Journal of Cellular Biochemistry 103:1309–1325 (2008)

A PP2A Active Site Mutant Impedes Growth and Causes Misregulation of Native Catalytic Subunit Expression

Donna L. Lizotte, Joshua J. Blakeslee, Albert Siryaporn, Jeffrey T. Heath, and Alison DeLong*

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912

Abstract Activity of protein phosphatase 2A (PP2A) is tightly regulated and performs a diverse repertoire of cellular functions. Previously we isolated a dominant-negative active site mutant of the PP2A catalytic (C) subunit using a yeast complementation assay. We have established stable fibroblastic cell lines expressing epitope-tagged versions of the wild-type and H118N mutant C subunits and have used these cells to investigate mechanisms that regulate PP2A activity. Cells expressing the mutant C subunit exhibit a decreased growth rate and a prolonged G1 cell cycle phase. The mutant protein is enzymatically inactive, but extracts made from cells expressing the H118N C subunit show normal levels of total PP2A activity in vitro. The H118N mutant shows reduced binding to the regulatory A subunit, but binds normally to the α4 protein, a non-canonical regulator of PP2A. Expression of the H118N mutant interferes with the normal control of C subunit abundance, causing accumulation of the endogenous wild-type protein as well as the mutant transgene product. Our results indicate that the H118N mutant isoform retards C subunit turnover and suggest that PP2A C subunit turnover may be important for normal cell cycle progression. J. Cell. Biochem. 103: 1309–1325, 2008.

Key words: protein phosphatase 2A; catalytic site mutant; G1 phase delay; regulated protein turnover

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase that is found in all eukaryotic cells (reviewed in Janssens and Goris [2001]). This abundant enzyme is classically described as a heterotrimeric complex, consisting of the catalytic (C) subunit, a regulatory A subunit, and one of many different regulatory B subunit proteins. The two mammalian C subunit isoforms (α and β) are 97% identical in amino acid sequence but are encoded by distinct genes. The α isoform mRNA is more abundant than that of the β isoform in most tissues (reviewed in Mumby and Walter [1993]). The regulatory A subunit is composed of 15 alpha-helical HEAT repeats that provide a scaffold for the enzyme complex, and A subunit binding modulates catalytic activity [Kamibayashi et al., 1992; Ruediger et al., 1992; Turowski et al., 1997; Groves et al., 1999; Price and Mumby, 2000]. The C and A subunits form a core dimer that is ubiquitously expressed in mammalian cells. This heterodimeric complex associates with one of several B subunits, resulting in the classical heterotrimeric PP2A complex. Four unrelated B subunit types have been identified and each type is represented by different isoforms and/or splicing variants. B subunits are expressed in a tissue- and/or development stage-specific manner and perform distinct functions, dictating enzyme targeting and apparently controlling substrate specificity for the enzyme [Chen et al., 2004; Gentry et al., 2005; Arnold and Sears, 2006]. In addition to B subunits, a number of accessory proteins have been demonstrated to bind to the C subunit or the A/C heterodimer, yielding 'non-classical' PP2A enzyme complexes with altered specificity and function (reviewed in

Grant sponsor: National Science Foundation; Grant numbers: IBN-9986017, IOB-0446039; Grant sponsor: US Department of the Army; Grant number: DAMD17097-1-7113; Grant sponsor: NIH-NCRR; Grant number: RR15578; Grant sponsor: National Institutes of Health Predoctoral Training; Grant number: GM-07601.

Donna Lizotte's present address is Department of Science and Technology, Bryant University, Smithfield, RI 02917.

Albert Siryaporn's present address is Department of Physics and Astronomy, University of Pennsylvania, Philadelphia, PA 19104.

*Correspondence to: Alison DeLong, Sidney Frank Hall for the Life Sciences, Rm. 158, 185 Meeting St., Providence, RI 02912. E-mail: Alison_DeLong@brown.edu

Received 7 May 2007; Accepted 5 July 2007

DOI 10.1002/jcb.21514

Virshup [2000]). This diversity of PP2A enzyme composition allows participation in a broad array of cellular activities.

The α4 protein is a non-canonical regulatory subunit of PP2A that does not require the A/C heterodimer complex but can bind directly to the C subunit and alter its enzymatic activity [Murata et al., 1997]. Originally discovered as a phosphoprotein associated with the IG-α subunit in the B cell receptor complex [Inui et al., 1995], α4 also interacts with the catalytic subunits of other PP2A-like phosphatases such as PP4 and PP6 [Kloeker et al., 2003]. The regulatory effect of α4 binding is different for the PP2A and PP6 catalytic subunits [Prickett and Brautigan, 2006]. The *Saccharomyces cerevisiae* α4 homolog TAP42 also binds PP2A, PP4 and PP6 catalytic subunits [Di Como and Arndt, 1996; Wang et al., 2003] and acts downstream of the target of rapamycin (TOR) kinase in the rapamycin-sensitive pathway that connects nutritional status to the initiation of mRNA translation (reviewed in Zabrocki et al. [2002]). The roles of mammalian α4 and PP2A in the mTOR signaling pathway remain controversial. However, α4 binding mediates an interaction between the PP2A catalytic subunit and the microtubule-associated protein MID1, a RING-finger protein that plays a crucial role in ventral midline development in humans [Liu et al., 2001; Trockenbacher et al., 2001]. This interaction regulates ubiquitin-mediated proteolysis of a microtubule-associated PP2A catalytic subunit population. The pathology associated with MID1 defects may stem from abnormally high PP2A activity and consequent hypophosphorylation of microtubule-associated proteins [Trockenbacher et al., 2001].

Post-translational modification of the catalytic subunit influences both the enzymatic activity and the interactions of the C subunit with regulatory subunits and other proteins [Liu et al., 2001; Trockenbacher et al., 2001; Gentry et al., 2005]. Three sites of post-translational modification in the carboxy-terminal 'tail' of the PP2A C subunit have been identified. Phosphorylation of tyrosine 307 inhibits PP2A activity in vitro and occurs after growth factor stimulation in murine 10T1/2 fibroblast cells [Chen et al., 1992, 1994]. There is also evidence of inhibitory threonine phosphorylation, although the exact site(s) of phosphorylation have not yet been identified [Guo and Damuni, 1993]. Based on high sequence conservation, it is postulated that threonine 304 may be the target residue [Ogris et al., 1997; Evans et al., 1999]. In addition, the C-terminal leucine (leucine 309) is subject to methyl esterification (reviewed in Mumby [2001]). Methyl group addition and removal are catalyzed by PP2A-specific methyltransferase and methylesterase enzymes (PPMT and PPME/PME-1, respectively). A yeast strain lacking the methyltransferase activity exhibits partially disrupted PP2A holoenzyme formation in vivo, and *ppmt* and *ppme* mutants phenotypically resemble strains that lack regulatory subunit function [Wu et al., 2000; Gentry et al., 2005]. However, in vitro reconstitutions with recombinant mammalian subunits indicate that carboxy-terminal methyl esterification is not essential for holoenzyme formation with some B subunits [Xu et al., 2006].

Accumulation of C subunit protein also is regulated, and several reports have documented the difficulty of overexpressing the C subunit in mammalian cells (reviewed in Goldberg [1999]). For instance, NIH3T3 cells transfected with PP2A overexpression constructs exhibited a 10-fold increase in PP2A mRNA levels, but showed little change in the abundance of C subunit protein [Baharians and Schönthal, 1998]. Analysis of mRNA utilization in these cells revealed that PP2A transcripts showed reduced association with high molecular weight polysomes in the cells overexpressing PP2A mRNA, indicating inefficient translation. However, treatments that reduce PP2A activity can increase C subunit protein levels. Cells treated with the phosphatase inhibitor okadaic acid (OKA) show a dose-dependent increase in C subunit protein levels [Baharians and Schönthal, 1998]. Similarly, expression of the polyoma virus small t antigen, a protein inhibitor of PP2A, also leads to increased C subunit accumulation [Sontag et al., 1993]. These data reveal a homeostatic mechanism that controls the amount of active C subunit accumulating in cells, and suggest that regulation is mediated at least in part at the translational level.

We previously reported the isolation of dominant-negative alleles of the human PP2A C subunit using a yeast complementation system [Lizotte et al., 1999]. We have established stable fibroblastic cell lines that express epitope-tagged versions of the wild-type and mutant C subunits and have used these cells to investigate mechanisms that regulate PP2A

activity. Here we present data showing that expression of a dominant-negative C subunit allele abrogates the normal regulation of C subunit accumulation, allowing accumulation of the endogenous wild-type protein as well as the mutant transgene product. Our results suggest that the mutant isoform impedes turnover of C subunit protein. The mutant protein is enzymatically inactive and shows reduced binding to regulatory A subunits, but binds normally to the α4 protein. Cells expressing the mutant C subunit exhibit a decreased growth rate due to prolongation of the G1 cell cycle phase. Our data suggest that PP2A C subunit turnover is important for normal cell cycle progression.

