Receptor Interacting Protein RIP140 Inhibits Both Positive and Negative Gene Regulation by Glucocorticoids*

(Received for publication, November 23, 1998, and in revised form, March 22, 1999)

Nanthakumar Subramaniam‡, Eckardt Treuter§, and Sam Okret‡¶
From the Department of ‡Medical Nutrition, §Center for Biotechnology, Karolinska Institutet, Huddinge University Hospital, F60 Novum, SE-141 86 Huddinge, Sweden

Recent development in the field of gene regulation by nuclear receptors (NRs) have identified a role for cofactors in transcriptional control. While some of the NR-associated proteins serve as coactivators, the effect of the receptor interacting protein 140 (RIP140) on NR transcriptional responses is complex. In this report we have studied the effect of RIP140 on gene regulation by the glucocorticoid receptor (GR). We demonstrate that RIP140 antagonized all GR-mediated responses tested, which included activation through classical GRE, the synergistic effects of glucocorticoids on AP-1 and Pbx1/HOXB1 responsive elements, as well as gene repression through a negative GRE and cross-talk with NF-κB (RelA). This involved the ligand-binding domain of the GR and did not occur when the GR was bound to the antagonist RU486. The strong repressive effect of RIP140 was restricted to glucocorticoid-mediated responses in as much as it slightly increased signaling through the RelA and the Pit-1/Pbx proteins and only slightly repressed signaling through the Pbx1/HOXB1 and AP-1 proteins, excluding general squelching as a mechanism. Instead, this suggests that RIP140 acts as a direct inhibitor of GR function. In line with a direct effect of RIP140 on the GR, we demonstrate a GR-RIP140 interaction in vitro by a glutathione S-transferase-pull down assay. Furthermore, the repressive effect of RIP140 could partially be overcome by overexpression of the coactivator TIF2, which involved a competition between TIF2 and RIP140 for binding to the GR.

The glucocorticoid receptor (GR)1 belongs to the family of nuclear receptors (NRs) and selectively regulates a network of hormone responsive genes. This includes both stimulation and repression of target genes. Activation of target genes relies in most cases on the ability of the GR to bind to specific DNA sequences, termed glucocorticoid responsive elements (GREs) (1–4). These GREs act as hormone-regulated enhancer elements. Following the interaction of the GR with the GREs and in vivo most likely with other transcription factors and cofactors, it is thought that the GR directly or indirectly contacts the proteins present in the basal transcription machinery (4).

In some cases glucocorticoids stimulate gene expression independent of GR binding to the GREs. Instead, this effect is mediated through a protein-protein interaction between the GR and a second transcription factor (5). However, the outcome of the response, i.e. stimulation or repression of gene expression, is dependent on the composition of the transcription factor complex targeted by the GR. The best characterized example of this is the effect GR confers on AP-1 controlled target genes. In the case of a c-Jun homodimer binding to the AP-1 site, the interaction with the GR leads to stimulation of AP-1 mediated transcription (6). In contrast, a c-Jun/c-Fos heterodimeric AP-1 complex is repressed by the interaction with the GR (6–8). In addition, glucocorticoids enhance prolactin-stimulated transcription of the β-casein gene through the interaction of the GR with STAT5 (9). The GR can also stimulate STAT3 and C/EBPβ controlled transcription without contacting the DNA (10, 11). Furthermore, we have recently described potentiation of retinoic acid (RA) induced transcription through the Hoxb-1 promoter autoregulatory element b1-ARE, where this potentiation seems to involve a protein-protein interaction between the GR and the RA-induced Pbx1 and HOXB1 proteins binding to the b1-ARE. However, the role for GR interacting cofactors in this type of synergy between GR and other transcription factors is not known.

Glucocorticoids also repress transcription. In principle, this involves either binding of the GR to negative GREs (nGREs) leading to displacement of transcription factors or interference with their transcriptional activation, or alternatively binding of the GR to a second transcription factor independent of GR binding to the DNA (12, 13). Examples of nGREs are the PRL3 element in the bovine prolactin promoter and the human osteocalcin nGRE overlapping the TATA box (14–16). Examples of repression of target genes independent of GR binding to the DNA are the suppression of NF-κB signaling (17, 18) and AP-1 activity if the AP-1 complex is composed of a c-Jun/c-Fos heterodimer (see above).

