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Retinoblastoma/p107/p130 Pocket Proteins

PROTEIN DYNAMICS AND INTERACTIONS WITH TARGET GENE PROMOTERS

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The retinoblastoma (RB) tumor suppressor and its family members, p107 and p130, function by repressing E2F transcription factor activity to limit the expression of genes required for cell cycle progression. Traditionally, it is thought that the RB family proteins repress E2F target gene expression through complexing with E2F at gene promoters. However, whereas chromatin immunoprecipitation experiments have demonstrated p107 and p130 at E2F-responsive promoters, RB chromatin association has not been reliably observed. Here we used green fluorescent protein-tagged proteins to rigorously explore the mechanism of RB-mediated transcriptional repression relative to its p107 and p130 family members. The use of live cell fluorescent imaging demonstrated that RB, p107, and p130 exhibit similar nuclear dynamics. Although these findings suggest a similar engagement with nuclear structures, chromatin immunoprecipitation approaches with multiple independent antibodies failed to detect the association of RB with target gene promoters. However, by employing antibodies directed against green fluorescent protein, we could utilize the same antibody to assess RB, p107, and p130 engagement. This approach demonstrated RB association with target gene promoters in a fashion analogous to p107 and p130. Extension of this technology demonstrated that direct RB phosphorylation disrupts promoter association to regulate transcription. Thus, RB is associated with promoters in a manner similar to p107/p130 and that association is modulated by phosphorylation during cell cycle progression.

The retinoblastoma (RB)[3] tumor suppressor and its family members p107 and p130, collectively known as "pocket proteins", are involved in the coordinated regulation of cell cycle progression through modulation of the E2F family of transcription factors (1–4). Classically, RB functions as a transcriptional repressor. Correspondingly, microarray studies conducted with overexpression/activation of RB demonstrate significant attenuation of E2F-regulated genes, whereas conversely, genetic ablation of RB reveals elevation of these same target genes (5, 6). Multiple studies have been conducted to dissect the molecular mechanisms of RB-mediated repression, resulting in a general understanding of the regulation of RB repressor activity (7–9). In its hypophosphorylated form, RB is able to bind to E2F proteins, as well as co-repressors, such as Sin3, HDAC1, and SWI/SNF (10–12), resulting in transcriptional repression and the retention of cells in G_0/G_1 phases of the cell cycle (13, 14). However, in response to mitogenic signaling, RB is hyperphosphorylated by activated cyclin-CDK complexes resulting in the release of E2F, thus allowing expression of target genes required for cell cycle progression (15–17).

Historically, it has been assumed that pocket proteins repress the transcription of cell cycle genes through direct association with chromatin-bound E2F molecules at gene promoters. In fact, for p107 and p130 this appears to be true, with chromatin immunoprecipitation experiments detecting both p107 and p130 at a variety of E2F-responsive promoters (18–20). Specific studies failed to detect RB at these same promoters in the context of transcriptional repression, drawing into question the long held dogmatic view of RB-mediated repression (18, 19). Furthermore, RB association with promoters did not follow the long held belief that phosphorylation would disrupt the association at promoters (21, 22). Thus, although there are data in the literature supporting RB association with chromatin (*e.g.* Refs. 21, 23, 24), the emergence of negative data has spurred the proposal of alternative mechanisms through which RB can act in a fashion distinct from other pocket proteins in the absence of chromatin association (7, 25). Here we rigorously approached this question utilizing distinct methodology to specifically interrogate the specificity and regulation of RB function *versus* that of p107 and p130 at promoter elements.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfections—SAOS-2 and U2OS cultures were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with 100 units/ml penicillin/streptomycin and 2 mM L-glutamine at 37 °C and 5% CO_2. GFP-RB expression plasmid was described previously (26). Similarly, HA-p107 and p130 were subcloned into the pEGFP-C3 and pEGFP-C1 vectors (Clontech), respectively. β-Galactosidase, TS-Luc, and CycA-Luc were described previously (14, 27).

Immunoprecipitation and Immunoblot Analysis—Total cell lysates were resolved by SDS-PAGE and transferred onto

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[3] The abbreviations used are: RB, retinoblastoma; GFP, green fluorescent protein; BrdUrd, bromodeoxyuridine; FRAP, fluorescence recovery after photobleaching; ChIP, chromatin immunoprecipitation; IP, immunoprecipitation; PIPES, 1,4-piperazinediethanesulfonic acid; TS, thymidylate synthase; DHFR, dihydrofolate reductase; CDK, cyclin-dependent kinase.

