Retinoblastoma and the Related Pocket Protein p107 Act as Coactivators of NeuroD1 to Enhance Gene Transcription*

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Gene inactivation studies have suggested that the product of the retinoblastoma gene, Rb, is particularly limiting in pituitary pro-opiomelanocortin (POMC)-expressing cell lineages. Indeed, in Rb knock-out mice, these cells develop tumors with high frequency. To understand the implication of limiting Rb expression in these cells, we investigated the action of Rb and its related pocket proteins, p107 and p130, on POMC gene transcription. This led to the identification of the neurogenic basic helix-loop-helix transcription factor, NeuroD1, as a target of Rb action. Rb and to a lesser extent p107, but not p130, enhance NeuroD1-dependent transcription, and this activity appears to depend on direct protein interactions between the Rb pocket and the helix-loop-helix domain of NeuroD1. In vivo, NeuroD is found in a complex that includes Rb and also the orphan nuclear receptor NGFI-B, which mediates corticotropin-releasing hormone activation of POMC transcription. The formation of a similar complex in vitro requires the presence of Rb as a bridge between NeuroD and NGFI-B. In POMC-expressing AtT-20 cells, Rb and p107 are present on the POMC promoter and inhibition of their expression through small interfering RNA decreases POMC mRNA levels. The action of Rb and its related proteins on POMC transcription may contribute to the establishment and/or maintenance of the differentiation phenotype.

The Rb1 gene is known as a tumor suppressor, because its inactivation is linked to the development of many cancers. Current models propose that the anti-oncogenic functions of Rb result from its ability to arrest the cell cycle by the inhibition of E2F activity (1). However, Rb and the related “pocket” proteins p107 and p130 are not only negative cell cycle regulators; they also act positively on differentiation of many cell types. By its capacity to interact and to enhance activities of cell-specific transcription factors such as MyoD, NF-IL6, c/EBP/β, Chf-1, and AP-2, Rb acts as positive regulator of differentiation in myoblasts, adipoblasts, osteoblasts, and epithelial cells (1). In this context, it is noteworthy that the anti-oncogenic activity of Rb has been more often associated with late stage rather than onset of tumor progression, because Rb is mutated more frequently in dedifferentiated tumor cells (i.e. in carcinomas rather than adenomas), in several tissues, and particularly in the human pituitary gland (2, 3). Mice heterozygous for Rb mutations always develop pituitary tumors that arise from the pro-opiomelanocortin (POMC)-expressing melanotroph cells (3, 4). p107 and p130 also participate in developmental processes during limb development and osteogenesis (5, 6).

The POMC gene is expressed in two different pituitary lineages. In corticotrophs, POMC processing leads to synthesis of ACTH, whereas in melanotrophs, it produces α-melanotropin-stimulating hormone by an additional step of proteolytic cleavage (7). Within human pituitary adenomas, the ACTH-producing ones are the most frequent to progress to aggressive stages characterized by poorly differentiated invasive and non-secretory cells (8). This was correlated with the loss of Rb expression in corticotroph carcinomas (2). Thus and because we found that Rb can activate POMC transcription (50), we hypothesized that part of Rb function in corticotroph cells, including some anti-oncogenic activity, may be dependent on a positive effect on differentiation. To assess the role of Rb in the differentiation of POMC-expressing cells, we investigated the mechanisms by which Rb acts on transcriptional regulation of the POMC gene that is the hallmark of corticotroph function. Corticotroph-specific transcription of the POMC gene results from synergistic interactions between the homeobox factor Pitx1, the neurogenic bHLH factor NeuroD1/Beta2, and the T-box factor Tpit (10–12). The factors Tpit and NeuroD1 are restricted to the POMC lineages and are required for appropriate cell differentiation (13, 14). The Pitx1 factor is more widely expressed during early development (15) and is important for craniofacial and limb patterning (16) and, together with Pitx2, for early pituitary organogenesis (17). In corticotrophs, Pitx1 is implicated in cell-specific activity of the POMC promoter by direct interactions resulting in transcriptional synergism with Tpit (12), and with the NeuroD1 heterodimerization partners (11). POMC transcription also depends on the balance of stimulatory signals elicited by hypothalamic corticotropin-releasing hormone (CRH) and negative signals produced by glucocorticoids and their receptors. We have shown that a binding site for orphan nuclear receptors of the NGFI-B subfamily (which includes NGFI-B/Nur77, Nurrol, and NOR-1) is responsive to CRH (18). This Nur response element (NurRE) is a palindromic constituted of two half-sites with partial homology to a binding site for Nur monomers known as the NGFI-B-binding response element (19). The POMC NurRE is recognized by Nur factor.
homo- or heterodimers (20), and it was shown to be much more responsive to CRH than the NGFI-B-binding response element (18). We showed recently that in response to CRH the p160/SRC coactivators are recruited to Nur dimers at the NurRE and also to Tpit (21, 22). The Src/p160 coactivator family consists of three proteins designated SRC-1/NCoA-1, SRC-2/TIF2/GRIPI/NCoA-2, and SRC-3/p/CIP/RAC3/ACTR/AIB-1/TRAM-1 (23). SRC coactivators interact with transcription factors and mediate their transcriptional effects through their histone acetyltransferase activity or their interactions with other transcriptional regulators, such as CBP/p300, p/CAF, histone methyltransferase CARM1, and the SWI/SNF complex (23). We showed recently that Rb or related proteins also associate with nuclear receptor-SRC complexes, and particularly with the NGFI-B-SRC2 complex, to enhance the transcriptional response of the POMC promoter to CRH (50).

