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Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins

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The hormone-activated glucocorticoid receptor (GR), through its N- and C-terminal transcriptional activation functions AF-1 and AF-2, controls the transcription of target genes presumably through interaction(s) with transcriptional regulatory factors. Utilizing a modified yeast two-hybrid approach, we have identified the tumor susceptibility gene 101 (TSG101) and the vitamin D receptor-interacting protein 150 (DRIP150) as proteins that interact specifically with a functional GR AF-1 surface. In yeast and mammalian cells, TSG101 represses whereas DRIP150 enhances GR AF-1-mediated transactivation. Thus, GR AF-1 is capable of recruiting both positive and negative regulatory factors that differentially regulate GR transcriptional enhancement. In addition, we show that another member of the DRIP complex, DRIP205, interacts with the GR ligand binding domain in a hormone-dependent manner and facilitates GR transactivation in concert with DRIP150. These results suggest that DRIP150 and DRIP205 functionally link GR AF-1 and AF-2, and represent important mediators of GR transcriptional enhancement.

Keywords: DRIP coactivator complex/glucocorticoid receptor/transactivation/TSG101

Introduction

Steroid hormones regulate multiple metabolic and developmental processes through a family of intracellular receptor proteins, termed nuclear receptors (NRs) (Tsai and O'Malley, 1994). The glucocorticoid receptor (GR), the prototype for this family of transcriptional regulatory proteins, contains an N-terminal transcriptional regulatory domain, a central Zn^{2+} finger DNA binding domain and a C-terminal region responsible for hormone binding (Yamamoto, 1985; Tsai and O'Malley, 1994). Ligand binding releases GR from the inhibitory effects of the Hsp90-based molecular chaperone complex, allowing the receptor to bind to glucocorticoid response elements (GREs), and either activate or repress transcription of specific target genes in a hormone-dependent manner (Yamamoto, 1985). GR's stimulatory or inhibitory influences on gene expression are determined by the cell context, nature of the response element and composition of interacting sequence-specific transcription factors (Miner and Yamamoto, 1992; Lefstin *et al.*, 1994; Starr *et al.*, 1996).

It has been suggested that transcriptional activators such as GR use their transcriptional activation domains as surfaces to recruit chromatin remodeling factors, and to interact with general transcription factors (GTFs) or adaptor proteins that serve to link enhancer-bound transcription factors to the GTFs, thereby initiating transcription (Chen *et al.*, 1994). These adaptor proteins are often termed coactivators. Several lines of evidence support this model of transcriptional activation by GR. At least two regions of GR possess intrinsic transcriptional activation functions (AFs). AF-2, which maps to the C-terminus, is glucocorticoid dependent, with ligand binding promoting the formation of a surface that permits protein–protein contacts between AF-2 and additional transcriptional regulatory factors. In contrast, AF-1, located at the GR N-terminus, is glucocorticoid independent or constitutive, and cofactors that associate with the GR AF-1 remain largely undefined (Godowski *et al.*, 1987; Hollenberg and Evans, 1988).

Regions within the GR AF-1 important for transcriptional activation have been identified through deletion analysis and by examining the activity of fusion proteins in yeast and mammalian cells as well as in cell-free systems. GR AF-1 has been shown to activate minimal promoter constructs in a cell-free transcription system, suggesting a direct interaction with factors involved in transcription initiation (Freedman *et al.*, 1989; Bagchi *et al.*, 1990; Elliston *et al.*, 1990; Klein-Hitpass *et al.*, 1990; Tsai *et al.*, 1990). Consistent with this idea, *in vitro* squelching assays and protein–protein interaction studies have suggested contacts between GR AF-1 and components of the RNA polymerase II transcriptional apparatus (McEwan *et al.*, 1993). Specifically, GR AF-1 interacts with the TFIID complex and the TATA binding protein (TBP) *in vitro* (Ford *et al*., 1997). Finally, GR has been shown to enhance the formation of stable preinitiation complexes at target promoters *in vitro* (Elliston *et al*., 1990; Tsai *et al*., 1990). These findings support a model whereby GR AF-1 provides a surface that interacts with GTFs and possibly with as yet unidentified adaptor proteins.

A defined activation surface within GR AF-1, consisting of a 40-amino-acid 'core' between residues 208 and 247 (using the rat GR numbering scheme), has been delineated (Giguere *et al.*, 1986; Godowski *et al.*, 1988; Hollenberg and Evans, 1988; Dahlman-Wright *et al.*, 1994). This region functions as an activation domain irrespective of

its position in hybrid proteins (Hollenberg and Evans, 1988), consistent with the notion that this domain comprises an independent structural entity. Furthermore, Iñiguez-Lluhí *et al*. (1997) have identified a GR variant, termed 30IIB, that harbors three point mutations in AF-1 (E219K, F220L and W234R), which collectively abolish receptor transcriptional activation, but not transcriptional repression. Thus, the concept of a surface formed by AF-1 that recruits GTFs or novel adaptor proteins is an attractive model to define GR transcriptional activation.

Using a modified version of the yeast two-hybrid approach designed to isolate proteins that associate with transcriptional activators, we have identified proteins that associate with a functional GR AF-1. We examined the specificity of these interactions and characterized the functional effects of them on GR transcriptional enhancement in yeast and mammalian cells.

Results

A modified yeast two-hybrid approach to identify proteins that interact with GR AF-1

To identify proteins that interact with GR AF-1, we used a modified yeast two-hybrid system that allows for the selection of proteins that associate with transcriptional activators (see Materials and methods for details of the system) (Du *et al.*, 1996).

To focus our search on proteins that associate with GR in a manner dependent on the integrity of AF-1, we incorporated an additional tier of screening using the transcriptionally defective GR 30IIB mutant. Recall that this GR variant contains three point mutations in AF-1 (E219K, F220L and W234R) that selectively reduce receptor transcriptional activation by disrupting a distinct activation 'surface' while leaving the remaining architecture of the receptor intact. We observed a 95% reduction in GR AF-1-dependent transcriptional activation for 30IIB relative to wild-type (wt) GR (Figure 1A, top). Immunoblot analysis demonstrates that this decrease in GR transcriptional activation by 30IIB is not due to differences in receptor expression levels between the wt and the mutant receptor derivative (Figure 1A, bottom). Proteins that associate with the wtGR AF-1 were subsequently screened against the 30IIB mutant to identify cofactors that interact with a functional AF-1 activation surface. Out of 1.5×10^5 library transformants, 54 clones were isolated that interact specifically with the GR N-terminal activation domain, AF-1. From the 54 isolates that associated with GR, 20 displayed a reduced affinity for the 30IIB mutant (Figure 1B).