MATERIALS AND METHODS

Generation and Culture of Stable Cell Lines

A FLAG epitope tag was fused to the wild-type and H118N PP2A C subunit coding sequences of pDMC16 and pDMC27, the H118N derivative of pDMC16 [Lizotte et al., 1999], by PCR amplification. The DNA sequences of the epitope-tagged constructs were verified and the fusions were subcloned into the retroviral vector pLXSHyg and were packaged in the BOSC cell line [Pear et al., 1993]. Stable cell lines were derived from the TGR-1 parent by hygromycin selection, and were established as described by Prouty et al. [1993]. For cell proliferation assays, exponentially growing cells were seeded at an initial density of 55,000 cells (approximately 10% confluence) in 6-cm dishes. At each time point duplicate dishes were harvested with trypsin and diluted with PBS before counting, using a Coulter Counter™. For flow cytometric analysis, cells were grown to 50% confluence and harvested by trypsinization, then pelleted in ice cold PBS and prepared for FACS analysis [Shichiri et al., 1993]. Flow cytometric analysis was performed on a BD Biosciences FACS-Calibur instrument using CellQuest and Modfit software.

BrdU Incorporation Assays

In all BrdU incorporation assays, cells were labeled with BrdU and uridine and subjected to in situ histochemical staining for BrdU incorporation as described by Schorl and Sedivy [2003], using an anti-BrdU monoclonal antibody (Roche). Cells were maintained at 50–60% confluence to avoid contact inhibition. For pulse-labeling experiments, assays were performed in 6-cm culture dishes. Cells were seeded at same initial density (~50% confluence) and allowed to settle for 12–18 h before administering a 30-min pulse of BrdU. For S phase entry and restriction point analysis, exponentially growing cells were synchronized in mitosis by mitotic-shake off. Synchronized cells were plated in a 24-well microtiter plate and allowed to attach for 2 h in serum-supplemented medium before addition of BrdU. For restriction point analysis, cells were shifted to serum-free medium containing BrdU at specific time points, and all reactions were stopped with ascorbic acid 12 h after shake-off. Cells were subsequently fixed and processed to detect BrdU incorporation.

Microscopy and Data Analysis

Cells were observed using a Nikon TS100 inverted microscope. Random fields of histochemically stained cells were photographed using a Nikon Coolpix 995 digital camera at 200× magnification and subsequently counted by visual observation. Means and graphs were generated using Microsoft Excel™. The average total number of cells counted in each experiment is indicated in the corresponding figure legend.

Immunoblotting, Immunoprecipitation and Phosphatase Activity Assays

Whole-cell lysates of mammalian cells were prepared by washing with 50 mM Tris pH 7.5 and 150 mM NaCl twice, followed by harvesting in lysis buffer (50 mM Tris pH 7.5, 0.5% Triton X-100) supplemented with Complete Protease Inhibitor Cocktail (Roche). After a 10-min incubation on ice, extracts were subjected to a 10-min centrifugation at 4°C to remove large cell debris. Total protein concentrations in cleared protein extracts were assessed either by Bradford assay or Coomassie gel staining. For immunoblotting, proteins were resolved by SDS–PAGE and electrophoretically transferred to PVDF membrane (Millipore). Membranes were probed with the antibodies described below, then incubated with HRP-conjugated secondary antibodies and processed for chemiluminescent detection. Antibodies used were: Upstate Biotechnology 1D6 monoclonal PP2A catalytic subunit antibody (UBI, Lake Placid, NY); monoclonal anti-PP2A catalytic subunit antibody (Transduction Laboratories, Lexington, KY); Upstate Biotechnology polyclonal anti-PP2A catalytic subunit antibody

(UBI, Lake Placid, NY); monoclonal antibody 6F9 anti-A subunit hybridoma supernatant (gift of Gernot Walter); polyclonal anti-α4 antibody (gift of David Brautigan); monoclonal antibody anti-PARP antibody (Zymed). All antibodies were diluted according to the manufacturers' instructions.

Immunoprecipitation experiments employed M2 anti-FLAG agarose beads (Sigma), Protein A Sepharose (Sigma) or Protein G Dynabeads (Dynal). Dynabeads were washed twice with sodium phosphate buffer and incubated with 4.5 μg of total C subunit antibody for 2 h at 4°C. After this precapture step, the beads were washed twice with lysis buffer. Protein extracts containing 150 μg of total protein were mixed with the magnetic beads and incubated for 4–6 h at 4°C. Pellets were captured magnetically and were washed three times with lysis buffer. Immunoprecipitates were resuspended in Laemmli buffer, boiled for 3 min and subjected to SDS–PAGE.

For phosphatase activity assays, whole cell extracts or immunoprecipitation fractions were assayed for myelin basic protein (MBP) phosphatase activity using the Protein Serine/Threonine Phosphatase Assay System (New England Biolabs) according to the manufacturer's instructions. Whole cell extracts and immunoprecipitation supernatants were diluted to give total protein concentrations of 0.1–0.2 μg/μl to allow discrimination between PP2A-like and PP1 activities based on their differential okadaic acid (OKA) sensitivities [MacKintosh, 1993]. For each immunodepletion reaction, 100 μg aliquots of total protein were subjected to two rounds of immunoprecipitation with M2 anti-FLAG agarose beads or Protein A Sepharose beads. Supernatant fractions from the second-round immunoprecipitation were assayed, and the first- and second-round immunoprecipitation pellets were washed and pooled for activity assays and immunoblotting. Activity assays were performed in triplicate or quadruplicate and error bars represent standard deviations.

Metabolic Labeling Experiments

For each cell line assayed, 6-cm culture dishes were plated at ~40% confluence and cells were allowed 24 h to adhere to the dishes. To perform pulse-chase analysis, cells were washed twice with PBS and starved of methionine and cysteine for 1 h at 37°C in 2 ml of methionine/cysteine-free DMEM (Gibco-BRL) supplemented with 10% dialyzed calf serum. Cells were then washed with PBS and fed with methionine/cysteine-free DMEM and 10% dialyzed calf serum supplemented with 7 mCi of ^{35}S labeled methionine and cysteine (NEN) for 2 h at 37°C (250 μCi of ^{35}S per 6 cm dish assayed). After incubation with the radiolabeled amino acids, cells were washed twice with PBS and then returned to complete DMEM. Cells were harvested at the indicated time points by washing twice with PBS, followed by two washes with 50 mM Tris pH 7.5/150 mM NaCl. Cells were then harvested into 500 μl of lysis buffer and stored at −20°C. For rate of synthesis experiments, cells were incubated in similar supplemented medium containing the radiolabel and harvested directly from the radiolabeled medium as described above.

Northern Analysis and Real-Time PCR Analysis

Total RNA was prepared from confluent 15-cm dishes using the Trizol reagent (Life Technologies, Inc.) followed by chloroform extraction and isopropanol precipitation. RNA was quantitated by UV absorbance measurement and confirmed by ethidium bromide staining on an agarose gel. Ten micrograms of total RNA were separated on a 1% formaldehyde gel [Sambrook and Russell, 2001], transferred to Zeta-Probe® membrane (BioRad), and probed with ^{32}P-dCTP labeled C subunit probe. The 900 basepair probe was specific for the coding sequence of human PP2A catalytic subunit, and was prepared by gel purification and labeling via random priming [Feinberg and Vogelstein, 1984]. The membrane was hybridized with this probe at 60°C overnight. After hybridization, the membrane was washed stringently and signals were detected by phosphorimager. As a loading control, the same membrane was stripped and rehybridized with a ^{32}P-dCTP-labelled probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (kind gift of B. O'Connell and J. Sedivy).

Real-time reverse transcriptase PCR (RT-PCR) was performed on the ABI Prism 7700 Sequence Detection system (Applied Biosystems). Primers (5′-AGAGGCGAGCCACATGTCAC-3′ and 3′-ACTGCTGCTCTTCCCGATTC-5′) were designed according to the manufacturer's guidelines and prepared as 11 μM stocks. Trizol-prepared total RNA was treated with RNase-free DNase at 37°C

for 30 min. cDNA samples were prepared using poly dT (Taq-Man kit; Applied Biosystems). For real-time PCR analysis SYBR green fluorescence detection was used for all assays (Applied Biosystems). Each reaction was performed in triplicate and included a panel of primer pairs for the normalization standard, GAPDH [O'Connell et al., 2003].

