Characterization of Four Autonomous Repression Domains in the Corepressor Receptor Interacting Protein 140

Receptor interacting protein (RIP) 140 is a corepressor that can be recruited to nuclear receptors by means of LXXLL motifs. We have characterized four distinct autonomous repression domains in RIP140, termed RD1-4, that are highly conserved in mammals and birds. RD1 at the N terminus represses transcription in the presence of trichostatin A, suggesting that it functions by a histone deacetylase (HDAC)-independent mechanism. The repressive activity of RD2 is dependent upon carboxyl-terminal binding protein recruitment to two specific binding sites. Use of specific inhibitors indicates that RD2, RD3, and RD4 are capable of functioning by HDAC-dependent and HDAC-independent mechanisms, depending upon cell type.

The ability of nuclear receptors to regulate transcription from target genes depends on the recruitment of cofactors that initiate chromatin remodelling and the assembly of the transcription machinery. The remodeling of chromatin by coactivators and corepressors is a dynamic process catalyzed by enzyme complexes that can be divided into two distinct classes. The first class is composed of ATP-dependent complexes that are involved in the location and association of nucleosomes with DNA (1), and the second class is composed of enzymes that catalyze post-translational modifications in histones (2). Such modifications are proposed to constitute a “histone code” that represents an epigenetic marking mechanism for controlling gene transcription and other chromatin-regulated processes.

The best characterized coactivators for nuclear receptors are the p160 coactivators, which seem to serve as platforms for the recruitment of histone-modifying enzymes, including CREB-binding protein/p300 and methyltransferases that, respectively, acetylate and methylate residues in histones in the vicinity of target promoters (3). The p160 proteins have been found to interact by means of helical LXXLL motifs directly with an activation surface (called AF2) located on the ligand-binding domain of activated nuclear receptors (4, 5). The best characterized corepressors, nuclear corepressor protein and silencing mediator for retinoid and thyroid hormone receptor (SMRT), initially proposed that RIP140 may inhibit transcription by recruiting HDACs (11, 12) and/or carboxyl-terminal binding protein (CtBP) (13, 14).

In this paper, we have investigated the function of RIP140 as a corepressor by mapping the boundaries of autonomous repression domains and determining the contribution of HDAC and CtBP binding to their ability to repress transcription. We identified four distinct autonomous repression domains that function by HDAC-dependent and -independent mechanisms, depending upon the cell type.

EXPERIMENTAL PROCEDURES

Glutathione S-transferase (GST) Pull-down Assays—Expression vectors were transcribed and translated in vitro in the presence of [35S]methionine in reticulocyte lysate (Promega). GST fusion proteins were induced, purified, bound to Sepharose beads (Amersham Biosciences), and incubated with translated proteins, as described previously (7). After washing, the samples were separated by SDS-10% PAGE. Gels were fixed, dried, and the 35S-labeled proteins were visualized by autoradiography.

Cell Culture and Transient Transfections and Reporter Assays—Cells were transfected using FuGENE 6 transfection reagent (Roche). Mouse embryo fibroblast (MEF) cells, both wild type and null for both CtBP1 and CtBP2 (15), were transfected using LipofectAMINE Plus reagent (Invitrogen).

Cells were transfected with the reporter plasmids pGL3-2xERE-PS2 (16), pGL2-Lex-Gal-Luc (23), or a luciferase reporter gene cloned behind five Gal4 binding sites (17) as indicated in the legend to Fig. 1. The Gal4 DNA-binding domain (DBD) was fused to either full-length human RIP140 or a number of deletion mutants. For the trans-repression assay, the reporter was activated by pSG5-LexA-VP16. The effect of RIP140 on this activation of expression was tested using the pCIC-Gal4 RIP140 constructs. Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). Equal expression levels of each Gal-fusion protein was confirmed by Western blot analysis using anti-Gal4-DBD antibody (sc-577, Santa Cruz Biotechnology).