We sequenced the 20 partial cDNA clones that displayed

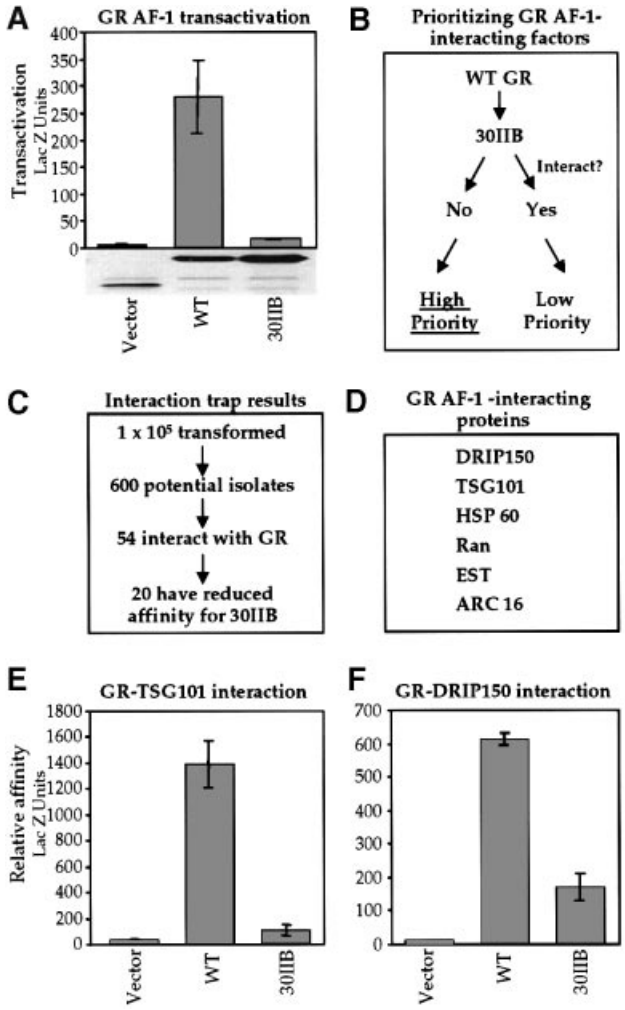

Fig. 1. Results of the modified yeast two-hybrid screen for GR AF-1 interacting factors. (**A**) Transcriptional activity of GR AF-1 derivatives. The GR wt and 30IIB mutants, in the context of the N-terminal residues 107–237, fused to the LexA DNA binding domain, were transformed into yeast along with a Lex-operator-linked β-galactosidase reporter gene. Cells were grown in selective media for 12 h at 30°C and β-galactosidase activity measured. Data represent the average of four independent clones and the standard deviation is shown. Immunoblot of extracts from strains expressing the Lex DNA binding domain (vector) or GR–LexA fusions (wt or 30IIB) were performed using a LexA-specific polyclonal antiserum. (**B**) Prioritizing GR AF-1-interacting proteins. Factors that interacted with $wtGR_{107–237}$ were screened against the GR transactivation-deficient mutant 30IIB. Isolates displaying reduced affinity for the functional activation domain as defined by the 30IIB mutant were given the highest priority. Proteins that interact with both wt and 30IIB were assigned the lowest priority. To ensure that these proteins interact specifically with GR and not with portions of the fusion protein encoded by sequences present in the vector, such as the nuclear localization signal, these clones were also screened against the activation domain construct lacking GR and showed no association with vector-encoded protein segments (not shown). The $GR_{107–237}$ bait does not associate with the LexA DNA binding domain (not shown). (**C**) Results of the interaction trap. Of the 1.5×10^5 individual HeLa cell library colonies examined for their ability to interact with GR AF-1, ~600 potential clones were selected as potential interactors ($Leu2^+/LacZ^+$); 54 associated with GR, while the remaining clones were self-activating false positives. Twenty of the 54 clones required a competent activation surface for their interaction, showing a reduced affinity for 30IIB relative to wtGR. (**D**) The 20 GR AF-1 interacting clones were partially sequenced and these sequences were used to search databases for homologies to known proteins. TSG101 and the DRIP150, along with HSP60, Ran1, ARC16 and five proteins of unknown identity represented in the EST database, interacted with wtGR, but not with 30IIB. (**E**) and (**F**) Quantitative analysis of TSG101 and DRIP150 interaction with GR AF-1 derivatives. $TSG101_{183–381}$ and $DRIP150_{1360–1454}$ expressed as fusion proteins to the LexA DNA binding domain were analyzed for their ability to interact with the vector, wtGR and 30IIB. The strength of interaction is determined by quantitative liquid β-galactosidase assays after a 12 h incubation in galactose-containing media at 30°C for TSG101 and 25°C for DRIP150, respectively. β-galactosidase activity shown represents the average from four independent colonies and the standard deviation.

