Rb Enhances p160/SRC Coactivator-dependent Activity of Nuclear Receptors and Hormone Responsiveness*

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The retinoblastoma tumor suppressor protein (Rb) is best known as a repressor of genes involved in cell cycle progression. Rb has also been implicated in activation of transcription, in particular by nuclear receptors (NRs) and by differentiation-related transcription factors, but the relevance of this activity is unclear. We show that Rb and the related proteins p107 and p130 enhance the activity of NRs related to NGFI-B (Nur factors) through direct interactions with NGFI-B and SRC-2. Although recruitment of SRC/p160 coactivators to the NGFI-B AF1 domain is independent of Rb, its presence enhances SRC-dependent transcription. Rb potentiation of SRC coactivators is exerted on a subset (Nur factors, hepatic nuclear factor-4, SF-1, and ER) but not all NRs. The levels of Rb-related proteins modulate hormone responsiveness of the NGFI-B-dependent pituitary pro-opiomelanocortin gene and HNF-4-dependent transcription during enterocyte differentiation. Increased Rb expression upon cell differentiation may promote differentiated functions, at least in part, by potentiation of NR activity.

The retinoblastoma tumor suppressor gene (RB1) encodes the Rb protein that is an important regulator of proliferation, differentiation, and cell death (1). To exercise its numerous functions, Rb acts as a transcriptional regulator interacting with many transcription factors. For example, Rb interacts and represses transcription factors of the E2F family, whereas it enhances activity of transcription factors required for cell differentiation, such as MyoD, C/EBP, c-Jun, AP-2, and Chf1 (2). Some functions of Rb are regulated by phosphorylation in a cell cycle-dependent manner. It is thought that dephosphorylated Rb inhibits E2F activity by binding E2F and recruiting histone deacetylases (2); this leads to repression of genes required for entry into S phase of the cell cycle. The phosphorylation of Rb at the end of the G1 phase by cyclin-dependent kinases causes disruption of Rb/E2F complexes and cell cycle progression. Rb phosphorylation is also regulated by cyclin-dependent kinase inhibitors like p16, and its activity is modulated by p300 acetylation (3).

Mice mutant for one copy of the Rb1 gene always develop tumors of pituitary pro-opiomelanocortin (POMC)-expressing cells (1), in contrast to humans where RB1 inactivation is associated with retinoblastoma and not directly with pituitary tumorigenesis. Despite the absence of direct mutation in the RB1 gene in human pituitary tumors, loss of Rb expression was linked to pituitary corticotroph tumor progression when adenomas were compared with poorly differentiated carcinomas (4).

To investigate the role of Rb in pituitary POMC cells and, particularly, in their transcriptional regulatory mechanisms, we determined whether Rb regulates transcription of the POMC gene itself. This led to identification of a subfamily of three orphan nuclear receptors (NRs) that are targeted by Rb. Indeed, this subfamily of orphan NRs includes the three Nur factors, NGFI-B (Nur77), Nur-related factor 1 (Nurr1), and neuron-derived orphan receptor 1 (NOR-1) (5–7). The Nur factors are immediate early response genes, and they are widely expressed. Nur77 and NOR-1 are implicated in the control of thymocyte apoptosis, whereas Nurr1 plays an essential role in the development and maintenance of midbrain dopaminergic neurons (reviewed in Ref. 7). Nur factors contribute to basal POMC transcription as well as to activation of transcription in response to hypothalamic corticotrophin-releasing hormone (CRH). The pituitary POMC promoter target of Nur factor action is the Nur response element (NurRE), a palindromic sequence bound by dimers formed between Nur family NRs (5, 7). The activity of Nur factor dimers is enhanced by coactivators of the steroid receptor coactivator SRC/p160 family, and this effect is mediated through the AF-1 domain of NGFI-B (8).

The SRC/p160 coactivator family consists of three proteins designated SRC-1/NCoA-1, SRC-2/TIF2/GRIP1/NCoA-2, and SRC-3/p/CIP/RAC3/ACTR/AIB-1/TRAM-1 (9). NRs contain two activation domains: one in the N terminus that is ligand-independent (AF-1), and the other in the C-terminal ligand-binding domain that is ligand-dependent (AF-2). AF-1 and AF-2 domains require coactivators such as SRCs to mediate their transcriptional effects. SRCs have histone acetyltransferase activity, and they also recruit CBP/p300 to enhance transcription.

Rb has been reported to modulate the activity of some NRs, possibly through interaction with other proteins. Indeed Rb

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The abbreviations used are: POMC, pro-opiomelanocortin; ER, estrogen receptor; TRO, triple knock-out; NR, nuclear receptor; Nurr1, Nur-related factor 1; NOR-1, neuron-derived orphan receptor 1; CRH, corticotrophin-releasing hormone; NurRE, Nur response element; PPARγ, peroxisome proliferator-activated receptor γ; CMV, cytomegalovirus; HA, hemagglutinin; GST, glutathione S-transferase; MEF, mouse primary fibroblast; WT, wild type; TRO, triple knock-out; siRNA, small interfering RNA; GFP, green fluorescent protein; ChIP, chromatin immunoprecipitation; HNF-4, hepatocyte nuclear factor-4; SF-1, steroidogenic factor-1; RAR, retinoic acid receptor; RXR, retinoid X receptor; DBD, DNA-binding domain; RbID, Rb-interacting domain; CICD, AD1/CBP-interacting domain; GR, glucocorticoid receptor.

