Differential Methylation and Altered Conformation of Cytoplasmic and Nuclear Forms of Protein Phosphatase 2A During Cell Cycle Progression

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Abstract. Protein phosphatase 2A (PP2A) appears to be involved in the regulation of many cellular processes. Control mechanisms that lead to the activation (and deactivation) of the various holoenzymes to initiate appropriate dephosphorylation events remain obscure. The core components of all PP2A holoenzymes are the catalytic (PP2Ac) and 63–65-kD regulatory (PR65) subunits. Monospecific and affinity-purified antibodies against both PP2Ac and PR65 show that these proteins are ubiquitously localized in the cytoplasm and the nucleus in nontransformed fibroblasts. As determined by quantitative immunofluorescence the core subunits of PP2A are twofold more concentrated in the nucleus than in the cytoplasm.

Detailed analysis of synchronized cells reveals striking changes in the nuclear to cytoplasmic ratio of PP2Ac-specific immunoreactivity albeit the total amounts of neither PP2Ac nor PR65 in each compartment alters significantly during the cell cycle. Our results imply that differential methylation of PP2Ac occurs at the G0/G1 and G1/S boundaries. Specifically a demethylated form of PP2Ac is found in the cytoplasm of G0 cells, and in the nucleus of S and G2 cells. In addition nuclear PP2A holoenzymes appear to undergo conformational changes at the G0/G1 and G1/S boundaries. During mitosis PP2A is lost from the nuclear compartment, and unlike protein phosphatase 1 shows no specific association with the condensed chromatin.

Protein phosphatase 2A (PP2A), one of the four major phosphoserine/threonine phosphatases, has been implicated in the modulation of many events including metabolic regulation (reviewed in reference 10), cell cycle progression (8, 16, 21, 31, 35, 41, 57), DNA replication (58), transcription (3, 59), and protein translation (4, 46). Along with this increasing evidence implicating PP2A in the regulation of all stages of membrane-to-nuclear signal transduction, PP2A has been shown to modulate both protein kinase and phosphatase activity (8, 20, 53). In addition, a possible role for PP2A in cellular transformation became evident when the dimeric holoenzyme of PP2A was identified to be one of the targets for the transforming activities of certain papovaviruses (reviewed in reference 45).

It is widely thought that protein phosphatase catalytic subunits exercise regulatory flexibility and differential substrate specificity through specific association with a variety of regulatory proteins (25, 39). There is no evidence that regulatory subunits are shared between more than a single class of catalytic subunits (5). This process of differential association gives rise to an extensive subset of oligomeric holoenzymes which is especially striking in the case of PP2A. A dimeric core holoenzyme consisting of a 36-kD catalytic subunit (PP2Ac) and a constant 65-kD regulatory subunit (PR65) systematically copurify. Additionally, other regulatory subunits with molecular masses of 54, 55 (PR55), 72 (PR72), and 74 kD have been described to be variably associated with this "core" dimer (reviewed in 39, 51). These different regulatory subunits are sufficient to modulate the specificity of PP2Ac in vitro and presumably in vivo (2, 15, 28). Molecular cloning has revealed an even greater complexity since for each subunit cloned to date more than one isoform has been shown to exist (21–23, 29, 38).

Recent data has shown that PP2A activity is further regulated by posttranslational modification of the catalytic subunit. Receptor and non-receptor tyrosine kinases were found to inactivate PP2Ac by phosphorylation of Tyr307 in vitro and in cultured cells (6, 7). Furthermore, inactivation of PP2A by phosphorylation on threonine residues has recently been reported (18). However, PP2A appears not only to integrate signals within phosphorylation cascades, but also to be the focal point of a distinct posttranslational modification...
system, namely reversible protein methylation. The carboxyl-terminal leucine of PP2Ac is the major substrate for a recently described, new type of methyltransferase (type IV) in vitro (34, 61) and in cultured cells (13).

The involvement of PP2A in the control of cell cycle progression, in particular its role during mitosis, has recently been revealed by biochemical and genetic studies. Several laboratories have reported a role for PP2A at the G1/M transition in particular in negatively regulating the p34cdc2 protein kinase (16, 35). Moreover, in Drosophila, the PR55 regulatory subunit of PP2A was found to be essential to the metaphase–anaphase transition (41). A role for PP2A during S phase has been suggested by studies on DNA replication using the papovavirus SV40. Indeed, in order to initiate replication the large T antigen needs to be dephosphorylated by an S-phase-specific form of PP2A (37, 58).