Proteasome Inhibition

Stable cell lines were seeded in 6-cm culture dishes and allowed to grow to 75–80% confluence in DMEM supplemented with 10% calf-serum. Cells were washed twice with warm PBS and returned to DMEM containing the proteasome inhibitor MG-132 at a final concentration of 1 μg/ml and incubated for 6, 12 or 18 h at 37°C. After incubation cells were carefully harvested by aspirating away supernatant, followed by pooling three PBS washes of the culture dishes. Cells were spun down for 5 min at 1,500 rpm and suspended in RIPA buffer supplemented with Complete Protease Inhibitor Cocktail and 1.25 mg/ml of NEM. Extracts were subjected to two 10-s periods of sonication, assayed for protein concentrations and processed by SDS–PAGE analysis.

RESULTS

Establishment of Stable Cell Lines

We previously reported the isolation of several dominant-negative mutants of the human PP2A catalytic (C) subunit [Lizotte et al., 1999]. Using a yeast complementation assay system, we demonstrated that the α isoform of the human C subunit (Cα) could complement a temperature sensitive (*ts*) mutation in the *S. cerevisiae* PP2A-C gene *pph21*. We showed that H118N and R89A mutants of Cα could not complement the *ts* phenotype and in addition prevented growth under permissive conditions [Lizotte et al., 1999]. To facilitate subsequent analysis, we added amino-terminal FLAG epitope tags to the wild-type and mutant C subunit alleles. Addition of the epitope tag did not change the ability of the wild-type allele to complement the yeast *ts* defect, nor did it alter the dominant-negative phenotypes of the H118N and R89A alleles (data not shown). For expression in mammalian cells, the wild-type (FLAG-Cα+) and dominant-negative (FLAG-CαH118N) mutants were inserted into the retroviral vector pLXSHyg and stably expressed in the Rat-1 fibroblastic cell line (see Materials and Methods). Cells infected with the empty vector were used as controls, and multiple clonal cell lines were established for each construct in the TGR1 background; TGR1 is a normal diploid rat fibroblastic cell line [Prouty et al., 1993].

G1 Phase Growth Defect in Cell Lines Expressing the Dominant-Negative C Subunit

We first assessed exponential phase proliferation rates of cell lines expressing PP2A transgenes. Proliferation of control and Cα+ cell lines was very similar. For four different FLAG-Cα+ cell lines, the average doubling time was 17.2 ± 2.4 h, closely matching two control lines (16.9 and 19.2 h). However, the doubling time of FLAG-CαH118N cells was significantly slower than the doubling times of both control and Cα+ cell lines ($P < 0.05$ and $P < 0.02$, respectively), averaging 23.5 ± 3.8 h with six different lines assayed. We also tested growth rates under limiting serum concentrations (2%). Although all cell lines tested proliferated at reduced rates, FLAG-CαH118N cells showed the slowest growth under these conditions (data not shown).

To determine whether slow growth is specific to a particular phase of the cell cycle, exponentially growing cells were fixed, stained with propidium iodide and subjected to flow cytometry. This analysis revealed that the mutant cultures had a significantly increased content of cells in the G1 phase (50–60% for FLAG-CαH118N cells vs. 30–35% for control cells and 30–40% for FLAG-Cα+; Fig. 1A). Using these FACS-derived cell cycle distributions and the doubling times determined above, we calculated the approximate duration of each cell cycle phase (Fig. 1B). While the S and G2 phases were very similar in all cell lines, the G1 duration was increased in FLAG-CαH118N cells.

We also used BrdU labeling to assess S phase content more accurately. The sensitivity of BrdU incorporation assays allows the unambiguous detection of cells in S phase even with short labeling periods (15–30 min). Thus pulse labeling with BrdU distinguishes S phase cells from those in late G1 or early G2 phase much more accurately than flow cytometry [Schorl and Sedivy, 2003]. In asynchronous, exponentially growing cultures a 30-min pulse with BrdU labeled 60–65% of the cells from control and

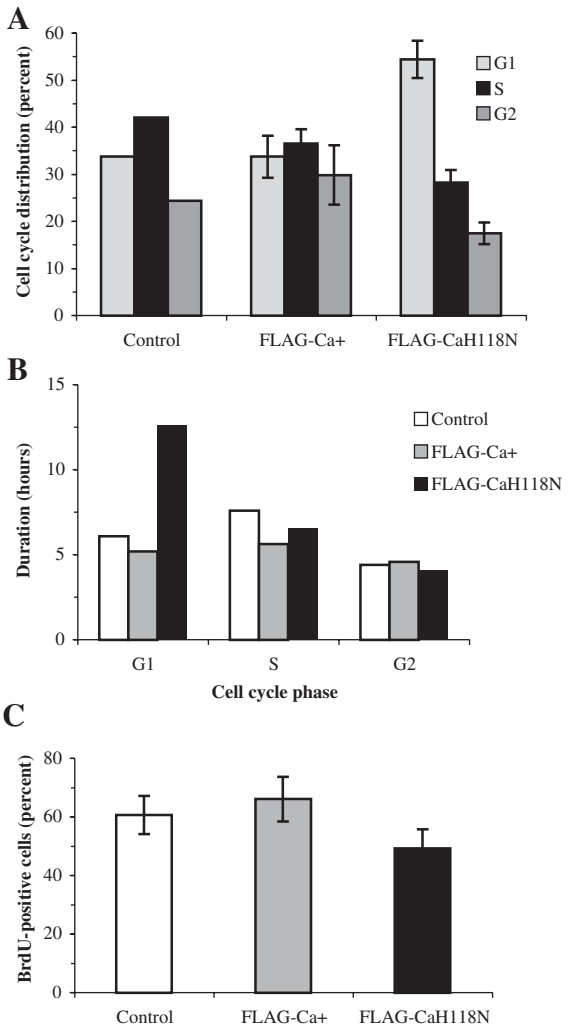

Fig. 1. G1 phase defect of cells expressing mutant PP2A-C. Exponentially growing cell lines were subjected to FACS analysis to determine cell cycle phase distribution (**A**) and phase duration (**B**). Each value shown represents the average for one (Control), two (FLAG-Cα+) or three (FLAG-CαH118N) cell lines assayed in duplicate; error bars are standard deviations. The duration of each cell cycle phase (B) was estimated by multiplying the percentage of cells in that phase by the doubling time of the cell line (FLAG-Cα+ and FLAG-CαH118N lines) or the average doubling time for control lines (control) as determined in cell proliferation assays. Exponentially growing cell lines (one per genotype) were pulse-labeled with BrdU to determine S phase content (**C**). Cells were pulse-labeled for 30 min, harvested immediately and stained for BrdU incorporation (see Materials and Methods). The labeling index denotes BrdU-positive cells as percent of total cells; values shown represent the averages for 10 dishes. The labeling index for FLAG-CαH118N cells was significantly reduced relative to the control ($P < 0.05$) and FLAG-Cα+ ($P < 0.005$) cells. The average total cell number counted per sample was 69 ± 19.

FLAG-Cα+ cell lines, and 50% of the cells from a FLAG-CαH118N cell line (Fig. 1C). Using these values and the doubling times determined above, we calculated S phase durations of 10.4 h for control, 11.3 h for FLAG-Cα+, and 11.7 h for FLAG-CαH118N cell lines. These data confirm that the length of S phase is not significantly affected by expression of FLAG-CαH118N.

To measure the length of G1 phase directly we synchronized cells in M phase by mitotic shake-off and determined their time of entry into S phase by BrdU incorporation. For control and FLAG-Cα+ cell lines, the midpoint of the S-phase transition was reached by 5–6 h after shake-off, and all cells were in S phase by 10–12 h (Fig. 2A,B). However, the mid-point of the transition to S in FLAG-CαH118N cells did not occur until 8 h after shake-off, and the labeling plateau was reached only after 16–20 h (Fig. 2C). These data confirm the increased length of G1 indicated by flow cytometry.

Early and late G1 phase events are demarcated by the restriction point (R), the transition between mitogen-dependent and mitogen-independent cell cycle progression. To define R temporally, cells were synchronized by mitotic shake-off, serum was withdrawn at subsequent time points, and entry into S phase was monitored by BrdU incorporation. R is typically defined as the point at which 50% of the cells in a culture become mitogen independent for entry into S phase. By this measure, the R point was reached in less than 2.5 h in control and FLAG-Cα+ cell cultures, and by 4 h in FLAG-CαH118N cells (Fig. 2A–C). Superimposition of the S phase entry and R point data demonstrated a clear prolongation of the pre-R point interval (M to R) in FLAG-CαH118N cells. These experiments indicate that expression of FLAG-CαH118N results in a significant lengthening of G1 phase, especially during the pre-R interval, while other cell cycle phases are relatively unaffected.