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19746
enables the activity of GR through interaction with the Brm/BRG1 sub-units of the SWI/SNF complex (10) and the activity of the androgen receptor independently of BRG1 (11). Rb also regulates negatively the activity of the thyroid hormone receptor by interacting with and inhibiting the coactivator Trip230 (12) and inhibits PPARγ-dependent transcription (13). Rb can also interact with the RIZ protein, which in turn interacts with the estrogen receptor (ER) in a ligand-dependent manner (14). These data have suggested that Rb may be an important regulator of NR activity.

In view of the particular, and apparently limiting, role of Rb in POMC-expressing lineages and of the present identification of Nur factors as Rb targets, we have investigated in detail the mechanism of Rb enhancement of NGFII-B activity and found that Rb acts as a potentiator of SRC/p160 coactivator function.

This action is mediated by direct interactions between Rb, NGFII-B, and SRCs. The RhSRC synergism is not restricted to Nur factors, operates on other nuclear receptors, and may constitute a paradigm for the action of Rb on various transcription factors.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—POMC promoter constructs, NurRE, GRE, SF1-RE reporter plasmids as well as SF-1, NGFII-B and its mutants, SRC-1, SRC-2, SRC-3, and CBP expression vectors were previously described (5, 8). SRC-2/TIF2 expression vectors were provided by Dr. Pierre Chambon (15). DR1, DR5, and ER reporter plasmids and pCMX-Eras and ERβ expression vectors were obtained from Dr. Vincent Giguère.

Expression vectors CMV-Rb, pSG5L-HA-Rb, and tumor-derived mutants were obtained from Dr. William Kaelin (16), whereas SV40-HA-mRh3p54 (Δp54) expression vector was from Dr. Paul Hamei (17). SV40-CMV reporter vectors were from Dr. Paul Robey (18), and CMV-p107 and CMV-p130 were from Dr. Sylvain Melcho. GST-Rb plasmids were described previously (19–21).

**Cell Culture and Transfection**—Cell culture and transfection for AtT-20 cells were described previously (8). WT and TKO MEFs (22) used at passages 3–10 were transfected with Effectene (Qiagen), and CV1, L, and C33A cells were transfected by the calcium phosphate method. Caco-2 cells were cultured as described (23). For co-transfection experiments (see Fig. 8), luciferase and GFP or with 100 pmol of each siRNA for Rb and p107, using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. For co-transfection experiments (see Fig. 8), luciferase and GFP or with 100 pmol of each siRNA for Rb and p107, using Oligofectamine (Invitrogen) according to the manufacturer’s instructions.

**Quantitative Real-time PCR**—Quantitative real-time PCR (Stratagene MX-4000 or Applied Abiprim 7000) was performed with the SYBR Green kit (Qiagen or Applied). The primers used were as follows: POMC promoter 5′-TCCATTGCCCCACACAGGCCC (−703) and 5′-GCTCT- GTTTGAGATTCTCTGGAG (−352); POMC exon 3, 5′-ACCCCGTGGA- TGTCCCTATGGGGAGG (−1612) and 5′-GGGCCAACGCTTGAAGAGGCG (−554); POMC 5′UTR-exon1, 5′-ACCAACCAACACCCTGGAATATAGAATA (−1227); Rb1 exon9-1-AT, 3′-GGGAGCTTGGCAGATG (−1710); p107 exon5-exon6, 5′-ATGAGCGGCTATCTGGGT (−1161) and 5′-AGGACAAAAGCTGCGGG (−9989); p130 exon2, 5′-CACCGGATCACTGTCAGGAG (−3047) and 5′-TTTGGGAGTAGTGTGGCAGT (−3180); p130 exon8-exon9, 5′-ATGAGGGGCTTACCGTTGGT (−1227); p107 exon5-exon6, 5′-ATGACGCTTGGAGTTCGAG (−1710); p107 exon5-exon6, 5′-ATGACGCTTGGAGTTCGAG (−1710); p107 exon5-exon6, 5′-ATGACGCTTGGAGTTCGAG (−1710); p107 exon5-exon6, 5′-ATGACGCTTGGAGTTCGAG (−1710); p107 exon5-exon6, 5′-ATGACGCTTGGAGTTCGAG (−1710); p107 exon5-exon6, 5′-ATGACGCTTGGAGTTCGAG (−1710).

**RESULTS**

**Rb Activates POMC Transcription through the NurRE**—To determine whether the POMC gene is a target of Rb regulation, we investigated the ability of Rb to modulate POMC promoter activity using transient co-transfection in POMC-expressing AtT-20 cells. Strong activation of the POMC promoter was
observed in the presence of Rb (Fig. 1A, lane 1). Using a panel of POMC promoter mutants, we found two different response elements that are targeted by Rb: one is the NurRE and the other is the Ebox\textsubscript{neuro}, that is the target of NeuroD1-containing basic helix-loop-helix dimers (25). As shown in Fig. 1A (lanes 2 and 4), mutagenesis of the NurRE abolished most of the Rb effect, whereas mutagenesis of another potential Nur factor target, the NBRE (5), did not affect Rb-dependent activity (lane 3). The importance of the NurRE for Rb activation was also shown using simple reporters that contain multimers of POMC regulatory elements. In this context, the NurRE confers Rb-dependent activity (Fig. 1B, compared lane 5 to lane 6). In both experiments, residual Rb-dependent activity (Fig. 1A, lanes 2 and 4) and B (lane 6) was ascribed to the Ebox\textsubscript{neuro} and to basic helix-loop-helix factors (25). Prior work indicated that the NurRE and the cognate Nur factors contribute to basal activity as well as to POMC promoter responsiveness to the hypothalamic hormone CRH (5, 7), whereas the Ebox\textsubscript{neuro} contributes to basal activity (26, 27).