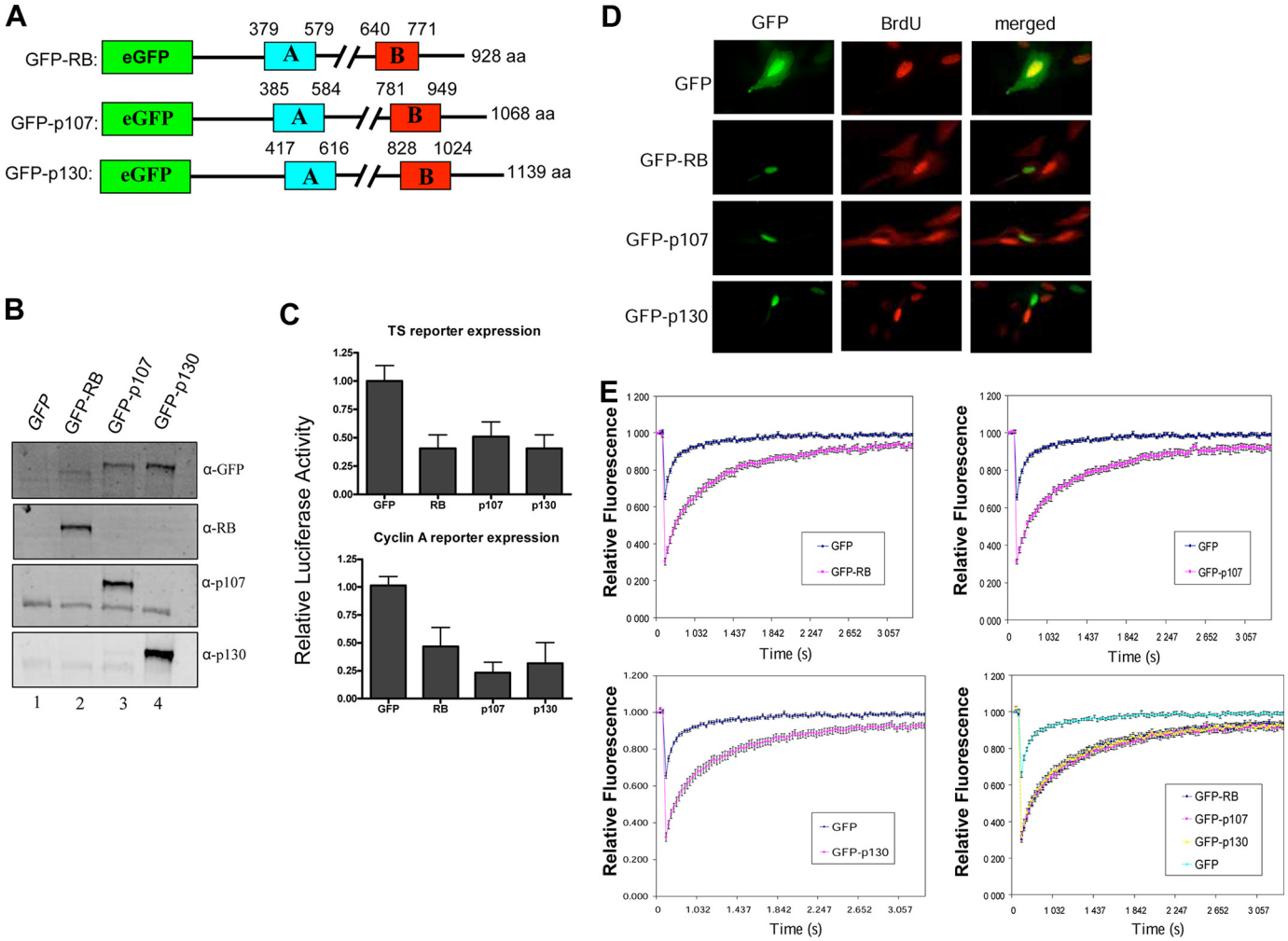


FIGURE 1. **Transfection of Saos-2 cells with GFP-RB, -p107, and -p130 constructs results in expression of functional GFP-tagged pocket proteins exhibiting similar protein dynamics.** *A*, eGFP was fused to the N terminus of wild-type RB, p107, and p130. *A* and *B domains*, which constitute the large pocket, are denoted. *aa*, amino acids. *B*, Saos-2 cells were transfected with GFP-RB, -p107, -p130, or vector control. Total protein was isolated 48 h post-transfection and immunoblotted for GFP, RB, p107, and p130. *C*, Saos-2 cells were co-transfected with GFP-pocket protein, cytomegalovirus β-galactosidase, and TS or cyclin A reporter constructs. Relative luciferase activity was normalized to β-galactosidase to control for transfection efficiency. *D*, Saos-2 cells were transfected with GFP-RB, -p107, -p130, or vector control. 48 h following transfection, cells were pulsed with BrdUrd (*BrdU*) for 4 h, fixed, and stained for BrdUrd incorporation. No cells transfected with either GFP-RB-, -p107-, or -p130-incorporated BrdUrd. *E*, Saos-2 cells were transfected with either GFP-RB, GFP-p107, or GFP-p130. 48 h post-transfection, cells were subjected to FRAP analysis. Following photobleaching of a small area of a transfected nucleus, fluorescence recovery was monitored over time. Recovery curves represent the average of 19 nuclei.

