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14. ABSTRACT
Work carried out under this grant showed that hyperphosphorylated Rb interacts with pp32 but not with the closely related proteins pp32r1 and pp32r2. Furthermore, pp32-Rb interaction inhibits the apoptotic activity of pp32 and stimulates proliferation. These results suggest a mechanism whereby cancer cells gain both a proliferative and survival advantage when Rb is inactivated by hyperphosphorylation. Further extension of these studies showed that pp32 increases androgen receptor-mediated transcription and the retinoblastoma protein modulates this activity. Furthermore, the results suggest that pp32 and the retinoblastoma protein may be part of a multiprotein complex that coordinately regulates nuclear receptor-mediated transcription and mRNA processing. Together, these findings indicate that the pp32-Rb interaction could serve as a drug target in prostate cancer.

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Introduction

The purpose of this Exploration – Hypothesis Generation proposal was to address the lack of effective chemotherapeutic agents in prostate cancer by determining whether pp32, a nuclear protein with pro-apoptotic properties, could serve as a target for development of novel and effective chemotherapeutic agents. The rationale derived from the observations that [1] pp32 is highly expressed in prostate cancers of Gleason score > 5, and that [2] pp32 is a multifunctional molecule whose inhibition leads to differentiation of a variety of human cancer cell lines. The experiments originally prposed to identify potential differentiation therapy targets through either physical association with pp32 or through siRNA depletion experiments to determine whether depletion of the candidates lead to differentiation.

Body

This section provides a synopsis of the key findings. Two publications are included in the appendices which provide full detail, commensurate with the report instructions.

As initially proposed, epitope-tagged pp32 constructs were successful in identifying a novel interaction of pp32. As shown in Figures 1 and 2 of Reference 1, these experiments demonstrated that pp32 interacts specifically with Rb. Importantly, the interaction occurs through a discrete region of pp32 since a short deletion in the active region abolished the ability of pp32 to interact with Rb. Likewise, the interaction required absolutely that Rb be phosphorylated on Thr 826. This interaction was shown to have significant functional consequences, since pp32 significantly increased E2F1-mediated transcriptional activity, while co-transfection of pp21 and Rb led to an inhibition of the pro-apoptotic activity usually associated with pp32.

A second series of studies, outlined in Reference 2, extended this work by demonstrating that [1] pp32 interacts specifically with the androgen receptor, that [2] a consequence of the interaction was to increase androgen receptor-mediated transcriptional activity, and that [3] when Rb interacts with pp32, it represses the ability of pp32 to activate androgen receptor-mediated transcription. PSF and nonO/p54nrb were also shown to be part of the complex. These results showed that pp32 coactivates the AR, in direct contrast to its actions on ERa and TRb. pp32 thus joins the growing list of bifunctional nuclear receptor coregulators which include PSF, nonO/p54nrb, cyclin D1, Zac 1,NSD 1, and RIP140.

Key Research Accomplishments

The key accomplishments of work performed under this proposal are the provision of a rationale for pp32 as a therapeutic target by showing that:

- pp32 interacts with Rb through a discrete, short region, and that this interaction leads to increased transcriptional activity that would be associated with proliferation (E2F1)
- The interaction of pp32 and Rb diminishes the pro-apoptotic (potentially anti—tumor) activity of pp32
- pp32 can bind to the androgen receptor and increase its transcriptional activity, however Rb interferes with this process.

Reportable Outcomes

Two publications are included as appendices:

Adebola O. and Pasternack G.R. Phosphorylated Rb complexes with pp32 and inhibits pp32-mediated apoptosis. Journal of Biological Chemistry. 280:15497-15502, 2005.

Adegbola O. and Pasternack G.R. A pp32-retinoblastoma protein complex modulates androgen receptor-mediated transcription and associates with components of the splicing machinery. Biochemical and Biophysical Research Communications. 334:702-708, 2005.

Work supported by this grant comprised a substantial portion of the PhD thesis of Dr. Onikepe Adegbola, who graduated from the Graduate Program in Pathobiology at Johns Hopkins in 2005.

Conclusion

As outlined in detail above and in the appendices, work performed with the support of this grant showed that the interaction of pp32 with Rb appears to contribute to the increased proliferation and decreased susceptibility of apoptosis that is characteristic of prostate cancer cells. This interaction thus becomes a rational target for future efforts at drug development.

References

1. Adebola O. and Pasternack G.R. Phosphorylated Rb complexes with pp32 and inhibits pp32-mediated apoptosis. Journal of Biological Chemistry. 280:15497-15502, 2005.

2. Adegbola O. and Pasternack G.R. A pp32-retinoblastoma protein complex modulates androgen receptor-mediated transcription and associates with components of the splicing machinery. Biochemical and Biophysical Research Communications. 334:702-708, 2005.

Appendices

1. Adebola O. and Pasternack G.R. Phosphorylated Rb complexes with pp32 and inhibits pp32-mediated apoptosis. Journal of Biological Chemistry. 280:15497-15502, 2005.

2. Adegbola O. and Pasternack G.R. A pp32-retinoblastoma protein complex modulates androgen receptor-mediated transcription and associates with components of the splicing machinery. Biochemical and Biophysical Research Communications. 334:702-708, 2005.

Supporting Data

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Phosphorylated Retinoblastoma Protein Complexes with pp32 and Inhibits pp32-mediated Apoptosis*

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The retinoblastoma gene product (Rb) is a tumor suppressor that affects apoptosis paradoxically. Most sporadic cancers inactivate Rb by preferentially targeting the pathway that regulates Rb phosphorylation, resulting in resistance to apoptosis; this contrasts with Rb inactivation by mutation, which is associated with high rates of apoptosis. How phosphorylated Rb protects cells from apoptosis is not well understood, but there is evidence that Rb may sequester a pro-apoptotic nuclear factor. pp32 (ANP32A) is a pro-apoptotic nuclear phosphoprotein, the expression of which is commonly increased in cancer. We report that hyperphosphorylated Rb interacts with pp32 but not with the closely related proteins pp32r1 and pp32r2. We further demonstrate that pp32-Rb interaction inhibits the apoptotic activity of pp32 and stimulates proliferation. These results suggest a mechanism whereby cancer cells gain both a proliferative and survival advantage when Rb is inactivated by hyperphosphorylation.

The retinoblastoma protein (Rb)[1] is a nuclear phosphoprotein that regulates proliferation, differentiation, and apoptosis. As a tumor suppressor, Rb inhibits proliferation by repressing E2F1-mediated transcription when hypophosphorylated. Hyperphosphorylation of Rb relieves E2F1 repression and allows cell cycle progression to occur (1). The importance of Rb is underscored by the fact that Rb function is disrupted in virtually all human cancers (2). Paradoxically and inconsistent with its role as a tumor suppressor, hyperphosphorylated wild-type Rb inhibits apoptosis in both cell culture and animal models (3–10). Because Rb inactivation is pivotal for carcinogenesis, this poses the problem of how cancer cells escape apoptosis when Rb function is disrupted.

Inherited cancers and cells in which Rb is inactivated by mutation have increased rates of both proliferation and apoptosis (11). Most sporadic cancers preferentially inactivate Rb by hyperphosphorylation, which may occur through mutation of cyclin D, cdk4, or p16. Such cancers are generally slow growing and resistant to apoptosis induced by chemotherapy or radiation (12). It is possible that in these cancers the tumor suppressor function of Rb is inhibited, whereas the anti-apoptotic function remains intact (13). Inactivation by hyperphosphorylation might promote proliferation by increasing free E2F1, as well as inhibit apoptosis by retaining the anti-apoptotic function of Rb. This is consistent with evidence suggesting that it is the hyperphosphorylated form of Rb rather than Rb *per se* that protects cells from apoptosis (14). The induction of apoptosis in various cell lines is accompanied by a shift in Rb from the hyperphosphorylated to the hypophosphorylated form (15, 16). Rb dephosphorylation, which has been shown to be required for apoptosis, occurs in the early stage of apoptosis (17, 18). Inhibition of Rb dephosphorylation prevents apoptosis, whereas induction of dephosphorylation leads to apoptosis (19). In DBA/2 mice, increased levels of hyperphosphorylated Rb appear to mediate apoptotic resistance (20). An increased level of hyperphosphorylated Rb is associated with a worse clinical outcome and greater chemoresistance as compared with Rb loss or normal levels of unphosphorylated Rb in patients with anaplastic large cell lymphoma (21). These observations all point to a pivotal role for hyperphosphorylated Rb in inhibiting apoptosis.

