted in a 29-fold activation of reporter gene activity in response to 10 μM TA (Ref. [25] and Windahl and McEwan unpublished observations). Significantly, in the presence of RIP 140 or SRC-1 transcriptional activation by GR$\Delta\tau_1$ was increased 6 to 7 fold (Fig. 4B and C, shaded bars). Thus, in yeast cells, both RIP140 and SRC-1 function as coactivator proteins to potentiate the hormone-dependent transactivation activity of GR$\Delta\tau_1$.

3.4. RIP140 inhibits GRΔτ$_1$ activity in COS-7 cells

To further determine the role of RIP140 in GR-dependent gene regulation, a mammalian cell transfection system was used. Transient transfection of COS-7 cells with the expression plasmid pCMV–GR$\Delta\tau_1$ resulted in a 7- to 10-fold activation of a reporter gene activity in a ligand dependent manner (Fig. 5A). Cotransfection of increasing amounts of a RIP140 ex-

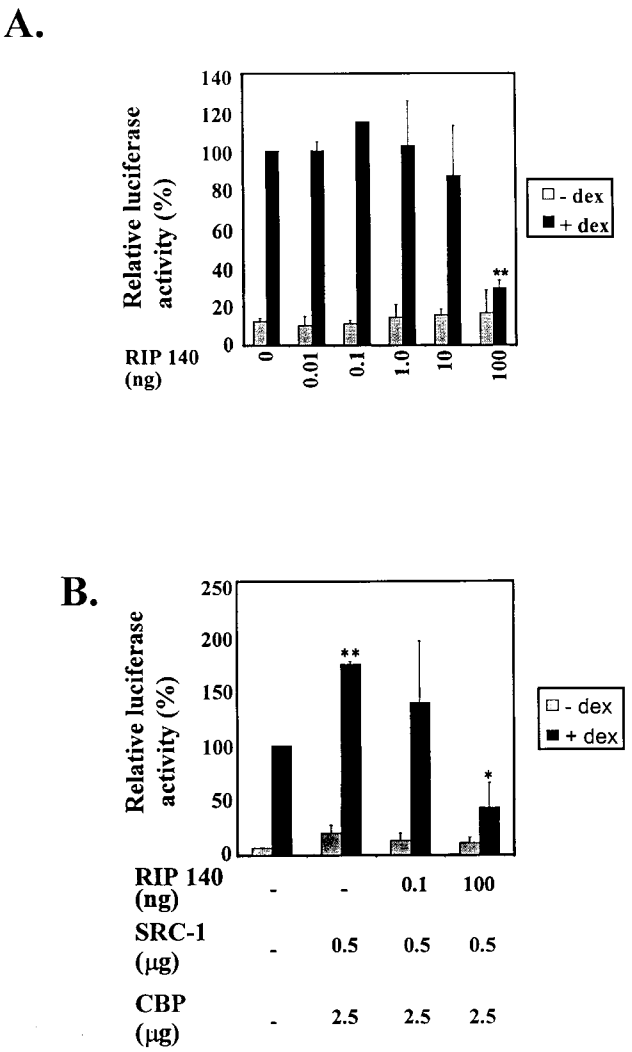

Fig. 5. The effect of RIP140, SRC-1 and CBP on GRΔτ$_1$ activity in COS-7 cells. A, COS-7 cells were transiently transfected on 50 mm plates with 0.3 μg vector containing GR with the strong N-terminal transactivation domain (τ$_1$) deleted (pCMV–GRΔτ$_1$), 5 μg reporter plasmid containing 2 × GREs (p19LucTK) and increasing amounts of expression vector for RIP140 (pSG–RIP140). The total amount of DNA was kept constant by transfecting the empty vector pSG5. The cells were incubated at 37°C for 24 h before induction with 1 μM dexamethasone (dex) or ethanol vehicle, and incubated for a further 24 h before harvesting. Luciferase activity was measured in duplicates. Data from at least two independent experiments, assayed in duplicate, were compared using the student *t*-test: $^{**}p < 0.01$. B, COS-7 cells were transiently transfected as described in part (A) with the indicated amount of expression vector for RIP140 (pSG–RIP140), SRC-1 (pSG–SRC-1) and CBP (pRc–CBP). Total transfected DNA concentration was kept constant at 8.3 μg with the empty vectors pSG5 and pRc–RSV. Data from at least two independent experiments, assayed in duplicate, were compared using the student *t*-test: $^{*}p < 0.05$ and $^{**}p < 0.01$.

pression plasmid (0.01 to 100 ng) did not significantly increase this activity. Instead, at concentrations above 10 ng RIP140 significantly inhibited dexamethasone-induced transactivation by GRΔτ$_1$ (Fig. 5A). In contrast, similar experiments with SRC-1 resulted in a modest stimulation (range 1.3- to 2.3-fold) of GRΔτ$_1$ activity, while CBP failed to modify the response of the receptor in this cell culture system (data not shown).

The failure of RIP140 and CBP to further stimulate GR activity, and the modest effects of SRC-1 in this system may reflect that the endogenous levels of these proteins in COS-7 cells are already optimal for receptor function, so that any further increase in any one factor has no or little positive effect. Alternatively, these proteins may function as part of a coactivator complex, such that increasing the concentration of one component would have little effect on the overall activity of the complex. Recently, there has been evidence for CBP, members of SRC-1/p160 family of proteins [32,33] and additional proteins [34] existing and functioning as such a complex. Therefore, to test this possibility with respect to the GR-mechanism of action, COS-7 cells were cotransfected with GRΔτ$_1$ and combinations of RIP140, SRC-1 and CBP.