For the mammalian two-hybrid assay, pACT-CtBP-VP16 (17) or pACT-VP16 was co-transfected with individual motifs or fragments of RIP140 fused to Gal4. In all transfections, the control vector pRL-CMV was included. Cells were harvested, and extracts were assayed for RIP, receptor interacting protein; CtBP, carboxyl-terminal binding protein; DBD, DNA-binding domain; GST, glutathione S-transferase; CHO, Chinese hamster ovary; TSA, trichostatin A; CREB, cAMP-responsive element-binding protein; MEF, mouse embryo fibroblast; ERE, estrogen responsive element; RD, repression domain.
FIG. 1. RIP140 contains four autonomous repression domains. The Gal4 DBD was fused to either full-length or fragments of RIP140, as indicated. 293T cells were co-transfected with pGL3–2xERE-PS2 (20 ng) (A) or Lex-Gal-Luc (20 ng) and Lex-VP16 (10 ng) (B and C) with increasing amounts (0.1, 1, or 10 ng) of the Gal4 fusion proteins. Gal4 corresponds to the Gal4 DNA-binding domain alone.

FIG. 2. Identification of CtBP-interacting motifs in RIP140. CtBP (A) or RIP140 fragments (C and D) fused to GST and GST alone used as a control were immobilized on glutathione beads and incubated with 35S-labeled RIP140 (A) or CtBP (C and D). Bound proteins were eluted and resolved by 10% SDS-PAGE, dried, and exposed to autoradiograph. B, to perform a mammalian two-hybrid assay, 293T cells were co-transfected with a reporter gene containing five Gal4 binding sites (20 ng), Gal-peptides encompassing the CtBP-interaction motifs (10 ng), and CtBP-VP16 (10 ng). VP16 alone was used as control.
luciferase activity using a dual luciferase reporter assay, as described previously (16).

RESULTS

RIP140 Contains Multiple Autonomous Repression Domains—We initially demonstrated that the ability of RIP140 to inhibit estrogen-stimulated transcription from an estrogen responsive element (ERE)-based reporter gene could be achieved by both the N-terminal (amino acids 1–528) and C-terminal halves (amino acids 535–1158) of the protein (Fig. 1A). In these experiments, we used RIP140 fragments fused to the Gal4 DNA-binding domain, which alone had no effect, so that we could exploit a transrepression assay to determine whether RIP140 contains autonomous repression domain(s). A luciferase reporter gene was constructed under the control of LexA and Gal4 binding sites upstream of an E1A TATA box. Basal transcription was markedly increased by lexA-VP16 expression (Fig. 1B), but this was reduced in a dose-dependent manner by full-length, N- and C-terminal RIP140 fragments but not by the Gal4 DBD or RIP140 alone. The equal expression level of each Gal4-RIP140 fusion protein was confirmed by Western blot analysis (data not shown). The ability of both halves of RIP140 to inhibit transcription from the reporter gene in trans indicates that the protein contains at least two autonomous repression domains.

The N-terminal repression domain had been shown to contain HDAC and CtBP binding sites (11, 13). Therefore, we generated additional fragments of RIP140 to discriminate between these binding sites. Accordingly, we found that residues 78–333, which are capable of binding class I HDACs (11) and residues 410–700 that binds CtBP (13) both inhibited transcription in the transrepression assay (Fig. 1C). In addition, we sub-divided the C terminus into residues 644–916 and 927–1158 and found that they too inhibited transcription, suggesting that there are at least four autonomous repression domains in RIP140, which we have named repression domains (RD) 1–4 (Fig. 1C).