Although the exact mechanism by which Rb inhibits apoptosis is unclear, it does not always require the inhibition of E2F1-mediated transcription by Rb. The increase in apoptosis seen in Rb-null embryos is only partially reversed in Rb and E2F1 double knock-outs (22). A caspase-resistant Rb mutant, Rb-MI, inhibits apoptosis in response to tumor necrosis factor α-induced apoptosis by interfering with caspase 3 activation (23). These results led to the postulate that Rb binds to and sequesters a nuclear factor that stimulates caspase 3 activation (24). Although Rb binds to >100 protein partners, the majority bind to the hypophosphorylated form (25); yet it is the hyperphosphorylated form that predominates in most sporadic cancers.

pp32 is a member of the ANP32 family of acidic, leucine-rich, nuclear phosphoproteins found in cells capable of self-renewal and in certain long-lived neuronal populations (26). pp32 has been implicated in a number of cellular processes, including proliferation (27), differentiation (28), caspase-dependent and caspase-independent apoptosis (29, 30), suppression of transformation *in vivo* (31, 32), inhibition of protein phosphatase 2A (33), regulation of mRNA trafficking and stability in association with HuR (34), and inhibition of acetyltransferases as part of the INHAT (inhibitor of acetyltransferases) complex (35).

At a biologic level, pp32 inhibits transformation of rat embryo fibroblasts (36), possibly through its pro-apoptotic activity. It accelerates caspase activation by stimulating the apoptosome, but the *in vivo* significance of this is unclear. In contradistinction to its pro-apoptotic and transformation inhibition functions, pp32 is highly expressed in cancer (37). In fact, pp32 is more highly

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[1] The abbreviations used are: Rb, retinoblastoma protein; pRb, phosphorylated Rb; LP, large pocket; LRR, leucine-rich region; WT, wild-type.

expressed in highly malignant prostatic adenocarcinomas with Gleason scores of ≥5 than in clinically indolent tumors with Gleason scores of <5 (38). These data suggest that high levels of pp32 might foster increased malignancy. We report here that hyperphosphorylated Rb and pp32 associate in a specific complex. The pp32-Rb interaction inhibits the apoptotic activity of pp32 and promotes increased proliferation.

EXPERIMENTAL PROCEDURES

Construction of Vectors—All PCR reagents were purchased from Qiagen. pp32 truncation constructs were generated via PCR amplification of desired pp32 sequences. Primers were designed using the Stanford primer program (genome-www2.stanford.edu/cgi-bin/SGD/web-primer). All pp32 constructs utilized a common upstream primer, and all downstream primers lacked a stop codon to facilitate COOH-terminal V5 epitope tagging. Following amplification from pp32, pp32r1, or pp32r2 plasmids, products were cloned into the expression vector pcDNA3.1/V5-His Topo TA (Invitrogen) according to the manufacturer's instructions. pp32Δ201–360 was constructed in two stages. Bases 1–201 and 360–747 were amplified separately. The upstream primer of bases 360–747 and the downstream primer of bases 1–201 had NheI sites at their 5′-ends. Both amplicons were NheI-digested and ligated, and pp32 upstream and downstream primers were used to amplify the ligation products. The PCR products were then cloned into pcDNA3.1/V5-His Topo TA (Invitrogen). All constructs were verified by sequencing.

E2F1 and pRb plasmids were kind gifts from Fikret Sahin (Johns Hopkins) and Robert Weinberg (Massachussetts Institute of Technology), respectively. The Rb large pocket constructs WT-LP and PSM2T-LP were kind gifts from Erik Knudsen (University of Cincinnati). The pE2F-TA-Luc vector was purchased from Clontech.

Cell Culture and Transfections—HEK 293, HeLa, and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were passaged 2–3 times/week. All DNA transfections were carried out using FuGENE 6 (Roche Applied Science) as described by the manufacturer.

Antibodies—The antibodies used for this study were anti-pp32, anti-V5 (Invitrogen), anti-E2F (KH95, BD Biosciences), anti-Rb G3-245 (BD Biosciences), anti-Rb G99-549 (BD Biosciences), anti-Rb G99-2005 (BD Biosciences), anti-Rb C-15 (Santa Cruz Biotechnology), and polyclonal anti-RB 851 (gift from Erik Knudsen). The anti-phospho-Rb antibodies used were anti-S795 (Cell Signaling) and anti-T249/252, anti-T356, anti-S612, anti-S780, anti-S807/811, anti-T821, and anti-T826 (all from BIOSOURCE).

Immunoprecipitation and Immunoblotting—1×10^6 HEK 293 cells were seeded onto a T-150 flask. 24 h later, cells were transfected with 15 μg of the indicated plasmids. 48 h post-transfection, cells were harvested, washed twice with cold PBS, and lysed with an M-PER mammalian protein extraction reagent (Pierce) containing 1× HALT protease inhibitor mixture (Pierce). Lysates were centrifuged at 4 °C for 30 min at 16,000 × *g* to remove particulate material. The supernatant was precleared for 2 h with protein A-agarose (Roche). The pre-cleared cell lysates were mixed with the indicated antibodies and protein A- or protein G-agarose (Roche Applied Science) and incubated at 4 °C overnight. The next day the reaction mixture was washed three times with cold phosphate-buffered saline, boiled for 3 min, and eluted in 2× SDS buffer. The eluted materials were subsequently analyzed by immunoblotting with the indicated antibodies.

Proteins were separated in NuPAGE 10% bis-Tris gel (Invitrogen) and electroblotted onto polyvinylidene difluoride membranes (Invitrogen). The immunoblot analysis used indicated specific antibodies and enhanced chemoluminescence (ECL)-based detection (Amersham Pharmacia Biotech). Where indicated, blots were stripped with Restore Western blot stripping buffer (Pierce) per the manufacturer's instructions.

Reporter Assays—For reporter assays, NIH 3T3 cells were transfected with 1 μg of E2F-TA-LUC (Clontech), 0.5 μg of E2F1, 0.5 μg of pRb, and 1 μg of pp32V5 or pp32Δ201–360V5 expression plasmids as indicated. In all of the samples, 50 ng of the reporter vector pRL-TK (Promega) was included for normalization of the transfection efficiency. Total transfected DNA was kept constant at 3 μg with pcDNA 3.1 when necessary. 24 h after transfections, cells were lysed and assayed for luciferase activity using the Dual luciferase kit (Promega) per the manufacturer's protocol.

Statistical analysis of the reporter data was carried out by one-way analysis of variance followed by a Tukey multiple comparison post-test to compare individual pairs of data sets. The analysis was performed using GraphPad Prism software, version 4.0 (www.graphpad.com).

Colony Formation Assays—1.5×10^5 NIH 3T3 or HeLa cells, as indicated, were seeded in 6-well plates overnight. 24 h later they were transfected with 1 μg of DNA containing the indicated plasmids. Total transfected DNA was kept constant at 2 μg with pcDNA 3.1 when necessary. 48 h after transfection, cells were split into a 100-mm dish containing Dulbecco's modified Eagle's medium and 1% penicillin/streptomycin supplemented with 1000–500 μg/ml G418 (Invitrogen). The cultures were fed every 3–4 days. After 2 weeks, the cells were fixed with 95% ethanol and stained with 0.5% crystal violet in 95% ethanol, the plates were photographed, and the colonies were counted.

Apoptosis Assays—1.5×10^5 HeLa cells were seeded in 6-well plates overnight. 24 h later they were transfected with 1 μg of DNA containing the indicated plasmids and 1 μg of vector control plasmid where necessary for a total of 2 μg of DNA. 48 h post-transfection cells were fixed with ice cold 100% methanol at −20 °C for 15 min. After fixation, cells were stained with 10 μg ml^{-1} Hoechst 33342 (Molecular Probes) for 10 min at 37 °C. Samples were mounted with mounting medium containing Prolong anti-fade reagent (Molecular Probes). Apoptotic cells were identified and counted using a Nikon microscope equipped with an epi-illuminator and appropriate filters. The percentages of apoptotic cells were determined from 300 cells counted in each of three independent experiments.

RESULTS

We hypothesized that pp32 might interact with other proteins, such as Rb, that are involved in the regulation of pathways determining cell fate. To test this hypothesis, we screened for interactions by immunoprecipitation and identified Rb as a protein interacting with pp32.