The GR contains a major transcriptional activation domain called activator function-1 (AF-1 or AF1) localized in the less conserved N-terminal region of the receptor (Ref. 19 and references therein). A second activation domain, the highly conserved ligand-dependent AF-2, is localized in the multifunctional ligand-binding domain (LBD) of the GR (20–22). During the past few years, so called coactivators and corepressors have been identified that modulate the transcriptional activity of NRs. With regard to the GR, several coactivators have been shown to interact with the GR and stimulate its transcriptional activity, including GRIP1/TIF2 (23, 24), SRC-1 (25), CBP/p300

* This work was supported by a grant from the Swedish Cancer Society (to S. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 46-8-5858-3728; Fax: 46-8-711-6659; E-mail: Sam.Okret@mednut.ki.se.
‡ The abbreviations used are: GR, glucocorticoid receptor; NRs, nuclear receptors; GREs, glucocorticoid responsive elements; RIP140, receptor interacting protein 140; ER, estrogen receptor; TR, thyroid hormone receptor; PPAR-γ, peroxisome proliferator-activated receptor γ; CAT, chloramphenicol acetyltransferase; LBD, ligand-binding domain; TRGE, TPA response element; Dex, dexamethasone; TPA, 12-O-tetradecanoylphorbol-13-acetate; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; nGRE, negative GRE; EC, embryonal carcinoma; tk, thymidine kinase.

‡ The authors wish to thank Dr. Karl-Olof Lundberg, Dr. Gunilla Karlsson, and Dr. Ture Eriksson for critical reading of the manuscript.
¶ N. Subramaniam and S. Okret, manuscript in preparation.
tor-activated receptor nuclear receptor TR2 (36). Furthermore, RIP140 may act as a prolactin and growth hormone promoter, respectively (35). RIP140 has also been shown to act as a coactivator for the cells were transfected by Lipofectin™ with 1 approximately 30% confluence in 6-well plates and the following day this interaction requires the binding of an agonist to the receptor (30–32). This correlates to a conformational change in the LBD and the ability of the NRs to transactivate (Ref. 3 and references therein).

One cofactor identified is the receptor interacting protein 140 (RIP140). This cofactor was first identified by in vitro protein-protein interaction assays using the hormone-binding domain of the estrogen receptor (ER) as a bait (32). The interactions of RIP140 with ER, retinoic acid receptor, and thyroid hormone receptor (TR) are all induced by their respective ligands and RIP140 weakly enhances in vivo transcription of these NRs (33). RIP140 has also been shown to act as a coactivator for the rat androgen receptor (34). However, more recent studies question a general coactivator role for RIP140 as RIP140 inhibited Pit-1/ER and Pit-1/TR synergistic transcription from the rat prolactin and growth hormone promoter, respectively (35). RIP140 also represses transcriptional activation of the orphan nuclear receptor TR2 (36). Furthermore, RIP140 may act as a coregulator of NRs rather than a coactivator as it antagonized the function of the coactivator SRC-1 on peroxisome proliferator-activated receptor γ (PPARγ) (37).

In this report we have investigated the effect of RIP140 on glucocorticoid responsive genes. This includes activation mediated through a classical GRE, activation through cross-talk with AP-1 and homeodomain proteins as well as glucocorticoid-regulated repression via an nGRE and NF-κB (RelA). We show that all glucocorticoid responses investigated are deregulated by RIP140 and that this most likely is mediated through an interaction between the GR and RIP140, abrogating GR function. The repressive activity of RIP140 could be explained by the ability of RIP140 to prevent binding of a “true” coactivator to the GR.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs—**The (GRE)2-tk-Luc reporter gene and the GR expression plasmid pSVGR1 have been described previously (38). The constitutively active rat pSVGR1LBD (amino acids 1–525) was kindly provided by K. R. Yamamoto (39). The luciferase reporter constructs pAdMLARE, 3xNF-κB(tk)Luc, and −73ColA-Luc have been described elsewhere (8, 17, 40). The CAT reporter plasmid PRL3CAT has also been described previously (41). The expression and translation of a GRE-controlled reporter gene ((GRE)2-tk-Luc) (Promega). The translated (35S)-labeled RIP140 or GR was incubated with GST, TIF2 fusion proteins was carried out as described previously (24, 37). The translated (35S)-labeled RIP140 or GR was incubated with GST, GST-GR, or GST-TIF2 fusion protein loaded on glutathione-Sepharose beads in a binding buffer containing 0.2 M NaCl (37). In the case of GST-TIF2-GST-GR interaction, an increasing amount of in vitro translated unlabeled RIP140 was added. Non-primed reticulocyte lysate was supplemented so that all incubations contained the same amount of lysate. After washing the beads, pelleted proteins were analyzed by SDS-PAGE (10%) and the amount of (35S)-labeled RIP140 or GR was detected by autoradiography.