Identification of CtBP Binding Sites in RIP140—Previous work demonstrated that CtBP was recruited to RIP140 by guest on July 25, 2018http://www.jbc.org/Downloaded from
through the sequence PIDLSCK (13, 14). To investigate whether this sequence was both necessary and sufficient for CtBP binding and transcriptional repression, we examined the effect of replacing PIDLS with PIAAS in functional assays. Surprisingly, the mutant version retained its ability to bind CtBP in GST pull-down assays (Fig. 2 A) and repressive activity, suggesting that RIP140 may contain additional CtBP binding sites. Inspection of its amino acid sequence for other sequences that fit the consensus for CtBP-interaction (17, 18) revealed that, in addition to PIDLS (motif 1), there are three
other potential binding sites, namely PINLS (motif 2) and SMDLT (motif 3) in RD2, and VRDLS (motif 4) in RD4 (Fig. 2B). In a mammalian two-hybrid assay, we found that the PIDLS and PINLS motifs in RD2 and the VRDLS motif in RD4 stimulated transcription in the presence of CtBP-VP16 (Fig. 2B), suggesting that they were distinct binding sites for CtBP. GST pull-down experiments confirmed the importance of these motifs in CtBP binding, and further suggested that motif 3 might also contribute to binding activity as judged by the interaction of different combinations of mutant versions. Thus, there was a progressive reduction in CtBP binding when motif 1 and motifs 1 and 2 were replaced, but the interaction was only abolished when all three motifs were replaced (Fig. 2C). There are two mammalian CtBPs, CtBP1 and CtBP2 (15), both of which we found could bind to RIP140 fragments encompassing the PIDLS/PINLS motifs in RD2 (RIP140–800) and the VRDLS motif in RD4 (RIP140, 753–1158), as shown in Fig. 2D.

Characterization of Repression Domain 2—The contribution of CtBP binding to repression by RD2 was investigated using mutant versions with different combinations of defective motif in the transrepression assay (Fig. 3A). Transrepression was reduced when PIDLS was replaced with PIAAS and similarly, but to a lesser extent, when PINLS was replaced with PIAAS, suggesting that both were required for optimum repression. There was a further slight reduction in transrepression when these two mutant motifs were combined, but the replacement of SMDLT had no additional effect. The data are consistent with results we obtained when we examined the relative contribution of the motifs to CtBP binding using the two-hybrid interaction assay. Thus, mutations in motif 1 reduced the ability of RD2 to interact with CtBP to a greater extent than motif 2, and combinations of mutant motifs had little further effect (Fig. 3B). That CtBP is essential for repression by RD2 was established by demonstrating that this domain no longer inhibited transcription in mouse embryonic fibroblasts devoid of both CtBP1 and CtBP2 (Fig. 3C).

Identification of Novel Repression Domains in RIP140—RD3, encompassed by residues 644–916, is a potent repressor of transcription that shows no significant sequence homology to other proteins, as analyzed by the Blast program at National Center for Biotechnology Information. Using a series of RIP140 fragments fused to Gal4, we identified amino acids 737–885 as the minimal region required for repression (Fig. 4A). This region does not contain any potential motifs for CtBP interaction and retained its ability to inhibit transcription in a cell line null for CtBP1 and CtBP2 using the transrepression assay (Fig. 4B). RD4, encompassed by residues 916–1158, contains a CtBP binding motif VRDLS; however, a mutant version in which this motif was replaced with VRAAS to abolish CtBP binding retained its function as a corepressor (Fig. 5A). Furthermore, RD4 was able to inhibit transcription in CtBP-null cells (Fig. 5B), indicating that RD4 functions as an autonomous repression domain by a CtBP-independent mechanism. Analysis of a series of RIP140 fragments (Fig. 5C) indicates that the minimal region responsible for repression is contained with residues 1118–1158. Finally, we investigated the possibility that the C terminus of RIP140 contains an additional CtBP-dependent repression domain by examining the repressive activity of residues 927–1118 lacking RD4 but encompassing the VRDLS motif; however, it was negligible (Fig. 5C).

Repression Domain Activity Varies According to Cell Type—We characterized the repression activities of the four repression domains in different cell types derived from the kidney (COS and 293T cells) and ovary (CHO and KK1 cells), because RIP140 is highly expressed in these tissues (Ref. 19 and data not shown). The repression activities of RD1, RD2, and RD3 were similar to one another, with slightly more inhibition observed in the kidney cell lines (Fig. 6A). RD4 was less active and exhibited negligible repressive activity in ovarian cells. Although the magnitude of repression observed in ovarian cells was less than that in kidney cells, we found that this was not a feature of all repressors, because CtBP, CTIP2, and Hey 1 repress transcription in ovarian cells to a greater extent than that in the kidney cells (data not shown).