When V5 epitope-tagged pp32 was expressed by transient transfection of mammalian cells, an interaction between endogenous Rb and pp32V5 was detected by Western blot analysis following immunoprecipitation with either an anti-V5 (Fig. 1*A*) or an anti-Rb antibody (Fig. 1*B*). The interaction between Rb and pp32 can also be demonstrated in untransfected mammalian cells, including HeLa cells (Fig. 1*C*), suggesting that it is physiologically relevant.

pp32 is made up of a nuclear localization signal, an acidic region, and a leucine-rich repeat (LRR) region that contains the nuclear export signal. Suppression of transformation and INHAT activity map to amino acids 150–174, slightly N-terminal to the acidic region. LRRs generally mediate protein-protein interactions (39), and the LRR of pp32 mediates its nucleocytoplasmic shuttling via binding to CRM1 (34). The Rb-binding region of pp32 was mapped using V5 epitope-tagged constructs lacking the acidic region, the LRR, or both (Fig. 1*D*). Whereas deletion of the acidic region had no effect, the deletion of nucleotides 201–360 encoding amino acids 67–120 in the LRR of pp32 abolished Rb binding (Fig. 1*E*). Because the expression of pp32V5–201 appeared to be low in the lysate shown in Fig. 1*E*, the experiment was repeated with a higher expression level (Fig. 1*F*), yielding the identical result.

As Rb functions are regulated by phosphorylation, we next determined which form of Rb (hypophosphorylated or hyperphosphorylated) interacted with pp32. V5 epitope-tagged pp32 was transiently expressed in HEK 293 cells, and the anti-V5 antibody was used to immunoprecipitate pp32V5. pp32 coimmunoprecipitated with hyperphosphorylated Rb because the V5 immunoprecipitates did not contain any hypophosphorylated Rb (Fig. 2, *A* and *B*).

Although not all Rb phosphorylation sites *in vivo* have been identified, Rb has at least 16 predicted cyclin-dependent kinase phosphorylation sites (40). Differentially phosphorylated forms of Rb appear to exist in cells (41), and there is evidence that differential phosphorylation of Rb may regulate its functions. Phosphorylation of Ser^{807} and Ser^{811} regulates binding of Rb to c-Abl, whereas phosphorylation of Thr^{821} and Thr^{826} regulate binding to L*XCX*E proteins (42). To determine the specific

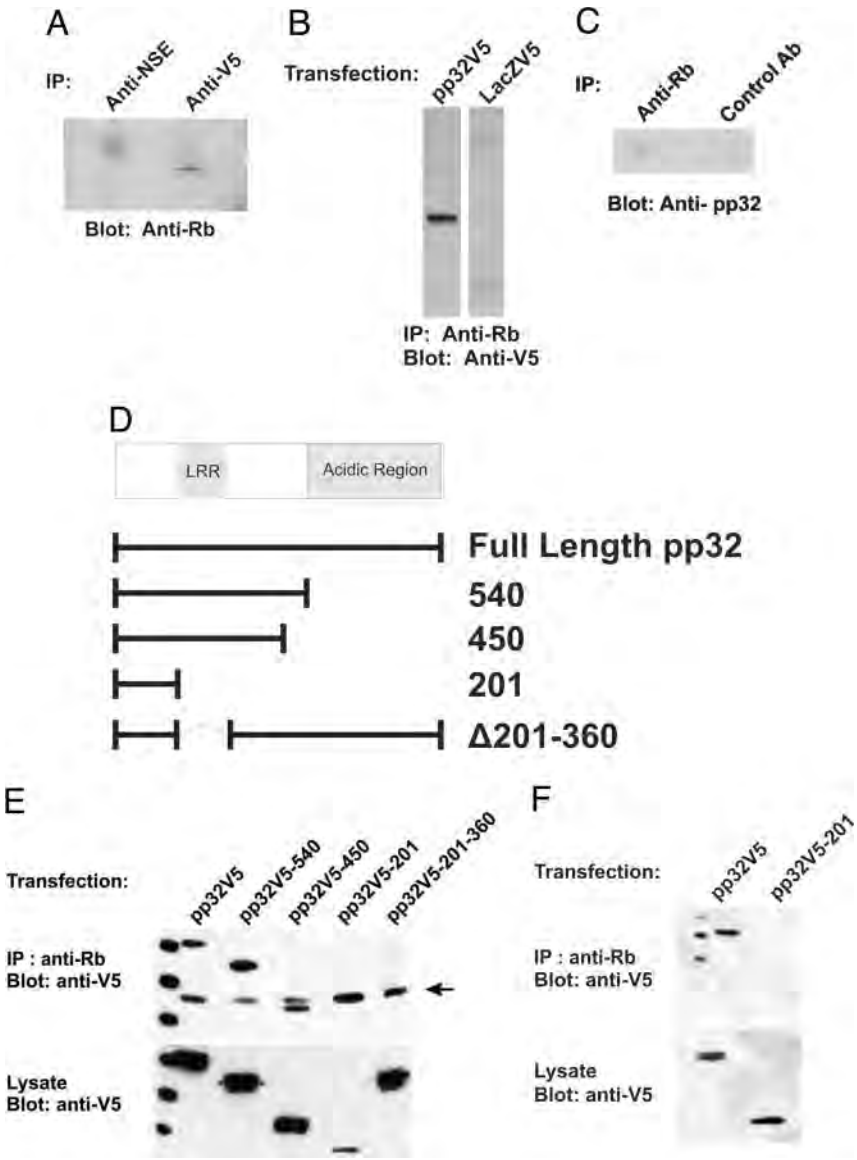

FIG. 1. **pp32 interacts with Rb via an LRR motif.** *A*, Rb co-immunoprecipitates with V5-tagged pp32. HEK 293 cells were transfected with pp32V5. Equal amounts of cell extracts were precipitated with an anti-V5 antibody or an anti-neuron-specific enolase antibody (*Anti-NSE*) as control, and the presence of Rb in the immunoprecipitates (*IP*) was visualized by Western blot analysis using an anti-Rb (G3-245) antibody. *B*, V5-tagged pp32 co-immunoprecipitates with Rb. HEK 293 cells were transfected with pp32V5 and lacZV5 as indicated. Equal amounts of cell extracts were precipitated with an anti-Rb (G99-2005) antibody, and the presence of V5 in immunoprecipitates was visualized by Western blot analysis using an anti-V5 antibody. *C*, endogenous pp32 co-immunoprecipitates with endogenous Rb. Equal amounts of HeLa cell extracts were precipitated with anti-Rb (G99-2005) or a proliferating cell nuclear antigen as the control antibody (*Control Ab*), and the presence of pp32 in immunoprecipitates was visualized by Western blot analysis using an anti-pp32 antibody. Identical results were obtained with HEK 293 and K562 cells. *D*, schematic diagram of pp32 mutants. The nucleic acid number of the domain boundaries is indicated. All contain a COOH-terminal V5 epitope tag. *E*, Rb interacts with the LRR of pp32. HEK 293 cells were transfected with the indicated V5 epitope-tagged mutants. In both the *top* and the *bottom sections* the *unlabeled lane* on the *left* represents molecular mass markers of 20, 30, and 40 kDa. *Top section*, equal amounts of cell extracts were precipitated with an anti-Rb (G99-2005) antibody, and the presence of V5 in immunoprecipitates was visualized by Western blot analysis using an anti-V5 antibody. The *arrow* indicates the position of the immunoglobulin light chain. *Bottom section*, cell extracts were subjected to anti-V5 Western blot analysis to confirm expression of the indicated V5 epitope-tagged mutants. *F*, replicate of the experiment shown in *panel E* restricted to pp32V5 and pp32V5-201 at a higher level of expression.

phosphorylated form of Rb that binds to pp32, various phospho-specific Rb antibodies were used to probe an anti-V5 immunoprecipitate of pp32V5. These immunoprecipitates specifically reacted with an anti-phosphoThr^{826} Rb antibody (Fig. 2*C*). To further confirm that Rb phosphorylation at Thr^{826} is necessary for pp32 binding, we used the Rb large pocket constructs WT-LP and PSM2T-LP, which have been described previously (42). WT-LP encodes the wild-type large pocket fragment of Rb (amino acids 379–928), and PSM2T-LP is a double T821A/T826A large pocket mutant. pp32V5 was cotransfected with either control, WT-LP, or PSM2T-LP constructs into HEK 293 cells, and the anti-V5 antibody was used to immunoprecipitate pp32V5 complexes. pp32 co-immunoprecipitated with WT-LP but not with PSM2T-LP, suggesting that pp32-Rb interaction requires Rb phosphorylation at Thr^{826} (Fig. 2*D*).