**RESULTS**

**RIP140 Antagonizes Glucocorticoid Receptor-mediated induction of a GRE Controlled Reporter Gene—**In order to investigate the effect of RIP140 on the ability of the GR to transactivate through GREs, a luciferase reporter gene ((GRE)2-tk-Luc) controlled by a heterologous promoter (the thymidine kinase promoter (tk) −105 to +52) and two GREs was co-transfected with a GR expression vector (SVGR1) with or without an expression vector for RIP140 (pSG-RIP140) into P19 EC cells. In the absence of RIP140, the synthetic glucocorticoid dexamethasone (Dex) induced reporter gene activity 12-fold (Fig. 1, lane 2). In contrast, when RIP140 was expressed in the cells, Dex-induced luciferase activity was abolished (Fig. 1A, lane 4). This suggests that RIP140 somehow interferes with the transcriptional activity of GR.

As it has been previously demonstrated that RIP140 interacts with the AF-2 domain located in the C-terminal LBD of NRs, we tested the ability of RIP140 to interfere with a GR that lacked its LBD. A GR devoid of its LBD harbors a constitutive ligand independent transactivation capacity due to the presence of a constitutive transactivation domain (τ1 or AF-1) in the N-terminal domain (39). Fig. 1B, lane 2, shows that the GR devoid of its LBD enhanced the reporter gene activity by about 5-fold in the absence of co-transfected RIP140, and no effect on the constitutive transcriptional activity was obtained in the presence of co-transfected RIP140 (compare lanes 2 and 3). This demonstrates that RIP140 is only able to antagonize the GR activity when it contains its LBD that includes the cofactor interacting AF-2 domain. Furthermore, this experiment showed that the ability of RIP140 to repress the activity of the wild type GR is not due to a general squelching of transcriptional activity, because the constitutively transactivating GR devoid of its LBD was unaffected.

**RIP140 Antagonizes the Enhancement by Glucocorticoids of Phorbol Ester and Retinoic Acid-induced Responses—**Since RIP140 antagonized GR-mediated gene activation through GREs, we were interested to know whether this effect of RIP140 also occurred on alternative glucocorticoid responsive genes whose expression are stimulated by glucocorticoids independent of binding of the GR to GREs. For this, we investigated the expression of RIP140 expression on glucocorticoid- and phorbol ester (TPA)-induced expression from a AP-1 responsive reporter gene. HE-La cells expressing endogenous GR were transfected with a TPA inducible collagenase A promoter, containing a TPA response element (TRE), coupled to a luciferase reporter gene (−73ColA-Luc) with or without the RIP140 expression vector. In the absence of transfected...
Inhibition of GR Signaling by RIP140

**FIG. 1.** RIP140 antagonizes wild type GR function but not the LBD deleted GR. A, the reporter gene (GRE)\(_2\)-tk-Luc (1 \(\mu\)g) was co-transfected with an expression vector for the wild type GR (pSVGR1, 250 ng) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the expression vector for RIP140 (pSG-RIP140, 100 ng) into P19 EC cells as described under "Experimental Procedures." Following transfection, cells were stimulated with or without 1 \(\mu\)M Dex for 16 h as indicated in the figure. B, (GRE)\(_2\)-tk-Luc was co-transfected into P19 EC cells with (lanes 2 and 3) or without (lane 1) an expression vector for a GR lacking LBD (pSVGR1\(\Delta\)LBD) in the presence (lane 3) or absence (lane 2) of pSG-RIP140 as described above. Following transfection, cells were refed with fresh medium. 16 h later cells were lysed and analyzed for luciferase activity. All luciferase activities were related to the activity for refed with fresh medium. 16 h later cells were lysed and analyzed for luciferase reporter gene in the absence of hormone (A) or in the absence of receptor (B). Each bar gives the mean ± S.D. from three experiments.

RIP140, TPA treatment of the cells stimulated luciferase activity 15–17-fold (Fig. 2A). Under our conditions, co-treatment with TPA and Dex caused a further enhancement of the luciferase activity to 33–35-fold stimulation. Dex alone caused a 5-fold induction of reporter gene activity. This is probably due to a small constitutive AP-1 activity in the cells.

Interestingly, RIP140 specifically inhibited the glucocorticoid-induced synergistic enhancement of TPA-stimulated activity, while it only modestly reduced the TPA response (Fig. 2A). Also the small Dex induced activation of the collagenase A-luciferase reporter gene in the absence of TPA treatment was abolished by co-transfection with RIP140. Similar results were obtained when the luciferase reporter gene was controlled by a heterologous promoter (tk promoter −105 to +52) and three TREs (data not shown). This demonstrates that RIP140 also inhibits the cross-talk between GR and AP-1 transcription factors.