In this present study, we show that Rb activates POMC transcription through its interaction with NeuroD1, in addition to its previously reported interaction with NGFI-B. Both Rb and the related p107, but not p130, can enhance NeuroD1-dependent POMC promoter activity. The transcriptional synergism between NeuroD1 and NGFI-B may be explained by the action of Rb as a protein bridge allowing co-recruitment of NeuroD1 and NGFI-B within the same protein complex.

**EXPERIMENTAL PROCEDURES**

**Immunohistochemistry**—Immunohistochemistry was performed as described (13, 15). Antibodies used were as follows: rabbit anti-POU5 1:500, rabbit anti-α-gtS1 1:500 (kindly provided by A. F. Parlow, Pituitary Hormones and Antisera Center, Torrance CA), and anti-Rb 1:100 (3-245, PharMingen) together with the tyramide signal amplification biotin system (PerkinElmer Life Sciences).

**Plasmid Constructs**—POMC promoter constructs and reporter plasmids as well as NGFI-B, NeuroD1/Beta2, and SRC-2 expression vectors were described previously (22, 24–26). Expression vectors for CMV-Rb, pSG51-hemagglutinin-Rb and tumor-derived mutants were described by Kaelin and co-workers (27), whereas CMV-mRbΔp34 (Δp34) vector was described by Hamel et al. (28), and CMV-p107 and CMV-p130 was obtained from Dr. Sylvain Meloche. GST-Rb plasmids were described previously (50). The CMV-RbΔ22 expression vectors were constructed by insertion of the BamH1 cDNA insert from SV-Rb into pBlueScript 2 (29) into pcDNA1 vector. Transfection experiments with pcDNA1-Rb gave similar results as with CMV-Rb.

**Cell Culture and Transfection**—Cell culture and transfection of AtT-20 cells were described previously (22). Wild type and triple knock-out (TKO) mouse embryo fibroblasts (30) used at passages 3–10 were transfected with plasmid DNA (Qiagen) and were transfected by the calcium phosphate method. Luciferase assays were assayed 2 days after transfection. Transfection efficiencies were normalized using a co-transfected CMV-β-galactosidase plasmid (10 ng). Each transfection in duplicate was repeated 3–10 times using different plasmid preparations.

**RNA Interference**—siRNA against mouse Rb and p107 genes were designed to target the following sequences, ctcgaacgagaaagata and ccatggtaaatagcttgatt and cgaagtattgacttttacagt for Rb and CAGCTTAATGTCGACTGATG and CAGAATGACTTAATGTCGAT for p107. The siRNA control targeted the GCAGCAGCCTCTTCCTACGA sequences in green fluorescent protein mRNA. All duplexes were synthesized at Proligo as 21-mers with dTdT 3′ overhangs. Each transfection was repeated at least 3 times in duplicate. AtT-20 cells were plated in 6-well plates and transfected with 400 pmol of siRNA green fluorescent protein or with 100 pmol of each siRNA for Rb and p107 using Lipofectamine (Invitrogen) according to manufacturer’s instructions. Two days after transfection, cells were harvested, and total RNA was extracted using phenol/chloroform method. RNA were treated 1 h with RNase-free DNase I (Roche Diagnostics), which was then heat-inactivated by a 10-min incubation at 70 °C. RNA isolation was done with 2 μg of this RNA, random hexanucleotides, and SuperScript II RT (Invitrogen) during 1 h. An aliquot of this reaction mixture was used for quantitative real time PCR.