Because hyperphosphorylated Rb is unable to repress E2F1-mediated transcription, we used an E2F-luciferase reporter plasmid to investigate whether pp32 could increase E2F1-mediated transcriptional activity. Overexpression of pp32 consistently resulted in 3–5-fold increased transactivation of the E2F-luciferase promoter in the presence of excess E2F1 ($p < 0.001$ for E2F1 plus pp32 *versus* control; Fig. 3). This increased transcriptional activation could be decreased by overexpression of Rb ($p < 0.001$ for E2F1 plus pp32 *versus* E2F1 plus pRB plus pp32) and completely abolished by disruption of the interaction between Rb and pp32 via the deletion of amino acids 67–120 ($p < 0.001$ for E2F1 plus pp32 *versus* E2F1 plus pp32Δ201–360). Cells transfected with the reporter plus pp32 alone, E2F1 alone, E2F1 plus Rb, E2F1 plus pRB plus pp32, E2F1 plus pp32Δ201–360, or E2F1 plus pRB plus pp32Δ201–360 did not differ significantly from the control or from one another ($p > 0.05$). These results suggest that pp32 is able to sequester hyperphosphorylated Rb and thereby increase free E2F1. At the doses used in these assays, pp32 did not increase E2F-luciferase transactivation in the absence of E2F1 overexpression. It is probable that because there was enough endogenous Rb to bind E2F1 and pp32, the sequestration of Rb by pp32 was only unmasked when excess E2F1 was added to the system. The fact that pp32 has an acidic domain found in transcriptional activators raised the possibility that pp32 directly interacts with E2F1 to increase E2F1-mediated transcription. pp32 was transiently overexpressed in HEK 293 cells, and cell lysates were immunoprecipitated with E2F1 and Rb antibodies. Although pp32 was detected in Rb immunoprecipitates, it was absent in E2F1 immunoprecipitates (data not shown). These results rule out both direct and indirect interactions between pp32 and E2F1.

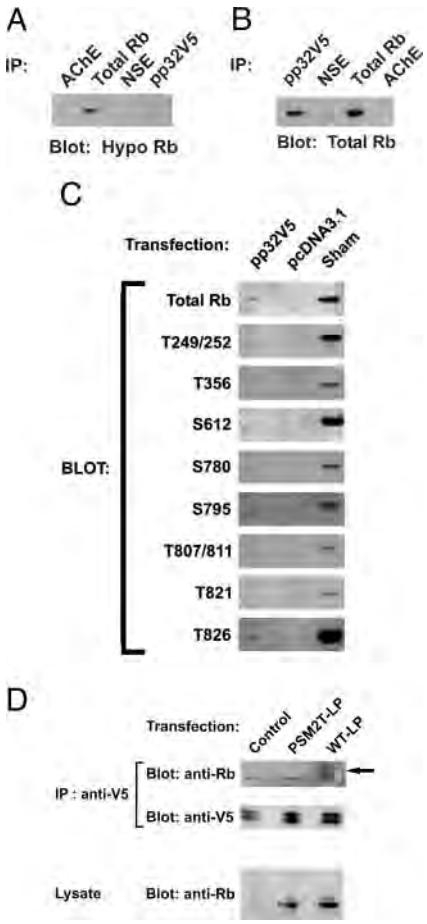

FIG. 2. **pp32 binds preferentially to Rb phosphorylated on Thr^{826}.** *A*, HEK 293 cells were transfected with pp32V5 or sham-transfected. Equal amounts of transfected cell extracts were precipitated with an anti-V5 antibody or an anti-neuron-specific enolase antibody (*NSE*) control as indicated. Equal amounts of sham-transfected cell extracts were precipitated with total Rb (C-15) or a control (*AChE*) antibody as indicated. The presence of hypophosphorylated Rb (*Hypo Rb*) in the immunoprecipitates (*IP*) was analyzed by immunoblotting using an antibody specific for hypophosphorylated Rb (G99-549). *B*, the blot in *panel A* was stripped and re-probed with total Rb (G3-245) antibody. *C*, HEK 293 cells were transfected with pp32V5 or LacZV5 (*pcDNA3.1*) or sham-transfected as indicated. Equal amounts of pp32V5- and LacZV5-transfected cell extracts were precipitated with anti-V5. As a positive control, sham transfected cell extracts were precipitated with antibody to total Rb (G3-245); this control, which precipitates considerably more Rb, is designated *Sham*. The presence of specific phosphorylated forms of Rb in the immunoprecipitates was analyzed by immunoblotting using the indicated anti-phospho-Rb antibodies. *D*, HEK 293 cells were cotransfected with the pp32V5 and control, WT-LP, or PSM2T-LP as indicated. Equal amounts of cell extracts were precipitated with an anti-V5 antibody. *Top*, the presence of WT-LP or PSM2T-LP in immunoprecipitates (*IP*) was probed by Western blot analysis using an anti-Rb antibody (851). The *arrow* on the *right* indicates the position of the Rb large pocket fragment. The lower band present in all three lanes is the immunoglobulin heavy chain, which serves as a loading control. *Middle*, anti-V5 immunoprecipitates were subjected to Western blot analysis with anti-V5 antibody to confirm pp32V5 immunoprecipitation. *Bottom*, cell extracts were subjected to Western blotting with an anti-Rb antibody (851) to confirm expression of WT-LP and PSM2T-LP.

Because pp32 is pro-apoptotic whereas Rb is anti-apoptotic, we investigated the effect of Rb on the apoptotic function of pp32. In mammalian cells, transient overexpression of pp32 resulted in increased apoptosis as assessed by Hoechst staining (Fig. 4*A*). The apoptotic effect of pp32 was abolished by the coexpression of Rb. To further evaluate the effect of Rb on the pro-apoptotic activity of pp32 in mammalian cells, we performed a colony formation assay. pp32 overexpression resulted

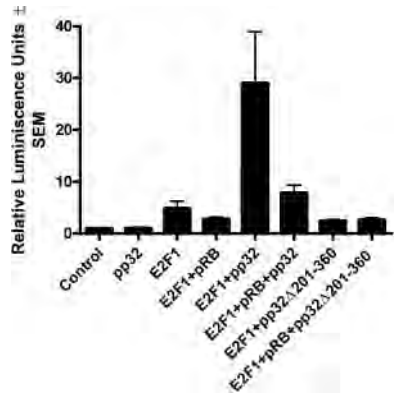

FIG. 3. **pp32 increases E2F1-mediated transcriptional activity.** NIH 3T3 cells were transiently transfected with E2F-luciferase reporter vector (pE2F-TA-Luc) and, where indicated, E2F1, pRb, pp32V5, or pp32Δ201–360V5 expression vectors. Data are presented as the mean ± S.E. from three independent experiments performed in duplicate.

in a decrease in colony formation compared with vector control. Cotransfection of Rb with pp32 abrogated the pp32-mediated decrease in colony formation (Fig. 4*B*). The pp32Δ201–360 construct yielded greatly diminished levels of apoptosis, which precluded the demonstration that it was insensitive to the addition of Rb; this experiment would have provided more direct evidence that the apoptotic effects of pp32 are inhibited by Rb rather than by another mechanism.

NIH 3T3 is a classic cell system for testing various transformation agents. Overexpression of the v-H-Ras protein in NIH 3T3 cells results in cellular transformation and the accelerated cell cycle progression associated with an increased level of cyclin D, which increases hyperphosphorylated Rb levels (43). To examine the effect of the pp32-Rb interaction on mitogenesis, we transfected activated H-ras, pp32, and pRb into NIH 3T3 cells. Coexpression of Ras and pp32 resulted in a slight decrease in colony formation, whereas coexpression of Ras, pp32, and Rb resulted in markedly increased colony formation compared with the Ras only control (Fig. 4*C*).

Whereas pp32 inhibits transformation, pp32r1 (ANP32C) and pp32r2 (ANP32D), both highly homologous to pp32 at the protein level (87.7 and 89.3% respectively), are tumorigenic (44). We therefore explored the possibility of an interaction between these family members and Rb. pp32r1 and r2 were V5 epitope-tagged and overexpressed in HEK 293 cells. Equal amounts of cell lysates were immunoprecipitated with anti-Rb and immunoblotted with anti-V5. Surprisingly, pp32r1 and pp32r2 did not interact with Rb (Fig. 5) despite high conservation within amino acids 67–120 of the LRR region (88.9 and 90.7% identical respectively), suggesting that the interaction between Rb and pp32 is highly specific.

DISCUSSION

Our data demonstrate an interaction between pp32 and hyperphosphorylated Rb. This interaction is mediated via the LRR of pp32, which also mediates its binding to CRM1 and nucleocytoplasmic shuttling. We have not established whether the pp32-Rb interaction is direct or whether it occurs through the intermediacy of other molecules. Nevertheless, we do show that pp32-Rb interaction is associated with increased E2F1 activity, inhibition of the apoptotic activity of pp32, and increased colony formation *in vivo*.