reduced affinity for the GR 30IIB mutant and subjected them to a database search using the BLAST program (Figure 1C). Six of the clones were identified as HSP60, five were identical to Ran1, two matched ARC16, and five were represented in the expressed sequence tag (EST) database. Ran1, a GTPase involved in nuclear import and export, has been shown previously to affect GR nuclear localization (Carey *et al.*, 1996). ARC16, a component of the ARP2/3 complex, is involved in nucleation of actin polymerization (Welch *et al.*, 1997), while HSP60 is a chaperonin with homology to GroEL (Venner *et al.*, 1990). In addition, we identified two clones potentially involved in transcriptional regulation, which is the focus of this study. The first is the tumor susceptibility gene 101 (TSG101), which interacts strongly with the wtGR but fails to interact with 30IIB (Figure 1E). TSG101 was initially identified in a screen designed to identify tumor suppressor proteins (Li and Cohen, 1996). Inactivation of TSG101 allows naive NIH 3T3 cells to grow in soft agar and form metastatic tumors in nude mice (Li and Cohen, 1996). The predicted TSG101 protein structure is suggestive of a transcription factor (Li and Cohen, 1996). The C-terminal region contains a coiled-coil domain, the central region of the protein contains a proline-rich segment reminiscent of a transcriptional activation domain, and the N-terminus contains a catalytically inactive ubiquitin-conjugating (UBC) domain. The second protein corresponds to vitamin D receptor-interacting protein 150 (DRIP150) and displays a reduced affinity for 30IIB by 68% relative to the wtGR (Figure 1F). DRIP150 is also identical to EXLM1 (Yoshikawa *et al.*, 1998), a gene product identified in a screen for factors that fail to undergo X-chromosome inactivation. DRIP150 is a component of several multiprotein complexes. (i) DRIP150 is part of the DRIP–TRAP complex, which binds in a ligand-dependent manner to the AF-2 regions of the vitamin D receptor (VDR) and thyroid hormone receptor (TR) *in vitro*, and is required for transcriptional activation by VDR and TR *in vitro* and *in vivo* (Fondell *et al*., 1996; Rachez *et al*., 1998). DRIP150 does not contact AF-2 of VDR directly, but rather is brought to the VDR AF-2 by another member of the complex, DRIP205 (Rachez *et al.*, 1999). (ii) DRIP150 is also a component of the NAT complex involved in transcriptional repression, and has been termed hRGR1 due to its homology to yeast RGR1, a component of the RNA polymerase holoenzyme mediator complex (Sun *et al.*, 1998). (iii) The DRIP complex is essentially identical to the activator recruited cofactor (ARC) complex, which binds to and is required for transactivation by other transcription factors, such as SREBP-1a, NF-κB p65 and VP16 (Näär *et al.*, 1998, 1999). (iv) DRIP150 is also a member of the smaller CRSP complex (CRSP150), which is required for Sp-1 activation in a purified transcription system (Ryu *et al.*, 1999). DRIP150 is also part of the SMCC transcriptional regulatory complex (Gu *et al.*, 1999). Thus, DRIP150/EXLM1/hRGR1/CRSP150 is a gene product that escapes X-chromosome inactivation and is found in multiprotein complexes involved in transcriptional regulation.

Specificity of TSG101 and DRIP150 interactions

To analyze the specificity of the TSG101 and DRIP150 interactions, we examined their ability to associate with a panel of transcriptional regulatory proteins in the modified yeast two-hybrid assay (Figure 2). The activation domains of the cyclic AMP response element binding protein (CREB), Sp-1 (Sp-1A and Sp-1B), VP16, the AF-1 regions of the androgen receptor (AR) and TR, the steroid receptor coactivator-1 (SRC-1) and the TBP associated factor 130 (TAF 130) were utilized as baits. As shown in Figure 2, TSG101 associates with the GR, AR and TR AF-1 regions, whereas DRIP150 interacts exclusively with GR, indicating that these proteins are specific for the AF-1 domains of NRs.

GR AF-1 interacts with the coiled-coil domain of TSG101

In an attempt to localize the region of TSG101 that interacts with GR, we created a series of TSG101 C-terminal truncations. TSG101 derivatives containing amino acids 1–82, 1–121, 1–206, 1–240, 1–339 and 1–381, the full-length TSG101, were expressed as fusion proteins with LexA and their ability to interact with GR AF-1 in the modified yeast two-hybrid assay analyzed. The original TSG101 clone identified in the yeast two-hybrid screen encompasses amino acids 183–381. GR associates with the full-length $TSG101_{1–381}$ and $TSG101_{1–339}$, but not with any of the smaller TSG101 derivatives (Figure 3A). In the absence of GR AF-1 expression, on glucose plates, neither full-length TSG101 nor any of the C-terminal truncations activate transcription when bound to DNA. Immunoblot analysis using antibodies against LexA indicates that all of the TSG101 derivatives are expressed at similar levels, with the exception of $TSG101_{1–339}$ which is expressed at an ~4-fold lower level (Figure 3B), suggesting that the association between GR and $TSG101_{1–339}$ may be greater than represented in this assay. These results indicate that the region of TSG101 that interacts with GR AF-1 localizes to amino acids 241–339, a region that overlaps with the coiled-coil domain of TSG101 (Li and Cohen, 1996; Koonin and Abagyan, 1997).

DRIP150 interacts with a functional GR AF-1 in vitro

The ability of DRIP150 to interact with GR AF-1 was also tested *in vitro* using wtGR and 30IIB expressed as glutathione *S*-transferase (GST) fusion proteins. In this

Regulatory Factors	$TSG101_{183-381}$	$DRIP150_{1360-1454}$
$GR_{107-237}$	+	+
AR_{18-500}	+	-
$CREB\text{-}N_{3-296}$	-	-
$TAF\ 130_{270-700}$	-	-
$SP1\ A_{83-262}$	-	-
$SP1\ B_{263-542}$	-	-
$SRC\text{-}1_{374-800}$	-	-
$T_3R\beta2_{1-159}$	+	-
$VP16_{412-456}$	-	-

Fig. 2. Specificity of GR–TSG101 and GR–DRIP150 interactions. Interaction of GR with $TSG101_{183–381}$ or $DRIP150_{1360–1454}$ and with other transcriptional regulatory proteins was analyzed using the modified yeast two-hybrid assay. The strength of interaction is determined by qualitative plate β-galactosidase assays after an 18 h incubation on galactose–X-gal plates at 30°C. Strong interactions (+) represent dark blue colonies and (–) represents no interaction above background 'vector only' control (white colony). TSG101 interacted with the AF-1 domains of GR, androgen receptor (AR) and thyroid receptor (TR), whereas DRIP150 associates with GR AF-1 exclusively.

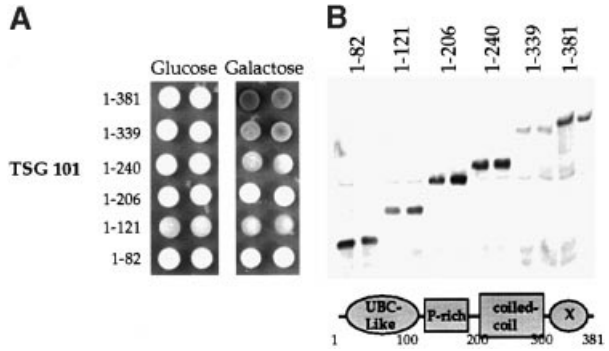

Fig. 3. GR interacts with the TSG101 coiled-coil domain. (**A**) A series of C-terminal truncations of TSG101 were fused to the LexA DNA binding domain and analyzed for their ability to interact with $GR_{107–237}$ in the yeast two-hybrid system. Expression of the β-galactosidase reporter gene was compared on glucose–X-gal versus galactose–X-gal plates. The interaction is observed when GR is expressed in the presence of galactose, but not glucose, demonstrating that the transcriptional activation is dependent on GR–TSG101 interactions. (**B**) Extracts in duplicate from strains expressing the indicated LexA DNA binding domain–TSG101 fusion proteins were probed with a LexA-specific antibody. A schematic representation of the TSG101 protein is based on structural prediction and includes an N-terminal catalytically inactive ubiquitin-conjugating (UBC-like) enzyme domain, a central proline-rich segment (P-rich) reminiscent of a transcriptional activation domain as well as a C-terminal coiled-coil domain.