Glucocorticoids enhance transcription not only through AP-1 transcription factors but also via a second class of proteins, namely homeodomain containing proteins. We have observed that glucocorticoids enhance the RA-induced expression of a luciferase reporter gene controlled by the Hox-B1 gene promoter autoregulatory element b1-ARE.\(^2\) The b1-ARE is recognized by two homeodomain containing proteins, Pbx1 and HOXB1, both induced by RA in P19 embryonal carcinoma (P19 EC) cells. Results suggest that the GR enhances the RA-induced transcription from the b1-ARE by directly contacting the Pbx1 and HOXB1 proteins without binding to the DNA itself. In order to investigate whether RIP140 will also interfere with glucocorticoid enhancement of RA-induced expression from the b1-ARE, the reporter gene pAdMLARE and the GR expression vector were co-transfected into P19 EC cells with or without the expression vector for RIP140. Treatment of the cells with RA induced b1-ARE luciferase gene activity 20-fold (Fig. 2B, lane 3). Dex in itself had no effect on the b1-ARE luciferase gene activity, but co-treatment with RA and Dex caused a synergistic activation (35-fold stimulation). Transfection with RIP140 abolished this Dex/RA mediated synergy on the b1-ARE element (Fig. 2B, compare lanes 6 and 8). This demonstrates that a separate GR controlled cross-talk pathway, in addition to AP-1, is affected by RIP140. To be noted is that the RIP140 does not significantly affect the RA-induced expression of the b1-ARE luciferase gene, demonstrating a specificity in the inactivation function of RIP140.

---

**FIG. 2.** RIP140 prevents agonist but not antagonist induced synergy. A, the TPA responsive reporter gene −73ColA-Luc (1 \(\mu\)g) was co-transfected without (lanes 1–4) or with pSG-RIP140 (lanes 5–8) into HeLa tk− cells. After transfection, cells were treated with or without 1 \(\mu\)M Dex and/or 100 ng/ml TPA for 16 h as indicated in the figure. B, pAdMLARE (1 \(\mu\)g) was co-transfected with wild type pSVGR1 (250 ng), in the absence (lanes 1–4 and 9–10) or presence (lanes 5–8 and 11–12) of pSG-RIP140 (100 ng) into P19 EC cells. After 6 h of incubation, cells were stimulated with or without 1 \(\mu\)M Dex and/or 1 \(\mu\)M RA or 100 nM RU486 and/or 1 \(\mu\)M RA for 16 h as indicated in the figure. All luciferase activities were related to the activity for −73ColA-Luc or pAdMLARE in the absence of hormone treatment. Each bar gives the mean ± S.D. from two and three experiments, respectively.

---

\(^2\) The b1-ARE is recognized by two homeodomain containing proteins, Pbx1 and HOXB1, both induced by RA in P19 embryonal carcinoma (P19 EC) cells. Results suggest that the GR enhances the RA-induced transcription from the b1-ARE by directly contacting the Pbx1 and HOXB1 proteins without binding to the DNA itself. In order to investigate whether RIP140 will also interfere with glucocorticoid enhancement of RA-induced expression from the b1-ARE, the reporter gene pAdMLARE and the GR expression vector were co-transfected into P19 EC cells with or without the expression vector for RIP140. Treatment of the cells with RA induced b1-ARE luciferase gene activity 20-fold (Fig. 2B, lane 3). Dex in itself had no effect on the b1-ARE luciferase gene activity, but co-treatment with RA and Dex caused a synergistic activation (35-fold stimulation). Transfection with RIP140 abolished this Dex/RA mediated synergy on the b1-ARE element (Fig. 2B, compare lanes 6 and 8). This demonstrates that a separate GR controlled cross-talk pathway, in addition to AP-1, is affected by RIP140. To be noted is that the RIP140 does not significantly affect the RA-induced expression of the b1-ARE luciferase gene, demonstrating a specificity in the inactivation function of RIP140.
The synergistic activity between Pbx1/Hoxb1 and GR on the b1-ARE is also observed when the GR is bound to the glucocorticoid antagonist RU486 (Fig. 2B, lane 10). In contrast to the ability of RIP140 to abolish the Pbx1/Hoxb1 and GR synergy when the GR was bound to an agonist (Dex), RIP140 was unable to abolish the synergy when the GR was bound to the antagonist RU486 (lane 12). This suggests that RIP140, like several other cofactors, only interacts with NRs bound to agonists (30, 32). This also supports the previous conclusion that RIP140 does not act as a general inhibitor of transcription.