To delineate the roles of specific PP2A holoenzymes and their regulation, we have examined the localization of PP2A core enzyme subunits during the cell cycle of mammalian fibroblasts using a combination of immunofluorescence and biochemical techniques. PP2Ac and the constant regulatory subunit, PR65, were both found at high levels in the cytoplasm and the nucleus. However, the ratio of nuclear to cytoplasmic immunofluorescence intensity of PP2Ac varied significantly in a cell cycle-dependent manner. This observation appears to be attributable, at least in part, to epitope masking brought about by differential methylation and conformational changes in the PP2A holoenzyme(s).

Materials and Methods

Preparation of Antisera

Peptides were obtained from Multiple Peptide System, Cambridge Research Biochemicals Inc. (Wilmington, DE) or were synthesized on a Pep-Tech synthesizer 9050 (Milligen/Biosource, Burlington, MA). All antisera were prepared in rabbits as previously described (24). If not stated differently the IgG fraction of all antisera was routinely isolated on protein A-Sepharose 4B (Pharmacia LKB Nuclear, Gaithersburg, MD) using standard procedures (19). Anti-PP2Ac antibodies: AbC108 were generated against recombinant PP2Ac. The a-isoform of the catalytic subunit (PP2Ac-a) was expressed in E. coli (strain JM109/DE3) using the T7 promoter driven expression plasmid pRK172 (43), and inclusion bodies were isolated as described previously (24). Subsequently, the protein was solubilized in 8 M urea, 30 mM Tris/Cl, pH 7.5, 1 mM EDTA, 10 mM DTT followed by extensive dialysis against TBS. The solubilized protein was more than 80% pure and ~250 μg was used for each injection. Monospecific AbC108 were purified using immunoaffinity, recombiant PP2Ac-a as previously described (24).

Anti-PR65 antibodies: a peptide corresponding to amino acids 177 to 196 of the PR65 (22) was used to prepare Ab195177/196. Recombinant human PR65α, expressed in JM109/DE3 using pRK172 (43) as previously described (24), was used to generate Ab195177/196. The soluble PR65α protein was purified to homogeneity by several chromatographic steps (24). Approximately 100 μg of this preparation was used for each injection. Immobilized, recombinant PR65α was further used for the affinity purification of Ab195177/196 and Ab195177/196 as previously described (24).

Cell Culture and Microinjection

Rat embryo fibroblasts REF-52 (42) and human fibroblasts HS68 (CRL-1365) were cultured as described elsewhere (33). Cells were subcultured 2–3 d before use onto either 25-mm-diam glass coverslips (Schott Laborteknik, Göttingen, Germany) for microinjection studies, or 12-mm acid-washed glass coverslips (Schott Labor) were obtained by 36–48 h of serum deprivation and refeeding (33). Cells were injected in PBS, blocked with 0.2% BSA in PBS, and incubated with anti-PP2A antibodies at 37°C for 40–60 min. In competition experiments the diluted antibodies were preincubated for 30 min with 100 μM of the corresponding peptide or, for AbC108, with ~0.1 nM of PP2Ac protein isolated from rabbit skeletal muscle (54). Following incubation with the primary antibody cells were washed in PBS containing 0.2% BSA and then incubated for 30 min with biotinylated goat anti-rabbit antibodies (Amersham Corp., Arlington Heights, IL) diluted 1:100. Subsequently, a primary-secondary antibody complexes were visualized using streptavidin coupled to Texas Red (Amersham) at a dilution of 1:200 (20 to 30 min). Microinjected cells were identified by containing with FITC conjugated anti-mouse antibodies (Organon Tecknika, Rockville, MD) diluted 1:20. Cells were stained for DNA using Hoechst 33258 (1 μg/ml bisbenzimid) or 4,6-diamidino-2-phenylindole (0.1 μg/ml), mounted, and photographed as described elsewhere (33).