Abnormal Accumulation of Inactive C Subunit Protein

We assayed the levels of C subunit accumulation by immunoblot analysis of exponentially growing cultures of one control, four Cα+, and five CαH118N cell lines. The FLAG epitope tag causes a 1 kDa shift in SDS–PAGE gel migration, distinguishing FLAG-C from the native C subunit proteins, which run at ca. 36 kDa. The abundance of native C and FLAG-C in cells

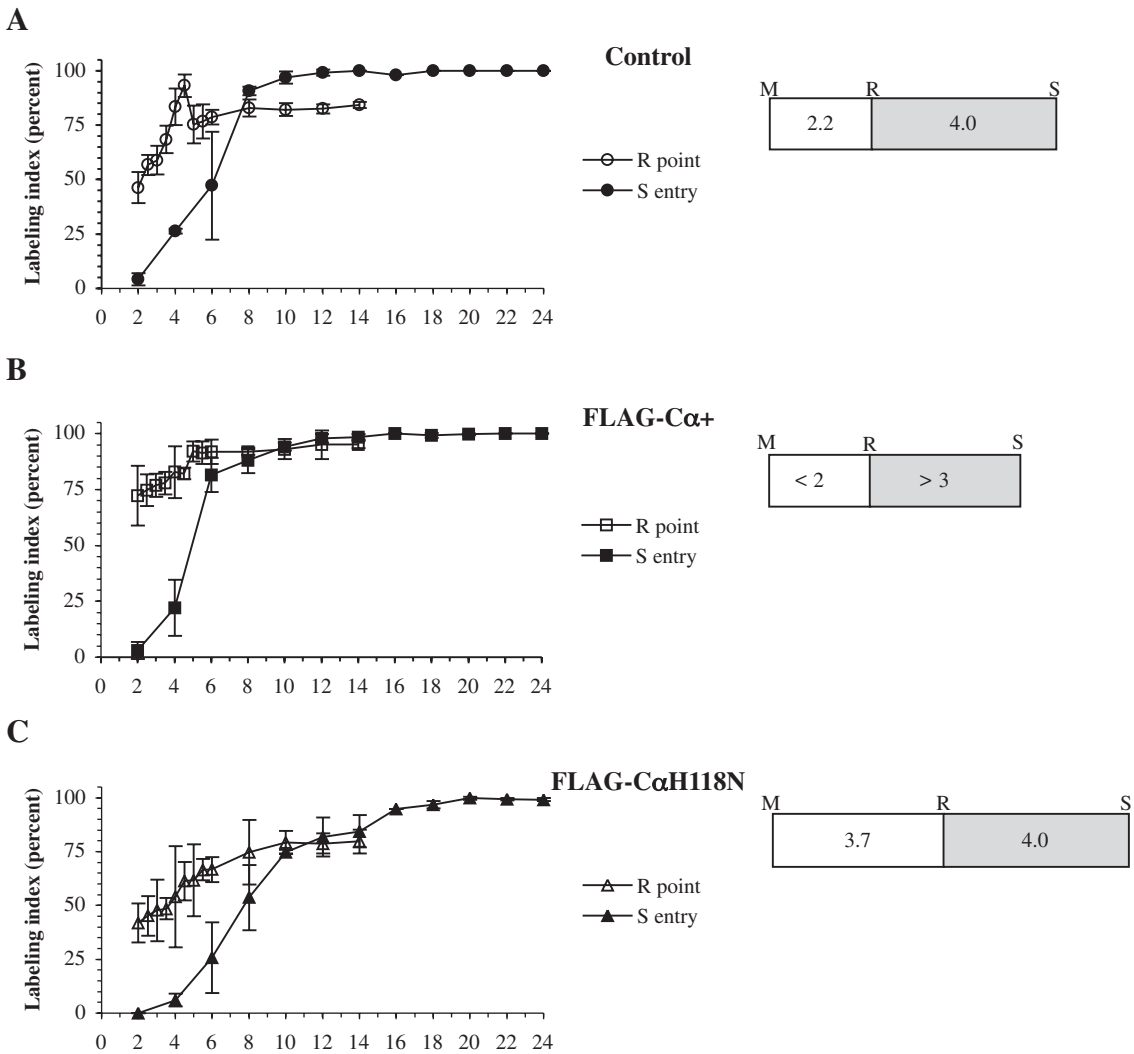

Fig. 2. Determination of S phase entry time and G1 phase restriction point. To determine times of S phase entry (filled symbols), control (**A**), FLAG-Cα+ (**B**) and FLAG-CαH118N (**C**) cells were synchronized by mitotic shake-off and then labeled with BrdU. Labeling reactions were terminated at the time points indicated, and cells were stained for BrdU incorporation (see Materials and Methods). To determine restriction (R) points (open symbols), cells were synchronized by mitotic shake-off and plated in 10% calf serum. BrdU-containing medium was added after 2 h, and serum stimulation was withdrawn at the time points indicated. Incubation in the presence of BrdU continued until the cells were harvested and stained for BrdU incorporation (see Materials and Methods). The labeling index denotes BrdU-positive cells as a percentage of total cells. S phase entry data for each cell line were overlaid with the restriction point data, and the restriction point and S phase entry times were determined. The bars at right show the approximate lengths (in h) of the pre-R and post-R segments of the G1 phase. The average total cell number counted per sample was 81 ± 42.

expressing FLAG-Cα+ was very similar to the abundance of native C subunit in the control cell line (Fig. 3). In contrast, the FLAG-CαH118N protein was present at much higher levels in all cell lines examined. Furthermore, the endogenous C subunit protein in these cell lines accumulated to levels much higher than in the control and FLAG-Cα+ cells (Fig. 3). These data show that the CαH118N protein is expressed at a relatively high level and that the endogenous C subunit accumulates to abnormally high levels in cells expressing this allele.

Our complementation assays in yeast indicated that the CαH118N protein is catalytically inactive [Lizotte et al., 1999]. To verify these results we tested the enzymatic activity of the wild-type and mutant C subunits. Anti-FLAG antibodies were used to pull down the epitope-tagged proteins from mammalian cell extracts, allowing measurement of immunoprecipitated PP2A activity. The activity of immunoprecipitated FLAG-Cα+ was

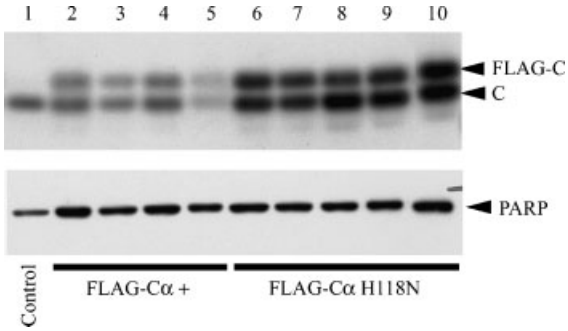

Fig. 3. Accumulation of C subunit proteins. Cell lysates were prepared from cell lines carrying the pLXSH vector (**lane 1**), FLAG-Cα+ (**lanes 2–5**) or FLAG-CαH118N (**lanes 6–10**) and subjected to SDS–PAGE and immunoblotting with polyclonal anti-C subunit antibody or with anti-PARP antibody to control for protein loading.

well above background, while that of the FLAG-CαH118N matched the negative control (Fig. 4A). Similar results were obtained using the same assay system on transiently transfected COS cells (data not shown). These results are consistent with previous reports showing negligible activity for CαH118N and CαH118Q mutant isoforms [Evans et al., 1999; Ogris et al., 1999a].