**GST Pull-down Assays**—GST fusion proteins and ΔN30 labeled Rb, NGFI-B, and NeuroD1/Beta2 mutants were produced as described (29). Labeled proteins were incubated with 1 μg of immobilized GST or GST-Rb constructs in 150 μl of TENN50 (50 mM Tris, pH 7.5, 5 mM EDTA, 50 mM NaCl, 0.1% Nonidet P-40) with 1 mM phenylmethylsulfonyl fluoride and 0.5% bovine serum albumin for 2 h at 4 °C. Beads were washed at 4 °C twice in TENN250 and twice in TENN125. Bound proteins were resolved on SDS-PAGE, stained with Coomassie Blue to ensure that similar amounts of fusion proteins were recovered, and then autoradiographed.

**Co-immunoprecipitation Assays and Western Blots**—C33A cells (10-cm plate) were transfected with 7 μg of each expression plasmid and harvested 48 h later. These and AtT-20 cells were harvested in cold phosphate-buffered saline and extracted for 30 min at 4 °C in TENN250 with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors. After centrifugation, supernatants corresponding to 107 cells were immunoprecipitated at 4 °C for 4 h with α-FLAG M2 (Sigma), α-hemagglutinin (sc-805, Santa Cruz), α-Rb, α-p107 (C-18, Santa Cruz), oNeuroD1/Beta2 (rabbit polyclonal (26)) and isotype-matched non-immune IgG (Sigma) as a control. Immunoprecipitates obtained after 1 h of incubation with protein A/G-agarose beads (Santa Cruz) saturated with bovine serum albumin were washed twice with TENN250 and thrice with TENN125. After SDS-PAGE, Western blots were revealed with antibodies against FLAG M2, hemagglutinin, Rb, NGFI-B (554088, PharMingen) and NeuroD1/Beta2. Western blot analysis was performed as described (29).

**Chromatin Immunoprecipitation (ChIP)**—AtT-20 cell extracts were prepared for ChIP as described (21). Supernatants corresponding to 107 cells were immunoprecipitated to overcome spurious signals. Each μg of antibodies against NGFI-B, Rb, p107, NeuroD1, and isotype-matched non-immune IgG as the negative control. Immunoprecipitates were collected with protein A/G-agarose beads saturated with bovine serum albumin and tRNA. Beads were washed as described by Upstate Biotechnology. Quantitative PCR was performed by quantitative real time PCR or on serial 4-fold dilutions of DNA extracted from immunoprecipitates. These PCR products were separated on agarose gels and revealed by hybridization with appropriate DNA probes. Quantification of signals for different dilutions was performed with PhosphorImage.

**Primers and Quantitative Real Time PCR**—Quantitative real time PCR (Stratagene MX-4000 or Applied Abiprism7000) was used with the SYBR Green kit (Qiagen or Applied). The primers were used as follows: POMC promoter 5′-ATGGAATCCCTTGGCATGGCT (−703) and 5′-GCTATCCAGAAAACCC-703) and 5′-GGGGCAAGGAGGTTGAGA-AATG (−5746); POMC 5′-untranslated region-exon 1 5′-ACCCACCC-AACCTCCTGCAATATAAA and 5′-TGGCCTTCCTTGGACTCTAGCTTCTT; β-actin (exon 3) 5′-GTATGTTGGAGATTGGTGCAA and 5′-TTCCATGTCGTTTCAATGTTGTA; B2-globulin TGCATTAACGAAAAACCTCTCA (−109) and 5′-GCGGGTGAGTTGAGCTTGAG (−209); Rb exon 18-exon 19 5′-ATGGGAATCTTTGCTGGAGCT (−1612) and 5′-AGGACAGGTTTCAAGGTAATGCC (−1710); p107 exon 21-exon 22 5′-AGGCACTGCTGTCGTAAGGT (−3047) and 5′-TTGGAGGTTGAGCTTGACTGCT (−3181). PCR efficiency and comparative quantification were calculated as described previously (32, 33) and are expressed relative to the β-actin transcript or DNA template, for real time or ChIP assays, respectively.