To assess the importance of Rb and its related “pocket” proteins in \textit{vivo}, we used a small interfering (si) RNA approach in AtT-20 cells. Because these cells express significant levels of Rb and p107, we used a mixture of four siRNAs complementary to these mRNAs (a pair for each). The siRNAs produced significant reductions of mRNAs for both Rb and p107, but not for \(\beta\)-2-microglobulin (Fig. 1C). The decrease of Rb and p107 mRNAs resulted in reduced basal POMC expression and in complete abrogation of POMC gene activation by CRH (Fig. 1C). Rb-related proteins thus appear important for POMC expression.

\textbf{Rb Synergizes with NGFI-B and with SRC/p160 Coactivators—}Because the NurRE is the target of orphan NRs of the Nur family, we tested the effect of Rb on NGFI-B activity. Using a NurRE reporter in AtT-20 cells, we show that Rb acts as a coactivator of NGFI-B-dependent activity in a concentration-dependent fashion (Fig. 2A). Because we have previously shown enhancement of NGFI-B activity by the SRC/p160 coactivators (8), we tested a putative collaboration between Rb and these coactivators. As shown in Fig. 2B, Rb further enhanced NGFI-B- and coactivator-dependent transcription assessed using the NurRE reporter (top), whereas no effect was observed with a reporter devoid of NurRE (Min, bottom). The major effect of Rb was to enhance the activity stimulated by the SRC coactivators SRC-1, SRC-2, or SRC-3. Indeed, Rb increased the steepness of the NGFI-B dose-response curve beyond the effect of each SRC coactivator (Fig. 2C), suggesting that Rb acts synergistically with SRC coactivators to enhance NGFI-B activity. In these experiments, the levels of expression of Rb, NGFI-B, or SRC coactivators were not affected by co-expression of each other (Fig. 2, B (bottom) and D). Equivalent results were obtained in CV1 or L cells and in cells that are deficient in Rb protein and C33A and MDA-MB468 cells (data not shown). However, the SRC coactivator effect on NGFI-B-dependent activity appeared larger in Rb-positive cells (CV1 or L) compared with Rb-defective cells (C33A and MDA-MB468). Conversely, the effect of Rb on NGFI-B- and SRC-2-dependent (NGFI-B/SRC-2) transcription was greater in Rb-defective compared with Rb-positive cells (data not shown).

\textbf{Rb-related Proteins Potentiate NGFI-B/SRC Activity but Are Not Essential—}Rb is the founding member of the “pocket protein” family that also includes p107 and p130 (1). To determine whether Rb or related proteins are essential for SRC coactivator effects, we used primary fibroblasts (MEF) from triple knock-out (TKO) mice deficient in all pocket proteins (22). TKO MEFs and control (WT) MEFs had very similar transfection efficiency (not shown). NGFI-B stimulated NurRE-dependent transcription in both cells, and these activities were enhanced by SRC-2 (Fig. 2E). Thus, Rb-related proteins are dispensable for SRC enhancement of NGFI-B activity. However, WT MEFs exhibited higher activity and enhancement compared with TKO MEFs, consistent with the enhancement effect of Rb proteins (Fig. 2E). This was confirmed by increased NGFI-B- and SRC-2-dependent transcription after addition of Rb in TKO cells (Fig. 2F). All pocket proteins enhanced NGFI-B activity with or without SRC-2 in TKO and AtT-20 cells (Fig. 2G). These results show that pocket proteins are not essential for SRC coactivator function although they synergize with SRC coactivators.

\textbf{Nuclear Receptor Selectivity of Rb Potentiation—}Although Rb synergy with SRCs was not recognized previously, its effect on some NRs, such as GR, androgen receptor, and PPAR\(\gamma\), was known. We therefore wanted to determine whether SRC coactivator activity is subject to Rb enhancement for all NRs. Using a representative panel of orphan- and ligand-dependent NRs together with reporters for their cognate DNA targets, we identified three subsets of NRs with respect to Rb action: one is subject to Rb enhancement of SRC-2 activity (Fig. 3, A–D), another is insensitive to Rb (Fig. 3, E–G), whereas the third is
repressed by Rb (Fig. 3, H and I). In the first group, the two NGFI-B-related factors, Nurr1 and NOR-1, as well as the orphan NRs, hepatocyte nuclear factor-4 (HNF-4) and steroidogenic factor-1 (SF-1), and the ligand-dependent estrogen receptors (ERα and ERβ) were all enhanced by Rb. In contrast, the SRC-2-dependent activities of GR, RAR, and RXR were not sensitive to Rb, despite the positive effect of Rb on GR-dependent activity (10). Finally, the SRC-2-dependent activities of COUP-TFI and PPARγ (Ref. 13) were repressed in the presence of Rb. Similar results were obtained in TKO MEFs, C33A, CV1, or L cells. These results indicate that only a subset of NRs is sensitive to Rb potentiation of SRC activity.

