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The $\alpha 4$ -Containing Form of Protein Phosphatase 2A in Liver and Hepatic Cells

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Abstract

The Ser/Thr phosphatase PP2A is a set of multisubunit enzymes that regulate many cellular processes. In yeast, the PP2A regulatory subunit Tap42 forms part of the Target of Rapamycin (TOR) signaling pathway that links nutrient and energy availability to cell growth. The physiological intersection between the mammalian orthologs of Tap42 and TOR, $\alpha 4$ and mTOR, has not been fully characterized. We used two *in vivo* models of liver growth in the rat, late gestation fetal development and regeneration after partial hepatectomy, to explore the regulation of the $\alpha 4$ -containing form of PP2A. The $\alpha 4$ /PP2A catalytic subunit ($\alpha 4$ /PP2A-C) complex was present in both fetal and adult liver extracts. There was a trend towards higher levels of $\alpha 4$ protein in fetal liver, but the complex was more abundant in adult liver. Fractionation of extracts by ion exchange chromatography and transient transfection of the AML12 mouse hepatic cell line indicated that $\alpha 4$ associates with PP2A-C but that these complexes have low catalytic activity with both peptide and protein substrates. $\alpha 4$ was able to associate with forms of PP2A-C that were both methylated and non-methylated at the carboxy-terminus. The mTOR inhibitor rapamycin did not block the formation of $\alpha 4$ /PP2A-C in liver or hepatic cells, nor did it appear to modulate PP2A activity. Furthermore, sensitivity to the growth inhibitory effects of rapamycin among a panel of hepatic cell lines did not correlate with levels of $\alpha 4$ or $\alpha 4$ /PP2A-C. Our results indicate that the yeast Tap42/TOR paradigm is not conserved in hepatic cells.

Keywords

Protein phosphorylation; mTOR; rapamycin; regeneration; cell growth

PP2A is a major Ser/Thr phosphatase that plays a significant role in the regulation of many cellular processes, including metabolism, transcription, translation, cell cycle progression, cell growth and apoptosis [Janssens and Goris, 2001; Mumby and Walter, 1993; Sontag, 2001; Zolnierowicz, 2000]. The activity of such a versatile enzyme must therefore be tightly controlled *in vivo*. Indeed, the catalytic subunit of PP2A (PP2A-C) is regulated at many levels. The C-terminus of PP2A-C is post-translationally modified by phosphorylation at Y307 and methyl esterification at L309. Phosphorylation results in inhibition of PP2A activity [Chen et al., 1992; Guo and Damuni, 1993] while methylation is thought to play an important function in regulatory subunit binding [Bryant et al., 1999; Tolstykh et al., 2000; Wu et al., 2000; Yu et al., 2001]. More specifically, PP2A-C methylation can influence the association of PP2A-C with specific regulatory subunits, thereby serving to determine the

composition, subcellular localization and function of PP2A-C holoenzymes in a variety of cell types [Gentry et al., 2005; Longin et al., 2007; Nunbhakdi-Craig et al., 2007].

The prototypical PP2A enzyme is a heterodimer that consists of the catalytic C subunit and scaffolding A subunit. The substrate specificity and subcellular localization of PP2A are thought to be defined by an array of B-type regulatory subunits that bind to the AC dimer to form a variety of heterotrimeric complexes [Mumby and Walter, 1993]. Among various B-type subunits, B56 $\alpha/\beta/\epsilon$ complexed with the A and C subunits localize to the cytoplasm, whereas heterotrimeric forms of PP2A that include B56 $\delta/\gamma1/\gamma3$ are concentrated in the nucleus [McCright et al., 1996]. Likewise, the two regulatory subunits Cdc55p (a B-type subunit) and Rts1p (a B'-type subunit) of *Saccharomyces cerevisiae* display distinct, dynamic localization patterns that support the hypothesis that regulatory subunits determine localization of PP2A [Gentry and Hallberg, 2002]. Recent structural studies further support the role of the B subunit in determining substrate specificity by showing that regulatory B' subunit interacts with the PP2A-C subunit near the active site and that the B' subunit is responsible for the ultimate tertiary structure of the catalytic subunit [Cho and Xu, 2007; Xu et al., 2006].

Studies in yeast have established a model in which PP2A, through its interaction with Tap42, functions as a component of the rapamycin-sensitive TOR signaling pathway, a pathway required for initiation of protein synthesis in response to nutrient availability. In this model, TOR phosphorylates Tap42 to promote association with the catalytic subunits of several Ser/Thr phosphatases, including the yeast homologs of PP2A-C [Jiang Y and Broach JR, 1999]. Under nutrient-rich conditions, phosphorylated Tap42 binds to and inhibits PP2A activity towards downstream effectors of TOR, such as the transcriptional regulator GLN3 [Beck and Hall, 1999] and the Ser/Thr kinase NPR1 [Schmidt et al., 1998]. In contrast, nutrient starvation or rapamycin block the phosphorylation of Tap42, inducing the dissociation of Tap42 from PP2A-C [Di Como CJ and Arndt KT, 1996]. Thus, the overall model suggests that TOR regulates protein phosphorylation in yeast by indirectly restraining the activity of PP2A through phosphorylation of Tap42.

In mammalian cells, PP2A-C has been found to associate with the homolog of Tap42, a novel regulatory protein termed $\alpha4$ [Murata et al., 1997; Prickett and Brautigan, 2004]. $\alpha4$ binds to PP2A-C independent of the A and B subunits. Thus, $\alpha4$ /PP2A-C is considered to represent a unique, heterodimeric form of the phosphatase [Jiang Y and Broach JR, 1999].