Experiments with phosphatase inhibitors have indicated that PP2A inhibition can prolong the G1 cell cycle phase [Yan and Mumby, 1999], suggesting that inhibition of endogenous PP2A activity might contribute to the retarded growth of cells expressing CαH118N. We therefore assayed total PP2A activity in extracts isolated from exponentially growing cells expressing

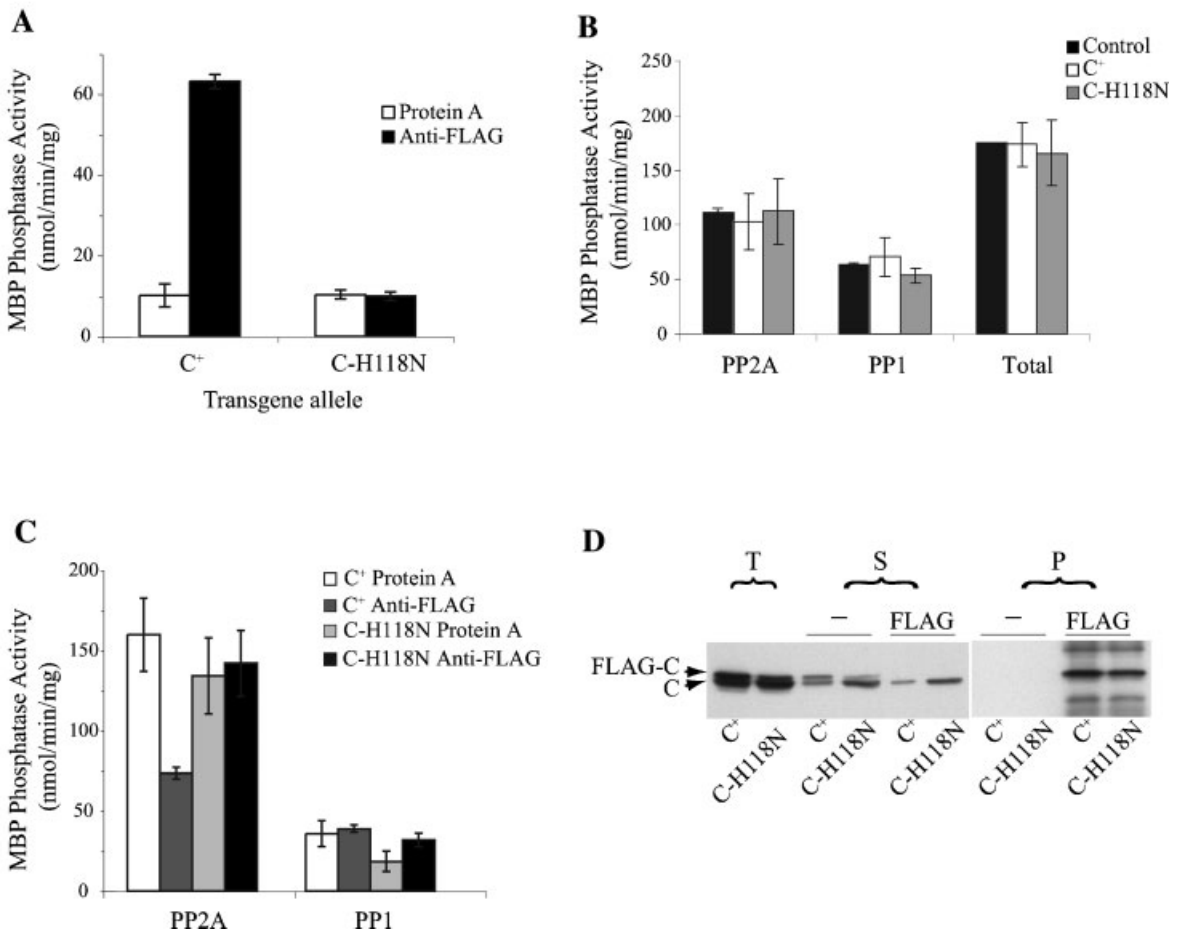

Fig. 4. Phosphatase activity assays. **A**: FLAG-tagged catalytic subunits were immunoprecipitated from cell extracts and assayed for protein phosphatase activity using ^{33}P-labeled myelin basic protein (MBP) as substrate in the absence or presence of 1 μM okadaic acid (OKA). Values shown represent the total OKA-sensitive MBP phosphatase activity. **B**: MBP phosphatase activities from whole cell extracts were measured in the absence or presence of 1 nM or 1 μM OKA to determine PP2A-specific activity. **C**: Supernatants from Protein A or anti-FLAG immunoprecipitation reactions were assayed for MBP phosphatase activities in the absence or presence of 1 nM or 1 μM OKA to determine PP2A-specific activity. **D**: Fractions from the protein A (–) or anti-FLAG (FLAG) immunoprecipitation reactions assayed in panels A and C were subjected to SDS–PAGE and immunoblotting to verify the depletion of FLAG-C in the anti-FLAG supernatants. T, Total extract; S, supernatant fraction; P, pellet fraction.

mutant or wild-type C subunits. Cells were harvested and activity assays were performed using ^{33}P-MBP as substrate in the absence or presence of 1 nM or 1 μM OKA. Both total and PP2A-like (OKA-inhibitable) MBP phosphatase activities were very similar in all cell lines (Fig. 4B). These data indicate that expression of a catalytically active C subunit does not increase total PP2A activity. Furthermore, expression of the inactive H118N subunit does not detectably decrease total PP2A activity. To confirm these results, we determined the total PP2A activity remaining in extracts after immunodepletion of the epitope-tagged C subunits (Fig. 4C,D). As expected, immunodepletion of FLAG-Cα+ clearly decreased the PP2A activity of the lysates, showing that the activity measured in total extracts is a combination of endogenous and transgene products. In contrast, immunodepletion of FLAG-CαH118N did not alter PP2A activity, indicating that activity in the FLAG-CαH118N cell lines is contributed by the endogenous C subunit alone. Thus it is unlikely that the slow growth of cells expressing the H118N protein is due to inhibition of overall PP2A activity in these cells.

Regulatory Subunit Binding by Mutant Catalytic Subunits

Given the relatively high abundance of the CαH118N subunit, competition for PP2A regulatory subunits may explain the slow growth of cells expressing this protein. We used co-immunoprecipitation assays to compare the ability of epitope-tagged wild-type and mutant C subunits to bind regulatory subunits in vivo. Epitope-tagged C subunits were immunoprecipitated from cell lysates with anti-FLAG antibody and processed for immunoblot analysis. The 37 kDa FLAG-C band was observed in the immunoprecipitated pellet fraction as well as the supernatants and the total extracts from cells expressing FLAG-Cα+ and FLAG-CαH118N (Fig. 5A). As expected, no bands were observed in the pellet fraction for the control extracts, indicating that the immunoprecipitation was specific for the FLAG-tagged proteins. Endogenous C subunit bands were observed in the total extract and supernatant fractions for all cell lines tested. We used a monoclonal antibody against the A subunit amino-terminus to assay for co-immunoprecipitation of A subunit proteins. We detected the A subunit in the immunoprecipitated pellet fractions for all

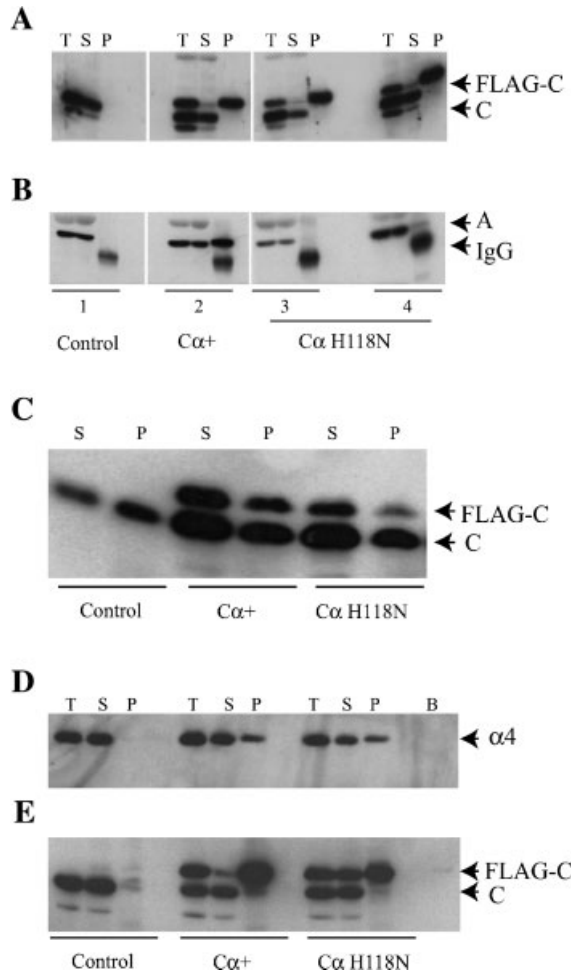

Fig. 5. Interaction of PP2A catalytic subunits with regulatory subunits. **A**,**B**: Cell lysates from a control cell line (**set 1**), FLAG-Cα+ cell line (**set 2**), and FLAG-CαH118N cell lines (**sets 3** and **4**) were immunoprecipitated with anti-FLAG antibody beads and analyzed by SDS–PAGE followed by immunoblotting with anti-C subunit antibody 1D6 (A) and with anti-A subunit antibody 6F9 (B). **C**: Extracts were immunoprecipitated with anti-A subunit antibody (6F9) and protein G-Sepharose beads and immunoblotted with anti-C subunit antibody (1D6). **D** and **E**: Anti-FLAG immunoprecipitates were prepared as described above and subjected to immunoblotting with anti-α4 antibody (D) and with anti-C subunit antibody 1D6 (E). T, Total extract; S, Supernatant fraction; P, Pellet fraction; B, beads alone pellet fraction.