**RESULTS**

**Rb Enhancement of POMC Promoter Is Cell-specific**—We have previously shown that Rb enhances the activity of the POMC promoter, and a part of this action was due to interactions with the nuclear receptor NGFI-B (50). When the effect of Rb was tested on the intact POMC promoter, it was found that Rb enhances POMC promoter activity in POMC-expressing AtT-20 cells (Fig. 1A) but not in other cells (Fig. 1B). This effect was observed with reporters containing the upstream regulatory sequences of the POMC promoter (34, 35) but not with reporters devoid of these sequences (Fig. 1A), suggesting that cell-specific recognition elements of the POMC promoter are required for Rb action. To determine whether endogenous Rb or related proteins such as p107 contribute to basal POMC expression, we used siRNAs complementary to Rb and p107 (two oligonucleotides complementary to each) to inhibit their expression in AtT-20 cells. Reduction of Rb and p107 expression was accompanied by a decrease in POMC mRNA but not of β2-microglobulin mRNA used as control (Fig. 1C). Thus, constitutive POMC expression is partly dependent on Rb-related protein. We next ascertained that Rb is expressed in...
Rb as Coactivator of NeuroD1 Action on POMC

normal pituitary corticotroph cells. Previous work had shown co-expression of Rb and p107 throughout pituitary development (36). We therefore used double immunohistochemistry against Rb and ACTH to reveal co-expression of these two proteins in corticotrophs. At day 14.5 of mouse embryonic development, the pattern of Rb-positive cells partly overlapped with ACTH immunoreactivity (Fig. 1C). This indicates that POMC-expressing corticotrophs express Rb. However, strong Rb expression was also observed in the rostral tip of the pituitary that contains αGSU-expressing cells and these were also shown by co-labeling to be Rb-positive (Fig. 1D). These results show wide Rb expression in the pituitary and particularly in corticotrophs.

Corticotroph-specific Sequences Are Targets of Rb—Using a panel of POMC promoter deletion constructs, Rb enhancement was only observed with reporters containing cell-specific regulatory sequences (Fig. 2A). Cell-specific activity requires regulatory sequences present in the distant and central regions of the promoter (34). To define more precisely the Rb-response elements, we used a series of linker-scanning mutations of the POMC promoter (26, 34). This analysis showed that binding sites for the cell-restricted factors Pitx1, Tpit, and NeuroD1 (Eboxneuro) were essential for Rb enhancement (Fig. 2B). It showed that the NurRE and distal region-1 regulatory elements are also essential for Rb action. The loss of Rb responsiveness is not always associated with loss of promoter activity, because other POMC promoter mutants (with decreased basal activity) are still responsive to Rb. Thus, previously described factors, which act on cell-specific regulatory sequences, such as Pitx1, Tpit, and NeuroD1 (26, 34), are potential targets of Rb action. In addition, the Nur factors that recognize the NurRE may also be Rb targets. The role of distal region-1 binding transcription factors in this regulatory complex remains unexplored.

Rb Targets NeuroD1—To identify POMC transcription factor(s) targeted by Rb, we used reporter constructs dependent on individual regulatory elements in transfection experiments. This analysis (Fig. 3A) confirmed the action of Rb on the composite distal regulatory element containing the NurRE and Eboxneuro (construct 3) and also showed that the Eboxneuro on its own conferred Rb responsiveness (construct 4). In contrast, an Eboxneuro mutant (construct 5) and the distal region-1 element (construct 2) were unresponsive to Rb as was the central region of the promoter that contains the binding sites for Pitx1 and Tpit (Fig. 3A, construct 1). Because the Eboxneuro is targeted by heterodimers of NeuroD1 and ubiquitous bHLH factors such as Pan1 (11), we assessed more directly in a reconstituted system the action of Rb on NeuroD1-dependent activity. The Rb-deficient C33A cells used in these experiments do not express Tpit, Pitx1, or NeuroD1 (data not shown). Although Rb had no effect in absence of NeuroD1, it enhanced NeuroD1-dependent activity on the POMC promoter (Fig. 3B) and on the Eboxneuro reporter (Fig. 3E). An EboxUbi reporter, which is dependent on ubiquitous bHLH factors (11, 26), did not exhibit Rb responsiveness even in presence of Pan1, the ubiquitous dimerization partner of NeuroD1 (Fig. 3D). Moreover, Rb did not affect the synergistic action of Pitx1 on the Pan1-dependent activity (Fig. 3D) (11). Similarly, and in accordance with results obtained with the central region-containing promoter (Fig. 3A), a Pitx1-Tpit reporter plasmid did not exhibit responsiveness to Rb (Fig. 3C). NeuroD1 was the only