Immobilon-P membrane (Millipore Corp., Billerica, MA). Specific proteins were detected using the antibodies p107 (catalog number sc-318, Santa Cruz Biotechnology, Santa Cruz, CA), p130 (catalog number sc-317, Santa Cruz Biotechnology), and RB (catalog number 554136, Pharmingen). Total cell lysates were used for immunoprecipitation experiments; in the case of GFP-pocket protein-transfected cells, lysates were obtained 72 h post-transfection. Lysates were immunoprecipitated using antibodies against RB (catalog number sc-102, Santa Cruz Biotechnology; catalog number 554136, Pharmingen; catalog number SA-188, Biomol Research Laboratories) or by antibodies directed against GFP (catalog number A11121, Molecular Probes, Eugene, OR). A small amount of lysate was retained for the total lysate control. Antibody-bound proteins were precipitated using protein A beads (GE Healthcare). Immunoprecipitated proteins and lysed controls were resolved by SDS-PAGE.

Reporter Assays—Transfected cells were harvested for reporters 48 h post-transfection. Reporters were carried out using cell lysis buffer and luciferin (Promega, Madison, WI), and β-galactosidase activity was determined using GalactoStar reagents (ABI, Foster City, CA). Luciferase activity was normalized to β-galactosidase activity to account for differences in transfection efficiency, and numbers were plotted relative to the GFP-transfected control.

BrdUrd Incorporation and Immunofluorescence—Prior to harvesting, cells were pulsed with BrdUrd (Amersham Biosciences) for 8 h. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.4% Triton X-100 in phosphate-buffered saline. Cells were stained with rat anti-BrdUrd antibody (OBT0030CX, Accurate Scientific) and rhodamine-conjugated donkey anti-rat antibody 1:100 (712-295153, Jackson ImmunoResearch). DNA was stained with 4′,6-diamidino-2-phenylindole (Invitrogen). GFP-positive cells were scored for BrdUrd incorporation.

FRAP Analysis—Cells were seeded on 25-mm coverslips and transfected with GFP-RB, -p107, -p130, or GFP vector

control. Transfected cells were placed in a water-jacketed stage chamber and used for live cell imaging 48 h post-transfection. Fluorescence recovery after photobleaching (FRAP) was carried out on a Zeiss LSM510 laser-scanning confocal unit connected to a Zeiss inverted microscope. For each condition, 19 nuclei were analyzed with 30 iterations and 5-ms cycle delay. Bleached regions were monitored for fluorescence recovery. Similar results were obtained from three independent experiments.

Chromatin Immunoprecipitation Assays (ChIP)—SAOS-2 cells were cultured in 15-cm dishes and transfected as indicated. Transiently transfected cells were processed for ChIP assay 48–72 h post-transfection or untransfected cells at ~80% confluent. Cells were cross-linked with formaldehyde to a final concentration of 1% for 10 min at room temperature. The cross-linking reaction was stopped by the addition of glycine to a final molarity of 0.125 M. The cells were harvested and washed three times with ice-cold Dulbecco's buffered phosphate solution buffer containing 5 mM EDTA. Cells were incubated in Cell Lysis Buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 100 ng of leupeptin and aprotinin per ml) for 30 min and centrifuged. Nuclei were isolated via centrifugation and resuspended in nuclei lysis buffer (50 mM Tris-Cl (pH 8.1), 10 mM EDTA, 1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, 100 ng of leupeptin and aprotinin per ml) for 30 min, and cell debris was removed by centrifugation. The chromatin was sonicated seven times to generate DNA fragments with a range of 100–1000 bp. The sheared chromatin concentration was measured; ~10% of the chromatin was kept as an input, and the rest of the chromatin was diluted 3-fold. Chromatin was precleared with blocked protein A slurry (GE Healthcare), and then equal amounts of precleared chromatin from all conditions were immunoprecipitated with 1 μg of antibody as follows: p107, p130, and Dbf4 were obtained from Santa Cruz Biotechnology (catalog numbers 318, sc-317, and sc-11354, respectively); RB antibodies were obtained from Pharmingen (catalog number 554136), Santa Cruz Biotechnology (catalog number sc-102), and Biomol (catalog number SA-188); GFP antibodies were obtained from Molecular Probes, Eugene, OR (catalog number A11121), and Abcam, Cambridge, MA (catalog number ab 290). IPs were carried out in 500 μl of RIPA buffer containing sheared chromatin + protease inhibitors for 3 h at 4 °C. 20 μl of protein A slurry was added overnight. IPs were washed three times with RIPA + protease inhibitors and two times with TE before incubating beads with extraction buffer (0.1 M $NaHCO_3$, 1% SDS, 0.3 M NaCl, 10 mg/ml RNase A) at 65 °C overnight to decross-link. Immunoprecipitated DNA was purified using the PCR purification kit (Qiagen, Valencia, CA) and resuspended in 50 μl of sterile water. The purified DNA was PCR-amplified using semiquantitative or quantitative real time PCR.