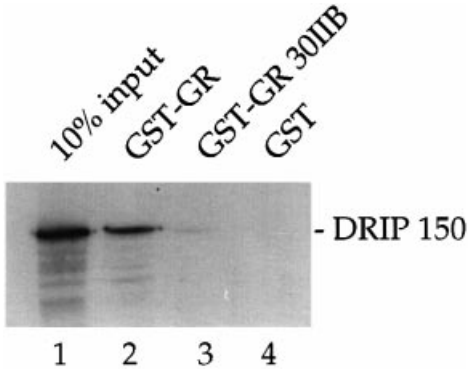

Fig. 4. DRIP150 interacts with GR AF-1 *in vitro*. Interaction of DRIP150 with the GR AF-1 'surface' *in vitro*. To examine GR–DRIP150 interactions *in vitro*, a full-length DRIP150 cDNA was translated *in vitro* in the presence of [^{35}S]methionine (lane 1; 10% input) and incubated with Sepharose beads containing bound GST–$GR_{107–318}$ (lane 2), GST–$GR30IIB_{107–318}$ (lane 3) or GST (lane 4). Bound DRIP150 was resolved by SDS–PAGE and visualized by autoradiography.

GST pull-down assay, *in vitro*-translated full-length DRIP150 bound GST–wtGR, but did not interact efficiently with GST–30IIB or GST (Figure 4). These results substantiate the DRIP150–GR AF-1 interaction observed in the yeast two-hybrid assay, and indicate that DRIP150 interacts with a functional GR AF-1 surface.

Differential effects of TSG101 and DRIP150 on GR-dependent transcriptional activation

Because TSG101 and DRIP150 require a competent GR AF-1 for interaction, we anticipated that these factors would play a role in GR-dependent transcriptional regulation. The effect of full-length TSG101 expression on GR-dependent transcriptional activation was examined in yeast. Yeast were transformed with expression vectors encoding GR AF-1 derivatives fused to the LexA DNA

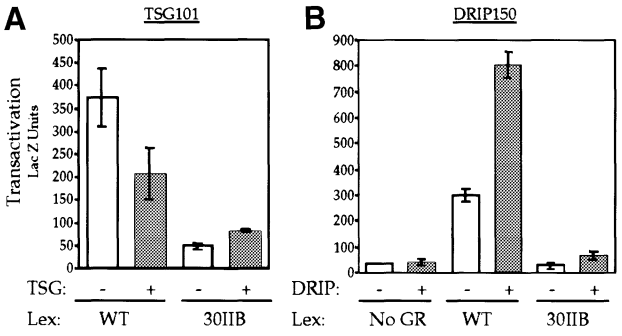

Fig. 5. TSG101 represses, whereas DRIP150 enhances, GR-dependent transcriptional activation in yeast. $TSG101_{1–381}$ (**A**) or $DRIP150_{40–1454}$ (**B**), under the control of a galactose-inducible promoter, were transformed into yeast expressing $GR_{107–237}$wt or 30IIB fused to the LexA DNA binding domain along with a Lex-operator-linked β-galactosidase reporter gene. Transcriptional activity is determined by quantitative liquid β-galactosidase assays in the presence and absence of TSG101 or DRIP150 after a 12 h incubation in galactose- or glucose-containing media, as indicated, at 30°C. β-galactosidase activity shown represents the average from four independent colonies and the standard deviation is shown.

binding domain and TSG101 under the control of a galactose-inducible promoter or the vector alone, along with a Lex-operator-linked β-galactosidase reporter plasmid. As shown in Figure 5A, GR AF-1 transcriptional activation is reduced in the presence of TSG101. These results suggest that TSG101 functions as a transcriptional repressor that binds to and inhibits GR AF-1-dependent transcriptional activation.

To assess the effect of DRIP150 expression on GR transcriptional activation, another set of yeast strains was created that contain the GR derivatives fused to the LexA DNA binding domain, along with a near full-length DRIP150 derivative ($DRIP_{40–1454}$) under the control of a galactose-inducible promoter or the vector alone, and a Lex-operator-linked reporter gene. Expression of DRIP150 in yeast enhanced transcriptional activation by wtGR nearly 3-fold (Figure 5B). In contrast, co-expression of DRIP150 had little effect on 30IIB, indicating that activation of GR AF-1 by DRIP150, as is its ability to interact, is dependent upon the integrity of the GR AF-1 activation surface. Thus, DRIP150 increases GR AF-1-dependent transcriptional activation in yeast, suggesting that DRIP150 represents a novel GR AF-1 coactivator.

To assess whether TSG101 and DRIP150 overexpression affects GR-dependent transcriptional activation in mammalian cells, we transiently transfected HeLa cells, containing endogenous GR, with increasing concentrations of expression vectors encoding either TSG101 or DRIP150 along with a mouse mammary tumour virus (MMTV)-luciferase reporter gene. Overexpression of TSG101 repressed GR-dependent transcriptional activation from the MMTV promoter in a dose-dependent manner, while increasing amounts of DRIP150 resulted in enhanced transcriptional activation by GR (Figure 6A and B). The effect of DRIP150 on GR was not restricted to a single cell type, since overexpression of DRIP150 in CV-1 cells also results in a dose-dependent increase in GR transcriptional enhancement (Figure 6C). Thus, TSG101 represses, whereas DRIP150 enhances, GR-dependent transcriptional activation in mammalian cells.