FLAG-Cα+ lines tested (Fig. 5B). However, A subunit protein was present at very low levels or was completely absent in the pellet fraction for the FLAG-CαH118N lines (Fig. 5B and data not shown). Similar results were obtained in co-immunoprecipitation experiments using extracts from COS cells that were transiently transfected with the FLAG-Cα+ and FLAG-CαH118N constructs (data not shown).

Immunoprecipitation assays also were performed using anti-A subunit antibodies, and

immunoprecipitated fractions were assayed for presence of the C subunit via immunoblot analysis (Fig. 5C). Again, the FLAG-Cα+ protein co-immunoprecipitated with the A subunit, while FLAG-CαH118N was present at very low levels in immunoprecipitates. For control, FLAG-Cα+ and FLAG-CαH118N cell extracts, native catalytic subunit protein was observed in the pellet fraction, indicating normal binding of the endogenous C and A subunits (Fig. 5C). These results show that CαH118N binds only weakly to the A subunit, but the mutant subunit does not interfere with A subunit binding to endogenous C subunits.

Reduced A subunit binding by CαH118N indicates reduced ability to act in heterotrimeric complexes. To test binding of the mutant C to another known regulatory subunit, we assayed for co-immunoprecipitation of α4 protein with Cα+ and CαH118N. For both the Cα+ and the CαH118N anti-FLAG immunoprecipitates, a band corresponding to α4 was observed in the total extract, supernatant and pellet fractions (Fig. 5D). No α4 band was observed in the pellet fraction from control cell extracts. Comparison of the immunoprecipitated pellet fractions suggests that the CαH118N subunit binds α4 at least as well as Cα+ does (Fig. 5D,E). This demonstrates that the H118N mutant C subunit is not generally defective in binding interactions.

Transgene mRNA Abundance is Variable

The striking difference in C subunit protein levels that we observed in our CαH118N cell lines suggested that regulation of C subunit accumulation is impaired. We performed RNA blot analysis to determine whether increased C subunit protein correlates with increased mRNA accumulation. Total cellular RNA was isolated from three FLAG-Cα+ lines, three FLAG-CαH118N lines and three control lines, including the parental TGR1 lines. A coding sequence probe that hybridized to the expected 1.8 kb native C subunit transcript also recognized an abundant transcript of 3.0 kb in RNA extracted from FLAG-Cα+ and FLAG-CαH118N cell lines (data not shown). Quantification of hybridization signals (normalized against a GAPDH probe) showed that that transgene transcript abundance is high and variable in both the FLAG-Cα+ and FLAG-CαH118N lines (Fig. 6A). Furthermore, transcript abundance did not correlate with protein

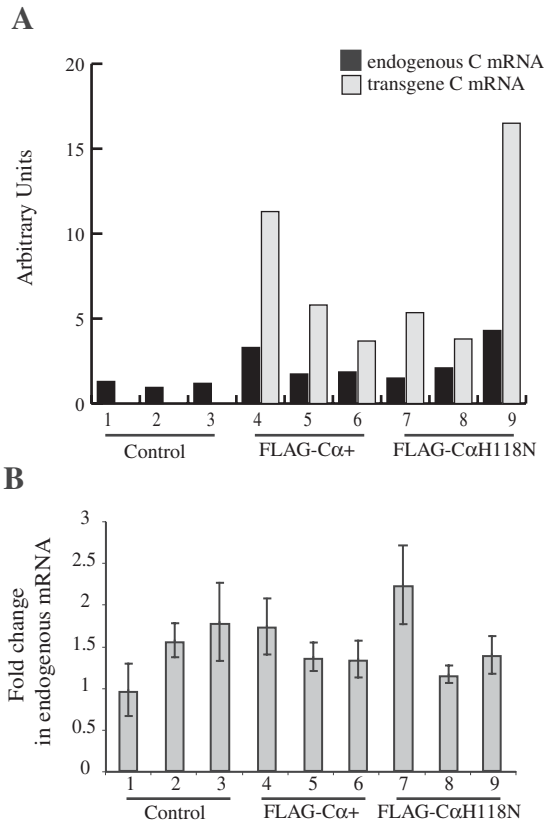

Fig. 6. Abundance of transgene mRNA products. Total RNA was prepared from confluent 15cm dishes of control, FLAG-Cα+ and FLAG-CαH118N cell lines and subjected to RNA blot (**A**) and real-time PCR (**B**) analysis. A ^{32}P-labeled C subunit probe was used to detect mRNA for both the endogenous and transgene messages, and the filter was also hybridized with a probe for GAPDH. A: Quantitation of C subunit hybridization signals, normalized to the GAPDH signal using a phosphorimager. Black bars, endogenous C subunit mRNA; gray bars, transgene mRNA. B: Real-time PCR results using a primer set designed to recognize only the endogenous catalytic subunit message. All reactions were performed in triplicate. Error bars represent standard deviations.

levels (compare Fig. 6A and Fig. 3). To determine whether expression of the transgene altered accumulation of endogenous C subunit message, we employed real-time PCR analysis using a primer set specific for the 3′ UTR region of the endogenous form of the message (see Materials and Methods). Two independent real-time PCR analyses were performed and both experiments showed that native C mRNA levels were variable but similar in control, Cα+ and CαH118N cell lines (Fig. 6B and data not shown). There was no correlation between mRNA level and transgene allele present in these cell lines.

Synthesis and Turnover of PP2A Catalytic Subunit Proteins

Protein accumulation might be increased in FLAG-CαH118N cell lines by accelerating the rate of C subunit synthesis or by decreasing turnover rate. To address the former hypothesis, we measured the rate of C subunit synthesis using metabolic labeling. Cultures were starved to deplete their endogenous amino acid pools and subsequently fed medium containing ^{35}S-labeled methionine and cysteine. Cells were harvested at time points every 20 min for a total of 2 h. The ^{35}S-labeled cell lysates were immunoprecipitated with anti-C subunit antibodies and processed by SDS–PAGE. Phosphorimaging was used to quantitate the amount of radioactive signal in each band. As expected, one band corresponding to the endogenous C subunit was observed in control lines while two bands were observed in the FLAG-Cα+ and FLAG-CαH118N lines. Immunoblot analysis confirmed that the observed bands were the PP2A catalytic subunits (data not shown). For the endogenous C subunit protein, similar synthesis rates were observed in all three lines (Fig. 7). However, both transgenic proteins incorporated label