Rb and SRC2 Act through AF-1 Transactivation Domain of NGFI-B—We previously showed that SRC coactivators exert their effects through the AF-1 N-terminal domain of NGFI-B (8, 28). This AF-1 domain was shown to interact physically with SRC-2 (29). The AF-1 is subdivided into two regions comprised between amino acids 20 and 36 and between amino acids 74 and 174 (8). When NGFI-B deletion mutants were assessed for their ability to support Rb enhancement (Fig. 4A), all Rb-dependent enhancement was lost upon deletion of N-terminal sequences between amino acids 17 and 37. Accordingly, deletion of the Q-rich domain (∆Q) of SRC-2/TIF2, which is known to interact with the AF-1 domain (15), resulted in the loss of SRC-2-dependent activation both in presence and absence of Rb (Fig. 4B). In contrast, mutation of the LXXLL motifs (m123) in the NR interacting domain, which are required for AF-2-dependent activity, did not affect SRC-2 activation of NGFI-B activity, nor Rb enhancement (Fig. 4B). These results indicate that the interaction between SRC-2 and the AF-1 domain is necessary for synergy between Rb and SRC-2.

Multiple Rb domains are required for its potentiator effect on NGFI-B and SRC-2—To test whether Rb mutations identified in human tumors affect Rb’s potentiator effect on NGFI-B/SRC activity, we tested them in Rb-defective C33A cells. In limiting concentrations, all tumor-associated Rb mutants were less active than intact Rb for NGFI-B-dependent activity with or without SRC-2 (Fig. 4C, E). However, when expressed at saturating levels, all mutant Rb proteins were able to enhance NGFI-B and SRC-2-dependent transcription (not shown). These results are similar to those obtained for enhancement of Cbfa1 activity (24).

To identify Rb domains necessary for enhancement of NGFI-B/SRC activity, we used a series of Rb deletion mutants that
are expressed at similar levels ([18] and Fig. 4F). The Rb N-terminal domain (N) was inactive in itself; however, its deletion (ΔN or ABC) decreased activity markedly (Fig. 4D) indicating that it contributes to Rb activity, in particular the short region defined by Δex4 and ΔN1. The Rb large pocket (ABC or ΔN) had low activity; interestingly, these mutants fully mimicked the activity of Rb at higher concentrations (not shown), suggesting that the pocket contains the essential features for Rb enhancement of NGFI-B transcription. This was not observed upon deletion of the A (ΔA), B (ΔB, ΔB) or C (AB) domains of the pocket. Thus, all sub-domains of the pocket are required. Within the C domain, the region defined by the ΔC3 mutant appeared most important. Interestingly, this region is subject to acetylation by p300 (3).

Considering the importance of Rb phosphorylation for cell cycle control, we assessed the activity of the p34 mutant that has mutations in eight p34cdc-dependent Rb phosphorylation sites and that is no longer subject to phosphorylation-dependent E2F de-repression (17). This mutant was fully active for Rb enhancement of NGFI-B transcription. This was not observed upon deletion of the A (ΔA), B (ΔB, ΔB) or C (AB) domains of the pocket. Thus, all sub-domains of the pocket are required. Within the C domain, the region defined by the ΔC3 mutant appeared most important. Interestingly, this region is subject to acetylation by p300 (3).

Localization of Interaction Domains on Rb, NGFI-B, and SRC-2—The simplest model to account for the synergistic effects of Rb and SRCs on NGFI-B-dependent transcription would be direct interaction between these proteins. We have defined these interactions in vitro. The Rb mutagenesis results have suggested that many Rb regions are implicated in the NGFI-B/SRC-2 synergy (Fig. 4D). To address this, in vitro pull-down assays using GST fusion proteins encoding various Rb domains were performed with NGFI-B and SRC-2(TIF2): both proteins interacted with the B region of the Rb pocket (Fig. 5A). SRC-2 also bound efficiently to the Rb N terminus. The Rb pocket was previously shown to interact with the LX-CXE motif present in oncoproteins (2); consistent with the absence of this motif in either NGFI-B or SRC-2, the C706F Rb mutant bound both as well as Rb (data not shown).

We also identified the NGFI-B DNA-binding domain (DBD) for interaction with the B region of Rb (Fig. 5B) or to the ABC pocket (not shown). Unexpectedly, we noted that the C-terminal region of Rb retained the DBD or NGFI-B deleted of the N-terminal domain, although GST-Rb C was unable to bind full-length or C terminus-deleted NGFI-B. This may suggest a putative interaction for the Rb C terminus following NGFI-B conformation changes.