Fluorescence measurements were made as follows: cells were fixed and stained as described above. Afterwards, images were acquired on a Leica CLSM without changing the pinhole size, laser power or the photomultiplier sensitivity. The latter were set such that the brightest image values were within the maximum threshold level (12 bit). Subsequently, the internal cellular fluorescence in the cytoplasmic or the nuclear compartments was calculated using Imagic fluorescence sensitivity software (developed by Ned J. C. Lamb, details upon request) on a Silicon Graphics Indigo 2 workstation.

Cell Fractionation

After washing in PBS once, cells were allowed to swell on ice in buffer A (10 mM Hepes–NaOH, pH 7.5, 10 mM KCl) for 10 min. All subsequent steps were performed at 0–4°C. The cells were scraped into cell extraction buffer A consisting of buffer A supplemented with 1 mM DTT, 1 mM EDTA, 1.5 mM MgCl2, 25% glycerol, and a complex mixture of protease inhibitors (0.1 mM tosyl-L-phenylalanine-chloromethylketone, 0.1 mM tosyl-L-lysine-chloromethylketone, 3 μM pepstatin A, 2 μg/ml aprotinin, 1 mM benzamidine, 4 μM leupeptin [all Fluka Chem. Corp., Ronkonkoma, NY] and 0.5 mM PMSF [Boehringer Mannheim Corp., Indianapolis, IN]). Cell lysis was achieved either by the addition of NP-40 to a final concentration of 0.1% and 10s vortexing, or alternatively by passing the cell suspension through 18-gauge and five times through 22-gauge needles. Interestingly, the use of NP-40 yielded less PP2A in the nuclear fraction, suggesting that, even low concentrations (0.1%) of nonionic detergents cause leaking of PP2A from the nuclear compartment. Completion of cell lysis was monitored by phase contrast microscopy and was always ~99%. After washing in PBS once, cells were allowed to swell on ice in buffer B resuspended in buffer B and resedimented at 20,000 g. The pellet was resuspended in nuclear extraction buffer C (25 mM Hepes–NaOH, pH 7.5, 300 mM KCl, 1 mM DTT, 1 mM EDTA, 1.5 mM MgCl2, 25% glycerol, and the above described mixture of protease inhibitors). The suspension was rocked for 30 min and pelleted by centrifugation at 20,000 g. The resulting supernatant represented the soluble cytoplasmic fraction.

When the methylation status of PP2Ac was studied, PMSF was added.
Table I. Polyclonal Antibodies Were Raised against the Indicated Antigen as Described in Materials and Methods

| PP2A Subunit          | Antibody name       | Antigen                                                                 |
|-----------------------|---------------------|-------------------------------------------------------------------------|
| Catalytic subunit     | AbC<sup>709/306</sup> | 20<sup>H</sup>HVR<sup>T</sup>RTPDYFL<sup>109</sup> (carboxyl-terminal peptides, common to α- and β-isoforms) |
| (PP2Ac)               | AbC<sup>203/306</sup> | 30<sup>R</sup>RTPDYFL<sup>309</sup> Human, recombinant PP2Ac-α            |
|                       | AbC<sup>recomb</sup> |                                                                         |
| 65-kD regulatory subunit | Ab65<sup>171/196</sup> | 17<sup>D</sup>TTPMVRRAAASKLGEFKVL<sup>196</sup> (internal peptide, common to α- and β-isoforms) |
| (PR65)                | Ab65<sup>recomb</sup> | Human, recombinant PR65α                                                 |

* Numbers at the beginning and the end of peptide sequences refer to their position within human PP2Ac-α (55) and PR65α (22).

Blotting onto Immobilon-P membrane (Millipore Corp., Bedford, MA) was carried out at 15 V constant voltage in a Trans-Blot (Bio-Rad Labs.) semi-dry electrophoretic transfer cell in 25 mM Tris, 192 mM glycerol, 20% methanol plus 0.1% SDS when the carboxyl-terminal modification was studied. Immunoblotting was performed as described before (24) using 5% skimmed milk or 1% BSA in PBS containing 0.2% Triton X-100 and 0.1% Tween 20. Competition experiments were performed as described above for immunofluorescence. To detect primary antibodies, peroxidase coupled donkey anti-rabbit IgG (Amersham) were used at a dilution of 1:2,000 as second antibodies, followed by analyses using Phospholmage and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Alternatively, fluorographs, exposed in the linear range of the film, were densitometrically scanned.