Semiquantitative PCR—Purified DNAs from chromatin immunoprecipitation experiments were amplified by semiquantitative PCR using the following E2F target gene promoter primers: plk-1 sense, GGT TTG GTT TCC CAG GCT AT, and antisense, GCT GGG AAC GTT ACA AAA GC; RNR2 sense, GAG GCA TGC ACA GCC ATT, and antisense, GAC ACG GAG GGA GAG CAT AG; TopoIIa sense, TCT AGT CCC GCC TCC CTA AC, and antisense, GGA GAG CTC CAC TTG AAC CTT; TS sense, CTC CGT TCT GTG CCA CAC C, and antisense, TGG ATC TGC CCC AG TAC T; DHFR sense, CGT AGA CTG GAA TCG GCT CAA, and antisense, AGT TTG GCG CGA AAT TGT GG; cyclin A sense, CCC CAG CCA GTT TGT TTC T, and antisense, AGT TCA AGT ATC CCG CGA CT; albumin sense, CAG GGA TGG AAA GAA TCC TAT GCC, and antisense, CCA TGT TCC CAT TCC TGC TGT. Dbf4 antibody and the albumin promoter served as negative controls. The PCR-amplified products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide visualized under UV transilluminator and documented by using eagle eye system.

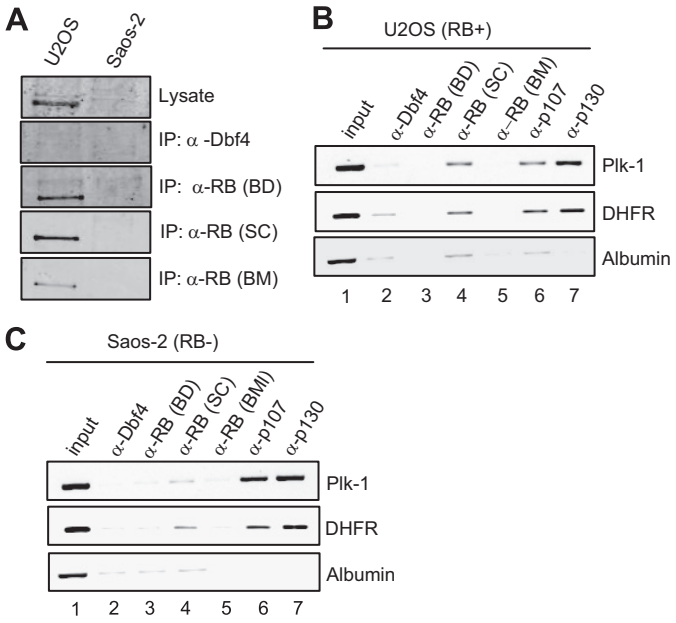


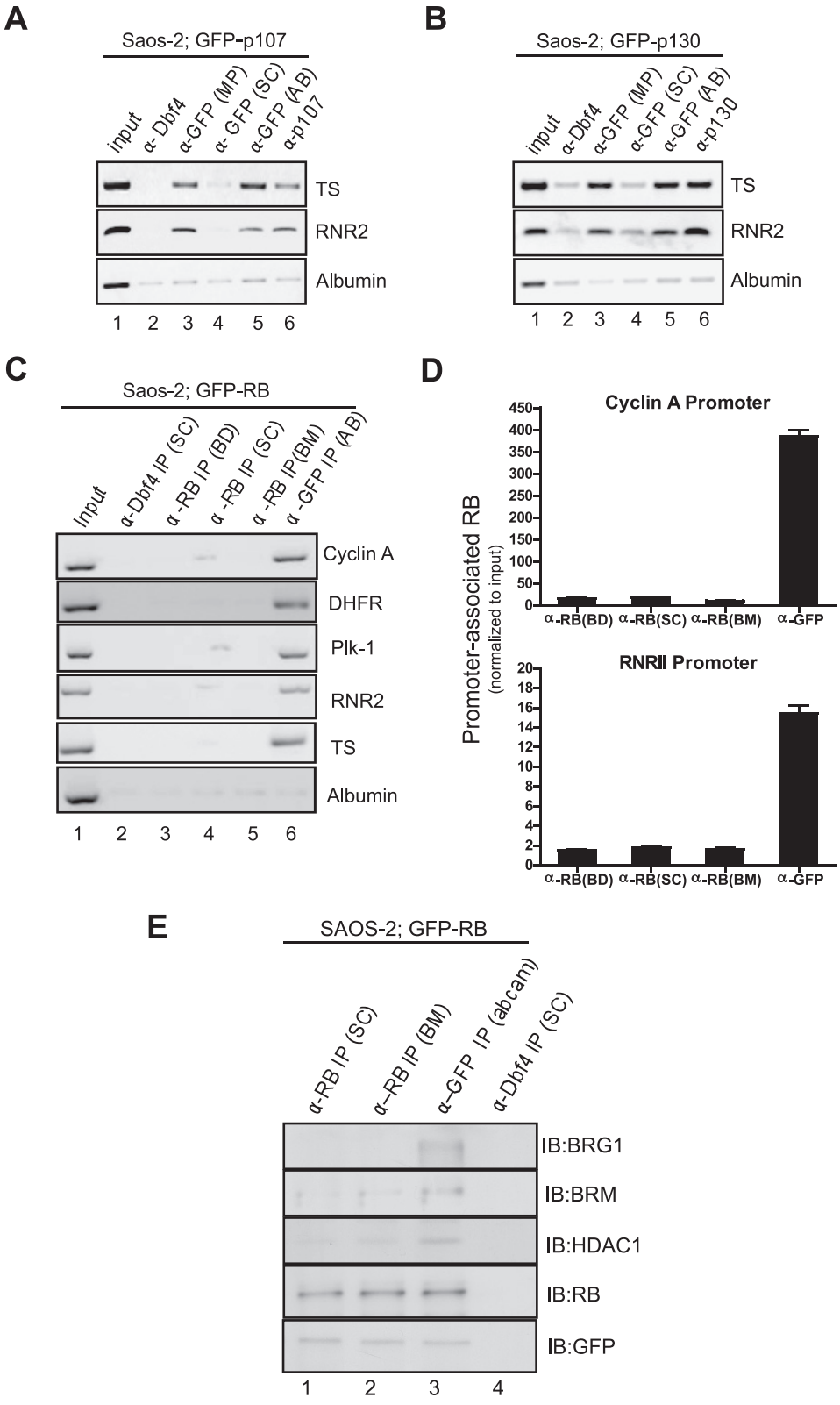
FIGURE 2. **Standard ChIP procedures fail to specifically detect RB at target gene promoters.** *A,* lysates derived from RB-proficient U2OS cells were used for immunoprecipitation of RB by commercially available RB antibodies. Lysates from RB-deficient Saos-2 cells served as a negative control. Precipitated proteins were immunoblotted for RB using the Pharmingen (*BD*) antibody. *B,* chromatin obtained from U2OS cells was used for ChIP assays to detect RB, as well as its family members p107 and p130 at target gene promoters. Immunoprecipitation was performed using three antibodies for RB (as in *A*) as well as antibodies for p107 and p130. Immunoprecipitation using a Dbf4 antibody served as a negative control. Input and immunoprecipitated DNA were amplified by PCR using primers specific for the Plk-1 and DHFR promoters. *C,* ChIP assays were performed as in *B*, using chromatin isolated for the RB-deficient Saos-2 cell line. *SC*, Santa Cruz Biotechnology; *BM*, Biomol.

