Nucleic Acids Research, 1996, Vol. 24, No. 22 **4379–4386**

Two receptor interaction domains in the corepressor, N-CoR/RIP13, are required for an efficient interaction with Rev-erbAα and RVR: physical association is dependent on the E region of the orphan receptors

Michael Downes, Les J. Burke, Peter J. Bailey and George E. O. Muscat*

University of Queensland, Centre for Molecular and Cellular Biology, Ritchie Research Laboratories, B402A, St Lucia, 4072 Queensland, Australia

Received September 6, 1996; Revised and Accepted October 7, 1996

ABSTRACT

Rev-erbAα and RVR/Rev-erbβ/BD73 are orphan steroid receptors that have no known ligands in the 'classical sense'. These 'orphans' do not activate transcription, but function as dominant transcriptional silencers. The thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) act as transcriptional silencers by binding corepressors (e.g. N-CoR/RIP13 and SMRT/TRAC-2) in the absence of ligands. The molecular basis of repression by orphan receptors, however, remains obscure, and it is unclear whether these corepressors mediate transcriptional silencing by Rev-erbAα and RVR. Recently, two new variants of N-CoR have been described, RIP13a and RIP13Δ1. The characterisation of these splice variants has identified a second receptor interaction domain (ID-II), in addition to the previously characterised interaction domain (ID-I). This investigation utilised the mammalian two hybrid system and transfection analysis to demonstrate that Rev-erbAα and RVR will not efficiently interact with either ID-I or ID-II separately from RIP13a or RIP13Δ1. However, they interact efficiently with a domain composed of ID-I and ID-II from RIP13a. Interestingly, the interaction of Rev-erbAα and RVR is strongest with ID-I and ID-II from RIP13Δ1. Detailed deletion analysis of the orphan receptor interaction with RIP13/N-CoR rigorously demonstrated that the physical association was critically dependent on an intact E region of Rev-erbAα and RVR. Over-expression of the corepressor interaction domains (i.e. dominant negative forms of N-CoR/RIP13) could alleviate orphan receptor-mediated repression of *trans*-activation by GALVP16. This demonstrated that these regions could function as anti-repressors. In conclusion, these data from two independent approaches demonstrate that repression by Rev-erbAα and RVR is mediated by an interaction of ID-I and ID-II of N-CoR, RIP13a and Δ1 with the putative ligand binding domain of the orphan receptors.

INTRODUCTION

Members of the steroid/thyroid hormone nuclear receptor (NR) superfamily bind specific DNA elements and function as ligand activated transcription factors (1,2). This group includes the 'orphan receptors' which have no known ligands in the 'classical sense' and appear to be the ancient progenitors of this receptor superfamily. The Rev-erb family of proteins are orphan members of the receptor superfamily. Two isoforms of the Rev-erb family have been isolated from mammalian genotypes, Rev-erbAα (3) [also known as Ear-1 (4)] and RVR (5) [also known as Rev-erbβ (6,7) and BD73 (8)]. Major differences between the two isoforms occur within the hyper-variable A/B and D regions of the proteins (8). Both isoforms are expressed in a wide range of tissues and are present in all major organs. Rev-erbAα mRNA is upregulated during adipocyte differentiation but repressed during myogenesis (9,10). These orphan receptors are closely related to the ROR/RZRα gene family (retinoic acid receptor related orphan receptor) and the *Drosophila* orphan receptor, E75A, particularly in the DNA-binding domain (DBD) and the putative ligand-binding domain (LBD) (5,7,8). RVR and Rev-erbAα bind as monomers to an asymmetric $(^{A}/_{T})_6$RGGTCA motif (8,11). The Rev-erb family has also been demonstrated to bind as homodimers to novel HREs consisting of two tandemly arranged AGGTCA motifs, separated by 2 bp with unique 5′ flanking and spacer nucleotides (RevDR-2) (12,13). Reports on the transcriptional properties of the Rev-erb family were initially conflicting. Rev-erbAα was first reported to act as a constitutive activator of transcription through its cognate monomeric asymmetric motif (11). Recently, we and other groups have demonstrated that members of the Rev-erb family are, in fact, dominant repressors of transcription (5,7,8,10,12,14). We have further characterised the repression domain of RVR and Rev-erbAα to a minimal region [~35 amino acids (aa)] in the E-domain, that is highly conserved between Rev-erbAα and β (97%). This region spans the LBD-specific signature motif, (**F**/**WAK**XXXX**F**XX**L**XXX**DQ**X-X**LL**), helix 3, Loop3–4, helix 4 and 5 (identified in the crystal structures of the steroid receptor LBDs) (14,15).

The ability of classical steroid receptors to repress basal transcription has long been established (16–18). The recent

* To whom correspondence should be addressed. Tel: + 61 7 3365 4492; Fax: +61 7 3365 4388; Email: g.muscat@mailbox.uq.oz.au

characterisation of the co-repressors, N-CoR and SMRT/TRAC-2 that interact with unliganded thyroid hormone receptor (TR) and retinoic acid receptor (RAR), has shed some light on the mechanism of NR repression (19–22). However, the molecular basis of repression by these orphans remains obscure. Furthermore, it is unclear whether these corepressors are involved in transcriptional silencing by Rev-erbAα and RVR. Very recently, two variant forms of N-CoR have been identified, RIP13a and RIP13Δ1, that are products of alternate promoter utilisation and alternate splicing (23). Detailed analysis has identified two interaction domains (ID-I and ID-II) in N-CoR/RIP13 that interact with nuclear receptors (23). Thus the present studies investigated whether the Rev-erb family interacted *in vivo* with either ID-I or ID-II from N-CoR/RIP13a and Δ1. We demonstrated that the physical association of the orphan receptors with N-CoR/RIP13 requires both interaction domains and the E-region of Rev-erbAα and RVR. Furthermore, expression of dominant negative forms of N-CoR/RIP-13 could override repression of *trans*-activation by the Rev-erb gene family and function as anti-repressors.