Repression of Gene Transcription Confounded by the GR Is Also Inhibited by RIP140—In order to explore whether the inhibitory activity of RIP140 also applied to other pathways repressed by the GR, two negatively regulated response elements were investigated. In the first case we examined the effect of RIP140 on gene repression by the GR via the interaction of the GR with a negative GRE (nGRE) from the bovine prolactin gene (PRL3 nGRE (43)). We have previously shown that repression via this nGRE requires the interaction of the GR with the PRL3 nGRE (15). Binding of the GR to the PRL3 nGRE involves displacement in pituitary cells of the pituitary-specific transcription factor Pit-1 and a ubiquitously expressed Pbx protein (14, 15). When the PRL3nGRE-CAT reporter gene was transfected into pituitary GH3 cells, Dex repressed transcription by about 40% (Fig. 3A). However, when RIP140 was co-transfected, GR was no longer able to repress the transcriptional activity from the PRL3 element. Interestingly, RIP140 slightly but significantly ($p < 0.05$) increased the basal activity from the PRL3 element in the absence of glucocorticoid treatment. Identical results were observed in COS-7 cells, in which cells GR also represses transcription from the PRL3 nGRE (data not shown). In non-pituitary cells Oct-1 replaces the function of Pit-1 (14).

Glucocorticoids also repress NF-$\kappa$B controlled target genes by the ability of GR to interfere with the transcriptional activity of NF-$\kappa$B proteins (17). This mainly occurs via a protein-protein interaction between the GR and NF-$\kappa$B proteins independent of the ability of GR to interact with the DNA. To assess whether repression of NF-$\kappa$B signaling by glucocorticoid is relieved by RIP140, we transfected a luciferase reporter gene containing three NF-$\kappa$B sites from the human intercellular adhesion molecule-1 promoter and a minimal (~105 to +52) tk promoter together with expression vectors for the GR and the NF-$\kappa$B family member RelA with or without the expression vector for RIP140 into COS-7 cells. Transfection with RelA stimulated the transcription from the 3xNF-$\kappa$B(1)tk-luciferase gene 10-fold (data not shown) and treatment with Dex repressed this activity by 50% (Fig. 3B). Interestingly, co-transfection with RIP140 not only derepressed the GR-dependent inhibition but also slightly but significantly ($p < 0.05$) enhanced the RelA-mediated transactivation of the luciferase reporter gene. These results show that in addition to inhibiting GR-mediated enhancement of transcription, RIP140 also blocks GR-mediated repression, both from target genes which involves binding of the GR to nGREs and through mechanisms that involve a protein-protein interaction between the GR and other transcription factors. The effect is specific for the GR, since RIP140 showed a slight stimulatory effect on the activity of RelA and Pit-1/Pbx in the absence of glucocorticoids.

RIP140 Interacts with the Glucocorticoid Receptor in Vitro—Our transfection data demonstrating the ability of RIP140 to inhibit all tested types of GR-mediated responses in several cell lines while leaving other responses unaffected or only slightly affected, suggest that RIP140 mediates it effects by acting directly on the GR. This may be explained by a direct interaction between RIP140 and GR. In order to find support for such a protein-protein interaction, we performed a pull-down assay in which the GR fused to GST or GST alone were incubated with 35S-labeled RIP140. The presence of 35S-labeled RIP140 in the pellet was analyzed by SDS-PAGE and the amount of 35S-labeled RIP140 was detected by autoradiography. As can be seen in Fig. 4, RIP140 strongly interacted with the GR-GST reporter gene and the GR and RIP140 expression vectors with or without an expression vector for the coactivator TIF2. As observed before, GR in the presence of Dex activated the (GRE)$_2$-tk-Luc reporter gene and this activation was completely abolished by RIP140 (Fig. 5A). Transfection of TIF2 in the absence of RIP140 potentiated GR activation of the reporter gene approximately 2.5-fold. Interestingly, co-transfection with TIF2 par-
Inhibition of GR Signaling by RIP140

Fig. 4. GR physically interacts with RIP140 in vitro. In vitro translated 35S-labeled RIP140 was incubated with GST or GST-GR fusion protein bound to glutathione-Sepharose beads. After washing the beads, precipitated proteins were analyzed by SDS-PAGE and 35S-labeled RIP140 was visualized by autoradiography. Lane 1 represents the input of the labeled RIP140.

Discussion

Recent results have revealed that the function of the ubiquitously expressed receptor interacting protein RIP140 in NR transactivation might be more complex than initially described. RIP140 has been shown to be a weak coactivator of ER, retinoic acid receptor, and TR, binding to the AF-2 domain of these NRs (32, 33). RIP140 also stimulates transcription of the AR (34). RIP140 interacts with additional NRs, including PPAR and the orphan nuclear receptor TR2 (36, 37). However, in contrast to other coactivators, RIP140 does not stimulate transcriptional activation of the PPAR, rather it counteracts coactivators that are able to enhance PPAR transcription (37). RIP140 also acts as a corepressor of TR2 activity (36). In addition, it was recently demonstrated that RIP140 can both repress or stimulate Pit-1/NR transcriptional synergy, depending on the absence or presence of defined domains of the Pit-1 protein in a promoter dependent way (35). Thus, the consequence of RIP140 interaction with NRs is complex in that it can act both as a coactivator and an inhibitor of transcription.