Real Time PCR—Purified DNAs from immunoprecipitation with RB antibodies (Pharmingen, Santa Cruz Biotechnology, and Biomol) and GFP antibody (Abcam) as mentioned before were subjected to real time PCR as described by Link *et al.* (28). Quantification of the data was performed by quantitative real time PCR on an ABI Step-One apparatus using Power SYBR Green Master Mix and primers directed against the E2F targets as follows: cyclin A sense, CCC CAG CCA GTT TGT TTC T, and antisense, AGT TCA AGT ATC CCG CGA CT; RNRII sense, GGG AGA TTT AAA GGC TGC TGG AGT G, and antisense, ACA CGG AGG GAG AGC ATA GTG GA. Input reactions and negative control (α-Dbf4) immunoprecipitations were used to assess relative recruitment of RB at E2F target gene promoters. Fold induc-

tion of RB ChIP signals were presented over α-Dbf4 (negative control).

RESULTS

Although the p107 and p130 members of the retinoblastoma family have been reproducibly and reliably detected at E2F target gene promoters by ChIP, the detection of RB at such promoters has been the subject of significant controversy (18, 21, 22). Because ChIP represents a static approach to define biochemical behavior, the complementary approach of utilizing live cell dynamics was employed (26). Specifically, GFP was fused to the N-terminal region of RB, p107, and p130 (Fig. 1*A*). To assess the function of GFP-tagged pocket proteins, SAOS-2 cells, which are RB-deficient and known to be sensitive to ectopic RB expression, were transiently transfected with GFP-RB, -p107, -p130, or empty vector control (Fig. 1*B*). Following transfection, GFP proteins were examined for their ability to repress transcription and halt cell cycle progression. Expression of GFP-RB, GFP-p107, or GFP-p130 significantly repressed TS and cyclin A reporter activity (Fig. 1*C*). Additionally, whereas cells transfected with the GFP vector control maintained the ability to proliferate, as indicated by BrdUrd incorporation, all cells transfected with GFP-RB, -p107, or -p130 were BrdUrd-negative, indicating a potent cell cycle arrest following pocket protein expression (Fig. 1*D*). These analyses indicate that the GFP tag did not alter the transcriptional repression or cell cycle inhibition mediated by pocket proteins.