We next assessed whether a TSG101 derivative lacking

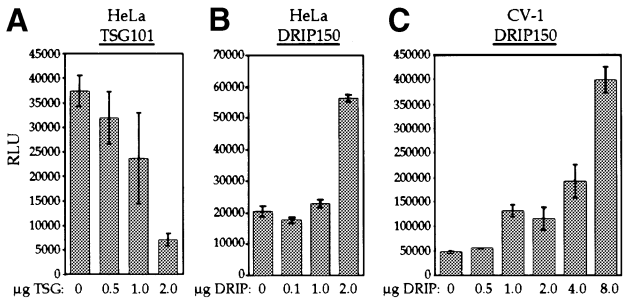

Fig. 6. TSG101 represses, whereas DRIP150 enhances, GR-dependent transcriptional activation in mammalian cells. HeLa cells were transfected with expression plasmids for full-length TSG101 (**A**) or full-length DRIP150 (**B**), at the indicated concentrations along with the GR-responsive, MMTV-Luc reporter plasmid and the total amount of DNA per dish was equalized with an empty expression vector. CV-1 cells were transfected with DRIP150 (**C**) at the indicated concentrations along with a GR expression vector and an MMTV-Luc reporter plasmid. Adding empty expression vector equalized the total amount of DNA per dish. Cells were treated with 100 nM dexamethasone for 12 h and GR transcriptional activity was assayed as described in Materials and methods, normalized to protein concentration and expressed as relative luminescence units (RLU). The average of three independent experiments is shown with the standard deviation.

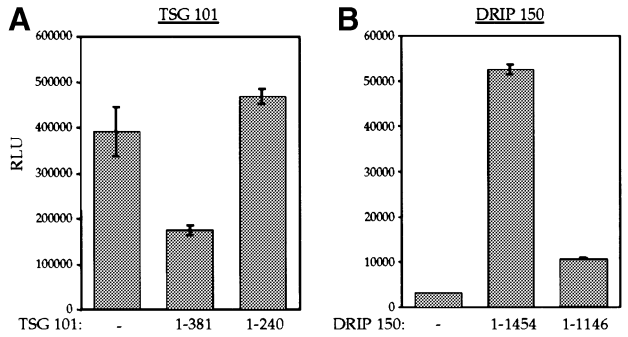

Fig. 7. The effects of TSG101 and DRIP150 on GR transcriptional regulation are dependent on the GR-interacting regions. (**A**) Repression of GR transactivation is associated with the TSG101 GR-interacting region. HeLa cells were transfected with $GR_{1–556}$, a constitutively active GR derivative lacking the LBD, and either 1.5 µg of the empty expression vector (–), full-length $TSG101_{1–381}$ (1.5 µg) or a C-terminal deletion of $TSG101_{1–240}$ (1.5 µg) that does not interact with GR. (**B**) Enhancement of GR transactivation is associated with the DRIP150 GR-interacting region. HeLa cells were transfected with $GR_{1–556}$ and either an empty expression vector (1 µg), full-length $DRIP150_{1–1454}$ (1 µg) or a C-terminal deletion $DRIP150_{1–1146}$ (1 µg) derivative, and GR activity was determined as described in Figure 6. The average of three independent experiments is shown with the standard deviation.

the GR-AF-1-interacting region was capable of affecting GR-dependent transcriptional activation. HeLa cells were transfected with an MMTV-luciferase reporter gene along with either an empty expression vector, full-length TSG101 (1–381) or a C-terminal deletion of TSG101 (1–241) incapable of interacting with GR AF-1 (see Figure 3). Full-length TSG101 is capable of repressing GR transcriptional activation, whereas the C-terminal TSG101 truncation is not (Figure 7A). Therefore, the inhibition of GR transcriptional activation by TSG101 requires the TSG101 GR-interacting region.

We also examined whether a DRIP150 derivative lacking the GR-AF-1-interacting region was able to increase GR transactivation. Full-length DRIP150 (1–1454) or a C-terminal deletion derivative, DRIP150 (1–1146), lacking the GR-interacting region was transfected into HeLa cells and GR-AF-1-dependent transcriptional activation was measured. Transcriptional activation of GR is increased 10-fold in the presence of full-length DRIP150 overexpression, whereas the DRIP150 (1–1146) derivative lacking the interacting region elicits an ~2-fold increase in GR enhancement (Figure 7B). These results indicate that the enhanced GR transactivation observed upon DRIP150 overexpression is largely dependent upon the DRIP150 GR-interacting region.

DRIP205 interacts with GR LBD in a hormone-dependent manner

Since association of the DRIP complex with NRs was originally discovered as a ligand- and AF-2-dependent process through a single subunit (Rachez *et al.*, 1999), DRIP205, we also examined whether DRIP205 is capable of interacting with the GR ligand binding domain (LBD) and whether this interaction is hormone dependent. We *in vitro* transcribed and translated full-length GR, as well as GR N- and C-terminal deletion derivatives (Figure 8A), and precipitated them using GST–DRIP205 in the absence and presence of dexamethasone. Remarkably, DRIP205 bound full-length GR in a ligand-dependent manner (Figure 8B). GR LBD (GR-C) also efficiently associated with DRIP205 in the presence but not the absence of dexamethasone. In contrast, the GR N-terminus (GR-N), the target of DRIP150 association, did not interact with DRIP205, indicating that the GR LBD is sufficient for ligand-dependent interaction with DRIP205.

Interestingly, *in vitro*-translated full-length GR bound GST–DRIP150 in a dexamethasone-independent fashion, consistent with DRIP150's interaction with the glucocorticoid-independent AF-1 (Figure 8C). GR LBD failed to associate with GST–DRIP150 either in the presence or absence of dexamethasone (Figure 8C). These results establish that DRIP150 interacts with GR AF-1, but not AF-2. Together, our data indicate that DRIP205 associates with GR LBD in a hormone-dependent manner, whereas DRIP150 interacts with GR AF-1, thereby linking AF-1 to AF-2 via the DRIP coactivator complex.

To assess whether DRIP205 and DRIP150 overexpression affects GR-dependent transcriptional activation, we transiently transfected HeLa cells with expression vectors encoding either DRIP205 or DRIP150, individually or together, and an MMTV-luciferase reporter gene. Expression of DRIP205 had little effect on GR transcriptional activation, whereas expression of DRIP150, under the conditions examined, increased receptor-dependent transcriptional activity ~10-fold (Figure 8D). Co-expression of DRIP205 and DRIP150 further augmented GR-dependent transactivation 20-fold, lending support to the idea that components of the DRIP coactivator complex are key regulators of GR transcriptional enhancement.

Discussion

We have identified TSG101 and DRIP150 as proteins that interact with GR AF-1 and differentially affect GR transcriptional activation. TSG101 expression results in a reduction of GR transcriptional activation in both yeast and mammalian cells, whereas overexpression of DRIP150 increases GR transcriptional enhancement in these systems.