Whether mTOR regulation of $\alpha4$ /PP2A-C plays a role analogous to that in yeast is controversial. Some studies suggest that mTOR can directly phosphorylate its effectors, such as S6K1 and 4E-BP1, while others claim that the mTOR-dependent phosphorylation of S6K1 and 4E-BP1 is an indirect result of the inhibition of PP2A [Gingras AC et al., 2001a; Gingras AC et al., 2001b]. PP2A is able to dephosphorylate S6K1 *in vitro* [Ferrari et al., 1991], and okadaic acid, a potent PP2A inhibitor, antagonizes dephosphorylation of TOR substrates (i.e. S6K1 and 4E-BP1), promoting S6K1 activation and cell growth in *Drosophila* [Cygnar et al., 2005]. A role for PP2A is supported by studies showing a direct interaction of PP2A with S6K1 in mammalian cells [Peterson RT et al., 1999; Westphal et al., 1999].

The $\alpha4$ protein has been found to associate with the catalytic subunits of PP2A, PP4 and PP6, making it a common regulator of multiple PP2A family members [Chen et al., 2004; Kloecker et al., 2003; Murata et al., 1997]. Mutations in PP2A-C that induce the loss of binding to A subunit enhance its binding to $\alpha4$, whereas mutations that eliminate the $\alpha4$ association increase AC content [Prickett and Brautigan, 2004]. This suggests a competitive and mutually exclusive association of A subunit and $\alpha4$ with PP2A-C in cells. Furthermore,

previous studies show that single mutations of PP2A-C at the site of phosphorylation (Y307F) or methyl esterification (L309Q) result in recovery of only AC dimers whereas double mutation of both residues favor the formation of $\alpha 4$ /PP2A-C [Chung et al., 1999]. This suggests a role for PP2A-C post-translation modification in the association with $\alpha 4$. Recent structural studies reveal that the tetratricopeptide repeat-like fold in Tap42/ $\alpha 4$ may have a scaffolding function that promotes the dephosphorylation of substrates recruited to the C-terminal region of PP2A-C [Yang et al., 2007]. It has been proposed that $\alpha 4$, rather than simply inhibiting PP2A activity, may protect mTOR downstream effectors from being dephosphorylated by modulating the activity of other undefined phosphatases [Inui et al., 1998]. It has been shown that $\alpha 4$ interacts with S6K1 through PP2A-C in a rapamycin-sensitive manner [Yamashita et al., 2005]. Recent studies in *Drosophila* have suggested that PP2A, but not other members of this subfamily, is the major S6K phosphatase [Bielinski and Mumby, 2007]. Studies in *Drosophila* led to the interesting observation that Tap42 induces nuclear accumulation of PP2A-C but loss of TOR has no effect on PP2A-C localization [Cygnar et al., 2005]. This suggests that Tap42 may act independently of TOR and regulate the localization of PP2A, similar to the B regulatory subunits.

A number of questions regarding the function and regulation of the mTOR/ $\alpha 4$ /PP2A system remain unanswered. Rapamycin sensitivity of the interaction between $\alpha 4$ and PP2A-C has been observed in some systems [Inui et al., 1998; Kong et al., 2004; Murata et al., 1997], but in others this association is unaffected by rapamycin [Kloeker et al., 2003; Nanahoshi et al., 1998; Nien et al., 2007]. In addition, the issue of whether $\alpha 4$ has a positive or negative effect on PP2A activity has not been definitively determined.

Early studies indicated that yeast strains that overexpressed Tap42 mutants were almost completely resistant to rapamycin [Di Como CJ and Arndt KT, 1996]. $\alpha 4$ transfection in Jurkat cells was shown to result in rapamycin resistance [Inui et al., 1998]. Conditions that promote the association of Tap42 with the yeast homologs of PP2A-C, such as deletion of *TPD3* (yeast A subunit) or *CDC55* (yeast B subunit), induce partial rapamycin resistance [Jiang Y and Broach JR, 1999]. These relationships between $\alpha 4$ and resistance to the anti-proliferative effects of rapamycin have been relatively unexplored in mammalian models for rapamycin resistance.

In the present studies, we have explored the *in vivo* relationship between mTOR, $\alpha 4$ and PP2A-C in liver. We used two *in vivo* models of liver growth, the rapamycin-resistant hepatocyte proliferation that occurs in the late gestation fetal rat, and the rapamycin-sensitive process of liver regeneration induced by 2/3 partial hepatectomy in the adult rat [Curran et al., 1993; Fausto and Webber, 1993; Gruppuso et al., 1997]. We also utilized *in vitro* hepatic cell models in an effort to facilitate the study of the functional regulation of PP2A. The normal diploid nontumorigenic rat hepatic epithelial line (WB-F344, abbreviated as WB) was originally isolated from an adult Fischer rat [Tsao MS et al., 1984]. We have utilized a spectrum of cell lines derived from the WB line or from other sources [Campbell et al., 2000; Hixson et al., 1985] that we have found exhibit varying degrees of rapamycin sensitivity. Based on the limited transfection efficiency seen with these rat hepatic cell lines, we also utilized a hepatic cell line derived from mice transgenic for human TGF α , AML12 [Wu et al., 1994]. Overall, our studies have found that the regulation of the association between PP2A-C and $\alpha 4$ and the effect of rapamycin on the interaction in hepatic cells do not follow the yeast paradigm.