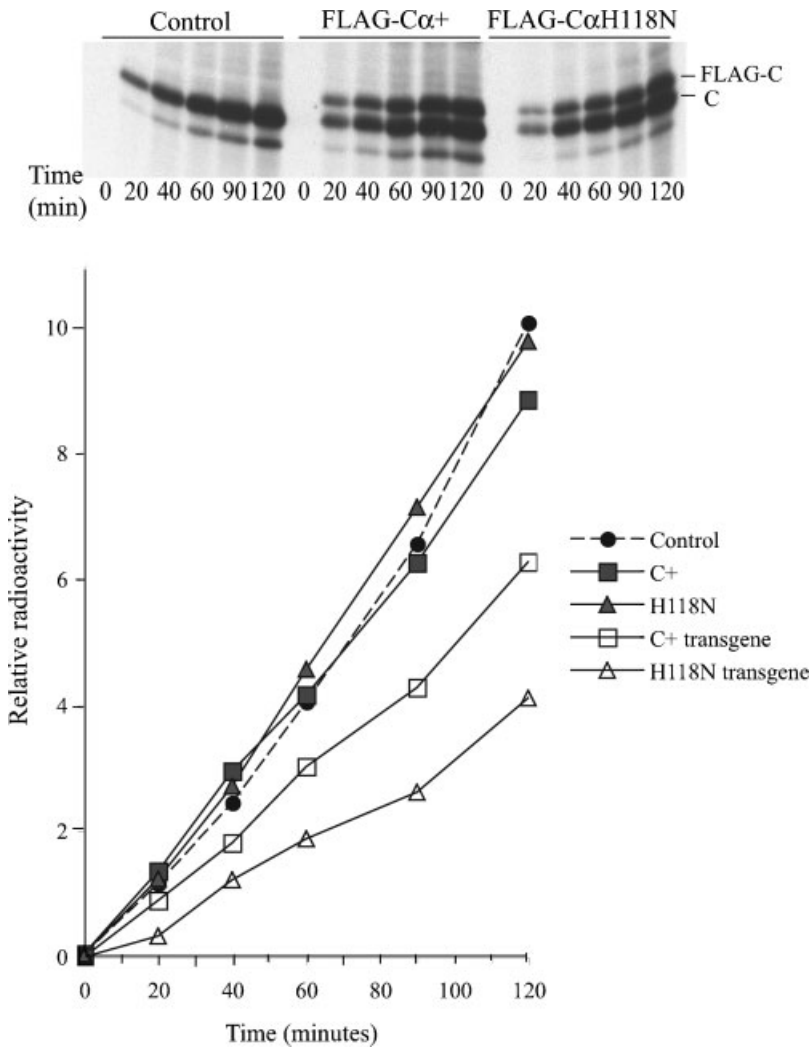

Fig. 7. Catalytic subunit synthesis rates. Cells were seeded in 6-cm tissue culture dishes and allowed to settle for 24 h. Cells were incubated in the presence of ^{35}S-labeled methionine and cysteine, then harvested and lysed at the indicated time points. C subunits were immunoprecipitated and subjected to SDS–PAGE (see Materials and Methods). Rates of radioactive decay in each band were quantitated using a STORM imaging system. Control cell line, circles; FLAG-Cα+ line, squares; FLAG-CαH118N lines, triangles; endogenous C, filled symbols; FLAG-C, open symbols.

more slowly than the endogenous protein, suggesting less efficient synthesis or instability of the transgene product. Less efficient synthesis of transgene-encoded C subunits was observed in experiments using a transiently transfected wild-type PP2A gene under the control of a murine leukemia virus (MLV) promoter [Baharians and Schönthal, 1998]. Transcripts driven from this construct were under-represented in the polysome-associated mRNA population, suggesting less efficient translation of the transgene mRNA [Baharians and Schönthal, 1998]. Our results are consistent with a translational mechanism that limits C subunit protein synthesis even in the presence of elevated mRNA levels; however, they do not explain the increased C subunit levels observed in our CαH118N cell lines.

To determine whether regulated protein turnover might contribute to C subunit protein accumulation phenotypes, pulse-chase experiments were performed using FLAG-Cα+, FLAG-CαH118N and control cell lines. These experiments followed a similar procedure to that used in the rate of synthesis analysis, except that time points were taken every 6 h for a total of 30 h (Fig. 8). The C subunit half-life observed in control cells was 13 h, while the endogenous C subunit in the FLAG-Cα+ line turned over faster, with a half-life of 5–6 h. As in the rate of synthesis experiments, the transgene C subunit proteins labeled poorly. However, the apparent half-life of the FLAG-Cα+ protein was 7 h, closely matching that of the endogenous protein in these cells. These data suggest that expression of the wild-type transgene accelerates turnover. In contrast, the half-life of the endogenous C subunit protein in the FLAG-CαH118N cell line was much longer (21 h), indicating that the rate of turnover is slower. The FLAG-CαH118N protein half-life was 9–10 h, suggesting that turnover of the FLAG-CαH118N protein is slightly slower than that of the FLAG-Cα+ protein. More strikingly, expression of the mutant C subunit isoform appears to retard turnover of the endogenous C subunit. These results support the hypothesis that accumulation of the PP2A catalytic subunit is tightly regulated, and that C subunit activity contributes to its regulation [Baharians and Schönthal, 1998].

To ascertain whether C subunit turnover is influenced by activity of the proteasome, cells were cultured in the presence of the proteasome inhibitor MG132 for 6, 12 or 18 h. Protein extracts were harvested in the presence of NEM to inhibit the hydrolysis of conjugated ubiquitin moieties. Immunoblot analysis with an antibody specific for the catalytic subunit revealed the accumulation of more slowly migrating C subunit species in cells treated with MG-132 (Fig. 9). In control cell extracts a single major band at ca 44 kDa was detected, while extracts from cells carrying FLAG-C transgenes exhibited a second major band at 46 kDa. The apparent molecular weights of these bands were consistent with the predicted sizes of mono-ubiquitinated forms of C and FLAG-C proteins, and the intensities of the lower mobility bands increased with longer exposure to the MG132 drug. These data indicate that proteasome inhibition leads to accumulation of modified forms of both the endogenous and transgene C subunits.

DISCUSSION

Regulation of PP2A expression and activity has proven to be a key aspect of its cellular versatility, yet this regulation has posed obstacles in the analysis of PP2A functions. In this study we report the establishment of fibroblastic cell lines that stably express either an epitope-tagged wild-type human catalytic subunit (FLAG-Cα+) or an epitope-tagged active site mutant (FLAG-CαH118N) [Lizotte et al., 1999]. Although transgene expression in these cell lines is driven by a strong viral promoter and transgene mRNA levels are high, cells carrying the wild-type transgene express only a modest amount of the epitope-tagged C subunit protein. Cells carrying the mutant transgene, however, exhibit higher transgene accumulation levels, and also exhibit an increase in endogenous catalytic subunit protein levels. Our data suggest that although the C subunit is a relatively stable protein, its accumulation is regulated at the level of turnover. Enzyme activity assays show that both Cα+ and CαH118N cell lines exhibit total PP2A activities matching that of the parental cell line. Immunodepletion data show that while epitope-tagged Cα+ contributes to the active PP2A pool, the CαH118N allele does not affect activity of the endogenous PP2A population either positively or negatively (Fig. 4). These observations suggest that the mechanisms limiting C subunit accumulation and activity

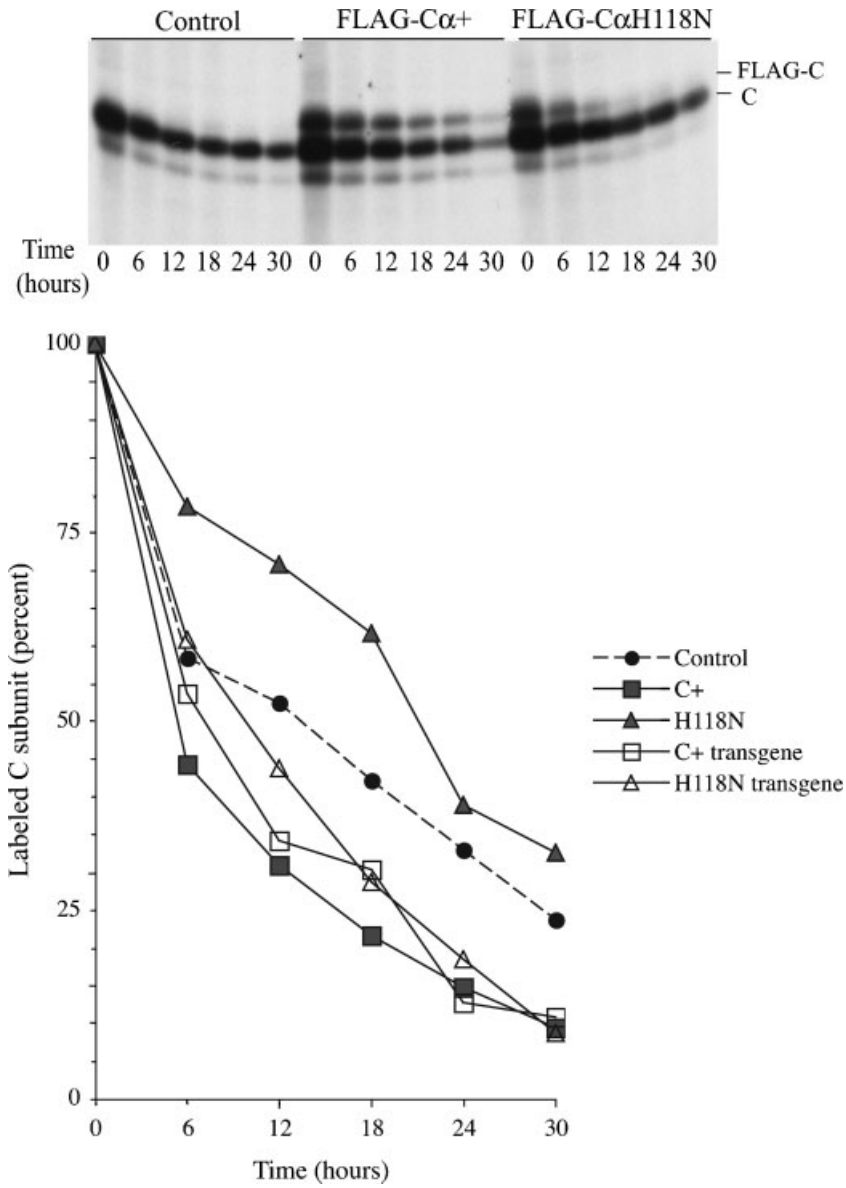

Fig. 8. Catalytic subunit protein turnover. Cells were seeded in 6-cm tissue culture dishes, labeled for 2 h in medium containing ^{35}S-methionine and cysteine, then at timepoint zero washed and shifted into unlabeled medium. Cells were harvested and lysed at the time points indicated, and C subunit proteins were immunoprecipitated and subjected to SDS–PAGE (see Materials and Methods). Labeling of each C subunit protein at time zero was defined as 100%, and labeling at subsequent timepoints was calculated relative to this value. Disappearance of the labeled band was quantitated using a STORM imaging system. Control cell line, circles; FLAG-Cα+ line, squares; FLAG-CαH118N lines, triangles; endogenous C, filled symbols; FLAG-C, open symbols.

contribute to a homeostatic system maintaining stable overall PP2A function under normal growth conditions. Even when C subunit accumulation is increased (as in cells expressing the CαH118N allele), overall PP2A activity levels remain constant.