Figure 5B shows that cotransfection of SRC-1 together with CBP resulted in a modest but reproducible stimulation of transactivation by GRΔτ$_1$. No further increase in GRΔτ$_1$ activity was observed when all three receptor interacting proteins were cotransfected. Indeed, at a higher concentration of RIP140 expression plasmid, stimulation of GRΔτ$_1$ activity by SRC-1 and CBP was inhibited (Fig. 5B). Interestingly, the degree of inhibition by RIP140 was the same as observed in the absence of SRC-1 and CBP (70–75%, compare Fig. 5A and B). Thus in a mammalian cell background RIP140 could have a repressive effect on GR-dependent gene regulation.

4. Discussion

In this study we have demonstrated a direct interaction between the GR and the receptor interacting protein RIP140. This is the first report of an interaction between the GR and RIP140, which was originally identified as an ER-binding protein [21] and subsequently found to bind to the RAR, RXR and the TR [30]. More recently, RIP140 has also been shown to bind to the PPARα and γ, and to modulate receptor-dependent transactivation [22]. As with these previous interactions, the binding of RIP140 to the GR required the receptor ligand-binding domain (LBD) and was dependent on the presence of ligand in vivo and in vitro. In a yeast transactivation assay the activity of a truncated GR, containing the ligand-dependent AF-2 transactivation function, was further stimulated by coexpression of RIP140. This is consistent with a role for RIP140 as a coactivator of GR AF-2 function and confirms recent reports [30] identifying

the presence of a transactivation function within the C-terminal region of RIP140. Furthermore, in a similar yeast transactivation assay, RIP140 was demonstrated to enhance transcriptional activation by the ER and RAR [35]. In contrast, in a mammalian cell-culture system no significant enhancement of ligand-dependent transactivation by the GR was observed by cotransfection of RIP140. However, increasing amounts of transfected RIP140 expression plasmid DNA did result in a significant inhibition of transcription. Similar results have been reported when RIP140 was cotransfected with the ER [21] and PPAR [22] as well as for the receptor interacting proteins TIF1 and GRIP1/TIF2 with the RXR and GR respectively [36–38]. This is thought to be due to endogenous levels of the putative coactivator being already optimum for receptor function in the cell line used, such that any additional increase in coactivator levels leads to an inhibition of transcription through squelching. Thus, the observed inhibition would be indirect and result from an increase in the concentration of the coactivator. While this remains a formal possibility, a consideration of the very low amounts of RIP140 expression plasmid required to observe inhibition, compared to the amount needed for SRC-1 and CBP, which were also expressed from strong promoters, suggests to us that the inhibition of GR dependent transactivation may be direct and not the result of squelching (see also below).

It is becoming clear that regulation of gene expression by steroid receptors involves multiple-protein–protein interactions (see Introduction). In the case of the GR a number of different target factors have been implicated in receptor-dependent transactivation, including members of the SRC family of proteins, SRC-1a [26,39], GRIP1/TIF2 [37,40] and ACTR [41], the promiscuous coactivators CBP/p300 [31,32], the Ada/Gcn5 coactivator complex [42,43], GRIP170 [44], Rsp5/hRPF1 [45] and components of the general transcription machinery [15,46]. Evidence to-date from both yeast and mammalian cell based transactivation assays support the model that SRC proteins and CBP mediate ligand-dependent activation involving the AF-2 of the receptor, while the Ada complex is involved in the activity of the AF-1/τ_1 domain. Interestingly, although these different factors can be thought of as 'bridging proteins' linking the DNA-bound receptor with the transcriptional machinery there is growing evidence indicating that some of these factors have intrinsic enzymatic activities that suggest a more direct role in transactivation. Thus CBP [47,48] and possibly members of the SRC family [41,49] have histone acetyltransferase activity, while TIF1α, a factor originally identified as a coactivator for the RXR [36], has recently been reported to be a protein kinase [50]. Thus receptor coactivator proteins need not necessarily perform a static role as bridging proteins but may indeed actively participate in the transcription reaction by modifying chromatin structure and/or the activities of components of the transcriptional machinery.

What is the function of RIP140? A number of properties of RIP140 are consistent with a role as a nuclear receptor coactivator protein. These include the ability to bind in a ligand-dependent manner with both steroid ([21] and the present study) and nonsteroid receptors [22,30], the requirement for an intact receptor transactivation function [21,22,30] and the ability to enhance receptor-dependent transactivation under certain conditions ([35,51] and the present study). However, while there is growing evidence to support the role of the SRC family of proteins and CBP as coactivator proteins, the precise function of RIP140 remains less clear. In this study we have shown a ligand-dependent interaction between the GR and RIP140 and demonstrated in a yeast transactivation assay that RIP140 could act as a coactivator for the receptor AF-2 transactivation function. Significantly, in mammalian cells RIP140 inhibited transactivation by GR alone and in the presence of cotransfected SRC-1 and CBP, which in the absence of RIP140 stimulated GR activity. This suggests that RIP140 may modulate GR-dependent transactivation by competing for binding of positive coactivators. Evidence for such a mode of action has recently been obtained in studies demonstrating that RIP140 could compete for binding of SRC-1 to PPARγ [22]. Thus, the observed inhibitory action of RIP140 could result from direct competition for binding to the receptor of SRC-1 and/or CBP and preventing the formation of productive complexes. Additional experiments will be required to address if this is the mechanism by which RIP140 modulates GR function. Furthermore, although previous studies have eliminated interaction of RIP140 with the general transcription factors the TATA-binding protein (TBP) and TFIIB [21], it remains a formal possibility that RIP140 binds to other components of the transcriptional machinery, resulting in the assembly of abortive preinitiation complexes. Thus, further studies will be required to identify specific targets for RIP140, as this will be essential in order to resolve the role of this receptor interacting protein in GR-dependent gene regulation and nuclear receptor function in general.

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