Two SRC-2 regions, from amino acids 940 to 1010 and the AD2 domain, were implicated in interaction with the N domain of Rb (Fig. 5C). The 940–1010 region also interacted with the B domain. This Rb-interacting domain (RbID) is distinct from AD1/CBP-interacting domain (CID) (30). The RbID was not previously associated with a known function or with protein interactions. Because the Rb and CID's of SRC-2 are contiguous, we tested whether Rb and CBP may act jointly to enhance the SRC-2 effect on NGFI-B-dependent transcription. Indeed, Rb and CBP had additive effects on NGFI-B activity (Fig. 5D). This effect was enhanced in the presence of SRC-2. The greater effect of Rb in MEF TKO, compared with AtT-20 cells, is con-
In Vivo Rb Complexes with NGFI-B and SRC-2 and Recruitment to the POMC Promoter—We next investigated the formation of NGFI-B/SRC-2/Rb complexes in vivo. Co-immunoprecipitation experiments were performed using lysates from C33A cells overexpressing FLAG-tagged NGFI-B, HA-tagged Rb, and SRC-2 proteins. After immunoprecipitation with anti-FLAG antibodies, Western blot analyses revealed Rb and SRC-2 in the same complexes (Fig. 6, compare lanes 3 and 5 with 2 and 4). Endogenous SRC-2 was detectable in these samples (lane 3) and increased upon SRC-2 overexpression (lane 5). These interactions were confirmed in a reverse experiment using antibodies against HA fused to Rb. Both NGFI-B and SRC-2 were detected in the HA-Rb immunoprecipitate (lane 9). To confirm the existence of these complexes in cells without overexpression, cell extracts from AtT-20 cells treated or not with CRH were subjected to immunoprecipitation with anti-Rb (Fig. 6B) or with anti-NGFI-B (Fig. 6C). Endogenous NGFI-B co-immunoprecipitated with Rb (Fig. 6B, compare lanes 3 and 4 with 5 and 6), but CRH treatment did not affect the total cellular amount of these complexes. Immunoprecipitation of nuclear NGFI-B could bring down p107, particularly in CRH-treated cells (Fig. 6C, lane 4), but Rb could not be detected because of its low abundance. The recruitment of p107 upon CRH stimulation (lane 4), is consistent with prior data showing CRH-dependent enhancement of nuclear NGFI-B.
DNA-binding activity without change in total nuclear NGFI-B protein (8).

We next assessed the in vivo recruitment of these proteins to the POMC promoter by chromatin immunoprecipitation (ChIP). Protein recruitment to the POMC promoter was compared with POMC exon 3 sequences used as control. None of these sequences contain recognizable E2F binding sites. In untreated cells, NGFI-B and Rb appeared to be present on the promoter, whereas SRC-2 and p107 were not detectable (Fig. 6D). However, CRH stimulation led to marked recruitment of NGFI-B, Rb, p107, and SRC-2. Co-recruitment of these proteins is consistent with their joint action on transcription in response to CRH, in agreement with prior work on the role of SRC coactivators in NGFI-B-mediated signaling (8). Although both Rb and p107 were recruited in response to CRH, only Rb appeared to be present on the POMC promoter in basal conditions; this would be consistent with a preference for Rb over p107 for interaction with NGFI-B (Fig. 2G) and with the other POMC promoter target of these proteins, NeuroD1 (25).

Co-recruitment of Rb and Rb during Enterocyte Differentiation—Because we identified other NRs that are potentiated by Rb, we wanted to test the generality of our model in another system. To this end, we investigated the HNF-4-dependent activation of the α1-AT gene that occurs during enterocyte differentiation of CaCo-2 cells (23). We first showed co-immunoprecipitation of Rb with HNF-4 in transfected cells (Fig. 7A) in agreement with their synergism in transcription (Fig. 3B). Enterocytic differentiation of CaCo-2 cells is marked by expression of the α1-AT gene (Fig. 7B) and induction of HNF-4 expression (Fig. 7C) as previously shown (23). During this, Rb levels were not markedly affected, although Rb dephosphorylation occurred (Fig. 7C). ChIP analysis of occupancy at the α1-AT promoter revealed a striking recruitment of Rb and SRC-2 together with HNF-4 at the promoter but not in the 3′-untranslated region (Fig. 7D). This recruitment is highest early (day 5) in the differentiation process at the time of onset of HNF-4 expression. Thus, co-recruitment of Rb with NR (HNF-4) and SRC coactivators is not unique to pituitary cells and may reflect a general paradigm.

Rb Potentiates Hormone Action through the NurRE—The present work suggests that Rb-related proteins should modulate/enhance responsiveness to NRs. Because the NurRE confers responsiveness of the POMC promoter to the hypotalamic...
hormone CRH (5), we tested whether the stimulatory effect of CRH on NurRE-mediated transcription is enhanced by Rb. As shown in Fig. 8A, the activity of the NurRE reporter was modestly induced by Rb alone and by increasing doses of CRH. However, the response of the NurRE reporter to CRH was markedly potentiated by Rb. Thus, limiting amounts of Rb or its related proteins could significantly modulate the hormone response to CRH.

To demonstrate the in vivo importance of Rb-related proteins in hormone responsiveness, we used siRNA to decrease the levels of Rb-related proteins. Toward this end, we first assessed the relative expression levels of Rb, p107, and p130 in hormone-responsive AtT-20 cells. Experiments with antibodies (Fig. 6, A–D) had suggested that p107 may be more abundant than Rb in those cells, and quantitative RT-PCR supported this conclusion (Fig. 8B). We therefore targeted p107 for knock-down using siRNA (Fig. 8C). In comparison to control siRNA, which did not affect p107 levels (Fig. 8C), the decrease in p107 led to dose-dependent reduction in CRH responsiveness of the NGFI-B-dependent reporter (Fig. 8D).

These experiments clearly suggest that the levels of Rb-related proteins may serve to modulate hormone responsiveness and in this way, to promote a differentiation phenotype.