Materials and Methods

Materials

Antibodies to HA, full-length PP2A-C and the p85 subunit of phosphatidylinositol 3-kinase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). C-terminus PP2A-C antibody and PP2A assay reagents were obtained from Millipore Corporation (Billerica, MA). FLAG antibody was from Sigma-Aldrich (St. Louis, MO). Antibodies directed towards ribosomal protein S6 and phosphorylated S6^{Ser235/236} were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Mid-portion PP2A-C antibody and $\alpha 4$ antibody were prepared as described previously [Chen et al., 1994; Prickett and Brautigan, 2004]. Myelin basic protein from bovine brain (Sigma-Aldrich Corp., St. Louis, MO) was used as substrate in a PP2A assay. It was first phosphorylated using recombinant, full length, active human MAP Kinase1/Erk1 from Millipore Corporation and [γ -³²P]-ATP (3000 Ci/mmol; Perkin Elmer Life and Analytical Sciences, Boston, MA). Lipofectamine and Plus reagents were purchased from Invitrogen Corporation (Carlsbad, CA). The MonoQ HR5/5 column was purchased from GE Healthcare (Piscataway, NJ). Rapamycin was purchased from LC Laboratories (Woburn, MA).

Animals and Preparation of Liver Extracts

Adult Sprague-Dawley male rats and pregnant rats of known gestational age (term being 21 days) were used for all studies (Charles River Laboratories, Wilmington, MA). Adult male rats (125-175 g) were anesthetized with pentobarbital (50 mg/kg body weight, administered by intraperitoneal injection) and exsanguinated prior to removal of the liver. For pregnant rats, cesarean sections were performed under pentobarbital anesthesia and fetal (embryonic day 19) livers were harvested before sacrifice. Two-thirds partial hepatectomy was performed on adult male rats (125-175 g) under isofluorane anesthesia as previously described [Sanders and Gruppuso, 2005]. For rapamycin studies, the animals were injected with DMSO vehicle or 2.5 μ g rapamycin/g body weight prior to sacrifice at the times specified. Except where noted, livers were flash-frozen in liquid nitrogen and stored at -70°C until use. Wherever replicate analyses are referred to, samples were prepared from separate adult livers or from separate pools of fetal livers. Fetal livers were taken from multiple dams. All animal studies complied with guidelines set by the Rhode Island Hospital Institutional Animal Care and Use Committee. Rat liver extracts were prepared as previously described [Yoo et al., 2007].

Cell Lines and Preparation of Extracts

AML12 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) medium containing 10% fetal bovine serum and supplemented with 0.1% gentamicin (Invitrogen Corporation, Carlsbad, California), insulin, transferrin and selenious acid (ITS; Becton Dickinson, Franklin Lakes, NJ) and 0.04 μ g/ml dexamethasone. The WB, GN5, WB311 and GP6 cell lines were routinely cultured in DMEM/F-12 containing 10% FBS, 2 mM L-glutamine, gentamicin and ITS. H4-II-E cells were cultured in MEM with 5% FBS. 1682-A, THC 252 and H5D cells were routinely cultured in Weymouth's medium containing 10% FBS.

Where noted, cell lines were treated with rapamycin at various concentrations (0 to 200 nM) and for various durations (10 min to 24 hrs). Analysis for [³H]thymidine incorporation was performed as previously described [Curran et al., 1993]. Cell lysates were prepared using a buffer composed of 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 1 mM DTT, 0.1% NP-40 and 10% glycerol plus protease inhibitors (10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 34.4 μ g/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride). Cell lysates were

clarified by centrifugation for 10 min at 14,000 rpm. Supernatants were frozen at -70°C until analysis.

Determination of PP2A Activity

PP2A activity in liver extracts, cell lysates and immunoprecipitates was determined using a peptide-based assay. The reaction mixture (50 μl) included 5 μl of 1 mM peptide [KR(pT)IRR] and sample (5 μg liver extract protein, 3 μl column eluate or immunoprecipitate derived from 100 μg lysate protein). Immunoprecipitates were analyzed while bound to antibody beads. In all cases, the reaction was allowed to proceed for 30 min at 30°C . Following centrifugation, 25 μl of the reaction mixture was added to 100 μl of the Malachite Green Phosphate Detection Solution. As per the manufacturer's instructions, the color was allowed to develop for 30 min following which the absorbance was read at 650 nm. Results, representing phosphate released from substrate, were quantified relative to a phosphate standard curve.

PP2A activity was also determined as the release of ^{32}P from radiolabeled MBP. Preparation of the ^{32}P -MBP substrate was carried out as follows. A kinase reaction mixture containing 25 mM Tris/HCl, pH 7.5, 0.02 mM EGTA, 412.5 μg MBP, 1.5 μg recombinant Erk1, 10 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 100 μM ATP and 50 μCi [γ - ^{32}P]-ATP was incubated 30°C for 3 days. The labeling reaction was terminated by adding trichloroacetic acid to a final concentration of 20% and incubating on ice for 90 min. The resulting pellet was collected by centrifugation at $16,100 \times g$ for 5 min and washed with 10% ice-cold trichloroacetic acid. The pellet was diluted in 100 μl of 0.1M NaOH followed by the addition of 150 μl of Substrate Dilution Buffer (20 mM MOPS, pH 7.5, 50 mM NaCl, 1 mM MgCl_2 , 1 mM dithiothreitol, 0.1% B-mercaptoethanol, 10% glycerol, and 0.1 mg/ml bovine serum albumin). The substrate was further diluted to 2 μM ^{32}P -ATP and stored at -20°C until use. For the phosphatase assay, sample (10 μl) plus substrate (10 μl) were incubated at 30°C for 10 min. The reaction was stopped by adding 100 μl of 25mg/ml BSA and 880 μl of 20% TCA. Following centrifugation at $16,100 \times g$, 0.9 ml of the supernatant was counted in 5 ml of Optifluor (Perkin Elmer Life and Analytical Sciences) to determine the amount of ^{32}P released from the substrate.