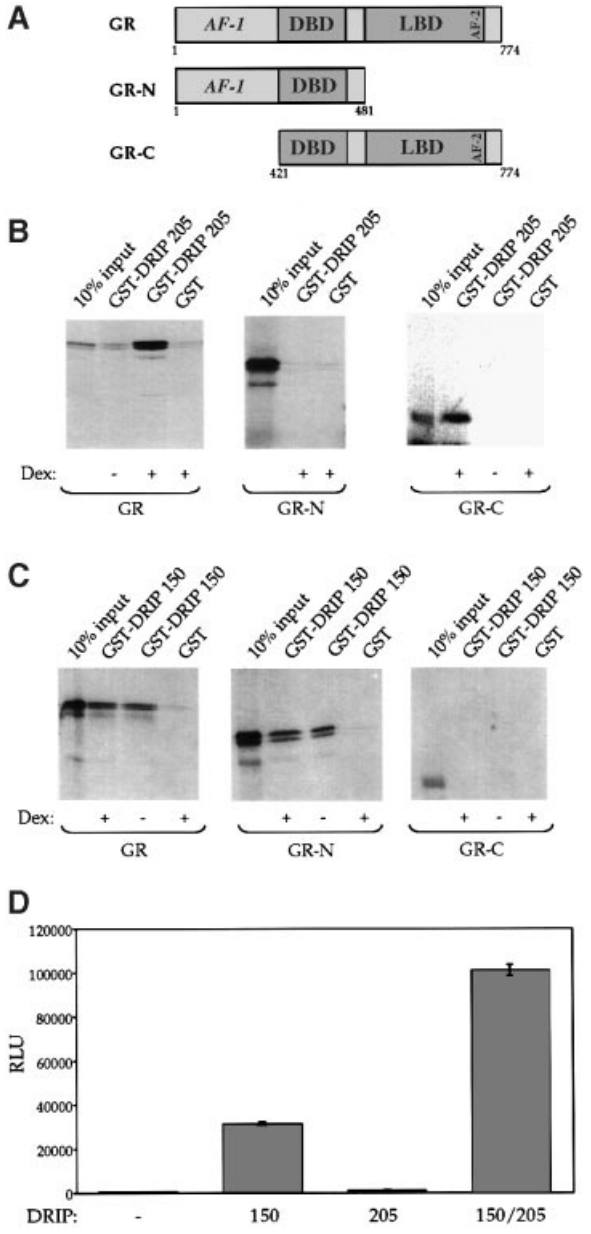

Fig. 8. The GR N- and C-termini associate with DRIP150 and DRIP205, respectively. (**A**) Schematic representation of full-length human $GR_{1–774}$ (GR), $GR_{1–481}$ (GR-N) and $GR_{421–774}$ (GR-C) derivatives. (**B**) Ligand-dependent interaction of DRIP205 with GR *in vitro*. To examine GR–DRIP205 interaction, full-length GR was transcribed and translated *in vitro* in the presence of [^{35}S]methionine (lane 1; 10% input) and incubated with Sepharose beads containing bound GST–DRIP205, in the absence or presence of dexamethasone, or GST. The GR-N and GR-C derivatives were translated *in vitro* and incubated with Sepharose beads containing bound GST–DRIP205, in the absence or presence of dexamethasone, or GST. Bound GR was resolved by SDS–PAGE and visualized by autoradiography. Note the hormone-dependent interaction of GR and GR-C with DRIP205. (**C**) Ligand-independent association of DRIP150 with the GR *in vitro*. GR, GR-N or GR-C were translated *in vitro* in the presence of [^{35}S]methionine (lane 1; 10% input) and incubated with Sepharose beads containing bound GST–DRIP150 in the absence or presence of dexamethasone, or GST. Bound GR was resolved by SDS–PAGE and visualized by autoradiography. Note the ligand-independent interaction of GR and GR-N with DRIP150. (**D**) Enhancement of GR transactivation by DRIP150 and DRIP205. HeLa cells were transfected with either 3.0 μg of the empty expression vector (–), DRIP150 (1.5 μg), DRIP205 (1.5 μg) or DRIP150 (1.5 μg) and DRIP205 (1.5 μg) and assayed as described in Figure 6, and GR transactivation was measured and expressed as RLU.

The differential effects of TSG101 and DRIP150 on GR transcriptional activation are dependent upon the DRIP150 and TSG101 GR-interacting regions, since deletion of these domains renders both cofactors less capable of affecting GR transcriptional activation. Thus, DRIP150 and TSG101 represent a new class of GR AF-1-associated cofactors that regulate receptor transcriptional activation in a reciprocal manner. To our knowledge, this is the first example of steroid receptor association with DRIPs.

We also demonstrate a ligand-dependent interaction between DRIP205 and GR LBD. Although overexpression of DRIP205 had little effect on GR transcriptional enhancement, co-expression of DRIP150 and DRIP205 augments GR transcriptional activation to a degree greater than either molecule alone, suggesting that DRIP205 and DRIP150 cooperate in promoting GR transcriptional activation. Thus, DRIP150 and DRIP205 associate with GR N- and C-termini, respectively, functionally linking GR AF-1 to AF-2.

TSG101 was originally identified in a screen for genes that, when inactivated, promote neoplastic transformation (Li and Cohen, 1996). Our results suggest that TSG101 functions as a transcriptional repressor protein, decreasing GR-dependent transcriptional activation by binding and repressing AF-1. TSG101 also interacts with the AF-1 regions of AR and TR, and expression of TSG101 decreases AR-dependent transcriptional activation (not shown). Although Watanabe *et al.* (1998) demonstrated previously that TSG101 suppresses the transcriptional activity of a variety of NRs, including GR, AR and TR, our findings link TSG101 specifically to AF-1 domains in the receptors.

The ability of TSG101 to bind to GR AF-1 and inhibit transactivation is reminiscent of the interaction between another tumor susceptibility gene, Rb, and the transcription factor it negatively regulates, E2F-1. Rb is capable of recruiting a histone deacetylase (HDAC) to E2F-1, thereby repressing E2F-regulated promoters (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Conceivably, TSG101 functions in an analogous fashion, recruiting a HDAC to GR and repressing its transcriptional activity through changes in chromatin structure. This idea is also consistent with the ability of TSG101 to repress a wide variety of activators and promoters when overexpressed. Additional experiments will be required to determine the mechanism of TSG101-mediated suppression of GR transactivation and whether it is mediated by HDACs.