**DISCUSSION**

The present work supports a model of Rb action as a transcriptional activator that acts thru its enhancement of SRC/p160 coactivator function. Indeed, we have shown that Rb, and the related proteins p107 and p130, enhance the activity of SRC-2 on NGFI-B-dependent transcription (Fig. 2). This action relies on protein-protein interactions that occur spontaneously after cell confluence. a1-AT mRNA levels were quantitated by quantitative real-time PCR and compared with those of acid ribosomal phosphoprotein (ARP-PO) as control. C, expression of Rb and HNF-4 during Caco-2 cell differentiation was assessed by Western blotting. D, ChIP analysis of HNF-4, Rb, and SRC-2 recruitment to the a1-AT promoter during Caco-2 cell differentiation. None of these proteins were recruited to the a1-AT 3′-untranslated region or to the ARP-PO promoter that served as negative control.

**FIG. 7.** Rb recruitment by HNF-4 during Caco-2 cell differentiation. A, co-immunoprecipitation of Rb with HNF-4. The experiment was performed as described in A. B, induction of a1-antitrypin (a1-AT) gene expression during differentiation of Caco-2 cells that occurs spontaneously after cell confluence. a1-AT mRNA levels were quantitated by quantitative real-time PCR and compared with those of acid ribosomal phosphoprotein (ARP-PO) as control. C, expression of Rb and HNF-4 during Caco-2 cell differentiation was assessed by Western blotting. D, ChIP analysis of HNF-4, Rb, and SRC-2 recruitment to the a1-AT promoter during Caco-2 cell differentiation. None of these proteins were recruited to the a1-AT 3′-untranslated region or to the ARP-PO promoter that served as negative control.

**FIG. 6.** In vivo recruitment of Rb and SRC-2 together with NGFI-B and HNF-4. A–D, Rb forms in vivo complexes with NGFI-B and SRC-2 and is recruited to the POMC promoter. A, co-immunoprecipitation of Rb and SRC-2 with NGFI-B. C33A cells were transfected with expression vectors for FLAG-NGFI-B, HA-Rb, or SRC-2 as indicated. Whole cell extracts (WCE) were used in immunoprecipitation using anti-FLAG-antibody to bring down FLAG-NGFI-B (lanes 3 and 5) or anti-HA-antibody to bring down HA-Rb (lanes 8 and 9), or isotype-matched non-immune IgG as control (lanes 2, 4, and 10). Following SDS-PAGE, Western blotting was used to reveal SRC-2, Rb, and NGFI-B. Double arrows are used to indicate Rb proteins that are either hypo- (bottom band) or hyper- (top band) phosphorylated. B, co-immunoprecipitation of NGFI-B with Rb from WCE of AtT-20 cells treated (+) or not (−) with CRH 10−7 M for 2 h. After immunoprecipitation with anti-Rb antibody (lanes 3 and 4) or non-immune IgG (lanes 5 and 6), Rb and NGFI-B were revealed by Western blot. C, co-immunoprecipitation of p107 with NGFI-B from nuclear extracts (NE) of AtT-20 cells treated (+) and not (−) with CRH 10−7 M. D, chromatin immunoprecipitation (ChIP) was used to show recruitment of NGFI-B, SRC-2, p107, and Rb to the POMC promoter in presence or absence (veh) of CRH treatment. Enrichment of POMC promoter sequences is shown relative to sequences within exon 3 of the gene. ChIP and PCR amplifications were each performed in three separate experiments.

The present work supports a model of Rb action as a transcriptional activator that acts thru its enhancement of SRC/p160 coactivator function. Indeed, we have shown that Rb, and the related proteins p107 and p130, enhance the activity of SRC-2 on NGFI-B-dependent transcription (Fig. 2). This action relies on protein-protein interactions that occur in vitro (Fig. 5) and in vivo (Fig. 6). The Rb/NGFI-B interaction (Fig. 5B) is not
Rb Is a Potentiator of Coactivator Function

The recruitment of SRC coactivators.

which is required for DNA binding, the formation of dimers and

pathway, the serine dephosphorylation of the NGFI-B DBD,

these cofactors on the POMC promoter would follow the previ-

ous experiments performed in duplicates. 

Assembly of 

D

co-recruitment of Rb and/or p107 with NGFI-B and SRC-2 on 

the POMC promoter upon CRH action (Fig. 6

). Assembly of 

D

co-recruitment of Rb and CBP (Fig. 5

).

mRNAs. C, Western blot analyses of p107 and glyceraldehyde-3-phosphate dehydrogenase in AtT-20 cell extracts transfected or not (NT) with siRNA (25 nM) targeting p107 or random sequences (Ctl). Cells were co-transfected with CMV-GFP and 3 x 10^6 GFP-positive cells were sorted by fluourescence-activated cell sorting for analysis. D, knock-down of p107 using increasing concentrations (nanomolar) of specific siRNA, but not control siRNA (Ctl), decreased CRH (10^{-7} M) response of NurRE reporter plasmid in transfected AtT-20 cells.