MATERIALS AND METHODS

Primer sequences

GMUQ251 5′-CGCGGATCCCACCATGGAGCTGAACGCAGGAGG-3′
GMUQ252 5′-CGCGGATCCTTAAGGATGAACTTTAAAGGC-3′
GMUQ265 5′-CGCGGATCCGTTCACGAGATGCTGTTCGAT-3′
GMUQ297 5′-GCGAATTCACCNC$^{A}/_{T}{}^{A}/_{G}$TCN$^{G}/_{C}{}^{A}/_{T}$NA$^{A}/_{G}$NGT$^{T}/_{C}$TC$^{G}/_{A}$TA$^{T}/_{C}$TG-3′
GMUQ301 5′-GCGCGTCGACATATG$^{T}/_{A}$CTG$^{G}/_{T}{}^{A}/_{G}$CA$^{T}/_{G}$GA$^{A}/_{G}$ATCTGGGAAG-3′
GMUQ302 5′-GCGTCTAGATGA$^{A}/_{C}$GCAAA$^{T}/_{G}$CG$^{T}/_{C}$ACCAT$^{T}/_{C}$A$^{A}/_{G}{}^{A}/_{C}$A-3′
GMUQ303 5′-GCGCGTCGACATATGTTTGC$^{A}/_{C}$AA$^{G}/_{A}{}^{A}/_{C}{}^{G}/_{A}GAT^{T}/_{C}CC^{T}/_{C}$GGC-3′
GMUQ304 5′-GCGTCTAGAAGC$^{T}/_{C}$TT$^{T}/_{A}$A$^{A}/_{G}$CAG$^{A}/_{G}{}^{T}/_{G}$T$^{G}/_{C}$ACCTG-3′
GMUQ330 5′-GCG GAA TTC ACC ATG CCC CAG ATG GAT GTT TCC-3′
GMUQ331 5′-GCG GAA TTC TCA CTC ATA GGG CTC TGA TGG-3′

Plasmids

The expression plasmids pGAL0 (24), pNLVP16 (25), pGALVP16 (10) and pG5E1b-CAT (26) have been described elsewhere. pGAL0 contains the GAL4 DBD, pNLVP16 contains the acidic activation domain of VP16 and pGALVP16 contains the GAL4 DBD linked to the acidic activation domain of VP16.

The construction of the following GAL4 and VP16 chimeric expression vectors have been described elsewhere (15), GAL4-ID-I [GAL-NCoR (ID)], VP16-mRXRγ, VP16-cTRα, VP16-Rev (VP16-Rev aa 21–614), VP16-Rev CDE (VP16-Rev aa 107–614), VP16-Rev DE (VP16-Rev aa 290–614), VP16-Rev E (VP16-Rev aa 437–614), VP16-Rev E-509 (VP16-Rev aa 509–614), VP16-Rev (aa 455–488).

For construction of the following VP16-Rev chimeric expression vectors, the following primers were used to amplify regions of Rev-erbAα from GV-Rev aa 437–614 (10) using *Pfu* DNA polymerase (Stratagene); VP16 Rev aa 437–488 (GMUQ301 and GMUQ302); VP16 Rev aa 437–476 (GMUQ301 and GMUQ304). These fragments containing primer-derived 5′ *Sal*I and 3′ *Xba*I were digested with *Sal*I/*Xba*I and ligated to *Sal*I/*Xba*I-digested pNLVP16. The remaining VP16 chimeras were created by inserting fragments of Rev-erbAα into the pNLVP16 vector. To construct VP16-Rev AB (VP16-Rev aa 21–125), pGAL4-Rev (aa 21–125) (10) was cleaved with *Sal*I/*Xba*I generating a 353 bp insert, which was then cloned into *Sal*I/*Xba*I-digested pNLVP16. To construct VP16-Rev C (VP16-Rev aa 107–199), VP16-Rev CDE (VP16-Rev aa 107–614) (15) was cleaved with *Sal*I/*Xba*I generating a 277 bp insert, which was cloned into *Sal*I/*Xba*I-digested pNLVP16. To construct VP16-Rev CD (VP16-Rev aa 107–290), VP16-Rev CDE (VP16-Rev aa 107–614) (15) was cleaved with *Nde*I–*Eco*RV; the resulting 548 bp fragment was then end filled with Klenow and ligated into *Xho*I-digested Klenow end filled pNLVP16.

Two primers, GMUQ251 and GMUQ252, were used to amplify the 1731 bp open reading frame of RVR from the parent plasmid pCMXRVR (5) with *Ult*ma DNA polymerase (Perkin Elmer). This fragment containing primer-derived *Bam*HI ends was cloned into *Sma*I-digested pBS and was called pBS-RVR. VP16-RVR chimeras were created by inserting fragments of RVR into pNLVP16. To create VP16-RVR (VP16-RVR aa 1–576), the 1745 bp fragment of *Bam*HI-digested pBS-RVR was end-filled with Klenow and ligated with *Sal*I-digested, Klenow end filled pNLVP16. To construct VP16-RVR AB (VP16-RVR aa 1–88), the 1745 bp fragment of *Bam*HI-digested pBS-RVR was digested with *Hin*fI and the 273 bp fragment was end filled with Klenow and cloned into *Sal*I-digested, Klenow end filled pNLVP16. VP16-RVR ABCD (VP16-RVR aa 1–276) was created by inserting the Klenow end filled, 837 bp fragment of a *Sph*I/*Bgl*II digestion of the 1745 *Bam*HI fragment from pBS-RVR into *Sal*I-digested, Klenow end filled pNLVP16. To construct VP16-RVR DE (VP16-RVR aa 170–576), a PCR fragment was amplified from pCMX-RVR with *Ult*ma DNA polymerase using the primers GMUQ265 and GMUQ252. This fragment was digested with *Bam*HI and cloned into *Bam*HI-digested pBSK^{+} and was called pBSK-RVR DE (aa 170–576). VP16-RVR DE was prepared by ligating the end filled, 1236 bp *Bam*HI fragment of pBSK-RVR DE (aa 170–576) into *Xho*I-digested, Klenow end filled pNLVP16. To construct VP16-RVR D (VP16-RVR aa 178–353) and VP16-RVR E (VP16-RVR aa 355–576), the 1236 bp insert generated by *Bam*HI digestion of pBSK-RVR DE (aa 170–576) was digested with *Eco*RI and the 564 and 675 bp fragments were end filled with Klenow and cloned into *Xho*I-digested, Klenow end filled pNLVP16.