FIG. 1. Rb enhances POMC promoter activity and is expressed in pituitary POMC cells. A, dose-dependent activation by Rb of the full-length POMC promoter (−480) but not of the minimal promoter (−34) in AtT-20 corticotroph cells. POMC-expressing AtT-20 cells were transfected with POMC promoter constructs fused to the luciferase reporter (350 ng) and with indicated amounts of Rb expression vector or with empty vector. Results are the averages (±S.E.) from at least three sets of experiments performed in duplicate and are expressed as -fold activation by Rb. B, absence of Rb effect in other cell types than AtT-20 cells. CV1, L, P19, and C33A cells were transfected as described previously with Rb expression vector (100 ng). C, requirement of Rb-related proteins Rb and p107 for transcription of the endogenous POMC gene. AtT-20 cells were transfected with siRNA against green fluorescent protein as negative control or with a pool of siRNAs against Rb and p107. Relative mRNA abundance from endogenous genes encoding Rb, p107, POMC (using two set of primers amplifying the 5′-untranslated region (UTR)-exon 1 (ex1) and exon 3 regions), and β2-microglobulin (β2-μG) were measured relative to β-actin mRNA using real-time quantitative PCR. All results are averages of 3–10 different experiments performed in duplicates. D and E, Rb nuclear brown (peroxidase) labeling immunohistochemically analysis of pituitary from embryonic day 14.5 mouse embryo showing that all POMC-expressing corticotroph cells contain Rb in their nucleus. Inset shows position of enlarged field. D, co-localization of Rb and corticotroph-specific ACTH is revealed by cytoplasmic blue labeling (alkaline phosphatase). E, co-localization of Rb- and αGSU-expressing cells revealed by cytoplasmic blue labeling.
factor whose activity was enhanced by Rb (Fig. 3E). These results strongly suggest that NeuroD1 itself is the target of Rb action within the POMC cell-specific regulatory complex.

Rb-related Protein p107, but Not p130, also Potentiates POMC Promoter Activity—Rb is the founding member of the pocket protein family that also includes p107 and p130 (37). We assessed in AtT-20 cells the ability of these proteins to activate the POMC promoter or a multimer construct containing all regulatory elements required for corticotroph-specific transcription. Fig. 4 shows that only p107, but not p130, is able to stimulate activity of these promoters. Although p130 potentiates NGFI-B activity as well as Rb or p107 (50), the ability to enhance corticotroph-specific transcription of the POMC promoter is strongest for Rb followed by p107 (50), probably reflecting the ability of these two proteins to enhance NeuroD1 activity.

Rb Activation of POMC Transcription Depends on the Pocket Domain, but Not on Rb Phosphorylation Status—To test whether Rb mutations found in tumors also affect the activation of the POMC promoter by Rb, we tested in AtT-20 cells the effect of the Δex22 pocket mutant, which is unable to bind E2F, to arrest cells in G1 and to promote differentiation (27). This mutation reduced by ≈50% the capacity of Rb to induce the POMC promoter (Fig. 4) suggesting that the pocket domain of Rb is implicated at least in part in this action of Rb. Also, considering the importance of Rb phosphorylation for cell cycle control, we assessed the activity of the Δp34 mutant that has mutations in eight putative p34cdc-dependent Rb phosphorylation sites shown to be important for regulation of E2F-dependent activity (28).
and that is no longer subject to phosphorylation-dependent E2F derepression (28). Because AtT-20 cells have mostly hyperphosphorylated Rb as a result of the loss of p16 expression (38), a requirement for dephosphorylated Rb for activation of POMC would result in greater activity of the p34 mutant compared with the wild-type protein, because the latter could be inactivated by phosphorylation. As shown in Fig. 4, this mutant had similar activity compared with Rb for enhancement of POMC promoter activity. These results suggest that p34cdc-dependent phosphorylation sites are not relevant for the function of Rb as an activator of POMC transcription and that the function(s) of Rb in cell cycle control may be separate from its functions in cell-specific transcription, as shown previously (27).