We demonstrate that RIP140 acts as an inhibitor of all tested GR mediated activities, which include positive regulation through classical GREs, synergy through cross-talk with AP-1 and Pbx1/HOXB1, and negative regulation via nGRE and cross-talk with RelA. These inhibitory effects are apparently independent of promoter context and cell context as inhibition was seen in four different cell lines. No or only minor effects were seen on the transactivating activities of AP-1, RelA, Pit-1/Pbx, and Pbx1/HOXB1, demonstrating that RIP140 is not a general squelcher of transcriptional activities. In fact, RIP140 may act as a weak coactivator of RelA or the Pit-1/Pbx complex (Fig. 3, A and B). Furthermore, RIP140 did not significantly influence retinoic acid receptor induction of Pbx1/HOXB1-mediated transcription (Fig. 2B), suggesting that RIP140 is not an inhibitor of all NRs in P19 EC cells.

An explanation for the repressive effect on all types of glucocorticoid responses is that RIP140 interacts with the GR, partially restoring the ability of the GR to activate the (GRE)2-tk-Luc reporter gene in the presence of RIP140. This suggested a model where RIP140 prevents the interaction of GR with coactivators by competing for binding to the GR. This possibility was further investigated by a GST competitive pull-down assay. GST-TIF2 was incubated with in vitro translated 35S-labeled GR with or without in vitro translated unlabeled RIP140. As can be seen from Fig. 5B, increasing the amount of RIP140 reduced binding of the GR to TIF2 (compare lanes 4–5 to lane 3). This decrease in GR binding was specific and not due to the presence of an increasing amount of reticulocyte lysate, as all incubations contained the same amount of lysate. These experiments support a model in which RIP140 inhibits GR activity by preventing binding to the GR of a coactivator.

Fig. 5. A, TIF2 partially rescues RIP140-mediated repression. The reporter gene (GRE)2-tk-Luc (1 µg) was co-transfected with an expression vector for wild type GR (pSVGR1, 100 ng) and TIF2 (pCMV5-TIF2, amounts indicated in the figure) in the absence (lanes 1–4) or presence (lanes 5–14) of the expression vector for RIP140 (pSG-RIP140, 50 ng) into COS-7 cells as described under “Experimental Procedures.” Following transfection, cells were stimulated with or without 1 µM Dex for 16 h as indicated in the figure. All luciferase activities were related to the activity for (GRE)2-tk-Luc in the absence of hormone. Each bar gives the mean ± S.D. from two experiments. B, RIP140 prevents binding of the GR to TIF2 in vitro. GST-TIF2 (lanes 3–5) or GST (lane 2) bound to glutathione-Sepharose beads were incubated with in vitro translated 35S-labeled GR without (lanes 2 and 4) or with 2 µl (lane 4) or 4 µl (lane 5) of in vitro translated unlabeled RIP140. Non-primed reticulocyte lysate was added to all incubations to obtain an equal volume of lysate. PhosphorImager analysis revealed that the formation of GST-TIF2-GR complexes were reduced by 44% (lane 4) or 88% (lane 5) as compared with complex formation in the absence of RIP140 (lane 3) when nonspecific GR binding to GST alone (lane 2) was deducted. Lane 1 shows 50% of the input of 35S-labeled GR.

preventing true coactivators to bind to the GR. Support for this assumption comes from our transfection and pull-down experiments (Figs. 4 and 5). The competition model is further substantiated by our previous observation where we demonstrated that RIP140 competed with SRC-1 for binding to the AF-2 domain of PPAR-γ (37). The same mechanism has been proposed for RIP140 inhibition of Pit-1/ER and Pit-1/TR synergy and TR2 activity (35, 36). The competition model for cofactor binding to a common site in the GR can also explain the ability of RIP140 to inhibit negative gene regulation by the GR. It has been suggested that repression of gene activity by the GR
Inhibition of GR Signaling by RIP140

involves competition for a limiting coactivator (CBP) common to the GR and a second positively acting transcription factor complex, e.g. AP-1 or NF-κB (31, 44). Thus, binding of GR to RIP140 would then prevent GR to titrate out CBP. An alternative explanation for the ability of RIP140 to prevent GR mediated responses is that RIP140 functions as a steric obstacle hampering GR-DNA or protein-protein interaction, both being important in positive and negative gene regulation by the GR (12). Interestingly, it was recently reported that another coactivator, RAP46, is a negative regulator of GR transcriptional activity and acts by preventing GR binding to a GRE (45).