Finally, we investigated the contribution of HDAC enzymatic activity to repression by the four domains by testing the ability of the HDAC inhibitors trichostatin A (TSA) (Fig. 6B) and valproic acid (data not shown) to reverse transcriptional inhibition. We found that repression by RIP140 itself was unaffected by TSA, but that of RD2, and to a lesser extent RD3 and RD4, was reversed. However, this effect was restricted to COS cells, suggesting that repression is mediated by both HDAC-dependent and -independent mechanisms that vary according to cell type.
DISCUSSION

RIP140 is a ligand-dependent corepressor of nuclear receptors that may inhibit transcription from target genes by competing with essential coactivators or by active repression following the recruitment of additional proteins. In this paper, we have characterized four distinct autonomous repression domains (Fig. 7A): RD1, which interacts with HDACs (11); RD2, which binds CtBP (13, 14); and RD3 and RD4, which may interact with proteins that have yet to be identified. Thus, it seems that RIP140 is a bridging protein that docks to nuclear receptors by means of LXXLL motifs (4, 7) and provides a platform for the recruitment of one or more corepressors involved in chromatin remodelling in the vicinity of target genes.

Sequence comparisons indicate that RIP140 is highly conserved in mammalian, bird, and fish species, but it does not seem to be related to other known transcriptional repressors. RD1 is the most highly conserved region with greater than 50% conservation between mammalian and fish species. The key residues required for CtBP binding (17) that encompass the three motifs in RD2 are also highly conserved (Fig. 7B), and this observation extends to the VRDLS motif in the C terminus of the protein (data not shown). Although this motif was dispensable for repression by RD4, it is conceivable that it is capable of recruiting CtBP in the context of full-length RIP140. RD3 and RD4 are also similar in human, mouse, and chicken (Fig. 7, C and D).

RD1 has been shown to interact with HDACs (11), but we found that it is insensitive to the effects of the inhibitors TSA or valproic acid, indicating that it can repress transcription by an HDAC-independent mechanism. It was reported (11) that the ability of RIP140 to inhibit the transcriptional activity of retinoic acid receptors in COS cells was reversed by TSA treatment, but the repression domains involved were not mapped. It is possible that one of the other repression domains and not RD1 was responsible for this inhibition, because we found that their repressive activity, and particularly that of RD2, was reversed by TSA treatment.

Our study confirms the original observation of Goodman and coworkers (13) that repression by RD2 is mediated by CtBP, although we demonstrate two CtBP binding sites in RIP140 that are both required for optimum binding and repression. The precise mechanism by which CtBP functions as a corepressor is unclear, and previous reports (18) suggest that it may repress transcription from certain target promoters by HDAC-dependent mechanisms and from other promoters by HDAC-independent mechanisms involving the polycomb complex. Similarly, we find that, whereas CtBP is essential for repres-
sion by RD2, it can function by HDAC-dependent or -independent mechanisms, depending upon cell type. We were unable to demonstrate a role for CtBP in repression by RD3 or RD4 and, given that TSA only partially reversed their ability to inhibit transcription, we conclude that they function by novel mechanisms.

Multiple repression mechanisms have been reported to account for the repressive activity of ligand-dependent corepressor (8). Repression domain function may vary depending on the nuclear receptor to which RIP140 is recruited or the cell type. For example, the contribution of RD2 to the repressive activity of RIP140 in COS cells seems to be minimal because TSA reverses inhibition by the isolated RD2 but not full-length protein in these cells. Alternatively the mechanism utilized may vary according to the physiological status of the cells. For example, the interaction of CtBP with the PXDLS sequence in E1A was reported to be modulated by NAD and NADH, and so repression by CtBP (20, 21) might be regulated by cellular redox states. Because RIP140 plays a distinct role in ovulation (19, 22) and adipose biology (data not shown), it may be necessary to control its function as a corepressor by different mechanisms in specific cell types or in different physiological circumstances.

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