For construction of the following GV-RVR chimeras, primers were used to amplify regions of RVR from GALVP16-RVR E (GV-RVR aa 355–576) (14) with *Pfu* DNA polymerase (Stratagene); VP16-RVR (aa 394–449) (GMUQ301 and GMUQ302); VP16-RVR (aa 394–437) (GMUQ301 and GMUQ304); VP16-RVR (aa 416–449) (GMUQ303 and GMUQ302). These fragments containing primer-derived 5′ *Sal*I and 3′ *Xba*I ends were digested with *Sal*I/*Xba*I and ligated to *Sal*I/*Xba*I-digested pNLVP16.

ID-II and ID-IIΔ1 were amplified using *Pfu* DNA polymerase from RIP13a and RIP13Δ1 (23), respectively, using the primers GMUQ 330 and GMUQ 331. ID I+II and ID I+IIΔ1 were amplified using *Pfu* DNA polymerase from N-CoR/RIP13a and RIP13Δ1 using primers GMUQ 330 and GMUQ 297. The resulting products were cleaved with *Eco*RI and ligated with *Eco*RI-cleaved pGAL0 or pSG5 (Stratagene). All GAL, VP16 and GALVP16 and GAL-N-CoR constructs were sequenced to confirm the reading frame.

Mammalian two hybrid assay

Plasmids [pG5E1bCAT reporter (3 μg) and GAL-IDs (1 μg)] were co-transfected/expressed into human choriocarcinoma JEG-3

cells with either VP16 or VP16-Rev or VP16-RVR plasmids (1 µg), then assayed with respect to their ability to *trans*-activate the reporter (pG5E1bCAT). JEG-3 cells were cultured for 24 h in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% charcoal stripped foetal calf serum (FCS). Each six well dish of JEG-3 cells (60–70% confluence) was transiently transfected with plasmid DNA by the DOTAP (Boehringer Mannheim)-mediated procedure as described previously (27,28). The DNA/DOTAP mixture was added to the cells in 3 ml of fresh medium. After 24 h, fresh medium was added and the cells were harvested for the assay of CAT enzyme activity 24 h after the addition of fresh medium. Each transfection experiment was independently performed at least three times to overcome the variability inherent in transfections.

COS-1 transfection

Each 35 mm dish (Falcon) of COS-1 cells (60–70% confluence) was transiently transfected with 3 µg of reporter plasmid DNA (pG5E1bCAT) expressing chloramphenicol acetyl transferase (CAT), 1 µg of GALVP16 chimeras and 1 µg of pSG5 expression vectors by the DOTAP-mediated procedure above. The DNA/DOTAP mixture was added to the cells in 3 ml of fresh medium. After 24 h, fresh medium was added to the cells and cells were harvested for the assay of CAT enzyme activity 36–48 h after the transfection. Each transfection was performed at least three times to overcome the variability inherent in transfections.

CAT assays

Cells were harvested, normalised to protein concentration (29), and CAT activity measured as previously described (30). Aliquots of the cell extracts were incubated at 37°C, with 0.1–0.4 µCi of [^{14}C]chloramphenicol (ICN) in the presence of 5 mM acetyl CoA in 0.25 M Tris–HCl pH 7.8. After a 1–4 h incubation period, the reaction was stopped by the addition of 1 ml ethyl acetate which was used to extract the chloramphenicol and its acetylated forms. Extracted materials were analysed on Silica gel thin layer chromatography plates as described previously (30). Quantitation of CAT assays was performed by an AMBIS β-scanner.

RESULTS

Efficient interaction of Rev-erbAα and RVR with N-CoR/RIP13 is dependent on both interaction domains (ID-I and ID-II)

N-CoR has been demonstrated to mediate inhibition of gene transcription by the thyroid hormone and retinoic acid receptors. Whether N-CoR interacts with orphans such as Rev-erbAα and RVR to mediate the potent transcriptional repression characteristics of these 'orphans' is unclear. To further characterise transcriptional regulation by the Rev-erb family, we investigated whether these orphan receptors interacted with N-CoR/RIP13 *in vivo*. Protein–protein interaction assay systems were developed initially in yeast, and refined further for the study of receptor interactions in transfected mammalian cells (15,31). In these experiments the yeast GAL4 DNA binding domain is fused to various receptor domains (e.g. AB or LBD) and expressed in transfected cells with a second type of hybrid receptor linked to the *trans*-activation domain of herpes simplex virus VP16. *Trans*-activation of a CAT reporter gene downstream of GAL4 binding sites fused to the E1b promoter is only achieved when the co-expressed receptors physically interact.