It has been shown that interaction with an agonist induces a conformational change in the LBD of NRs leading to the formation of an AF-2 domain capable of interacting with coactivators, including RIP140 (32, 33, 46). Like the case for other NRs, the interaction between RIP140 and GR is also likely to take place through the conserved AF-2 domain present in the LBD of the GR (20, 21). In line with this assumption, RIP140 was unable to inhibit the transcriptional activity of a GR devoid of its LBD (Fig. 1B). Opposite to the situation when NRs are bound to agonists, the AF-2 domain is not able to interact with coactivators when NRs bind antagonists (46). In our experiments, RIP140 did not inhibit the GR synergy with Pbx1/FOXB1 when the GR was bound to the glucocorticoid antagonist RU486 (Fig. 1B). This is in agreement with the suggestion that the agonistic activity of a mixed antagonist, like RU486, when bound to the receptor is mediated through the AF1 domain of the receptor, while RIP140 binds interferes with the function of the AF-2 domain (47). This would explain the lack of inhibitory activity of RIP140 on RU486-GR-Pbx1/FOXB1 synergy.

When HeLa cells that had been transfected with TRE-controlled reporter genes were treated with a combination of TPA and Dex, a Dexam stimulated enhancement of the TPA effect was observed (Fig. 2A). Most previous reports have demonstrated a Dexam-mediated repression in response to TPA (48). The reason for this discrepancy is unknown but could be due to different lines of HeLa cells. A synergistic effect of Dex and TPA can be obtained if our HeLa cells contain AP-1 complexes consisting mainly of c-Jun homodimers following TPA treatment. It has been demonstrated that the activity of c-Jun homodimers is activated by GR in contrast to the repression observed with c-Jun/c-Fos heterodimers (6).

Interestingly, we observed a small up-regulation of RelA and Pit-1/Pbx activities following co-transfection with RIP140 (Fig. 3, A and B). The mechanism behind this effect is unknown but it may be that RIP140 acts as a weak coactivator for these transcription factors, although it has so far not been reported that RIP140 can interact with transcription factors other than NRs. Alternatively, it may reflect a small constitutive GR repression of RelA and Pit-1/Pbx activities, which is relieved upon transfection by RIP140 (49).

There are several observations to suggest that RIP140 differs from true coactivators. RIP140 is generally less efficient in enhancing the AF-2 activity of NRs in mammalian cells compared with other coactivators (25, 33, 50). Most of the other coactivators function as bridging proteins between the basal transcriptional apparatus and the nuclear receptors but in contrast, the RIP140 has not been demonstrated to interact with members of the basal transcriptional machinery (31, 32). Furthermore, the lack of a histone acetyltransferase domain in RIP140, distances it from the CBP/p300, SRC-1, and P/CаF (51, 52). Most of the coactivators, but not RIP140, interact with histone acetyltransferase domain containing CBP/p300 to give a concerted activation function (51, 52). In summary, although RIP140 may act both as a coactivator and corepressor on different NRs or in different contexts, its main effect on glucocorticoid mediated responses is inhibitory. A dual role for coactivators is not unique as both the receptor interacting factors TIF1β and NSD1 exhibit characteristics of both corepressors and coactivators (27, 53, 54). Thus, the primary role for RIP140 may be to act as a coregulator fine tuning a complex network of genes.

Acknowledgments—We thank Drs. K. R. Yamamoto, F. Mavilio, J. Leers, and S. Wissink for providing plasmids. We also thank Dr. Gary Faulds for valuable comments on the manuscript.