PP2A-C and $\alpha 4$ Transfection

Plasmids encoding wild-type (WT) PP2A-C, L309Q-PP2A-C and $\alpha 4$ were generated as previously described [Chung et al., 1999; Murata et al., 1997]. AML12 cells were transfected with HA-tagged WT-PP2A-C or L309Q-PP2A-C, or with FLAG-tagged $\alpha 4$. For this procedure, 3×10^5 cells per well were seeded onto 6-well plates to achieve ~60% confluence. 4 μl of Plus reagent was added to 1 μg of DNA in total volume of 100 μl and incubated for 15 min at room temperature. This mixture was then combined with diluted lipofectamine transfection reagent (4 μl /100 μl) in 1:1 ratio and incubated for 15 min at room temperature. The final mixture (200 μl) was applied to cells in 1 ml of their usual media minus serum and incubated for 3 hr. The transfection was terminated by addition of fresh media containing 10% fetal bovine serum.

For immunoblotting, 100 μg of AML12 cell lysate protein, prepared as described above, was immunoprecipitated with 5 μg of FLAG antibody. The resulting immunoprecipitates were run on 10% SDS-polyacrylamide gel and analyzed using MD-PP2A-C, CT-PP2A-C, anti- $\alpha 4$, anti-HA and anti-FLAG antibodies. Lysates prepared from cells exposed to media only, exposed to media with lipofectamine reagents or transfected with empty vector were used as controls.

Western Immunoblotting

For analysis of liver extracts, 80 µg of total protein in Laemmli sample buffer was separated using a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). For analysis of cell lysates, 10-20 µg of extract protein was used. For all immunoblots, detection employed an enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ). Image acquisition employed the ChemiDoc-It Imaging System with Labworks 4.5 Image Acquisition and Analysis Software (UVP, Inc., Upland, CA).

Results

The $\alpha 4$ /PP2A Complex in Fetal and Adult Liver

Direct immunoblotting of triplicate samples of unfractionated homogenates showed that $\alpha 4$ content tended to be slightly higher in fetal liver than in adult liver (Fig. 1A). The immunoblot was also probed for the p85 subunit of PI3-kinase in order to demonstrate the consistency of transfer. Immunoprecipitation with $\alpha 4$ antibody followed by analysis of the immunoprecipitates by immunoblotting for $\alpha 4$ and PP2A-C showed that more PP2A-C was bound to $\alpha 4$ in adult compared to fetal liver (Fig. 1B). PP2A-C appeared as a doublet, as previously observed by us [Yoo et al., 2007] and by others [Turowski et al., 1995; Yu et al., 2001]. Quantification of the results of the direct immunoblotting and immunoprecipitation (Fig. 1C) showed that the trend towards higher $\alpha 4$ levels in fetal liver was not significant while the higher content of the $\alpha 4$ /PP2A-C complex in adult liver reached significance at the level of $P < 0.01$.

In order to examine the activity of $\alpha 4$ /PP2A-C complexes, liver extracts were fractionated by MonoQ chromatography as described previously [Yoo et al., 2007]. Fractions were analyzed for PP2A activity using a peptide substrate and ^{32}P -MBP (Fig. 2A). The activity profiles obtained with both substrates were similar, demonstrating two main peaks of activity. Our analysis of numerous such profiles by immunoblotting for regulatory subunits allowed us to assign the first and second peaks to the ABC and AC forms of PP2A, respectively [Yoo et al., 2007].

Immunoblotting of the MonoQ chromatography fractions for $\alpha 4$ showed a major peak that eluted early in the gradient (fractions 36 to 40) with continued elution through later fractions (up to fraction 46). The elution of PP2A-C occurred in two peaks that were in near alignment with the activity peaks and that corresponded to the ABC and AC forms of the enzyme. In order to determine the position of the $\alpha 4$ /PP2A-C complex, four fraction pools were analyzed by immunoprecipitation for $\alpha 4$ followed by immunoblotting for $\alpha 4$ and PP2A-C (Fig. 2B). Results showed that the first peak of $\alpha 4$ was not associated with PP2A-C. Two fraction pools from the later-eluting $\alpha 4$, one under the major activity peak and the second between the two peaks, showed the presence of $\alpha 4$ /PP2A-C. Given the relationship between activity and the position of the $\alpha 4$ /PP2A-C complex, it did not appear that this complex made a significant contribution to PP2A activity with either substrate. A pool derived from the end of the gradient served as a negative control, showing neither $\alpha 4$ nor PP2A-C in the immunoprecipitate.

The elution pattern of the $\alpha 4$ /PP2A-C complex was replicated in a second experiment (data not shown). Similar results were also observed in analyses of fetal liver extracts (data not shown).

The Effect of Rapamycin on $\alpha 4$ /PP2A-C

Previous studies in yeast and in mammalian cells have shown that $\alpha 4$ and PP2A-C dissociate in response to rapamycin [Gingras AC et al., 2001a; Inui et al., 1998; Murata et al., 1997]. We sought to determine if the same effect occurs in hepatic cells *in vivo*. Liver extracts were prepared from rats sacrificed 24 hr after intraperitoneal administration of rapamycin or DMSO vehicle as control (Fig. 3). Reduction of phosphorylated ribosomal protein S6 confirmed that rapamycin administration produced a marked inhibition of mTOR signaling. Liver PP2A-C content was unaffected, though the level of $\alpha 4$ was slightly reduced by rapamycin administration. Immunoprecipitation with $\alpha 4$ antibody followed by immunoblotting showed a modest increase in the content of the $\alpha 4$ /PP2A-C complex in animals that received rapamycin relative to those that were given vehicle alone. There was most certainly no evidence for a rapamycin-induced reduction in the content of the $\alpha 4$ /PP2A-C complex.