FIG. 8. Rb-related proteins modulate hormone responsiveness and transcription by NRs. A, Rb enhances CRH response. AtT-20 cells were transfected with multimerized NurRE reporter construct, Rb expression vector (black bars) or with plasmid control (gray bars). After 40 h, cells were stimulated for 6 h with increasing concentrations of CRH. Results are averages of three different experiments performed in duplicates. B, relative expression of Rb-related genes in AtT-20 cells. Quantitative real-time PCR was used to assess relative levels of Rb, p107, and p130 mRNAs. C, Western blot analyses of p107 and glyceraldehyde-3-phosphate dehydrogenase in AtT-20 cell extracts transfected or not (NT) with siRNA (25 nM) targeting p107 or random sequences (Ctl). Cells were co-transfected with CMV-GFP and 3 x 10^6 GFP-positive cells were sorted by fluorescence-activated cell sorting for analysis. D, knock-down of p107 using increasing concentrations (nanomolar) of specific siRNA, but not control siRNA (Ctl), decreased CRH (10^{-7} M) response of NurRE reporter plasmid in transfected AtT-20 cells.

The simplest model to account for these effects would be 

the AF-1 of NGFI-B (Fig. 4).

Conversely, the coactivator function of SRC-2 does not require 

Rb proteins (Fig. 2, E and F). However, the presence of Rb 

and/or p107 in complexes with NGFI-B and on the POMC 

promoter in vivo (Fig. 6, A–D), taken together with their effect 

on basal and CRH-stimulated POMC mRNA (Fig. 1C) and 

on responsiveness to CRH (Fig. 8, A–D) suggest that Rb-related 

proteins have a key modulatory role in hormone-inducible 

NGFI-B and SRC-dependent transcription. This activity is 

additive to that of CBP (Fig. 5D) and can be exerted on other NRs 

(Fig. 3, A–D) such as HNF-4 (Fig. 7, A–D). In this model (Fig. 

9A), Rb may potentiate the activity of coactivators by stabiliza-

tion/co-recruitment of protein complexes that include NRs 

and their coactivators.

A Mechanism for Rb Activation of Transcription—Activation 

of transcription by NRs requires coactivators and particularly 

those of the SRC/p160 family. Most often, SNC action depends 

on recruitment to the ligand-dependent C-terminal AF-2 do-

main of NRs. For NGFI-B dimers, the effect of SRCs is entirely 

mediated thru the N-terminal AF-1 domain (8). We now report 

that Rb is a cofactor for SRC-dependent transcriptional en-

hancement. Rb interacts with the NGFI-B DBD and with the 

SRC-2 RbID and AD2 domains. This is compatible with 

the recruitment of SRC-2 to the NGFI-B AF-1 domain. These 

multiple interactions leave the SRC-2 CID domain free to recruit 

CBP (Fig. 9A), and they are consistent with the additive effects 

of Rb and CBP (Fig. 5D).

The simplest model to account for these effects would be 

stabilization of NGFI-B/SRC-2 complexes by Rb. This model is 

consistent with the fact that Rb or its related proteins are not 

essential for NGFI-B-dependent transcription (Fig. 2, E–G). It 

is also consistent with a proposed role of Rb as potentiator of 

the transcriptional response to CRH (Figs. 1C and 8, A–D), by 

co-recruitment of Rb and/or p107 with NGFI-B and SRC-2 on 

the POMC promoter upon CRH action (Fig. 6D). Assembly of 

these cofactors on the POMC promoter would follow the previ-

ously documented (8) CRH activation of the protein kinase A 

pathway, the serine dephosphorylation of the NGFI-B DBD, 

which is required for DNA binding, the formation of dimers and 

the recruitment of SRC coactivators.

The molecular basis for the differential effects of Rb on 

different subgroups of NRs is not immediately obvious. The 

NGFI-B DBD that is the site of Rb interaction is as different by 

comparison to those of HNF-4, SF-1, and ER as those of GR, 

RAR, RXR, COUP-TF, or PPARγ, such that motifs for Rb 

interaction cannot be easily predicted. In contrast, the Rb re-

pression of PPARγ was attributed to an interaction with the 

PPARγ ligand-binding domain (13).

Structural Requirement for Activity of NGFI-B/SRC-2/Rb 

Complex—The AF-1 domain of NGFI-B is critical for activation, 

because it recruits SRC coactivators (Fig. 4, A and B). SRC-2 

carries two activation domains (Fig. 9A): the AD1 is a CBP-

interacting domain (CID) (15, 30) and the AD2 region has been 

shown to bind methyltransferases CARM1/PRMT1 (9). We 

show that the AD2 region also interacts with the Rb N termi-

nus, and we define a new Rb interaction domain (RbID) in 

SRC-2 that interacts with the B region of Rb (Figs. 5C and 9A). 

The RbID is distinct but very close to the CID. It is noteworthy 

that E2F, hBrm, and E1a also have nearly contiguous binding 

sites for Rb and CBP/p300 (31). It is also noteworthy that a 

region containing the RbID was recently implicated in trans-

repression (32).

The Rb pocket is the principal domain for Rb interaction with 

other cellular or viral proteins, including E2Fs, LT, E1a, and 

E7 (2). Rb interaction with NGFI-B and SRC-2 requires the B 

sub-domain of the pocket (Figs. 5A and 9A), in agreement with 

B domain deletion mutants (ΔB, Δb, and Δ22) that prevent Rb 

enhancement of NGFI-B/SRC-2-dependent transcription (Fig. 

4, C and D). This B sub-domain has also been implicated in in 

vitro interactions with c-Jun (20) and c-ski (33).

The Rb C-terminal domain is also important for its enhance-

ment function (Fig. 4D, mutant ΔC3), but there are no physical 

interactions between this region and SRC-2 or NGFI-B (Fig. 