Rb and p107 Interact with NeuroD1, but Not with Pan1—To test the hypothesis that Rb enhancement of NeuroD1 activity is dependent on direct interaction between these two proteins, we used an in vitro pull-down assay with GST fusion proteins encoding various Rb domains and 35S-labeled NeuroD1/Beta2 protein. As shown in Fig. 5A, NeuroD1 interacted efficiently with the large pocket of Rb (ABC) but not with its N-terminal domain, nor with GST alone. Two Rb subdomains, the B and C regions, interact independently with NeuroD1 (Fig. 5A). In accordance with the lack of Rb effect on Pan1 activity (Fig. 3C), we showed that Pan1 did not interact with Rb (Fig. 5B), although this protein was previously shown to be sufficient for in vitro interaction with Pitx1 (11).

To delineate NeuroD1 regions implicated in interactions with the B or C domains of Rb, we tested NeuroD1 deletions for binding to GST-Rb B and C (Fig. 5C). All NeuroD1 proteins containing an intact HLH dimerization domain bound GST-Rb B and C. Indeed, deletion of the NeuroD1 C terminus-(1–158) or a deletion in the basic DNA-binding domain (49–96) did not affect interactions with Rb. This interaction was totally abolished when the C-terminal part of the HLH domain is deleted (1–138), indicating that at least the 138–158 region of the HLH domain is important for binding both Rb B and C domains (Fig. 5C). This region does not share motifs similar to those found in oncproteins or E2F (1) that interact with the B subdomain. In agreement with this, the C706F Rb mutation that prevents E2F or oncprotein binding does not interfere with NeuroD1 binding (data not shown).

To confirm in vivo interactions between NeuroD1 and Rb-related proteins, we used coimmunoprecipitation experiments with extracts from AtT-20 cells that endogenously express these proteins. Because of the very low level of Rb protein in these cells, we were unable to detect endogenous Rb-NeuroD1 complexes in AtT-20 cells. However, the Rb-related protein p107 is much more abundant than Rb (~10-fold more by real
time quantitative PCR). Thus, immunoprecipitates produced with anti-NeuroD1 (Fig. 5D, lane 2) but not with non immune antibody control (IgG, lane 4) contained p107. Inversely, we detected NeuroD1 protein in p107 immunoprecipitates (Fig. 5D, lane 3). Moreover, we have also shown that Rb interacts in vivo with NeuroD1 in extracts of co-transfected cells (Fig. 6B, lane 4). These results demonstrated that Rb and p107 are present in the same complexes as NeuroD1 in living cells. The siRNA experiment (Fig. 1C) suggested that Rb and/or p107 contribute to basal POMC expression. If these proteins do so through direct protein interactions as suggested above, they should be present at the POMC promoter. This was indeed found to be the case when assessed by the ChIP method (Fig. 5E). Relative enrichment of Rb, and to a lesser extent p107, was observed on the POMC promoter relative to POMC exon 3 sequences that are located ~5-kb downstream in the gene. Similarly, NeuroD1 was found at the POMC promoter in agreement with its purported role in transcription.

Rb Acts as a Functional and Physical Bridge between NeuroD1 and NGFI-B—Previous studies (34, 35) have suggested that the activity of the NurRE and its cognate factors, such as NGFI-B, is dependent on the cell-specific transcription factors NeuroD1, Tpit, and Pitx1. However, the molecular basis for this dependence remains to be defined. In fact, the activity of the NurRE element is dependent on Eboxneuro activity, which is itself dependent on the Tpit/Pitx1 activity (39). The Eboxneuro-dependent activity of the NurRE can be reconstituted using a multimer construct containing the adjacent NurRE and Eboxneuro binding sites organized as they are found within the POMC promoter. This construct was poorly activated by NGFI-B or by NeuroD1 alone, but much more efficiently when both factors were added (Fig. 6A, lanes 1–4 and lanes 5–8). This result suggests that the NeuroD1 and NGFI-B activities are dependent on each other.