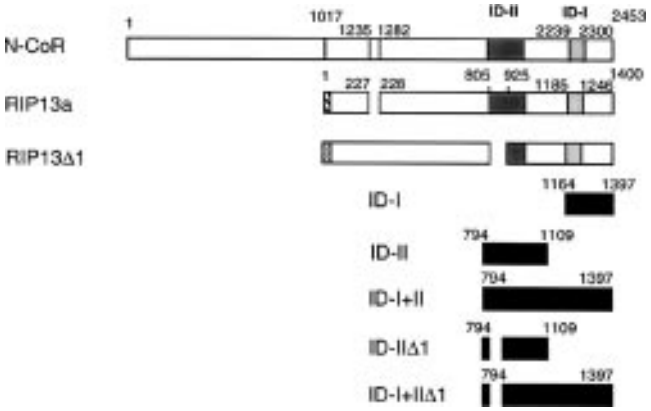

Figure 1. Alignment of the interaction domains (ID) with the similar regions of N-CoR, RIP13a and RIP13Δ1: schematic presentation of the IDs linked to the GAL4 DBD used in the mammalian two hybrid assay. The shaded regions indicate the position of ID-I and ID-II in the the C-terminus of N-CoR/RIP13. Striped areas in the N-termini of RIP13 and RIP13Δ1 represent unique N-terminal regions. Amino acids 1235–1282 in N-CoR are deleted in RIP13a. The region from aa 805 to 925 in RIP13a is deleted in RIP13Δ1. Regions of proteins containing the interaction domains of RIP13a and RIP13Δ1 used in the mammalian two hybrid system are shown in black. Amino acids in the ID-IIΔ1 and ID-I+IIΔ1 constructs refer to the corresponding amino acids in RIP13a.

We constructed chimeric GAL4 plasmids that contained interaction domain I (ID-I in N-CoR, RIP13a and RIP13Δ1 are identical), interaction domain II (ID-II) and ID-I+II. These plasmids were designated, GAL4-ID-I, GAL4-ID-II and GAL4-ID-I+II. We also constructed GAL4-ID-IIΔ1 and GAL4-ID-I+IIΔ1 (see Fig. 1 for specific details). ID-I corresponds to the region between amino acids 2218 and 2451 of N-CoR (19), and to amino acids 1164–1397 in RIP13a (Fig. 1). ID-II corresponds to the region between amino acids 1848 and 2163 of N-CoR and to amino acids 794–1109 in RIP13a. ID-II from RIP13Δ1 has an internal deletion of 120 aa, that lacks aa 805–925 from the RIP13a ID-II (Fig. 1). Seol *et al.* (1996) mapped/delimited the minimal ID-II domain between amino acids 1010/1089 and 2063/2142 in RIP13 and N-CoR, respectively, hence our ID-II region encompasses this minimal domain.

Initially, we examined the ability of these five chimeric plasmids that encoded GAL4-N-CoR/RIP13 hybrid molecules to regulate transcription of an E1b promoter cloned downstream of five copies of the GAL4 binding site linked to the CAT reporter. As shown in Figure 2A, ID-I alone weakly activated transcription (~10-fold) relative to the GAL4 DBD, whereas the other interaction domains failed to activate transcription.

We next examined the ability of co-expressed VP16-Rev-erbAα and VP16-RVR to *trans*-activate gene expression in this mammalian two hybrid system. We also transfected VP16-TRα and VP16-RXRγ as positive and negative controls, respectively, with respect to the ability of these receptors to interact strongly and weakly with N-CoR/RIP13, respectively. We observed that VP16-Rev-erbAα and VP16-RVR failed to interact effectively (i.e. <5-fold) with either ID-I or ID-II separately (Fig. 2B and C, respectively). However, we observed a significant increase (20–40-fold) in CAT activity when either VP16-Rev-erbAα or VP16-RVR was transfected with the GAL-ID-I+II (Fig. 2D). As