The GFP tag is powerful in allowing the analyses of protein behavior via live cell imaging. Specifically, utilization of FRAP analysis allows examination of the relative dynamics of RB, p107, and p130 (26). A small region of GFP-RB, -p107, or -p130 transfected nuclei was bleached and then monitored for fluorescence recovery. The rate of fluorescence recovery is directly related to the relative protein mobility. In this context, retention on chromatin is a key factor modulating protein dynamics (26, 29). The GFP protein is freely diffusible and exhibits very rapid dynamics, wherein the bleached area recovers exceedingly rapidly. In contrast, FRAP analyses revealed that p107 and p130 possess reduced dynamics, as revealed by greater bleach depth and delayed recovery (Fig. 1*E*). This dynamic behavior is presumably reflective of the complexes

and chromatin association that has been demonstrated previously for p107 and p130. Importantly, GFP-RB exhibited dynamic behavior that was overlapping with that of p107 and p130 suggesting that the complexes assembled by RB are similar to those produced by p107 and p130.

To directly address the ability of RB to associate with target gene promoters, a panel of RB antibodies was initially employed for ChIP analyses. Each of these antibodies effectively immunoprecipitate endogenous RB (Fig. 2*A*). These antibodies were then tested for their ability to detect chromatin-associated RB protein. For these analyses, both RB-proficient (U2OS) and RB-deficient (SAOS-2) cell lines were utilized to define the specificity of the ChIP signal. As expected, the RB family members, p107 and p130, were detected at two E2F target promoters (*Plk-1* and *DHFR*) in both U2OS and SAOS-2 cell lines (Fig. 2, *B* and *C*). However, only one of three RB antibodies was able to detect RB at these same promoters above background (Fig. 2*B*, *lane 4*). Importantly, this antibody also generated a comparable ChIP signal at a non-E2F target promoter (albumin) and also detected chromatin-associated RB protein in the RB-deficient SAOS-2 cell line (Fig. 2*C*, *lane 4*). Thus, whereas two of the antibodies fail to precipitate chromatin with RB, one antibody does enrich for chromatin. However, the precipitation of chromatin in this reaction is neither RB-dependent nor specific to RB/E2F-regulated genes.

Although attempts to chromatin immunoprecipitate endogenous RB were unsuccessful, it is appreciated that antibody affinity, epitope location, and steric considerations can effectively preclude antibody-antigen interactions. Such effects are particularly acute following formaldehyde fixation. Therefore, to rule out difficulties associated with antibody accessibility as an explanation for the lack of RB ChIP signal, we employed the RB, p107, and p130 proteins, which were all tagged in an analogous fashion with GFP (Fig. 1*A*). This allows the use of a single antibody to ChIP p107, p130, and RB. Because the association of p107 and p130 with target gene promoters has been well established by ChIP assay, immunoprecipitation of these two proteins via the GFP tag served as a positive control. Two of three GFP antibodies demonstrated a significant and specific enrichment of GFP-p107 and GFP-p130 at two target promoters, TS and ribonucleotide reductase 2 (Fig. 3, *A* and *B*, *lanes 3* and *5*). Furthermore, the ChIP signal generated using these GFP antibodies was comparable with that observed using p107 and p130 antibodies, thus demonstrating that GFP antibodies can be used to evaluate the ability of GFP-pocket proteins to assemble onto chromatin.

Having demonstrated the ability of GFP antibodies to determine promoter occupancy by GFP-p107 and GFP-p130, these same antibodies were used to address whether RB associates with chromatin at RB/E2F-regulated genes. As with p107 and p130, the GFP antibody was able to immunoprecipitate chromatin-associated GFP-RB specifically at E2F target promoters. In contrast, RB antibodies were unable to detect GFP-RB at these same promoters (Fig. 3*C*), in fact quantitative real time PCR experiments demonstrate a dramatic increase in RB ChIP signal following precipitation with a GFP antibody when compared with commercial RB antibodies (Fig. 3*D*). Furthermore, whereas the standard RB antibodies are able to detect immunoprecipitated GFP-RB, their ability to associate with known co-repressor proteins, HDAC1, BRG1, and BRM, is markedly diminished relative to co-immunoprecipitation studies utilizing a GFP antibody (Fig. 3*E*). Such experiments further support the idea that specific RB antibodies fail to effectively recognize RB-repressor complexes associating with target gene promoters and support the notion that GFP antibodies provide a significant and dramatic advantage in studies designed to demonstrate RB promoter occupancy.