It is attractive to speculate that Rb binding to the LRR of pp32 in the nucleus prevents binding of CRM1, thereby inhibiting the nucleocytoplasmic shuttling of pp32. The effect of Rb binding on the localization of pp32 has not been directly demonstrated. Because pp32 localizes preferentially to the nucleus,

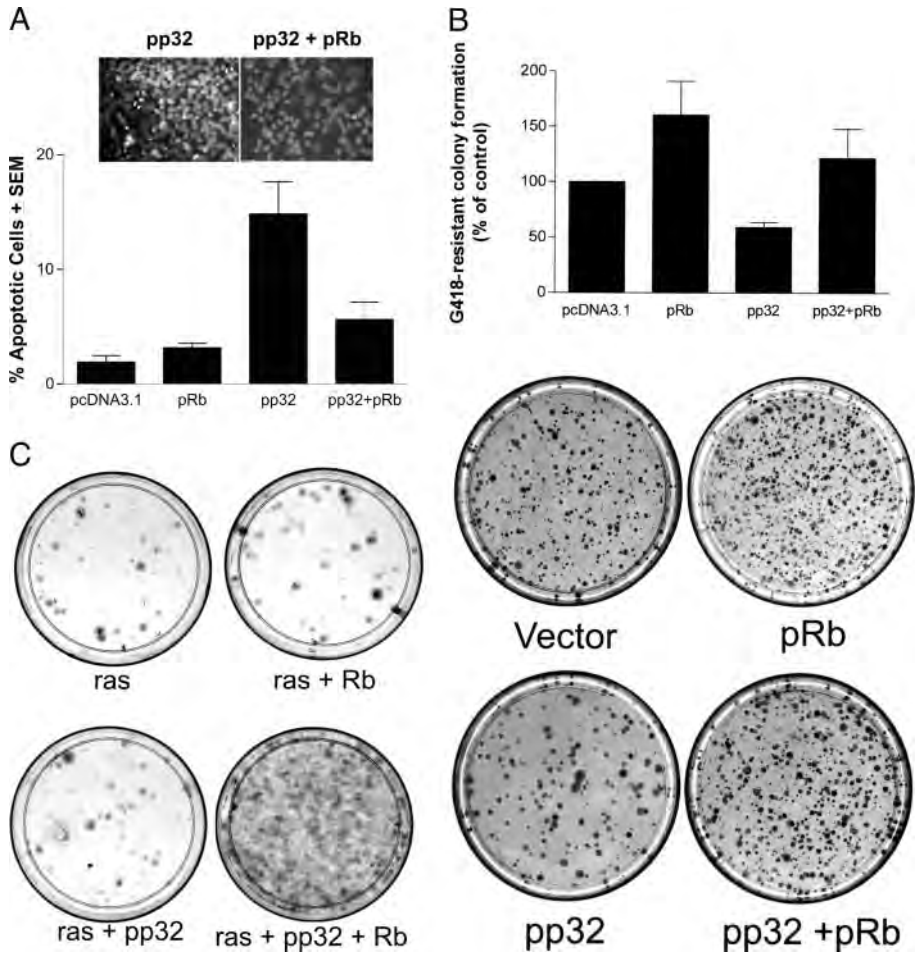

FIG. 4. **Association between Rb and pp32 correlates with the inhibition of pp32 apoptotic activity.** *A*, pp32-induced apoptosis is abrogated by Rb in mammalian cells. HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclei were stained with Hoechst stain and examined by immunofluorescence microscopy for characteristics of apoptosis (membrane blebbing, chromatin condensation, and pyknosis). Cell death was quantified in HeLa cells transfected with the indicated expression constructs. The data (mean ± S.E.) are the percentage of nuclei counted with apoptotic morphology (*n* equals at least three experiments). *B*, duplicate plates of HeLa cells were transfected with control, pp32, or pRb expression plasmids as indicated and subjected to colony formation assay. Plates were stained with methylene blue, and the total number of G418-resistant colonies was counted after 14 days of selection. A representative experiment is shown. The *bar graph* (mean ± S.E.) shows the percentage change in colony formation efficiency with the colony counts normalized against the vector only control (*n* equals at least three experiments in duplicate). *C*, duplicate plates of NIH 3T3 cells were transfected with Ras, pp32, or pRb expression plasmids as indicated and subjected to colony formation assay. Plates were stained with methylene blue and photographed after 14 days of G418 selection.

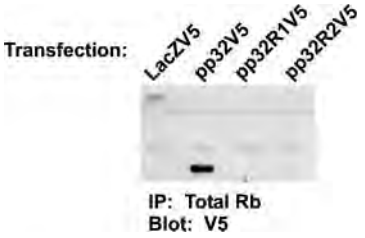

FIG. 5. **Rb associates with pp32 but not with other members of the ANP32 family.** HEK 293 cells were transfected with pp32V5, pp32r1V5, pp32r2V5, or LacZV5 expression vectors as indicated. Equal amounts of cell extracts were precipitated with anti-Rb (G99-2005) antibody and analyzed by Western blotting with anti-V5 antibody. *IP*, immunoprecipitation.

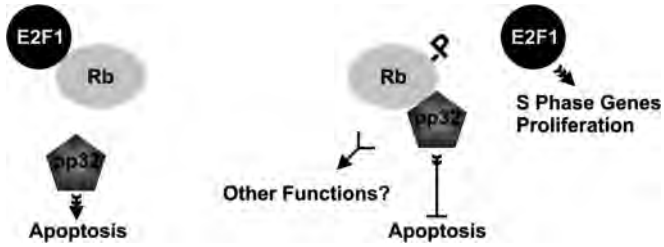

FIG. 6. **A proposed model of pp32-Rb interaction.** In normal cells, pp32 overexpression results in apoptosis. In cancer cells, high levels of hyperphosphorylated Rb sequester pp32, leading to inhibition of apoptosis, increased free E2F1, and increased proliferation.

shuttling has only been demonstrated in heterokaryon experiments (34). Direct demonstration would require the analysis of a series of heterokaryons with a number of stably transfected cell lines, where clear interpretation would be difficult. Based upon the available information, we propose a model whereby in normal cells increased pp32 is able to shuttle to the cytoplasm to promote apoptosis by stimulating the apoptosome, but in cancer cells increased hyperphosphorylated Rb sequesters pp32 in the nucleus, resulting in apoptotic resistance, increased free E2F1, and proliferation (Fig. 6). This model suggests a mechanism whereby cancer cells could escape apoptosis when Rb is inactivated by hyperphosphorylation. This model could also explain the seemingly paradoxical high level expression of pp32 in high grade cancer. Cancer cells would therefore preferentially inactivate Rb by hyperphosphorylation, gaining both growth and survival advantages by doing so.

Given the important role of apoptosis in tumorigenesis, our results suggest a mechanism whereby the inhibition of apoptosis by Rb might be exploited in cancer cells to promote the survival of transformed cells. Inhibitors of the pp32-Rb interaction might be useful for restoring apoptotic sensitivity to cancer cells, thereby potentiating chemotherapeutic agents.

Acknowledgment—We gratefully acknowledge Dr. Erik Knudsen for providing the Rb large pocket constructs and antibody and for very helpful discussions.

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A pp32–retinoblastoma protein complex modulates androgen receptor-mediated transcription and associates with components of the splicing machinery

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Abstract

We have previously shown pp32 and the retinoblastoma protein interact. pp32 and the retinoblastoma protein are nuclear receptor transcriptional coregulators: the retinoblastoma protein is a coactivator for androgen receptor, the major regulator of prostate cancer growth, while pp32, which is highly expressed in prostate cancer, is a corepressor of the estrogen receptor. We now show pp32 increases androgen receptor-mediated transcription and the retinoblastoma protein modulates this activity. Using affinity purification and mass spectrometry, we identify members of the pp32–retinoblastoma protein complex as PSF and nonO/p54nrb, proteins implicated in coordinate regulation of nuclear receptor-mediated transcription and splicing. We show that the pp32–retinoblastoma protein complex is modulated during TPA-induced K562 differentiation. Present evidence suggests that nuclear receptors assemble multiprotein complexes to coordinately regulate transcription and mRNA processing. Our results suggest that pp32 and the retinoblastoma protein may be part of a multiprotein complex that coordinately regulates nuclear receptor-mediated transcription and mRNA processing.

Keywords: pp32; Retinoblastoma protein; Androgen receptor; PSF; NonO/p54nrb

The androgen receptor (AR), a member of the nuclear hormone nuclear receptor (NR) superfamily, is a ligand-inducible transcription factor responsible for many physiological and pathological functions, such as development, differentiation, metabolism, and prostate cancer progression [1,2]. NRs contain six domains, A–F, based on amino acid sequence conservation and function [3]. The A/B domains, which possess constitutively active autonomous transcriptional activation function (AF-1), are poorly conserved among NRs and exhibit almost no amino acid sequence homology. In contrast, the E domain, which contains the ligand-dependent transcriptional activation functions (AF-2) and ligand-binding domain (LBD), is moderately well conserved, while the C domain, which contains the DNA-binding domain (DBD), is highly conserved.