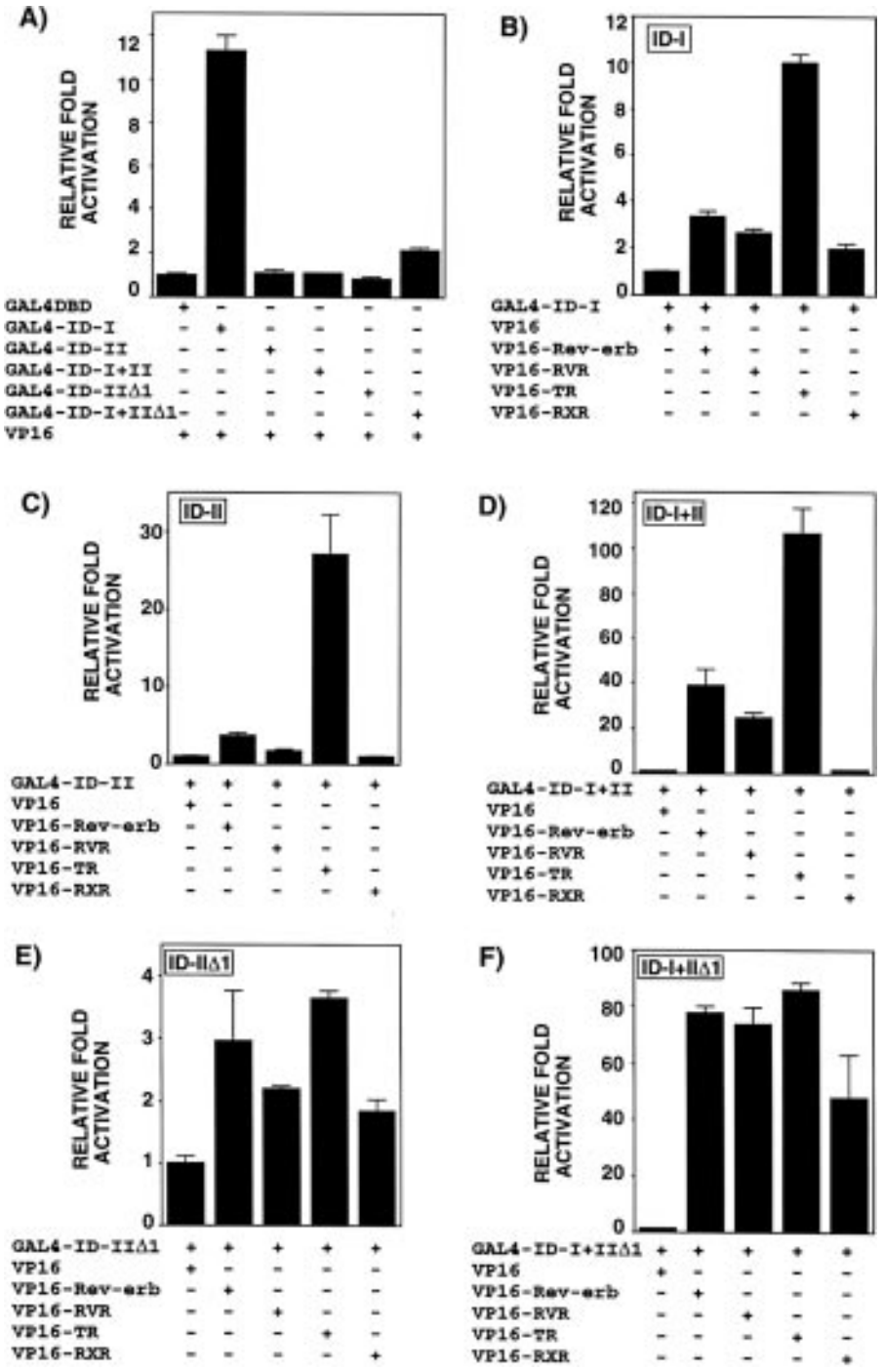

Figure 2. Analysis of the interaction of Rev-erbAα, RVR, TR and RXR with the N-CoR/RIP13 interaction domains. JEG3 cells were co-transfected with 1 µg of each GAL4 and VP16 chimeras as indicated, with 3 µg the reporter pG5E1bCAT. Results shown are mean ± standard deviation (SD) and were derived from at least three independent experiments. **(A)** Transcriptional activation by GALRIP13 ID chimeras. Fold activation is expressed relative to CAT activity measured after transfection with pGAL4 DBD alone. Interaction of Rev-erbAα, RVR, TR and RXR with ID-I **(B)**; ID-II **(C)**; ID-I+II **(D)**; ID-IIΔ1 **(E)**; ID-I+IIΔ1 **(F)**, respectively. Relative fold activation is expressed relative to the CAT activity measured after transfection with the appropriate GAL-ID chimera and VP16 vector alone arbitarily set to 1.0.

expected, neither Rev-erbAα nor RVR effectively interacted (i.e. <5-fold) with ID-IIΔ1 (Fig. 2E). Interestingly, we observed a dramatic interaction between the orphan receptors and ID-I+IIΔ1 that resulted in a 60–80-fold increase in CAT activity when either VP16-Rev-erbAα or VP16-RVR was transfected with the GAL-ID-I+IIΔ1 construct (Fig. 2F).

We saw ~10-, 30- and 100-fold increases in CAT activity when the VP16-TRα construct was transfected with the GAL4-ID-I, GAL4-ID-II and GAL4-ID-I+II constructs, respectively (Fig. 2B–D). This was consistent with the previous studies that demonstrated that TR interacts strongest with ID-I+II, but still interacts significantly with either interaction domain, indepen-

dently (23). As expected, the interaction of RXR with ID-I, ID-II, ID-I+II and ID-IIΔ1 was very weak (<5-fold) (Fig. 2B–E). Surprisingly, RXR significantly interacted with ID-I+IIΔ1 (Fig. 2F), co-transfection of VP16-RXR with GAL4-ID-I+IIΔ1 resulted in a 40-fold increase in CAT expression.

In conclusion, mammalian two hybrid experiments clearly demonstrate that Rev-erbAα and RVR significantly interact with ID-I+IIWT and Δ1. Furthermore, the data suggest that the region between aa 805 and 925 in RIP13a (that is deleted in the Δ1 isoform) may selectively discriminate between specific receptors (e.g. TR or RXR), and that N-CoR/RIP13 splice variants have different functions with respect to ligand-activated and orphan steroid receptors.

The E region of Rev-erbAα and RVR mediates the interaction of these orphan receptors with N-CoR/RIP13

To characterise further the interaction of Rev-erbAα and RVR with the ID-I+II interaction domain from N-CoR/RIP13, we investigated the potential of various domains from each of the orphan receptors to interact with N-CoR in the mammalian two hybrid assay. The chimeric construct consisting of the yeast GAL4 DNA binding domain fused to the interaction domain I+IIΔ1 (ID) was expressed in cells with a set of chimeric constructs containing full length or various deletions of the Rev-erbAα receptor linked to the *trans*-activation domain of VP16 (Fig. 3A). Consistent with the previous experiment, we saw a very strong increase in CAT activity when the VP16-Rev construct was transfected with the GAL4-ID-I+IIΔ1 construct. Furthermore, we observed very strong interactions between the Rev-erbAα CDE, DE and E (aa 437–614) domains with ID-I+IIΔ1, which suggested that the E-region is essential for binding to N-CoR/RIP13 (Fig. 3B). In agreement with the above data, little or no CAT activity was observed when we examined the ability of the Rev-erbAα AB, C, CD domains to interact with the N-CoR/RIP13 interaction domains (Fig. 3B). Detailed attempts to delimit the domain within the E region of Rev-erbAα that interacted with N-CoR/RIP13 were unsuccessful and suggested that a structurally intact ligand binding domain is critical for the interaction with the co-repressor (Fig. 3B).

We similarly analysed a set of chimeric constructs containing full length or various deletions of the RVR receptor linked to the *trans*-activation domain of VP16 (Fig. 4A) in the mammalian two hybrid assay to interact with ID-I+IIΔ1. Consistent with the previous experiment, we saw a very strong increase in CAT activity when the VP16-RVR construct was transfected with the GAL4-ID-I+IIΔ1 construct. Furthermore, we observed very strong interactions between the RVR DE and E (aa 357–576) domains with ID-I+IIΔ1, which suggested that the E-region is essential for binding to N-CoR/RIP13 (Fig. 4B). In agreement with the above data, little or no CAT activity was observed when we examined the ability of the RVR AB, ABCD and D regions to interact with the N-CoR/RIP13 interaction domain (Fig. 4B). Detailed attempts to delimit the domain within the E region of RVR that interacted with N-CoR/RIP13 were again unsuccessful, and suggested that a structurally intact E-region is critical for the interaction of RVR (like Rev-erbAα) with the co-repressor (Fig. 4B).