DRIP150 was initially isolated as a subunit of a multiprotein complex that interacts in a ligand-dependent manner with the AF-2 domain of VDR and enhances VDR transcriptional activation, indicating that the complex may have evolved to link NRs to the transcription apparatus. Recently, DRIP150 has been identified as a component of several multiprotein complexes, including ARC, CRSP, TRAP/SMCC and NAT, that regulate other transcriptional activators, suggesting a broader role for the DRIP complex in transcriptional regulation (Sun *et al.*, 1998; Näär *et al.*, 1999; Rachez *et al.*, 1999; Ryu *et al.*, 1999). In both yeast and mammalian cells, DRIP150 is capable of increasing GR-mediated transcriptional activation when overexpressed. DRIP205 binds directly to the LBDs of several NRs in a ligand-dependent manner. Here we demonstrate that GR LBD also interacts with DRIP205 in

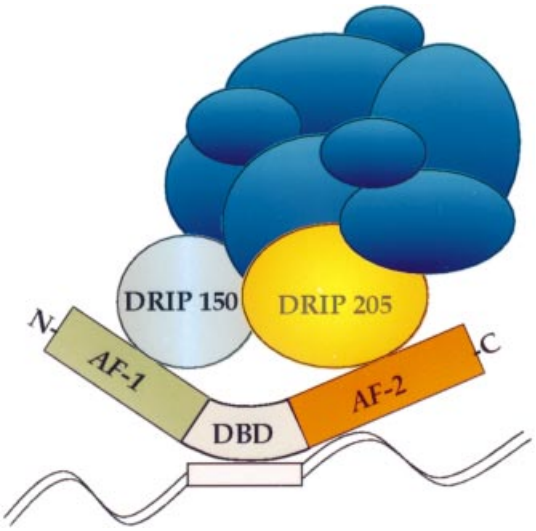

Fig. 9. A model for the regulation of GR by DRIP150 and DRIP205. Shown schematically is the full-length GR, which recruits DRIP150 (light blue oval) and DRIP205 (yellow oval) through an association with the GR N-terminus (green rectangle) and C-terminus (orange rectangle), respectively, thereby facilitating GR transactivation. The other subunits of the heteromeric DRIP complex are also depicted (dark blue ovals); however, their precise molecular interactions and effects on GR-mediated transactivation have not been determined.

a hormone-dependent manner. Although expression of DRIP205 has little effect on GR-mediated transcriptional activation, it appears to synergize with DRIP150 to facilitate GR-mediated transcriptional activation. From these results, we propose a model whereby DRIP150 and DRIP205 serve as a molecular bridge functionally linking the GR N- and C-terminal transcriptional activation functions, AF-1 and AF-2, thus facilitating transcriptional activation (Figure 9). Consistent with this view, recent findings from our laboratory suggest that distinct coactivators may promote the productive interaction between the GR N-terminus and LBD to facilitate GR transcriptional activation of the endogenous p21 promoter in SAOS2 cells (Rogatsky *et al.*, 1999). Although the N- and C-terminal regulatory regions of the estrogen receptor function synergistically to activate transcription via SRC1 (McInerney *et al.*, 1996), this is the first example whereby two members of the heteromeric DRIP coactivator complex, DRIP150 and DRIP205, associate with distinct GR domains to facilitate transcriptional enhancement. It is likely that the DRIP complex or subcomplexes containing DRIP150 and DRIP205 are important mediators of GR transcriptional activation. It is also conceivable that differences in the ratios of DRIP150 to DRIP205 within cells will contribute to cell- and enhancer-specific regulation by the GR. Thus, the ability of GR to target multiple proteins within the DRIP coactivator complex via distinct receptor domains likely represents a general and versatile means of regulating gene expression.

Materials and methods

Construction of plasmids

Yeast expression vectors for the LexA–GR fusion proteins (Lex–$GR_{107–237}$wt and Lex–$GR_{107–237}$30IIB) were created by digesting the respective full-length rat GR clones with *Nco*I, filling in the 5′ overhangs with DNA polymerase Klenow fragment to create a blunt end, followed by *Bgl*II digestion and subcloning the fragments into pEG202 digested with *Eco*RI, the 5′ end filled in, and *Bam*HI. Yeast two-hybrid 'bait' proteins, B42–$GR_{107–237}$wt and –30IIB, were constructed by subcloning GR *Eco*RI–*Xho*I fragments from pEG202:$GR_{107–237}$, wt and 30IIB, into the corresponding sites in pJG4-5. The LexA–HeLa cell 'prey' library was constructed by subcloning *Eco*RI–*Xho*I fragments from a pJG4-5:HeLa cell library into the pEG202 digested with *Eco*RI–*Xho*I. The LexA–TSG101 fusion protein was created by digesting the full-length TSG101 (a gift from S.Cohen, Stanford University, CA) with *Pvu*II, and subcloning the fragment into the *Bam*HI site of pEG202 also made blunt by filling in its 5′ overhang. An *Eco*RI–*Xho*I fragment from pEG202 was then subcloned into pJG4-6 to create a HA-tagged version of full-length TSG101. The TSG101 C-terminal truncations 1–82, 1–121, 1–206, 1–240 and 1–339 were created by digesting LexA–TSG101 with *Spe*I, *Bsp*HI, *Ava*II, *Ban*I or *Hin*dIII, respectively, filling in their 5′ overhangs with Klenow, digesting with *Eco*RI and subcloning into pEG202 digested with *Nco*I, filled in, and *Eco*RI. The mammalian expression vector pCDNA3:FLAG–TSG101 was created by subcloning the FLAG–TSG101 *Pvu*II fragment into pCDNA3 digested with *Bam*HI with its 5′ overhang filled in. pEG202:$DRIP150_{40–1454}$ (Lex–DRIP) and pJG4-6:$DRIP150_{40–1454}$ (HA–DRIP) were created by subcloning a *Not*I fragment (filled-in blunt) from pCDNA3:DRIP150 into pEG202 and pJG 4-6 digested with *Eco*RI–*Xho*I (filled-in blunt). pCDNA3:DRIP150(1–1146) was created by digesting pCDNA3:DRIP150 with *Nco*I, filling in the 5′ overhang, digesting with *Eco*RI and subcloning the fragment into *Eco*RI–*Xho*I (filled-in blunt) pCDNA3. pJG4-5:Sp-$1A_{83–262}$, pJG4-5:Sp-$1B_{263–542}$, pJG 4-5:TAF $130_{270–700}$, pJG 4-5:$VP16_{412–456}$ and pJG4-5:CREB-$N_{3–296}$ were provided by N.Tanese (New York University School of Medicine, New York). pJG4-5:SRC-$1_{374–800}$ and pJG4-5:T3Rβ$2_{1–159}$ were provided by H.Samuels (New York University School of Medicine, New York). pJG4-5:$AR_{18–500}$ was provided by S.Taneja (New York University School of Medicine, New York). The pJK103 reporter plasmid, which contains a single LexA operator linked to β-galactosidase, was used in all activity assays of the LexA fusion proteins and in the modified yeast two-hybrid assay. The pCMV:wtGR expression plasmid was used to produce rat GR, pMMTV:luciferase reporter was used to assay GR transcriptional activity, while pCMV:LacZ constitutively expresses β-galactosidase, a marker for transfection efficiency.