Upon ligand binding, AR dissociates from heat shock proteins and chaperones, dimerizes, binds to various cognate androgen response elements (AREs) in target genes and interacts with various coregulators to regulate gene transcription [4,5]. Coregulators are divided into coactivators and corepressors. Corepressors interact with NRs to repress the basal rate of transcription while coactivators enhance it. Coactivators, organized in multiprotein complexes, are essential for the transcriptional activation function of NRs and facilitate the access of nuclear receptors and the RNA polymerase II core machinery to their target DNA sequences by chromatin remodeling and histone modification [6–9].

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Increasing evidence indicates that coregulators also function to simultaneously regulate NR-mediated transcription and mRNA processing [10–12]. Two proteins implicated in coordinate regulation of NR-mediated transcription and splicing are PSF and nonO/p54nrb. NonO/p54nrb and PSF share 71% identity within a central region that includes two RNA binding (RRM) domains [13]. Together, nonO/p54nrb and PSF form an RNA- and DNA-binding heterodimer that exhibits multi-functional properties in a variety of nuclear processes, such as DNA unwinding for DNA replication and coupling of transcription and splicing [14,15].

pp32, a multifunctional nucleocytoplasmic shuttling phosphoprotein which is highly expressed in intermediate and high-grade prostate cancer, is a member of the ANP32 family of acidic, leucine-rich phosphoproteins found in cells capable of self-renewal [16]. pp32 consists of an NLS, a leucine rich repeat region (LRR) and an N-terminal acidic domain. pp32 has been implicated in a number of cellular and biologic processes, including proliferation [17], differentiation [18], apoptosis [19,20], inhibition of acetyltransferases as part of the INHAT complex [21], and regulation of mRNA trafficking and stability by binding to the RRM of the protein HuR [22].

pp32 has also been identified as a coregulator of NR-dependent transcription. Loven et al., showed that pp32 binds to the nuclear hormone receptors estrogen receptor alpha (ERα), thyroid receptor beta (TRβ), progesterone receptor B (PR-B), and peroxisome proliferator-activator receptor gamma (PPARγ). Loven et al. further showed that pp32 bound to both liganded and unliganded ERα and competed with the estrogen response element (ERE) for binding to the ERα. Although pp32 stabilized the ERα-ERE interaction, pp32 acted as a transcriptional corepressor of ERα and TRβ.

We recently identified an interaction between pp32 and the tumor suppressor, retinoblastoma protein (Rb). Rb is a multifunctional phosphoprotein that regulates proliferation, differentiation, and development [23]. Rb interacts with the nuclear hormone receptors glucocorticoid receptor (GR) and androgen receptor (AR) and potentiates GR- and AR-mediated transcription [24–26].

As pp32 is highly expressed in prostate cancer, and Rb functions as a coactivator of the AR, we examined the effect of pp32 on AR-mediated transcription, and what effect, if any, the pp32–Rb interaction had on AR-mediated transcription. We report here that pp32 acts as a coactivator for AR-mediated transcription and this activity is modulated by Rb. We also identify nonO/p54nrb and PSF, RRM containing proteins implicated in coordinate regulation of NR-mediated transcription and splicing as members of the pp32–Rb complex. We show that this complex is modulated during TPA-induced K562 differentiation. Taken together, these results suggest that pp32 and Rb assemble in multiprotein complexes that may serve to simultaneously regulate nuclear hormone receptor-mediated transcription and splicing.

Materials and methods

Construction of vectors. pp32 and pp32 truncation constructs have been previously described [27,28]. The AR plasmid and MMTV-ARE-luc (a reporter gene having an MMTV long terminal repeat promoter) were kind gifts from John Isaacs (Johns Hopkins) and Terry Brown (Johns Hopkins), respectively.

Cell culture and transfections. Cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEK 293 and LNCaP cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (P/S, Gibco). K562 cells were maintained in RPMI 1640 (Invitrogen) with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (P/S, Gibco). Cells were maintained in a humidified incubator at 37 °C and 5% CO_2 and passaged 2–3 times/week. For luciferase assays, LNCaP cells were grown for at least 3 days in DMEM free of phenol red, supplemented with charcoal-treated fetal bovine serum (HyClone). All DNA transfections were carried out using Fugene 6 (Roche) as described by the manufacturer.

Antibodies. The following antibodies were used for this study: anti-pp32 [29], anti-V5 (Invitrogen); anti-PSF (Sigma–Aldrich); anti-Rb G3-245, anti-Rb G99-2005, anti-nonO/p54nrb, and anti-androgen receptor G122-434 (BD Biosciences) and anti-phospho-Rb T826 (Biosource International).

Immunoprecipitation. LNCaP cells expressing endogenous AR were grown in DMEM without phenol red with 10% charcoal stripped serum (CSS) to near confluence. For HEK 293 cells, transfection was carried out as follows: 1×10^6 HEK 293 cells were seeded onto a T-150 flask. Twenty-four hours later, cells were transfected with 15 μg of the indicated plasmids (7.5 μg of each plasmid) using Fugene 6 (Roche). The cells were harvested 24 h after transfection. For harvesting, cells were washed twice with cold PBS, and lysed with 1 ml MPER (Pierce) containing 10 μl (1×) protease inhibitor cocktail (HALT, Pierce) as per manufacturer's instructions. Lysates were centrifuged at 4 °C for 30 min at 16,000*g* to remove particulate material. The supernatant was precleared for 2 h with 100 μl of protein A– or protein G–agarose (Roche). The pre-cleared cell lysates were mixed with the indicated antibodies and protein A– or protein G–agarose (Roche) and incubated at 4 °C overnight. The next day, the reaction mixture was washed three times with 1 ml of cold PBS, boiled for 3 min and eluted in 2× SDS buffer. The eluted materials were subsequently analyzed by immunoblotting with the indicated antibodies.

For immunoprecipitation of endogenous proteins, LNCaPs were grown in T-150 flasks to near confluence. Twenty-four hours later, cells received fresh medium containing 10% CSS with or without 1 nM R1881 (a synthetic androgen, Perkin-Elmer). All hormones were dissolved in ethanol. Cells were hormone treated for 24 h, and 48 h after transfection, cells were harvested as described above.

Immunoblotting. Proteins were separated in NuPAGE 10% Bis-Tris gel (Invitrogen) at 200 V for 50 min and electroblotted onto PVDF membranes (Invitrogen) at 14 V overnight. Membrane was blocked with blocking solution (5% non-fat dried milk and 0.1% Tween 20 in 1× PBS) for 1 h. The indicated antibodies were diluted in blocking solution and incubated with the membrane overnight. The next day the membrane was washed with washing solution (0.1% Tween 20 in 1× PBS) three times for 10 min each. Secondary

anti-rabbit antibody conjugated to horseradish peroxidase (Amersham/Pharmacia) was diluted 1:5000 with blocking solution and incubated with the membrane for 1 h at room temperature. Washing steps were repeated and immunoreactivity was detected using enhanced chemoluminescence (ECL) based detection. Where indicated, blots were stripped with Restore Western blot stripping buffer (Pierce) as per the manufacturer's instructions, blocked for 1 h at room temperature in blocking solution and immunoblotting was performed as described.

Reporter assays. LNCaP cells were maintained in DMEM without phenol red with 10% charcoal stripped serum (CSS) at least 3 days before transfection. Twenty-four hours before transfection, cells were plated at 2×10^5 cells/well in six-well plates. The next day each well was transfected with 500 ng ARE-luc and 500 ng AR plasmid as well as 10 ng of pRL-TK *Renilla* (Promega) to normalize for transfection efficiency. In addition, cells were transfected with 500 ng of the indicated plasmids. The total amount of DNA was kept constant in each transfection by adding empty pcDNA3.1 vector when appropriate.

At 24 h after transfection, cells were washed three times in PBS and fresh medium containing 10% CSS with or without 1 nM R1881 was added. All hormones were dissolved in ethanol. Medium without R1881 contained a corresponding volume of ethanol only. Cells were hormone treated for 24 h and harvested 48 h after transfection. Luciferase activities were detected using the Dual luciferase assay (Promega). Firefly luciferase activities were normalized to *Renilla* luciferase activities (plotted as F/R), assays were performed in duplicate, and the data are representative of at least three experiments.