In summary, the experiments indicated that E region from Rev-erbAα and RVR is necessary for the interaction of these orphan receptors with N-CoR/RIP13. Interestingly, the original N-CoR studies indicated that N-CoR/RIP13 interacted with the hinge regions of TR/RAR (19), our studies in the mammalian two hybrid system clearly indicate that the D region/hinge is not required, in agreement with the hypothesis put forward by Wurtz *et al*. (32).

Over-expression of the RIP13/N-CoR interaction domains alleviates Rev-erbAα/RVR-mediated repression of GALVP16 *trans*-activation: ID-I+II functions as an anti-repressor

We have previously demonstrated that the E regions from the orphan receptors, Rev-erbAα and RVR, when linked to the chimeric/potent *trans*-activator GALVP16, very efficiently repress its ability to constitutively activate the GAL4-dependent reporter, G5E1bCAT (10,14,15).

We investigated the ability of dominant-negative RIP13/N-CoR expression vectors (i.e. pSG5-ID-I+IIΔ1 and pSG5-ID-IIΔ1) versus native RIP13a and RIP13Δ1, to affect the orphan receptor-mediated repression of GALVP16. This would demonstrate that N-CoR/RIP13a mediated the repression of GALVP16 *trans*-activation by the orphan receptors, and that ID-I+II interacted with the orphan receptor E regions (by a different assay). The experiment demonstrated that pSG5-ID-I+IIΔ1 could function as an anti-repressor and partially alleviate (~30%) the orphan receptor-mediated repression of GALVP16 *trans*-activation (Fig. 5A). The plasmid, pSG5-ID-IIΔ1, could not alleviate the RVR-mediated repression of GALVP16, as expected from the mammalian two hybrid data. The native RIP13a and Δ1, that contained the functional interaction regions and repressor domains, did not function as anti-repressors, as expected. Furthermore, these dominant-negative and native N-CoR/RIP13 expression vectors had no effect on the *trans*-activation by wild-type GALVP16 (Fig. 5A). We then examined whether increasing amounts of the N-CoR/RIP13 interaction domain could restore >30% of the GALVP16 activity. As controls, we examined the effects of the increased levels of the N-CoR/RIP13 interaction domain on activation by GALVP16 and the GAL4 DBD, respectively (Fig. 5B). Increased levels (3-fold) of the dominant negative ID-I+IIΔ1 domain more efficiently alleviated RVR-mediated repression of GALVP16 *trans*-activation (Fig. 5B), whereas, the expression of the interaction domain had no effect on the *trans*-activation by GALVP16 or the GAL4 DBD. This suggested that transcriptional silencing by the Rev-erb family of orphan receptors involves N-CoR/RIP13.

DISCUSSION

N-CoR/RIP13 is involved in transcriptional silencing by Rev-erbAα and RVR

It has been demonstrated recently that the co-repressors, N-CoR and SMRT/TRAC-2, mediate transcriptional silencing by unliganded TR and RAR (19–23). These studies shed light on the mechanism of nuclear receptor repression, however, it was unclear whether these corepressors mediated transcriptional silencing by orphan steroid receptors, in particular, Rev-erbAα and RVR.

We had previously demonstrated that the interaction domain-I (aa 2218–2451) of the nuclear receptor co-repressor, N-CoR, did not efficiently associate with Rev-erbAα, and suggested that transcriptional silencing by Rev-erbAα was not mediated by an interaction with N-CoR (15). Our study did not rule out the