In order to repeat these analyses under conditions of maximal mTOR activation [Anand and Gruppuso, 2006], animals were fasted for 48 hr then injected with DMSO or rapamycin 15 min prior to a 1 hr refeeding period. The effectiveness of the rapamycin injection was again confirmed using immunoblotting for phosphorylated S6 as an indicator of mTOR signaling. Under these conditions, rapamycin administration did not change the amount of $\alpha 4$ associated with hepatic PP2A-C *in vivo* (data not shown).

We went on to examine $\alpha 4$ /PP2A-C in animals that underwent partial hepatectomy with or without prior administration of rapamycin. Animals were sacrificed 24 hr after partial hepatectomy. As expected, S6 phosphorylation was induced by partial hepatectomy and fully inhibited by the prior administration of rapamycin (Fig. 4A). Partial hepatectomy did not induce changes in the content of either PP2A-C or $\alpha 4$ proteins (Fig. 4A). The content of the $\alpha 4$ /PP2A complex recovered by $\alpha 4$ immunoprecipitation also was unaffected by partial hepatectomy or by rapamycin (Fig. 4B). Similar results were obtained in animals sacrificed 6 hr and 48 hr after partial hepatectomy (data not shown).

In Vitro Studies on the Regulation of $\alpha 4$ /PP2A-C in Hepatic Cells

Most hepatic cell lines show poor transfection efficiency. Among those that we tested, only AML12 cells showed adequate transfection efficiency to acquire interpretable data. Preliminary studies (data not shown) revealed that the AML12 cells are highly rapamycin-sensitive and that the transfection conditions used were associated with excellent cell viability and a transfection efficiency of approximately 10%.

AML12 cells were transfected with HA-tagged PP2A-C constructs that encoded the wild-type enzyme (WT) or one mutated at leucine 309 to an uncharged polar residue, glutamine (L309Q). The latter cannot be methylated. These plasmids were transfected alone or in combination with FLAG-tagged $\alpha 4$. Transfection with FLAG- $\alpha 4$ alone was also performed. Direct immunoblotting was carried out using antibodies directed towards HA, FLAG, PP2A-C and $\alpha 4$ (Fig. 5A). Immunoblotting for HA showed that WT-PP2A-C migrated at 45 kDa while L309Q-PP2A-C migrated at 43 kDa. Both antibodies detected a minor form at 41 kDa. Levels of both the WT-PP2A-C and L309Q-PP2A-C were enhanced by co-transfection with FLAG- $\alpha 4$. This suggested interaction between the proteins that resulted in stabilization of the PP2A-C. Immunoblotting with the antibody directed towards the mid-portion of PP2A-C detected both WT-PP2A-C and L309Q-PP2A-C as well as endogenous PP2A-C at 39 kDa. Levels of the endogenous protein were not affected by transfection.

We showed previously [Yoo et al., 2007] that immunoblotting with CT-PP2A-C antibody only detects unmethylated PP2A-C. Results of immunoblotting with this antibody indicated that the endogenous PP2A-C was predominantly in the methylated form. In addition, ectopic

WT-PP2A-C did not react with CT-PP2A-C antibody, indicating that this form also was methylated. As expected, L309Q-PP2A-C reacted with CT-PP2A-C antibody, consistent with its non-methylated status. Immunoblotting for $\alpha 4$ showed similar levels of the endogenous 42 kDa protein under all conditions.

In order to assess the interactions between transfected PP2A-C and $\alpha 4$, cell lysates were immunoprecipitated with $\alpha 4$ antibody. The immunoprecipitates were analyzed by immunoblotting. Results (Fig. 5B) showed that immunoprecipitation with $\alpha 4$ antibody yielded positive immunoblot results with both the MD-PP2A-C and CT-PP2A-C antibodies. This was interpreted as indicating that the transfected $\alpha 4$ was capable of binding to both the methylated and unmethylated forms of PP2A-C. We did not observe interactions between the transfected $\alpha 4$ and endogenous PP2A-C. This is likely attributable to the relatively low transfection efficiency that we achieved in these cells. While the levels of the transfected PP2A-C and endogenous enzyme appear similar in the immunoblots, the transfected cells are expressing high levels of the transfected protein relative to the endogenous protein. Therefore, the complexes that form with the transfected $\alpha 4$ predominantly include the more abundant co-transfected PP2A-C.

Transfected AML12 cells were treated with either DMSO vehicle or 20 nM rapamycin for 1 hr. Immunoblotting for phospho-S6 confirmed the efficacy of rapamycin in inhibiting mTOR signaling (Fig. 6A). Rapamycin did not affect the levels of ectopic PP2A-C or $\alpha 4$ (Fig. 7A). Most importantly, immunoprecipitation of $\alpha 4$ using anti-FLAG followed by immunoblotting for HA and FLAG showed that rapamycin had no effect on PP2A-C association with $\alpha 4$ (Fig. 6B).

In order to confirm our *in vivo* findings indicating the inhibitory effect of $\alpha 4$ on PP2A activity, we performed a phosphatase activity assay with immunoprecipitates from transfected AML12 cells. Immunoprecipitates were analyzed by immunoblotting (not shown) in order to correct activities for the recovery of the ectopic phosphatase. Results (Fig. 7A) showed that $\alpha 4$ co-transfection reduced the cell lysate specific activity of the WT-PP2A-C. Results with L309Q-PP2A-C showed a similar trend, though the effect did not achieve statistical significance. We extended our observations to the effect of rapamycin. Results (Fig. 7B) indicated that rapamycin did not induce a significant change in PP2A activity in cells co-transfected with either WT-PP2A-C or L309Q-PP2A-C and $\alpha 4$. This experiment was repeated in its entirety with similar results.