Affinity chromatography and immunoprecipitation. Ten T-150 of HEK 293 cells ($\sim 2 \times 10^9$ cells) were transfected with pp32V5-His for 48 h, or transfected with empty V5-His vector (control). Forty-eight hours post-transfection, cells were harvested by centrifugation at 4 °C and resuspended in 4 ml native binding buffer containing 1× protease inhibitor and phosphatase inhibitors. Cells were lysed using two freeze–thaw cycles. Resulting cell extracts were centrifuged at 14,000 rpm in a microcentrifuge at 4 °C. Prior to use, Ni–NTA resin (Qiagen) was washed four times with six bed-volumes of lysis buffer. Protein extract was mixed with the pre-equilibrated affinity resin. The suspension was rocked at 4 °C for 2 h. Unbound material was then removed by washing the affinity resin four times with wash buffer. K562 cell lysate (1×10^6 cell equivalents) in 1 ml of native binding buffer was then incubated for 2 h with nickel resin containing the attached pp32V5-His. Proteins were then eluted from the affinity column with 250 mM imidazole. Eluted fractions containing the pp32–Rb complex were pooled, and passed through an empty Bio-Spin chromatography column (Bio-Rad) to filter out residual resin beads. This filtered eluate was subjected to immunoprecipitation, for which we used monoclonal Rb antibody 554162 (BD Biosciences) and protein G–agarose beads (Pierce). The suspension was rocked at 4 °C overnight. The next day, the reaction mixture was washed three times with cold PBS, boiled for 3 min and eluted in 40 μl of 2× SDS buffer. The eluted materials were subsequently analyzed by separation with Mops buffer and NuPAGE 10% Bis-Tris gel (Invitrogen) at 150 V for 2 h. Immediately after running, the gel was stained as per the Protein Analysis Laboratory (PAL), University of Arizona protocol, bands of interest were cut out (antibody bands were identified using the control), put in 1.5 ml tubes and shipped to PAL for protein identification by mass spectrometry.

Differentiation assay. Exponentially growing K562 cells were differentiated to megakaryocytes by treatment with 100 nM phorbol 12-myristate 13-acetate (TPA, Sigma) for 0, 1, 2, 3 or 4 days. Differentiation was confirmed by microscopic examination of morphology. Cells were harvested at the indicated time points and immunoprecipitation was performed with anti-Rb monoclonal antibody (554162, BD Biosciences) as described above.

Results

pp32 interacts with the AR in vivo

pp32 binds to the nuclear receptors estrogen receptor alpha (ERα), thyroid receptor beta (TRβ), progesterone receptor B (PR-B), and peroxisome proliferator-activator receptor gamma (PPARγ). pp32 interacts with the ERα via the DBD, which is highly conserved amongst nuclear receptors [30]. To test whether or not the target nuclear receptors of pp32 extend to AR, we examined if pp32 interacted with AR in vivo via coimmunoprecipitation experiments performed in mammalian cells. When V5-His-epitope tagged pp32 is transiently cotransfected with AR into HEK 293 cells an interaction between AR and pp32V5-His, but not the LacZV5-His control, is detected by Western blot analysis following immunoprecipitation with anti-V5 (Fig. 1A). An endogenous interaction between AR and pp32 can also be demonstrated in untransfected LNCaP cells, suggesting that it is physiologically relevant (Fig. 1B). Although pp32 interacted with both liganded and unliganded AR (Fig. 1B), pp32's interaction with AR is decreased in the presence of ligand, similar to its interaction with the ERα [30].

pp32 is a coactivator of the AR

pp32 has been shown to function as a corepressor of the ERα and TRβ. To address the role of pp32 in AR-mediated transcription, we carried out transient cotransfection assays employing a luciferase reporter construct driven by the MMTV proviral promoter (i.e., MMTV-ARE-luciferase). Cotransfection of pp32 and AR into LNCaP cells enhanced AR-mediated transcription (Fig. 2). These results suggest that in contrast to its effects on the ERα and TRβ, pp32 is a coactivator of AR that activates AR-mediated transcription.

Rb represses pp32-mediated activation of AR-regulated transcription

Rb has been shown to function as a coactivator of the AR and increase AR-mediated transcription 2- to 3-fold via direct protein–protein interactions [25,26]. We have previously shown an interaction between Rb and pp32. To determine whether Rb can modulate pp32-mediated transcriptional activation of the AR, we carried out cotransfection experiments in mammalian cells. As shown in Fig. 2, Rb enhanced AR-mediated transcription 2- to 3-fold as previously described. However, the enhancement of AR-mediated transcription by pp32 was decreased by coexpression of Rb. This effect of Rb was not observed when wild-type pp32 was replaced by pp32Δ201–360, a pp32 deletion mutant that cannot bind to Rb (Fig. 2). These data indicate that Rb binding

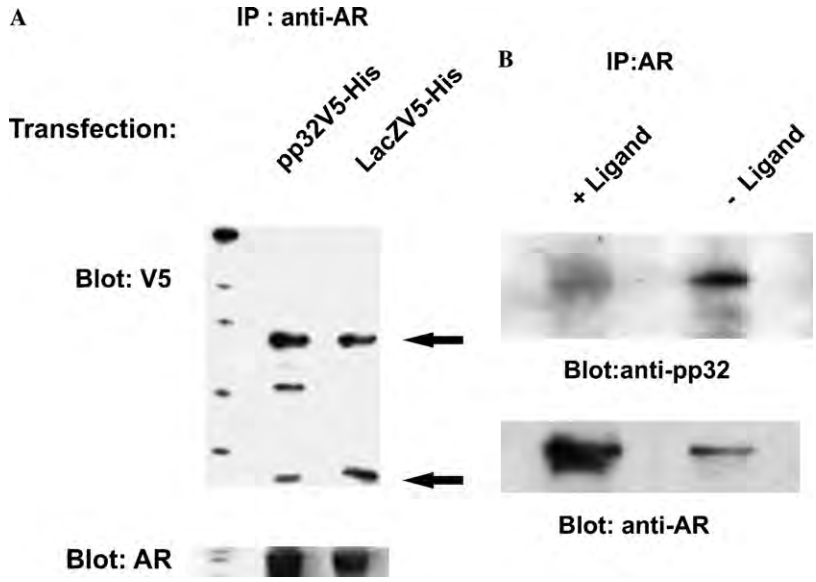

Fig. 1. pp32 interacts with the AR. (A) V5-His-tagged pp32 coimmunoprecipitates with AR. HEK 293 cells were transfected with AR and either pp32V5-His or LacZV5-His (control) as indicated. (Upper panel) Equal amounts of cell extracts were precipitated with anti-AR antibody G122-434 (BD Biosciences) and the presence of V5 in immunoprecipitates was visualized by Western blot analysis using anti-V5 antibody (Invitrogen). The unlabelled lane on the left shows molecular weight markers of 40, 50, 60, 80, and 220 kDa. The arrows indicate the position of immunoglobulin heavy and light chains. (Lower panel) The expression of AR in immunoprecipitates was determined by Western blotting using anti-AR antibody. (B) Endogenous pp32 coimmunoprecipitates with endogenous AR. Equal amounts of LNCaP cell extracts were precipitated with anti-AR antibody in the presence or absence of ligand (1 nM R1881). (Upper panel) Equal amounts of cell extracts were precipitated with anti-AR antibody and the presence of pp32 in immunoprecipitates was visualized by Western blot analysis using anti-pp32 antibody. (Lower panel) The expression of AR in immunoprecipitates was determined by Western blotting using anti-AR antibody.

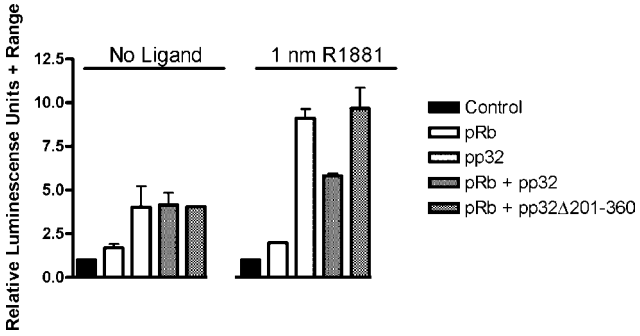

Fig. 2. pp32 increases AR-mediated transcriptional activity. LNCaP cells were transiently transfected with AR, MMTV ARE-luciferase reporter vector (MMTV ARE-Luc) and, as indicated, pRb, pp32V5-His or pp32Δ201–360V5-His expression vectors. Data are normalized to AR and MMTV ARE-luc only (control) and presented as the means ± SEM from three independent experiments performed in duplicate. Left, without ligand; right, with ligand, 1 nM R1881.

to pp32 can down-regulate pp32-mediated activation of AR-regulated transcription.

The pp32–Rb complex interacts with PSF and nonO/p54nrb

To identify other members of the pp32–Rb complex, we used K562 cells, a differentiable suspension leukemic cell line that can be easily grown in large quantities. K562 cell extracts were incubated with purified pp32V5-His and pp32–Rb complexes were purified by sequential affinity purification and Rb immunoprecipitation (Fig. 3A). Proteins bound to both pp32 and Rb were analyzed by SDS–PAGE and silver staining. Members of the pp32–Rb complex were identified by mass spectrometry (Fig. 3B) as nonO/p54nrb and PSF, RRM containing proteins implicated in coordinate regulation of transcriptional activation and splicing. PSF and nonO/p54nrb are also NR coregulators. Like pp32, PSF binds to the highly conserved DBD of NRs [31,32].