RB-mediated transcriptional repression can be disrupted following CDK-mediated phosphorylation, which has been shown to inhibit the association of RB-E2F complexes (16, 30). However, the influence of RB phosphorylation on chromatin association is less clear. To specifically define the impact of phosphorylation, SAOS-2 cells were co-transfected with cyclin E and either the wild-type large pocket of the RB protein (GFP-LP) or a mutant of this fragment with seven critical phosphorylation sites (PSM.7) in the C terminus mutated to nonphosphorylatable amino acids (GFP-LPΔCDK) (31). Overexpression of cyclin E resulted in accumulation of phosphorylated GFP-LP but had minimal effect on the mobility of the GFP-LPΔCDK protein (Fig. 4*A*). In addition, ChIP assays performed using a GFP antibody demonstrate a disruption of GFP-LP/chromatin association following overexpression of cyclin E (Fig. 4*B*). In contrast, the GFP-LPΔCDK remained associated with target promoters even in the presence of cyclin E (Fig. 4*C*). Thus, the phosphorylation status of RB directly impacts its ability to associate with chromatin at E2F-regulated promoters.

DISCUSSION

The retinoblastoma tumor suppressor and its family members p107 and p130 regulate cell cycle progression through modulation of E2F transcription factor activity. The pocket proteins bind to E2F, as well as co-repressors, resulting in the silencing of E2F target gene expression and inhibition of cell cycle progression (15, 16, 32). Traditionally, it has been thought that these pocket proteins bind to E2F and assemble repressor

FIGURE 3. **Antibodies recognizing GFP can be used to detect p107, p130, and RB at target gene promoters.** *A,* chromatin was isolated from Saos-2 cells 48 h following transfection with GFP-p107. ChIP assays were performed using three commercially available GFP antibodies as well as antibodies for p107 and Dbf4 as a positive and negative control, respectively. Input and immunoprecipitated DNA were amplified by PCR with primers specific for the promoters of two known E2F target genes (TS and RNR2) and one non-E2F target gene (albumin). *B,* chromatin was isolated from Saos-2 cells transfected with GFP-p130, and ChIP assays were performed as in *A*. *C,* chromatin was isolated from Saos-2 cells transfected with GFP-RB, and ChIP assays were performed as in *A* using the three RB antibodies (Pharmingen (*BD*), Santa Cruz Biotechnology (*SC*), and Biomol (*BM*)) and a GFP antibody (Abcam). Immunoprecipitated DNA was amplified by semiquantitative PCR with primers specific for Plk-1, TS, DHFR, RNR2, cyclin A, and albumin gene promoters. *D,* chromatin was isolated from Saos-2 cells transfected with GFP-RB, and ChIP assays were performed as in *A* using the three RB antibodies (Pharmingen, Santa Cruz Biotechnology, and Biomol) and a GFP antibody (Abcam). Immunoprecipitated DNA was amplified by quantitative real time PCR. ChIP signals are plotted as fold increase over α-Dbf4 IP. *E,* GFP-RB transfected cells were harvested, and lysates were immunoprecipitated with both RB (Santa Cruz Biotechnology and Biomol) and GFP antibodies (Abcam) with Dbf4 IP serving as a negative control. Precipitated proteins were probed for co-repressor proteins known to associate in RB repressor complexes.