As we have previously shown that Rb enhances NGFI-B-dependent transcription in collaboration with coactivators of the SRC/p160 family (50), we wanted to address the role of Rb or related proteins in the NGFI-B/NeuroD1 synergy. Toward this end, we used mouse embryonic fibroblasts derived from TKO mice for all Rb-related proteins (30) in co-transfection experiments. NeuroD1 and NGFI-B were not able to synergize efficiently in these cells (Fig. 6A, lanes 9–12) when compared with their activity in p107-positive Rb-negative C33A cells (Fig. 6A, lanes 1–4). Moreover, the SRC-2 coactivator that was shown to enhance NGFI-B activity (21), also restored some NGFI-B/NeuroD1 synergism even in absence of Rb-related proteins (Fig. 6A, lanes 17–20). But together, SRC-2 and Rb markedly enhanced the ability of NeuroD1 and NGFI-B to synergize (Fig. 6A, lane 24).

To understand why Rb is a limiting factor for the NeuroD1/NGFI-B synergy, we tested for potential interactions between
these proteins. Using a common immunoprecipitation assay with extracts of transfected cell, NGFI-B was co-immunoprecipitated with NeuroD1 only when Rb is co-expressed (Fig. 6B, lane 2 versus 3). Moreover, this was independent of other cellular proteins, because the formation of this Rb-dependent complex could be reconstituted using in vitro translated proteins. Indeed in absence of Rb (Fig. 6C, upper panel), NGFI-B and NeuroD1 did not co-purify in vitro but the addition of Rb (Fig. 6C, lower panel) sufficed to allow formation of a trimeric complex that could be immunoprecipitated with either NeuroD1 (lane 2) or Flag-NGFI-B antibodies (lane 3). These results show that in vitro Rb provides a sufficient bridge to bring together NGFI-B and NeuroD1 within a trimeric NGFI-B-Rb NeuroD1 complex. Formation of this complex provides a likely explanation for the transcriptional synergism observed between NeuroD1 and NGFI-B.

**DISCUSSION**

The present work supports a model of Rb (or p107) as a coactivator of NeuroD1 transcriptional activity. Indeed, we have shown that Rb and p107 enhance POMC promoter activity specifically in cells that express NeuroD1 such as in AtT-20 cells (Figs. 1A, 2, 3A, and 4) or after ectopic expression (Fig. 3B). Accordingly, knock-down of Rb and p107 using siRNAs in AtT-20 cells led to decreased basal POMC expression (Fig. 1C), and Rb, p107, and NeuroD1, could be shown to be present on the POMC promoter (Fig. 5E). Rb enhancement of POMC promoter activity depends on cell-specific regulatory elements (Fig. 2), particularly the Ebox\_neuro that is the target of NeuroD1 (Fig. 3). Rb and p107 interact directly with NeuroD1 (Fig. 5). Rb also potentiates the action of the SRC coactivators of NGFI-B-related nuclear receptors that mediate hormonal (CRH) stimulation of POMC transcription (50). Direct protein interactions involving Rb with NeuroD1 and Rb with NGFI-B (Fig. 6) could explain, at least in part, transcriptional synergism between NGFI-B and NeuroD1 (Fig. 7).

**Function of Rb in the NeuroD1-NGFI-B Complex**—The multiple protein interactions described in the present work and in a parallel paper on NGFI-B and SRC coactivators (50) suggest a mechanism by which the cell-specific activity of NeuroD1 can be coupled to the signal-dependent activity of NGFI-B for synergistic activation of POMC transcription. In this model (Fig. 7), the pocket and the C-terminal region of Rb interact directly with the NeuroD1 HLH domain, whereas the pocket supports interactions with the DNA binding domain of NGFI-B and with the Rb interactome domain of SRC2. In addition, the Rb N terminus interacts with the AD2 domain of SRC2. Although the concurrent occurrence of all these interactions has not been tested directly, the various interactions revealed in the present work, and the model suggests that they may indeed take place concurrently. The B domain of the Rb pocket is primarily involved in many of these interactions, and the same pocket domain has previously been shown to interact with many different proteins at the same time (40).

In basal conditions, recruitment of Rb to the promoter may occur through NeuroD1 and NGFI-B interactions. The preference for Rb over p107 would be consistent with the preferential action of Rb compared with p107 on the intact POMC promoter or on the simple reporter containing the Ebox\_neuro (Fig. 4). This conclusion is in agreement with the results of ChIP experiments that have shown presence of Rb on the POMC promoter, but only marginally of p107, in basal or unstimulated conditions (Fig. 5E).