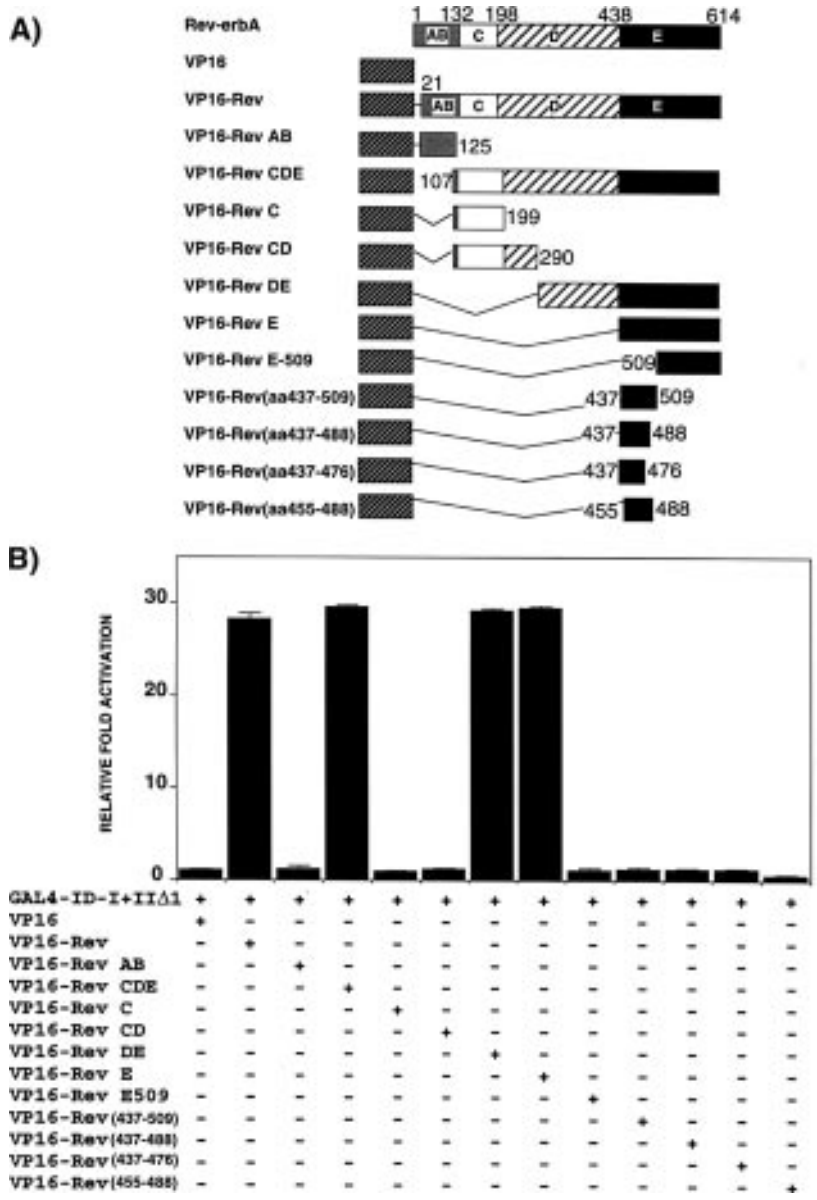

Figure 3. Analysis of the Rev-erbAα region that interacts with ID-I+II Δ1. **(A)** Schematic presentation of the Rev-erbAα regions linked to the VP16 *trans*-activation domain that were utilised in the mammalian two hybrid assay. Note that the E-region begins with Helix 3 as suggested by Wurtz *et al.* (32). **(B)** JEG3 cells were co-transfected with GAL-RIP13 ID-I+IIΔ1 and with the gamut of VP16-Rev chimeras as indicated in the presence of the reporter pG5E1bCAT. Results shown are mean ±SD and were derived from three independent experiments. Fold activation is expressed relative to CAT activity measured after transfection with GAL-ID-I+II Δ1 and VP16 vector alone arbitarily set to 1.0.

possibility that other domains of the co-repressor may interact with Rev-erbAα, or that novel co-repressors were involved in transcriptional repression by Rev-erbAα. During the course of the latter investigation it became evident that the co-repressors were a new family of regulators with alternately spliced variants (22). Two variant forms of N-CoR have been identified, RIP13a and RIP13Δ1, that are products of alternate promoter utilisation and alternate splicing (Fig. 1) (23). Although RIP13a and Δ1 have short unique N-terminal domains that substitute for the first ~1000 aa of N-CoR (that encode the two repression domains), they retain a domain that includes seven copies of a repeated motif, G-s-l-s/t-q-G-t-p, that is present in SMRT and is associated with repressor function (22,23). Detailed analysis of the splice variants identified a second interaction domain (ID-II), between aa 1010/1089 and 2063/2142 in RIP13a and N-CoR, respectively, that is located upstream of the previously characterised interaction domain (ID-I) (23). These latter studies prompted us to examine the molecular basis of Rev-erbAα and RVR transcriptional repression in the light of these exciting developments.

N-CoR/RIP13 interaction with the Rev-erb family requires two receptor interacting domains

Our study demonstrates clearly that N-CoR/RIP13 efficiently interacts with Rev-erbAα and RVR *in vivo*. Although we clearly show that each recently characterised interaction domain (ID-I

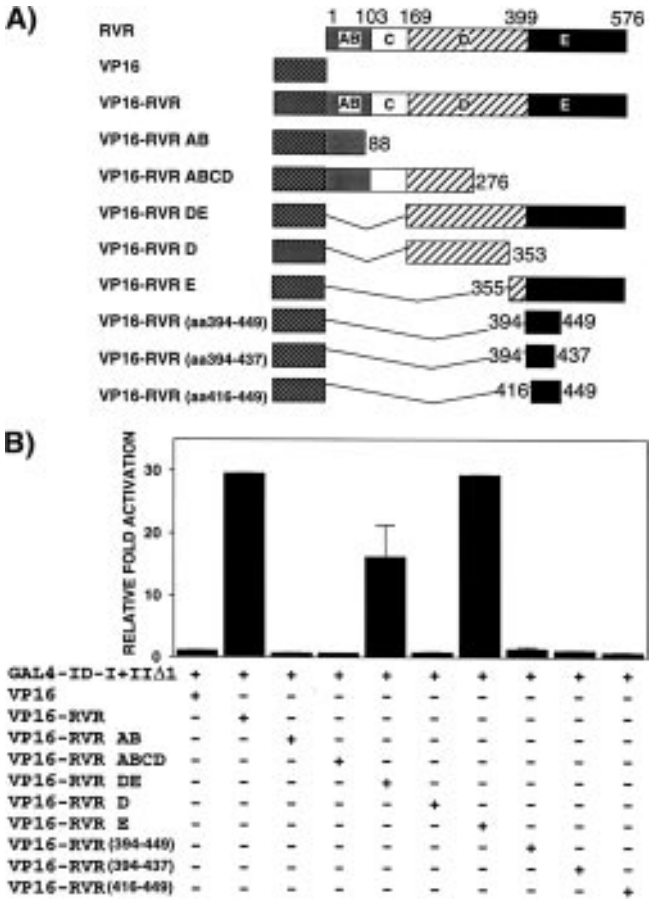

Figure 4. Analysis of RVR interaction with RIP13 ID-I+IIΔ1. (**A)** Schematic presentation of the RVR regions linked to the VP16 *trans*-activation domain that were utilised in the mammalian two hybrid assay. (**B)** JEG3 cells were co-transfected with GAL-RIP13 ID-I+IIΔ1 and VP-RVR chimeras as indicated in the presence of the reporter pG5E1bCAT. Results shown are mean ±SD and were derived from three independent experiments. CAT activity after transfection with GAL-ID-I+IIΔ1 and VP16 vector alone arbitarily set to 1.0.