The lack of correlation between transgene mRNA and protein product levels, and the poor labeling of both wild-type and mutant FLAG-C proteins are consistent with the previously documented translational regulatory mechanism [Baharians and Schönthal, 1998]. However, this mechanism fails to explain the differential protein accumulation phenotypes of the Cα+ and CαH118N cell lines. Increased accumulation of the H118N mutant C subunit also was observed in cultured insect cells; however, effects on endogenous C subunit protein accumulation and

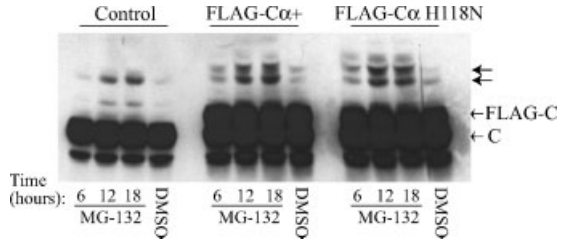

Fig. 9. The effect of proteasome inhibition on C subunit accumulation. Control, FLAG-Cα+ and FLAG-CαH118N were incubated with the proteasome inhibitor MG-132 or with DMSO for up to 18 h. Extracts were harvested and subjected to immunoblotting with anti-C subunit antibody (see Materials and Methods). MG-132 treatment leads to the appearance of additional C subunit bands (arrowheads). One band is observed in control lines and two bands are observed in the FLAG-C cell lines, and the intensity of these bands increases with longer treatment times.

activity were not assayed in that study [Myles et al., 2001]. The rates of synthesis of native C subunits are unchanged in the transgenic cell lines, explicitly arguing against a translational mechanism for the accumulation phenotypes of these cells. Instead, our data suggest that protein accumulation is regulated at the level of turnover, which is faster in cells expressing the wild-type allele and slower in cells expressing the H118N allele. While only minor differences in C subunit half-lives were observed in mass cultures of control and PP2A-transfected NIH-3T3 cells [Baharians and Schönthal, 1998], a later study showed that ubiquitin-mediated proteolysis is a key determinant of C subunit protein levels [Trockenbacher et al., 2001]. Retarded turnover of endogenous C subunits does not increase enzyme activity levels in CαH118N cell lines, indicating that 'excess' endogenous C subunits may be subject to inhibitory modification or interaction with inhibitory factors.

The dominant-negative phenotype of the H118N mutant in yeast cells had suggested that the mutant C subunit might compete for regulatory A subunit binding [Lizotte et al., 1999]. Regulatory A subunit binding by an H118Q mutant was found to be variable [Ogris et al., 1999a,b]. Transiently expressed HA-tagged H118N also interacts with the A subunit, although binding may be weaker [Prickett and Brautigan, 2004]. We also have observed variability; however, reciprocal co-immunoprecipitation data shown here, as well as additional data from immunoprecipitation experiments using yeast and COS cell extracts, demonstrate that CαH118N binding to endogenous A subunits is weaker than binding by wild-type C. Furthermore, cells expressing the CαH118N mutant show no decrease in A subunit binding by the native C subunit protein. We conclude that the mutant C subunit neither competes for A subunit binding nor interferes with A subunit binding by the endogenous C subunits. The recently determined crystal structures of PP2A suggest that the H118N mutation may indirectly impair interaction with the A subunit. H118, a known active site residue, lies in a loop between a surface residue (R110) that is involved in interaction with the A subunit [Xing et al., 2006; Xu et al., 2006; Cho and Xu, 2007] and a short motif (including residues R121, Q122, and Q125) that forms an interface with the B subunit [Xu et al., 2006; Cho and Xu, 2007]. The H118N substitution may partially disrupt the A subunit contacts made by R110. Indeed mutations at positions R110, R121, and Q122 all distort the catalytic site and reduce enzymatic activity [Prickett and Brautigan, 2004], suggesting that changes in this region affect both catalysis and regulatory subunit binding.

Weak A subunit binding does not indicate a gross conformational abnormality, since the mutant protein binds the α4 regulatory subunit at normal levels (Fig. 5D and Prickett and Brautigan [2006]). Indeed, the H118Q mutant binds the PME-1 methylesterase more stably than the wild-type protein does [Ogris et al., 1999b]. Furthermore, PME-1 co-purifies with an inactive form of the wild-type C subunit; the inactive C subunit can be reactivated by treatment with the PP2A phosphatase activator (PTPA) [Longin et al., 2004], a regulatory factor that plays a key role in controlling PP2A conformation and activity [Fellner et al., 2003; Chao et al., 2006; Jordens et al., 2006; Leulliot et al., 2006]. The conformation of active site mutants like H118Q and H59Q may resemble that of inactive C subunits in a dynamic and physiologically significant population that participates in an inactivation/reactivation cycle [Fellner et al., 2003; Longin et al., 2004; Chao et al., 2006; Jordens et al., 2006; Leulliot et al., 2006]. If this cycle is also linked to turnover of inactive species, non-productive interactions with the mutant protein might contribute to increased accumulation of inactive endogenous C subunits in cells expressing the CαH118N protein.

Cells expressing the CαH118N allele display a growth delay phenotype in the G1 phase of the cell cycle. Several cell cycle phase-specific roles of PP2A have been identified, including roles in the regulation of G1 cyclin-dependent kinases, initiation of DNA replication, entry into mitosis and segregation of chromosomes [Lee et al., 1991; Mumby and Walter, 1993; Yan and Mumby, 1999; Chou et al., 2002; Prickett and Brautigan, 2004; Dai and Wang, 2006]. Inhibition of PP2A by OKA treatment causes hypophosphorylation of Rb in Balb/c 3T3 cells [Yan and Mumby, 1999]. We have assayed for, but do not detect, hypophosphorylation of Rb and Rb family members in our CαH118N cell lines (D. Lizotte and A. DeLong, unpublished work). This result is consistent with our observation that expression of CαH118N does not inhibit overall PP2A activity—the growth delay in this case is not caused by reduced PP2A activity, and it is not mediated by the same mechanism. Instead, we propose that the slow growth phenotype of cells expressing CαH118N stems from the mutant protein's aberrant interactions with factors that are required for normal progression. For instance non-productive interaction with components of the ubiquitin-mediated proteolysis system may retard transit through G1 by interfering with proteolysis of a cell cycle regulator; normal G1 progression requires an active proteasome system [DeSalle and Pagano, 2001]. Alternatively, the CαH118N protein may stably bind a regulatory protein or a substrate molecule that is required in G1. The stability of the PME-1 interaction with the H118Q C subunit mutant provides a precedent for this model.

ACKNOWLEDGMENTS

We thank T. Serio, J. Singer and P. Gruppuso for comments on the manuscript, and J. Sedivy and K. Yeung for helpful discussions and advice. We thank D. Brautigan, R. Ruediger, G. Walter, D. Chatterjee, J. Wyche, B. O'Connell and J. Sedivy for gifts of antibodies and primers, and M. Grossel for help with phosphorimaging. We gratefully acknowledge the Singer and Sedivy laboratories for generously sharing reagents and ideas. This work was supported by grants from the NSF (IBN-9986017 and IOB-0446039), the US Department of the Army (DAMD17097-1-7113) and NIH-NCRR (RR15578). DLL was partially supported by National Institutes of Health Predoctoral Training Grant GM-07601. AS was partially supported by a UTRA fellowship.

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