Cell lysates derived from the above experiment were analyzed by immunoprecipitation with $\alpha 4$ antibody followed by the determination of phosphatase activity. In all cases, negligible activity was detected in these immunoprecipitates.

The Relationship Between the Rapamycin Sensitivity of Hepatic Cells and $\alpha 4$ /PP2A-C Content

We examined the $\alpha 4$ /PP2A-C complex in rapamycin-sensitive and resistant rat hepatic cell lines. These cell lines were characterized for rapamycin sensitivity by exposing them to various concentrations of rapamycin for 24 hr and measuring [3 H]thymidine incorporation during the last 6 hr of the incubation period. The IC₅₀ for rapamycin was determined for each cell line. The nontumorigenic H4-II-E and WB cells were highly sensitive to rapamycin, with IC₅₀ values in the range of 2 to 10 nM. The tumorigenic GN5 and 1682-A cells were also rapamycin-sensitive, whereas four other cell lines, WB311, GP6, H5D and THC-252 were highly resistant to rapamycin, showing an IC₅₀ of above 200 nM.

Among the eight cell lines, there was a greater than 5-fold difference in the level $\alpha 4$ and a 2 to 3-fold difference in levels of PP2A-C (Fig. 8A). The abundance of neither protein

corresponded to sensitivity of the cell lines to the anti-proliferative effects of rapamycin. Furthermore, rapamycin sensitivity was not associated with PP2A-C methylation, as indicated by reactivity with the MD-PP2A-C and CT-PP2A-C antibodies (Fig. 8A). PP2A-C association with $\alpha 4$ was examined in these cell lines by co-immunoprecipitation and immunoblotting. Results showed that the amount of the $\alpha 4$ /PP2A-C complex followed the level of $\alpha 4$ protein with no apparent correlation with rapamycin sensitivity or tumorigenicity (data not shown).

The effect of rapamycin on formation of the $\alpha 4$ /PP2A-C complex was studied in two of the cell lines, the rapamycin-sensitive WB cell line and a rapamycin-resistant counterpart, the WB311 cell line. These two particular lines were chosen because the WB311 cells were spontaneously derived from the WB cells. There was no change in the content of $\alpha 4$ or PP2A-C (not shown) or of the $\alpha 4$ /PP2A-C complex (Fig. 8B) for periods of rapamycin exposure ranging from 10 min to 6 hr.

Discussion

Although previous studies have identified the presence of $\alpha 4$ /PP2A complexes in yeast, plants and mammalian cells [Chen et al., 1998; Di Como CJ and Arndt KT, 1996; Harris et al., 1999; Inui et al., 1998; Kloeker et al., 2003; Kong et al., 2004; Murata et al., 1997; Nanahoshi et al., 1998; Nien et al., 2007; Prickett and Brautigan, 2004; Yamashita et al., 2005], information on the physiological control of this distinct form of PP2A in liver and hepatic cells is quite limited. Given the purported role of PP2A in cell growth, the possible link to mTOR and the limitations in the available data on the $\alpha 4$ /PP2A complex, we carried out studies in a well defined *in vivo* system, liver growth in the laboratory rat. The hypothesis was that the abundance of PP2A, in particular the $\alpha 4$ /PP2A-C complex, might be associated with differences in the rapamycin sensitivity and proliferative activity of fetal versus adult hepatocytes *in vivo*. We showed previously that PP2A activity and abundance were slightly higher in unfractionated E19 fetal compared to adult liver extracts [Yoo et al., 2007]. This was unexpected based on the reported inverse correlation between PP2A activity and cell growth and proliferation [Janssens et al., 2005; Janssens and Goris, 2001].

We detected the $\alpha 4$ /PP2A-C complex in unfractionated liver extracts and in $\alpha 4$ -containing fractions obtained by MonoQ chromatography. MonoQ fractionation also provided evidence that liver extracts contain $\alpha 4$ not bound to PP2A-C. This observation may be a function of the ability of $\alpha 4$ to associate with other PP2A-related phosphatases [Chen et al., 1998; Nanahoshi et al., 1999], or it may indicate the presence of a pool of free $\alpha 4$ in hepatocytes.

Previous studies have been interpreted as indicating that the A regulatory subunit is displaced by anti-HA antibody [Prickett and Brautigan, 2004]. This was demonstrated by showing that ectopically expressed HA-PP2A-C could associate with the endogenous A subunit in column chromatography fractions but that HA immunoprecipitates yielded only low co-precipitations with endogenous A subunit. In the present studies, the association between HA-PP2A-C and FLAG- $\alpha 4$ was maintained, thus allowing us to tentatively conclude that $\alpha 4$ exerts an inhibitory effect on the activity of the catalytic subunit. Overall, our findings are consistent with published studies showing that $\alpha 4$ has an inhibitory effect on PP2A activity towards ^{32}P -phosphorylated 4E-BP1 and *p*-nitrophenyl phosphate (*p*NPP) *in vitro* [Nanahoshi et al., 1998; Nanahoshi et al., 1999]. While our observations do not extensively address the issue of substrate specificity, analysis of liver extracts showed similar activity profiles using peptide and ^{32}P -MBP as substrate. It has been demonstrated that $\alpha 4$ enhances PP2A activity toward ^{32}P -MBP [Murata et al., 1997; Prickett and Brautigan, 2006]. In fact, $\alpha 4$ has been shown to have opposing effects on PP2A and PP6

activity [Prickett and Brautigan, 2006]. Our own data do not indicate that ^{32}P -MBP is a preferred substrate for the hepatic $\alpha 4$ /PP2A-C complex.