Upon CRH activation of the downstream signaling pathways (21, 22), the recruitment of NGFI-B to the NurRE sequence adjoining the Ebox\_neuro provides an additional target of Rb action. In this case, Rb recruitment and action take place through interactions not only with NGFI-B but also with its coactivator of the SRC family. We do not as yet know of NeuroD1 coactivators, and the SRCs were not found to contribute or interact with NeuroD1 (Ref. 41 and data not shown). However, the present demonstration that Rb can bridge complex formation in vitro between NeuroD1 and NGFI-B (Fig. 6, B and C) clearly suggests that the presence of Rb may contribute significantly to synergistic activation of transcription. It is interesting to note however that p107 is approximately nine times more abundant than Rb in the model AtT-20 cell line and that is consistent with both p107 and Rb being recruited to the POMC promoter upon CRH stimulation (50). Because neither Rb interaction with NeuroD1 nor with NGFI-B could be shown to depend on Rb phosphorylation status, it appears that the levels of Rb-related pocket proteins may be the most limiting factor for their action on POMC transcription. The molecular basis for NeuroD1 preferential interactions with Rb compared with p107 is not clear, but it may be the reason why Rb haploinsufficiency has a particularly penetrant phenotype in pituitary POMC-expressing cells (4, 37). It is also possible that another Rb posttranslational modification, such as p300 acetylation, may modulate this activity (42).

The action of Rb-related pocket proteins on POMC transcription may be related to an action of Rb proteins on expression of differentiation functions such as hormone gene expression in contrast, and possibly in opposition, to the role of Rb in the control of cell cycle and proliferation. It is noteworthy that increased levels of Rb expression have been associated with differentiation in many systems (43, 44). Although Rb enhances NeuroD1-dependent transcription, it is interesting that cyclin D1, a negative regulator of Rb action on E2F and cell cycle control (1) represses NeuroD1-dependent transcription (41). As for the effect of Rb on NeuroD1, the cyclin D1 action does not appear to be dependent on cell cycle-regulated kinases. The repressor effect of cyclin D1 on NeuroD1 activity appears to be mediated through an indirect association, possibly with p300 (41). These observations suggest another way in which expression of differentiation-related genes such as POMC may be regulated in an inverse relation by comparison to cell cycle control and proliferation. Similar interpretations have been proposed for Rb and cyclin D1 in relation to the myogenesis promoting action of myogenic bHLH proteins (45, 46). In addition, the mutually antagonistic interaction between Rb and Id2, an inhibitor of bHLH factor-dependent genes/function, may also contribute to a role of Rb in establishment of a differentiation phenotype (47, 48).
Functions of Rb in POMC Expression—The present work has shown that Rb may bridge interactions between NeuroD1 and NGFI-B leading to enhancement of POMC transcription. Although such interactions are possible in AtT-20 cells, which co-express NeuroD1 and CRH-stimulated NGFI-B, it is possible that Rb may rather act sequentially through one target than the other during differentiation of POMC corticotroph cells. Indeed, we have shown the importance of NeuroD1 early in differentiation of pituitary corticotrophs in a developmental window extending from embryonic day 12 to 15.5 of mouse development (14). Beyond this time, NeuroD1 protein is no longer very abundant in pituitary corticotrophs and hence, the primary target of Rb action may then be CRH-inducible NGFI-B. In such sequential model, the POMC promoter would always be sensitive to levels of Rb-related proteins. These models are consistent with the presence of Rb protein in early differentiating corticotrophs at embryonic day 14.5 of mouse development (Fig. 1, D and E).

There is no reason to believe that the action of Rb-related proteins on POMC transcription has anything to do in itself with pituitary cell tumorigenesis. Indeed, the tumorigenic effect of Rb haploinsufficiency in mice has been linked to the anti-oncogenic functions of Rb associated with the ability of Rb to repress E2F activity, because mice deficient in E2F1 have reduced prevalence of Rb haploinsufficiency-dependent pituitary tumors (49). On the other hand, the particular sensitivity of pituitary POMC cells to Rb-induced tumorigenesis is consistent with the interpretation that the levels of Rb-related proteins are limiting in these cell lineages, and hence, these levels may contribute to modulating POMC expression and the sensitivity of POMC response to CRH (50).

ACTH-producing pituitary adenomas account for 15% of all pituitary adenomas, and they have been associated with Cushings disease. These adenomas frequently progress to a more aggressive state characterized by less differentiated, invasive, and non-secreting cells (8). Rb expression is often lost in this process (2) consistent with the idea that the levels of Rb play an important role in maintenance of corticotroph cell differentiation status.

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