REFERENCES

1. Carlstedt-Duke, J., Wright, T., Gottlicher, M., Okret, S., and Gustafsson, J.-Å. (1995) in Endocrinology and Metabolism (Peleg, P., Baxter, J. D., and Frohman, L. A., ed) pp. 169–199, McGraw-Hill Book Co., New York.
2. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., and Chambon, P. (1995) Cell 83, 835–839.
3. Resche-Rigon, M., and Gromel, H. (1998) Curr. Opin. Chem. Biol. 2, 501–507.
4. Beato, M., and Sanchez-Pacheco, A. (1996) Endocr. Rev. 17, 587–609.
5. McEvans, I. J., Wright, A. P., and Gustafsson, J. A. (1997) BioEssays 19, 153–69.
6. Teurich, S., and Angel, P. (1995) Chem. Senses 20, 251–255.
7. Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Fuller, G. M. (1990) J. Biol. Chem. 265, 30607–30610.
8. Subramaniam, N., Cairns, W., and Okret, S. (1998) Biochemistry 37, 919–37.
9. Caldenhoven, E., Liden, J., Wissink, S., Van de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J.-Å., and Van de Saag, P. T. (1995) Mol. Endocrinol. 9, 401–412.
10. Liden, J., Delaunay, F., Rafter, I., Gustafsson, J.-Å., and Okret, S. (1997) J. Biol. Chem. 272, 21467–21472.
11. Almilof, T., Wallberg, A. E., Gustafsson, J.-Å., and Wright, A. P. (1998) Biochemistry 37, 9586–9594.
12. Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997) Curr. Opin. Cell Biol. 9, 222–232.
13. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Huang, C. L. (1996) Mol. Endocrinol. 10, 1167–1177.
14. Moras, D., and Gronemeyer, H. (1998) Curr. Opin. Cell Biol. 10, 384–391.
15. Eggert, M., Mows, C. C., Tripier, D., Arnold, R., Michel, J., Nickel, J., Schmidt, S., Beato, M., and Remizy, R. (1995) J. Biol. Chem. 270, 30753–30759.
16. Leers, J., Treuter, E., and Gustafsson, J. A. (1998) Mol. Cell. Biol. 18, 6001–6013.
17. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O Malley, B. W. (1995) Science 270, 1354–1357.
18. Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakanjima, T., Schulman, I. G., and Parker, M. G. (1996) EMBO J. 15, 21467–21472.
19. Kamei, Y., Xue, L., Heinzel, T., Torchina, J., Kurokawa, R., Glass, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) Cell 85, 403–414.
20. Cavaills, V., Daouvis, S., L’Horsot, F., Lopez, G., Hoare, S., Kusner, P. J., and Parker, M. G. (1995) EMBO J. 14, 3741–3751.
21. L’Horsot, F., Daouvis, S., Heery, D. M., Cavaills, V., and Parker, M. G. (1996) Mol. Cell. Biol. 16, 6293–6306.
22. Moras, D., and Schliep, D., Praygrent, D. J., Reese, J. C., Yates, J. T., and Kung, L. S. (1995) Mol. Cell. Biol. 15, 30753–30759.
23. Onate, S. A., Boonyaratavanakorn, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P., and O’Malley, B. W. (1998) J. Biol. Chem. 273, 12011–12019.
24. Leers, J., Treuter, E., and Gustafsson, J. A. (1998) Mol. Cell. Biol. 18, 835–839.
25. Godowski, P. J., Rusenki, S., Miesfeld, R., and Yamamoto, K. R. (1987) Nature 332, 365–368.
26. Di Rocco, G., Mavilio, F., and Zappavigna, V. (1997) EMBO J. 16, 3644–3654.
27. Subramaniam, N., Cairns, W., and Okret, S. (1997) DNA Cell Biol. 16, 153–163.
28. Meyer, T., Gustafsson, J.-Å., and Carlstedt-Duke, J. (1997) DNA Cell Biol. 16, 919–37.
B., Baeuerle, P. A., and van der Saag, P. T. (1997) J. Biol. Chem. 272, 22278–22284
43. Sakai, D. D., Helms, S., Carlstedt-Duke, J., Gustafsson, J.-A., Rottman, F. M., and Yamamoto, K. R. (1988) Genes Dev. 2, 1144–1154
44. Sheppard, K. A., Phelps, K. M., Williams, A. J., Thanos, D., Glass, C. K., Rosenfeld, M. G., Gerritsen, M. E., and Collins, T. (1998) J. Biol. Chem. 273, 29291–29294
45. Kullmann, M., Schneikert, J., Moll, J., Heck, S., Zeiner, M., Gehring, U., and Cato, A. C. B. (1998) J. Biol. Chem. 273, 14620–14625
46. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J.-Å., and Carlquist, M. (1997) Nature 389, 753–758
47. Smith, C. L., Nawaz, Z., and O’Malley, B. W. (1997) Mol. Endocrinol. 11, 657–666
48. Karin, M., Liu, Z., and Zandi, E. (1997) Curr. Opin. Cell Biol. 9, 240–246
49. Liu, W., Hillmann, A. G., and Harmon, J. M. (1995) Mol. Cell. Biol. 15, 1005–1013
50. Smith, C. L., Onate, S. A., Tsai, M. J., and O’Malley, B. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8884–8888
51. Struhl, K. (1996) Genes Dev. 12, 599–606
52. Giles, R. H., Peters, D. J., and Breuning, M. H. (1998) Trends Genet. 14, 178–183
53. Moosmann, P., Georgiev, O., Le Deurain, B., Bourquin, J.-P., and Schaffner, W. (1996) Nucleic Acids Res. 24, 4859–4867
54. Huang, N. W., Vom Baur, E., Garnier, J. M., Lerouge, T., Vonesch, J. L., Lutz, Y., Chambon, P., and Losson, R. (1996) EMBO J. 17, 3398–3412

Inhibition of GR Signaling by RIP140