The pp32–Rb complex is modulated by TPA-induced K562 differentiation

Recent results show that nonO/p54nrb remains on the promoter of the *MASH1* gene as part of both corepressor and coactivator complexes during differentiation [33]. To gain further insights into the pp32–Rb complex in vivo, we probed for the presence of the complex during TPA-induced megakaryocytic K562 differentiation. Total Rb was immunoprecipitated from K562 cells at the indicated time points after the addition of 100 nM TPA. The Rb immunoprecipitates were probed for the presence of Rb, Rb phosphorylated on T826, pp32, PSF, and nonO/p54nrb. Fig. 4 shows that, consistent with previous results, the phosphorylation state of Rb

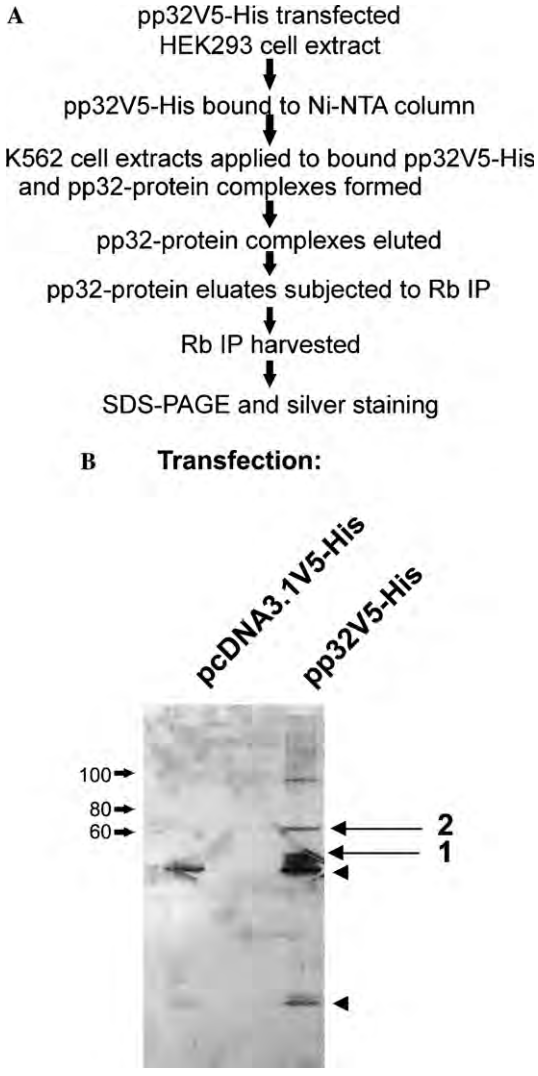

Fig. 3. Affinity purification of pp32–Rb complex interactants. (A) Purification scheme. Cell extracts from HEK 293 cells transiently transfected with pp32V5-His were applied to Ni–NTA columns for 2 h at 4 °C. The columns containing bound pp32V5-His were then extensively washed with wash buffer, and K562 cell extracts were applied to bound pp32V5-His for 2 h at 4 °C. After extensive washes, proteins bound to pp32V5-His were eluted with elution buffer. Eluates were immunoprecipitated with anti-Rb antibody 554162 (BD Biosciences). As a negative control, purification using HEK 293 cells transiently transfected with empty V5-His vector was performed concurrently. (B) Immunoprecipitates were boiled, separated by electrophoresis and analyzed by SDS–PAGE followed by silver staining according to the Proteomics Analysis Laboratory (PAL, Arizona) protocol. Molecular weight standards are shown to the left. Arrowheads indicate the immunoglobulin heavy and light chains. Proteins were identified by mass spectrometry as follows: 1, nonO/p54nrb; 2, PSF.

progressively changed from predominantly hyperphosphorylated to predominantly hypophosphorylated during differentiation. pp32 binds to Rb phosphorylated on T826, and T826 remained phosphorylated throughout differentiation, consistent with data showing that Rb mutants constitutively phosphorylated on T826 are

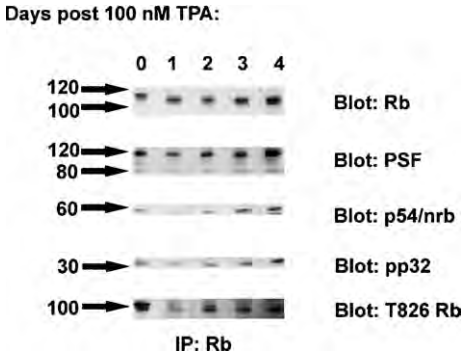

Fig. 4. Association of Rb with pp32, PSF, nonO/p54nrb during TPA induced K562 differentiation. After the addition of 100 nM TPA to exponentially growing K562 cells, cells were immunoprecipitated with anti-Rb followed by Western blotting with the indicated antibodies at the indicated time points.

competent for differentiation [34,35]. The pp32–Rb complex containing PSF and nonO/p54nrb was present throughout differentiation although the complex decreased on day 1 and day 2 post-addition of TPA.

Discussion

In summary, we have shown an interaction between pp32 and the androgen receptor (AR). We show that pp32 acts as a coactivator for the AR. Its coactivator function is modulated by its interaction with Rb, another AR coactivator. We identify PSF and nonO/p54nrb, RRM containing proteins implicated in coordinate regulation of transcriptional activation and splicing [36,14], as members of the pp32–Rb complex. PSF and nonO/p54nrb are also NR coregulators and PSF, like pp32, binds to the highly conserved DBD of NRs [31,32].

Our results indicating that pp32 coactivates the AR are in direct contrast to its actions on ERα and TRβ. pp32 joins the growing list of bifunctional NR coregulators which include PSF, nonO/p54nrb, cyclin D1, Zac 1, NSD 1, and RIP140 [37–41]. The switching mechanisms which regulate the bifunctionality of these coregulator proteins is not clear, but the coactivator or corepressor activity of these proteins may depend on the specific NR reporter gene promoter and cell type. It has been shown that promoter identity can influence the function of coregulators such that corepressors can become coactivators and vice versa. pp32 contains a C-terminal acidic domain seen in transcriptional activators [17] which may mediate its coactivator function. Also, as less pp32 binds to liganded AR, pp32 may act by stabilizing the AR–ARE interaction as it does the ERα–ERE interaction.

Although Rb acts as a coactivator for steroid receptors, coexpression with pp32 results in decreased pp32 coactivator activity. It has previously been

reported that Rb can interact with the thyroid hormone receptor (TR) coactivator Trip230 and decrease Trip230-mediated coactivation of the TR, although the direct effect of Rb on the TR was not examined [42]. There are two possible explanations for our results: overexpressed Rb may bind to pp32 and sequester it, thereby squelching pp32's coactivation of ERα as Rb had no effect on pp32 Δ201–360, a mutant unable to bind Rb. Another possibility is that Rb's interaction with pp32, PSF and nonO/p54nrb is part of the fine tuning and combinatorial control of NRs and coregulators to modulate differential target gene expression in various tissues during complex programs of differentiation and development. This is also possible as the pp32–Rb–PSF–nonO/p54nrb complex was regulated during differentiation in a manner consistent with the reported presence of nonO/p54nrb in both coactivator and corepressor complexes on the *MASH1* promoter during neuronal differentiation [33].

We also show that pp32 interacts with 2 RRM containing proteins, PSF and nonO/p54nrb. This is consistent with previous data showing that pp32 binds to the RRM domain of HuR to regulate mRNA trafficking and stability [22]. pp32's closest homologue in yeast is Lea1, a component of the spliceome. HuR, PSF, nonO/p54nrb are all RNA binding proteins which, like pp32, Rb, and NRs, are implicated in development, differentiation, and cancer.

It is becoming increasingly clear that NRs assemble multiprotein complexes to coordinately regulate and fine-tune transcription and splicing during complex programs of differentiation and development [12,43,44]. pp32 is a multifunctional protein highly expressed in prostate cancer and previously implicated in corepression of NR-mediated transcription. pp32 has also been implicated in mRNA processing via its interaction with the RNA binding protein HuR. pp32 may stabilize mRNA transcripts upregulated during AR-mediated differentiation [45,46]. We now show that pp32 functions as a coactivator for the AR and interacts in a complex with components of the splicing machinery. Taken together, these results suggest that pp32 and Rb may be part of a multiprotein complex that functions to coordinately regulate nuclear receptor-mediated transcription and mRNA processing.

Acknowledgments

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