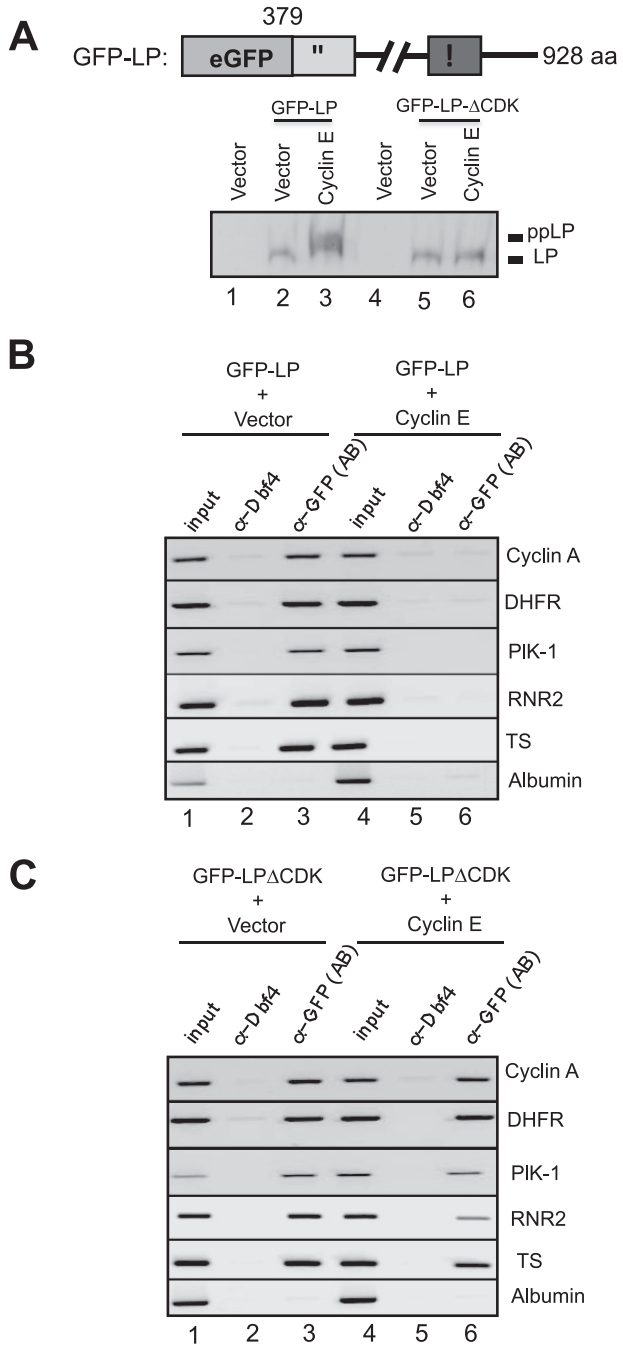


FIGURE 4. **Overexpression of cyclin E disrupts chromatin-bound RB complexes.** *A,* Saos-2 cells were transfected with GFP-WTLP, an active mutant (GFP-LP-ΔCDK), or vector control. Where indicated, cells were also co-transfected with a cyclin E expression vector. Cell lysates were immunoblotted for RB. *B* and *C,* cells were either co-transfected with GFP-WTLP and cyclin E, GFP-WTLP and vector control, GFP-LP-ΔCDK and cyclin E, or GFP-LP-ΔCDK and vector control. Following transfection, cells were harvested for ChIP assay using an antibody for GFP. Immunoprecipitated chromatin was amplified by PCR using primers for cyclin A, DHFR, Plk-1, RNR2, TS, or albumin. aa, amino acids.

complexes at target gene promoters. However, although this appears true for p107 and p130 (18, 20), recent studies have failed to detect RB at the promoters of genes repressed by RB activity (18–22). Such perplexing data have resulted in the proposition that RB functions in a fashion that is quite distinct from the other pocket proteins (7, 25). For example, it has been suggested that RB does not mediate the formation of repressor complexes at chromatin, but rather binds E2F in the nucleoplasm to affect changes in gene expression (25).

Here we sought to determine the relationship between pocket proteins in reference to transcriptional repressor function. Because the majority of published studies have utilized biochemical means to address this question, we initially utilized nuclear dynamics to compare the behavior of RB, p107, and p130. These analyses revealed that their respective dynamic properties are virtually identical, thereby suggesting that the macromolecular complexes elaborated by the pocket proteins are of similar composition. Furthermore, the reduced kinetics observed in such assays are largely attributed to associations with chromatin (26).

To directly evaluate whether RB could be detected at target gene promoters, we utilized a panel of commonly employed antibodies. These antibodies failed to provide specific ChIP signals when utilized under conditions identical to those used for p107 and p130. These findings are in agreement with similar investigations by other laboratories (18–20, 33). However, whether this observation reflects real differences in protein behavior or demonstrates effects related to antibody affinity or epitope masking was unclear. A strong indication that antibody accessibility may be an impediment was the finding that the association of RB with specific associated proteins is not readily detectable with a number of commonly utilized antibodies (37). Therefore, to rigorously examine this question, the use of the GFP-tagged pocket proteins was employed. Using the exact same antibody and ChIP conditions, we could clearly establish that RB does associate with chromatin in a fashion analogous to p107/p130. Thus, our data strongly indicate that many of the observations related to the failure of RB to be detected at promoters is a reflection of the reagents utilized. In keeping with this assertion, although all antibodies utilized efficiently precipitated the RB protein, the GFP antibodies recovered a ChIP signal that was 10–50-fold greater than that obtained with other antisera. Consistently, a recent publication revealed that whereas RB-chromatin association could not be observed utilizing normal fixation techniques, utilization of specialized fixation conditions allowed reliable detection of RB at E2F-responsive promoters (33). Combined, these findings indicate that the conformation of the RB-chromatin complex generally precludes antibody accessibility, whereas the expansive GFP moiety facilitates the detection and analyses of the RB complex.

By further employing this GFP-ChIP technique, it was observed that the large pocket fragment of RB, which is a minimal motif sufficient for transcriptional repression, does establish a stable complex at promoters. Furthermore, through the ectopic expression of cyclin E and RB mutants, we were able to directly define that phosphorylation does disrupt the complex of RB at promoters. Combined these studies show that the assembly of the promoter complexes are related to the functional effects on gene expression and biochemical properties of the RB protein that have been extensively validated.

With these findings established, it is highly likely that RB does function in a fashion akin to the other pocket proteins in modulating gene expression. Interestingly, whereas p107 and p130 have been identified in large repressor complexes (34–

36), RB has not been observed in these types of complexes. Potentially, this finding is again reflective of the limitations of technical approaches. A key example of such a possibility is that the association of RB with Sin3B is only detected by a single antibody of many antibodies utilized (37). Consistent with this observation, a number of proteins implicated in co-repression are significantly enriched in the context of immunoprecipitation with GFP *versus* RB antibodies. Thus, it is possible that such methodology will be crucial for deciphering the complexes assembled by pocket proteins.

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