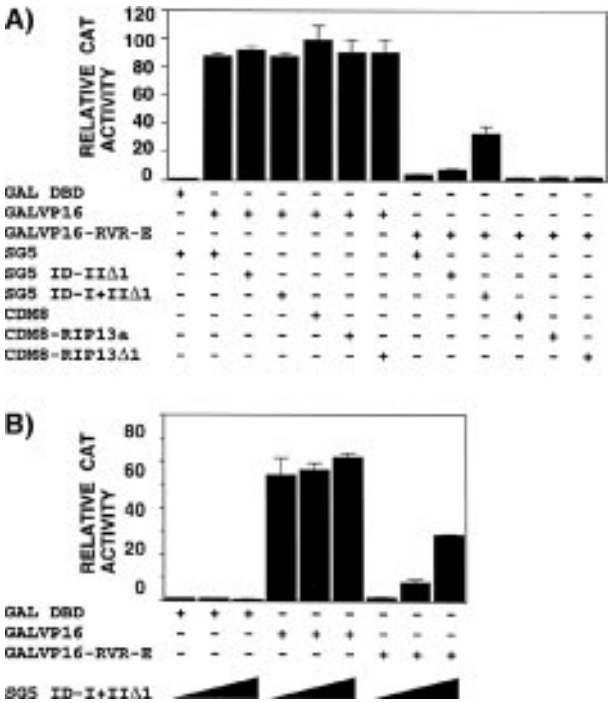

Figure 5. Alleviation of RVR repression of GAL-VP16 transactivation by overexpression of the N-CoR/RIP13 interaction domains. (**A)** COS-1 cells were co-transfected with 3 µg of pG5E1bCAT reporter, 1 µg of the GAL4 DBD or GAL-VP16 chimeras and 1 µg of pSG5 (i.e. vector alone), SG5-ID-IIΔ1, SG5-ID-I+IIΔ1, CDM8 (i.e. vector alone), CDM8-RIP13a and CDM8-RIP13Δ1 constructs as depicted and assayed for CAT activity. (**B)** COS-1 cells were co-transfected with 3 µg of pG5E1bCAT reporter, 1 µg of the GAL4 DBD or GAL-VP16 chimeras and 0–3 µg of pSG5, 0–3 µg of SG5-ID-I+IIΔ1 constructs as depicted and assayed for CAT activity. Results shown are mean ±SD and were derived from three to six independent experiments. Relative fold activation is expressed relative to CAT activity measured after transfection with the GAL4 DBD alone arbitarily set to 1.0.

and ID-II) in the co-repressor can independently interact with TR, both ID-I and ID-II are required for a significant interaction with the orphan receptors Rev-erbAα and RVR. Furthermore, the investigation revealed that the Δ1 splice variant very efficiently interacted with the orphan receptors and RXR. This suggested that the region between aa 805 and 925 in RIP13a (that is deleted in the Δ1 isoform) may selectively discriminate between specific receptors (e.g. TR or RXR), and that the splice variants have different functions with respect to ligand-activated and orphan steroid receptors. This indicates that the co-repressor splicing variants may have cell/receptor specific targets depending on spatio–temporal expression. N-CoR and SMRT/TRAC-2 are ubiquitously expressed proteins, however, the expression patterns of the splice variants RIP13a, RIP13Δ1 and TRAC-1 have not been studied. Furthermore, the expression of this expanding gene family during embryogenesis has not been investigated.

Physical association of N-CoR/RIP13 and the orphan receptors is dependent on an intact E region

The interaction of these orphan receptors with the co-repressors is critically dependent on an intact E-region of Rev-erbAα and RVR (the E-region begins with helix 3 as suggested by Wurtz *et al.*). Our investigation revealed that the hinge region did not physically associate with the co-repressors *in vivo*, neither was it required for an efficient interaction *in vivo*. Wurtz *et al.* (32) argued that they thought it was unlikely that N-CoR interacted with helix 1 in the hinge region, because the triple mutation used to map N-CoR binding would disrupt the interaction of helix 1 with the LBD core, dislodge H1 from its wild-type position and the specified amino acids that are engaged in internal contacts. Our data suggests that the interaction of N-CoR with Rev-erbAα and RVR does not require the domain of these orphans that would putatively form helix 1 and 2.

Interestingly, we found that deletion of the region between aa 437 and 509 from the entire E-region (aa 437–614) resulted in a domain that was unable to interact with N-CoR/RIP13 *in vivo*. We have previously demonstrated that aa 437–509 mediate the

repression of *trans*-activation by GALVP16. However, we note that independently, this short domain cannot interact with ID-I and ID-II of N-CoR/RIP13 *in vivo*.

Dominant-negative expression of N-CoR/RIP13 (i.e. the interaction domains) alleviates transcriptional repression by the Rev-erb family

Our study also demonstrated that over-expression of dominant negative forms of N-CoR/RIP13 could alleviate orphan receptor-mediated repression of GALVP16 *trans*-activation *in vivo*. This strongly suggested that transcriptional silencing by Rev-erbAα and RVR involves N-CoR/RIP13. Furthermore, this demonstrates that regulation of orphan receptor transcriptional silencing may involve splice variants, such as TRAC-1, that could putatively function as 'anti-repressors'.

Comprehensive analysis of repression will require a complete interaction analysis of all the putative co-regulators (e.g. N-CoR, RIP13a and Δ1, TRAC-1 and TRAC-2, p300/CBP, SRC-1, SUG-1/Trip-1 etc.) that may interact with the Rev-erb family. The transcriptional properties of the orphan receptors are probably regulated by a dynamic balance of positive and negative co-regulators that interact with the basal transcription machinery.

ACKNOWLEDGEMENTS

This investigation was supported by the National Health and Medical Research Council (NHMRC) of Australia. The Centre for Molecular and Cellular Biology is the recipient of a Australia Research Council (ARC) special research grant. We thank Drs W. Chin and V. Giguère for the Rev-erbAα and RVR cDNA clones. Special thanks to Drs David Moore and Wongi Seol who communicated data and plasmids prior to publication. All the authors contributed equally to this study.

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