5A). The ΔC3 (Δ839–892) deletion contains residues that are 

acytetylated by p300 (3). Thus, p300 acetylation may be impli-

cated in Rb enhancement, for example to start the cycle of 

transcription initiation as described for ER-mediated tran-

scription (34). The C-terminal region of Rb is also required for 

its growth arrest functions, but this was correlated with inter-

action of the tyrosine kinase c-Abl with the ΔC1 and ΔC2 

overlapping regions (35).
**Rb Function in Activation and Repression**—It is interesting to contrast the action of Rb in transcription enhancement and repression. In the context of E2F-dependent promoters, Rb plays a crucial role by recruitment of components of the repressor complex that includes histone deacetylases, DNMT1 and Suv39H (Fig. 9B) (2). This Rb activity is controlled through Rb phosphorylation as for its inhibitory effect on PPARγ (13). The involvement of Rb in transcription enhancement is quite different, because Rb is not an essential component of the transcriptional complex. Instead, Rb enhances the activity provided by other coactivators like SRC-2. Specific phosphorylation events have not been associated with this activity. Indeed, the Δp34 mutant of Rb (Fig. 4C), which cannot be phosphorylated during the cell cycle and which is more efficient to repress E2F activity (17), is as active as wild-type Rb for NGFI-B/SRC-2 enhancement. Further, co-immunoprecipitation with NGFI-B and Rb brought down mostly hypophosphorylated Rb, but also some hyperphosphorylated protein in C33A cells (Fig. 6a, lane 5) and mostly hyperphosphorylated Rb in AtT-20 cells (Fig. 6B), because these cells are deficient in p16 cyclin-dependent kinase inhibitor (36). Taken together, these data argue against a role of Rb phosphorylation in transcriptional enhancement. Similarly, the Rb/Sp1, Rb/Trip230, and Rb/Cbfa1 interactions were also shown to be independent of Rb phosphorylation (12, 24, 37). These results suggest a mechanism by which Rb, irrespective of its phosphorylation status, may be recruited to promoter sequences and play a significant role in transcriptional regulatory processes.

The activating effect of Rb could be associated with a positive role in promoting differentiated cell functions (Fig. 9B). This is clearly supported by the co-recruitment of Rb together with HNF-4 on the α1-AT promoter during CaCo-2 cell differentiation (Fig. 7, A–D). NRs are not the only targets of SRC-2 and Rb. For example, both Rb and SRC-2 have been shown to enhance MEF2-dependent function during myogenic differentiation (38, 39), and Rb activates MyoD-dependent transcription (40). Also, Rb was shown to enhance activity of the cell-restricted transcription factors C/EBP and Cbfα1 in adipocytes and osteoblasts, respectively (24, 41). C-Jun- and AP-2-dependent transcription are also activated by Rb in epithelial cells (19, 20). This action of Rb may be related to increases of Rb expression observed upon ex vivo cell differentiation in myoblast and hematopoietic cells (42) and in other systems in vivo (43). Thus, the total level of Rb-related proteins may contribute to establishment of differentiated phenotypes.

A modular role of Rb-related proteins on hormone (CRH) responsiveness of the POMC promoter is consistent with the limiting in vivo levels of Rb in pituitary POMC cells as revealed in Rb−/− mice (1). For NurRE-dependent and CRH-inducible transcription, the present work suggests that all three Rb-related proteins are equivalent (Fig. 2G) and that their additive levels may modulate CRH response (Figs. 1C and 8, A–D). However, Rb-related proteins also enhance basal POMC promoter activity through NeuroD1 interaction, and this effect also showed preference for Rb over p107 and no effect of p130 (25). This preference may account for Rb recruitment to the POMC promoter in unstimulated conditions (Fig. 6D) and for the particular dependence on Rb gene dosage in vivo. Thus, the preference for Rb over p107 in interactions with NGFI-B (Fig. 2G) and NeuroD1 (25) may account for its recruitment to the POMC promoter (Fig. 6D) despite the greater abundance of p107 in AtT-20 cells (Fig. 6B). Because differentiated hormone-producing adult pituitary cells undergo few cell divisions, the proposed role of Rb-related proteins as modulators of hormone responsiveness is not incompatible with other roles of these proteins in cell cycle control of proliferating cells. In this capacity, the levels of Rb-related proteins may serve to adjust hormone responsiveness in non-proliferating cells.

The model developed in the present work (Fig. 9, A and B) of Rb potentiation of SRC-2-dependent transcription may constitute a paradigm to consider the action of Rb on various NRs and possibly on other transcription factors. The identification of Rb as a component of a multicoregulatory complex may serve to integrate multiple pathways for differentiation, hormone response, and cell cycle control through coordination of cell-specific gene expression.

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**Fig. 9. Schematic representation of NGFI-B, SRC-2, and Rb complex.** A, this scheme recapitulates all interactions identified in vitro and is consistent with transfection data. B, dual function of Rb. In its cell-cycle control function, Rb represses E2F-dependent transcription by recruiting histone deacetylases and methyltransferases. As an activator of transcription, Rb potentiates the action of SRC coactivators on nuclear receptor-dependent transcription. This action may be associated with transcription factor and target genes that are linked to differentiated cell function.
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Rb Enhances p160/SRC Coactivator-dependent Activity of Nuclear Receptors and Hormone Responsiveness
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