We showed previously [Yoo et al., 2007] that a high proportion of PP2A-C in adult liver is unmethylated while fetal liver PP2A-C is predominantly methylated. Given the higher abundance of the $\alpha 4$ /PP2A-C complex in adult liver relative to fetal liver, we hypothesized that $\alpha 4$ preferentially binds to unmethylated PP2A-C. Instead, our studies on AML12 cells co-transfected with $\alpha 4$ and WT-PP2A-C and L309Q-PP2A-C indicated that $\alpha 4$ associates with both the methylated and unmethylated forms of PP2A-C. Previous studies using MonoQ chromatographic fractionation of COS-7 cell extracts [Chung et al., 1999] have shown that a mutation of Leu309 in PP2A-C results in the recovery of only AC and $\alpha 4$ /PP2A-C dimers and that a double mutation of Tyr307 and Leu309 favors the formation of $\alpha 4$ /PP2A-C. Our prior studies indicated an absence of PP2A-C phosphorylated at Tyr307 in liver homogenates [Yoo et al., 2007]. The present results indicate that the interaction of PP2A-C with $\alpha 4$ in hepatic cells is determined by factors other than the tyrosine phosphorylation or methylation state of the PP2A-C carboxy terminus.

Prior studies provided ample reason to anticipate that changes in hepatic mTOR signaling, whether induced by refeeding or partial hepatectomy or inhibited by rapamycin, might be associated with changes in the abundance of $\alpha 4$ /PP2A-C. While we found that the administration of rapamycin to fed rats induced a modest increase in the content of the $\alpha 4$ /PP2A-C complex, our studies provided no evidence that rapamycin could induce the reduction in $\alpha 4$ /PP2A-C formation that would be predicted by studies in yeast. In fact, we found that the *in vivo* administration of rapamycin could induce the dephosphorylation of S6 without changing the amount of $\alpha 4$ /PP2A-C. This makes it less likely that $\alpha 4$ mediates mTOR signaling in liver.

The absence of a direct effect of rapamycin on $\alpha 4$ /PP2A-C was corroborated by the transfection studies in AML12 cells. Of the studies examining the regulation of this complex in mammalian systems, several have shown that the interaction of PP2A-C with $\alpha 4$ is unaffected by rapamycin [Kloeker et al., 2003; Nanahoshi et al., 1998; Nien et al., 2007]. We also found that while co-transfection of $\alpha 4$ and PP2A-C resulted in decreased specific activity of the transfected PP2A-C, there was no apparent effect of rapamycin on activity of the complex.

Studies in yeast [Di Como CJ and Arndt KT, 1996; Jiang Y and Broach JR, 1999] have indicated that the conditions that promote formation of the $\alpha 4$ /PP2A-C complex induce partial rapamycin resistance. Our intent was to test the hypothesis that high levels of $\alpha 4$ /PP2A-C in fetal liver contribute to the resistance of fetal hepatocytes *in vivo* to the cell cycle inhibitory effects of rapamycin. We found that fetal hepatocytes *in vivo* have lower levels of $\alpha 4$ /PP2A-C than rapamycin-sensitive adult hepatocytes. We extended our observations to a panel of well characterized hepatic cell lines that in preliminary studies were found to have highly variable sensitivity to rapamycin. Similar to the *in vivo* model, we found no correlation between $\alpha 4$ content or $\alpha 4$ /PP2A-C content and sensitivity to the anti-proliferative effects of rapamycin.

Our studies have a number of limitations that should be taken into consideration. We did not directly examine the effect of $\alpha 4$ /PP2A-C overexpression on the ability of rapamycin to inhibit DNA synthesis in cultured hepatic cells. These studies were precluded by the effects of transfection protocols on rapamycin sensitivity. Our results do not rule out the possibility that the effect of rapamycin on $\alpha 4$ /PP2A-C is mediated by changes in the phosphorylation state of $\alpha 4$, PP2A-C or both. In the absence of data on the phosphorylation state of proteins that are endogenous substrates of $\alpha 4$ /PP2A-C, we cannot conclude the absence of an effect

of rapamycin on substrate-specific activity. Nonetheless, our results do lead to several conclusions. The methylation status of hepatic PP2A-C does not appear to be a primary determinant of the formation of $\alpha 4$ /PP2A-C in liver. The yeast paradigm in which modulation of TOR activity affects the association of $\alpha 4$ and PP2A-C does not appear to pertain to liver. Finally, the abundance of $\alpha 4$ or the $\alpha 4$ /PP2A-C complex is not a primary determinant of the rapamycin sensitivity of hepatic cells *in vivo* or *in vitro*.

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Fig. 1.

Content of $\alpha 4$ and $\alpha 4$ /PP2A-C in fetal and adult liver. **A:** Liver extracts were prepared from triplicate fetal and adult livers. Extracts (80 μ g protein per lane) were analyzed for $\alpha 4$ protein content by direct immunoblotting. Blots were stripped and reprobed for the p85 subunit of phosphatidylinositol 3-kinase to demonstrate consistency in the transfer. **B:** Extracts from triplicate fetal and adult livers were analyzed for content of the $\alpha 4$ /PP2A-C complex by immunoprecipitation of $\alpha 4$ followed by immunoblotting for PP2A-C. The blot was stripped and reprobed for $\alpha 4$ to verify equivalent immunoprecipitation in all samples. **C:** The results of the immunoblots in panels A and B were quantified. Results are shown as the mean + 1 standard deviation. *, $P < 0.01$ versus fetal results.

Fig. 2.