Modified yeast two-hybrid approach

The modified yeast two-hybrid approach is based on the LexA system as described in Bartel and Fields (1997) with certain modifications. EGY188 (*trp1 his3 ura3 leu2*) contains a chromosomally integrated leucine (*LEU*) reporter gene driven by a single LexA operator (*leu2::2* LexAop-*LEU2*), allowing for the selection of interacting proteins on galactose, leucine-deficient plates. EGY188 was transformed by the lithium acetate method (Gietz, 1995) with (i) pJG4-5:$GR_{107–237}$ bait, (ii) pEG202:HeLa cell cDNA library and (iii) pJK103, a β-galactosidase reporter gene with a single LexA operator. pJG 4-5, which contains the GAL1-10 promoter, expresses B42 activation domain fusion proteins grown in the presence of galactose, while pEG202, driven by the alcohol dehydrogenase promoter, constitutively expresses LexA DNA binding domain fusion proteins. Potential interacting proteins were selected by plating the cDNA library-expressing transformants onto galactose plates lacking leucine and containing the chromogenic substrate X-gal. As a majority of the colonies contained cDNAs that encode an activation domain (self-activating false positives) rather than a GR-interacting protein, a second screen was used. Colonies that grew on galactose in the absence of leucine and expressed LacZ (i.e. blue) were replica-plated onto glucose-X-gal plates. Since the expression of GR is under the control of the galactose-inducible, glucose-repressible Gal1-10 promoter, potential interactors are blue on galactose–X-gal plates, conditions where the GR bait is expressed, but white on glucose–X-gal plates. In contrast, false positives are blue on both glucose–X-gal and galactose–X-gal-plates, independent of GR expression, and were not analyzed.

Quantitative liquid β-galactosidase assay

Yeast were grown in selective liquid media containing 2% glucose for 10 h, pelleted, washed once in H_2O, normalized according to cell number and resuspended to an optical density (OD 600) of 0.15 in either 2% glucose or 2% galactose/1% raffinose, depending on the experiment. β-galactosidase assays were performed 12 h later as described previously (Garabedian, 1993).

GST pull-down assays

GST fusion proteins (20 μg), GR $wt_{107–318}$, GR $30IIB_{107–318}$, $DRIP150_{1345–1493}$ and $DRIP205_{527–774}$ or GST, immobilized on glutathione–Sepharose beads were preincubated in binding buffer [20 mM Tris pH 7.9, 170 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.05% Nonidet P-40 (NP-40), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 4 mg/ml bovine serum albumin (BSA)] for 30 min at 4°C. *In vitro*-translated, [^{35}S]methionine-labeled (Promega TNT reticulocyte lysate system) DRIP150 or GR was incubated with the immobilized fusion proteins for 1 h at 4°C. The beads were washed four times in wash buffer (20 mM Tris pH 7.9, 170 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.1% NP-40, 0.1 mM PMSF and 1 mM DTT), resuspended in 2× SDS sample buffer and boiled for 3 min; the associated proteins were resolved by SDS–PAGE and visualized by autoradiography.

Mammalian cell culture and transient transfection assays

Human cervical carcinoma cell lines (HeLa) and African green monkey kidney cells (CV-1) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), 50 U/ml each of penicillin and streptomycin (Life Technologies) and 2 mM L-glutamine (Life Technologies). For transfections, HeLa cells were seeded in 35 mm dishes at a density of 1.5×10^5, washed once with serum-free medium and transfected with 0.1 μg MMTV-Luc, 0.05 μg pCMV-LacZ, and the indicated concentrations of pCDNA3:FLAG–TSG101, pCDNA3:DRIP150 and pCDNA3:DRIP205, using 5 μl of lipofectamine reagent (Life Technologies) in a total volume of 1 ml of serum-free, phenol red-free DMEM per 35 mm dish according to the manufacturer's instructions. Three hours post-transfection, the transfection mix was removed, the cells were refed with 2 ml of DMEM–10% FBS, allowed to recover for 5–7 h, and were fed again with fresh DMEM–10% FBS supplemented with 100 nM Dex or an identical volume of 100% ethanol and incubated for 12 h. Transfected cells were washed twice in phosphate-buffered saline and harvested in 1× reporter lysis buffer (Promega) as per the manufacturer's instructions. CV-1 cells were transfected with 0.5 μg pCMV:GR, 1 μg MMTV-Luc, 0.5 μg pCMV-LacZ and the indicated concentration of pCDNA3:DRIP150 by the calcium phosphate method as described previously (Lemon and Freedman, 1996). Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml BSA, 1 mM DTT, using a Lumen LB 9507 luminometer (EG&G Berthold) and 1 mM D-luciferin (Analytical Luminescence Laboratory) as substrate. Lysates from the transfected cells were also assayed for β-galactosidase activity as described elsewhere (Alam and Cook, 1990).

Immunoblotting

Yeast protein extracts were prepared from 2 ml cultures and lysed using glass beads as previously described (Knoblauch and Garabedian, 1999). Extracts were normalized according to the Bradford protein assay (Bio-Rad) and separated on either 10 or 4–20% SDS–polyacrylamide gels (Novex) and transferred to Immobilon paper (Millipore). Membranes were probed with polyclonal antibodies for α-LexA (a gift from E.Golemis) or monoclonal antibodies to HA (12CA5; Boehringer Mannheim). The blots were developed using horseradish peroxidase-coupled donkey anti-rabbit or sheep anti-mouse antibodies and the enhanced chemiluminescence (ECL) substrate as per the manufacturer's instructions (Amersham–Pharmacia).

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