Chromatographic separation of adult liver extract PP2A complexes. **A:** Extract protein was applied to a MonoQ ion-exchange column and eluted with a linear salt gradient from 0 to 0.6 M. PP2A activity was determined in individual fractions using peptide substrate (filled circles/solid line) and ^{32}P -MBP (unfilled circles/dashed line). Samples of each fraction were then analyzed by immunoblotting with antibodies directed towards PP2A-C and $\alpha 4$. The previously determined locations of the ABC and AC forms of PP2A are shown above the major activity peaks. **B:** Fractions were combined to give four pools; fractions 35-37, 42-43, 44-46 and 52-53. An equal volume of each fraction pool (1 ml) was analyzed by $\alpha 4$ immunoprecipitation followed by immunoblotting for PP2A-C. The blot was then stripped and reprobed for $\alpha 4$.

Fig. 3.

The effect of rapamycin on formation of the $\alpha 4$ /PP2A-C complex in adult liver. Extract protein was prepared from livers taken from adult rats injected 24 hr previously with DMSO or rapamycin. **A:** The extracts (80 μ g protein per lane) were analyzed by direct immunoblotting for phosphorylated ribosomal protein S6 (P-S6), total S6, PP2A-C and $\alpha 4$. **B:** Extracts (1 mg protein) were analyzed by immunoprecipitation with $\alpha 4$ antibody, followed by immunoblotting for PP2A-C and $\alpha 4$. The ratio of PP2A-C to $\alpha 4$ in the immunoprecipitates is shown in the bar graph as the mean + 1 standard deviation. *, $P < 0.05$ versus DMSO control.

Fig. 4.

The effect of rapamycin on $\alpha 4$ /PP2A-C in regenerating liver. Adult rats were injected with DMSO vehicle or rapamycin 1 hr before partial hepatectomy. Control animals were not subjected to surgery. One animal underwent sham hepatectomy in which the liver was exteriorized but not ligated or excised. Animals were sacrificed and livers obtained 24 hr after surgery. **A:** The extracts (80 μ g protein per lane) were analyzed by direct immunoblotting for phosphorylated ribosomal protein S6 (P-S6), total S6, PP2A-C and $\alpha 4$. **B:** Extracts (1 mg protein) were analyzed by immunoprecipitation with $\alpha 4$ antibody followed by immunoblotting for PP2A-C and $\alpha 4$. The ratio of PP2A-C to $\alpha 4$ in the immunoprecipitates is shown for the control, partial hepatectomy/DMSO and partial hepatectomy/rapamycin groups in the bar graph as the mean + 1 standard deviation.

Fig. 5.

Formation of $\alpha 4$ /PP2A-C in AML12 cells. AML12 cells were transfected with 1 μ g of DNA encoding HA-tagged wild-type (WT)-PP2A-C, L309Q-PP2A-C and FLAG-tagged $\alpha 4$ alone or in combination. The lysates from untransfected cells, cells exposed to Lipofectamine/Plus transfection reagent (LF control) or cells transfected using empty vector were used as negative controls. **A:** Lysate protein (10 μ g) was analyzed by direct immunoblotting using antibodies against HA, MD-PP2A, CT-PP2A, $\alpha 4$, and FLAG. **B:** Lysate protein (200 μ g) was immunoprecipitated with $\alpha 4$ antibody followed by immunoblotting with HA, MD-PP2A, CT-PP2A, and FLAG antibodies.

Fig. 6.

In vitro effect of rapamycin on α4/PP2A. AML12 cells were transfected in the same manner as for Figure 5 but were exposed to DMSO vehicle or 20 nM rapamycin for 1 hr prior to preparing lysates. **A:** Lysate protein (10 μg) was analyzed by direct immunoblotting using anti-HA and anti-FLAG antibodies. Phospho-S6 and total S6 immunoblots were done to verify rapamycin effect. **B:** Lysate protein (200 μg) was analyzed by immunoprecipitation with anti-FLAG antibody followed by immunoblotting for HA and FLAG.

Fig. 7.

In vitro regulation of PP2A activity by α 4 and rapamycin. **A:** AML12 cells were transfected with HA-tagged WT-PP2A or L309Q-PP2A alone or with FLAG-tagged α 4. Cell lysates were immunoprecipitated with anti-HA antibody. PP2A activity was measured in each immunoprecipitate. The activity in pmol/min was normalized to the protein content of PP2A using densitometry values derived from a PP2A immunoblot (not shown). Analysis of triplicate lysates is shown as the mean + 1 standard deviation. *, $P < 0.01$. **B:** AML12 cells were transfected with HA-tagged WT-PP2A or L309Q-PP2A along with FLAG-tagged α 4 as for *Panel A*. Prior to preparing lysates, cells were exposed to 20 nM rapamycin or DMSO vehicle for 1 hr. The lysates were then analyzed by immunoprecipitation with anti-HA

antibody followed by measurement of PP2A activity in the immunoprecipitates. Results are shown as mean + 1 standard deviation (DMSO: solid bars; rapamycin: hatched bars). Differences between groups were not significant as determined by ANOVA.

Fig. 8. $\alpha 4$ /PP2A-C and rapamycin resistance in rat hepatic cell lines. A panel of rapamycin-sensitive (H4-II-E, GN5, 1682-A, WB) and rapamycin-resistant (WB311, GP6, H5D, THC-252) hepatic cells were studied. **A:** Lysate protein (20 μ g) from cells was analyzed by direct immunoblotting for content of $\alpha 4$ and PP2A-C. The latter employed antibodies directed toward the mid-portion (MD-PP2A) and carboxy-terminus (CT-PP2A) of PP2A-C. **B:** WB and WB311 cells were exposed to rapamycin for times varying from 10 min to 6 hr. Cell lysates were analyzed by immunoprecipitation with $\alpha 4$ antibody followed by immunoblotting for PP2A-C and $\alpha 4$.