Results

PP2Ac and PR65 Are as Abundant in the Nuclei as in the Cytoplasm of Mammalian Fibroblasts

We have generated and characterized several antisera against PP2Ac and the constant regulatory subunit, PR65. Their main features and nomenclature are summarized in Table I. The anti-PP2Ac antisera comprised the two previously de-
Figure 2. Immunocytochemical localization of PP2A subunits in REF-52 cells. REF-52 cells were grown on glass coverslips and formalin fixed. Cells were stained for PP2Ac using either AbC299/309 (A and B) or AbC recom. (C and D), and for PR65 using Ab65173/196 (E and F) as primary antibodies. In B, D, and F the antibodies were preincubated with their corresponding antigen before immunofluorescence. The resulting images were exposed and printed identically. The arrowhead in C indicates a representative nucleus with low immunostaining. Bar, 5 μm.
scribed peptide-specific antibodies, AbC\textsuperscript{299/309} (13, 24) and AbC\textsuperscript{307/309} (56). Both these anti-carboxyl-terminal antibodies gave identical results throughout and are therefore referred to as AbC\textsuperscript{299/309}. Additional antisera were generated against recombinant PP2Ac, produced, and partially purified from \textit{E. coli} (AbC\textsuperscript{recomb}). Initial studies examined the specificity of the different antisera on immunoblots of total REF-52 cell extracts. All anti-PP2Ac antisera exclusively recognized a doublet at \textasciitilde36 kD which comigrated with PP2Ac purified from rabbit skeletal muscle (Fig. 1 A). The signal of the peptide-specific antisera was competed by the corresponding peptide, as was the signal for the anti-recombinant antibody AbC\textsuperscript{recomb} using PP2Ac purified from rabbit skeletal muscle (data not shown).

In the light of the extensive conservation between serine/threonine protein phosphatase catalytic subunits (11), we carried out a number of control experiments to confirm the specificity of our antisera for PP2Ac. In conclusion neither AbC\textsuperscript{recomb} nor AbC\textsuperscript{299/309} exhibited significant cross-reactivity with recombinant PPlc or PPX, the closest relatives to PP2Ac. In addition, two-dimensional gel electrophoresis of extracts prepared from mammalian cultured cells followed by immunoblotting resulted in an identical pattern whatever antisera against PP2Ac was used. Together with our results using indirect immunofluorescence (see below), these antisera appeared to be specific reagents for studying PP2Ac in mammalian cultured cells.

We also generated antisera against the PR65 subunit. A 20-amino acid peptide comprising residues 177-196 present in both the \textalpha- and the \textbeta-isoforms of the protein (22), was used to generate peptide-specific antisera (Ab65\textsuperscript{177/196}). After affinity purification the monospecificity of these antisera was tested on immunoblots of total REF-52 cell extracts (Fig. 1 A). A single band at 63-65 kD was recognized, migrating slightly faster than recombinant human PR65\textalpha. The signal was competed by the corresponding antigenic peptide. In parallel we generated antibodies against recombinant PR65 (Ab65\textsuperscript{recomb}) and obtained identical results in immunoblots (Fig. 1 A).

To analyze the subcellular distribution of the two PP2A core subunits in asynchronously growing mammalian cells, cytoplasmic and nuclear extracts of REF-52 cells were prepared and subjected to immunoblotting with AbC\textsuperscript{recomb} and Ab65\textsuperscript{recomb}. When the cytoplasmic extract was compared with its corresponding nuclear extract (i.e., the cytoplasmic and the nuclear extracts were resuspended in the same volume) \textasciitilde80-90\% of the PP2Ac and PR65 was found in the cytoplasmic fraction (data not shown). Since the nucleus represents \textasciitilde10-20\% of total cell volume in REF-52 cells (N. J. C. Lamb, unpublished results) this suggested that the concentration of PP2A should be equal in both compartments. Indeed, when equal amounts of cytoplasmic and nuclear protein were analyzed, PP2Ac and PR65 could be detected with comparable intensities (Fig. 1 B). The catalytic subunit and the PR65 were 1.2- and 1.8-fold more concentrated in the cytoplasm than in the nucleus (average values determined from two scanned blots each). The peptide-specific antibodies AbC\textsuperscript{299/309} (see Fig. 4 E) and Ab65\textsuperscript{177/196} (data not shown) gave similar results.

To further precisely determine the intracellular localization of PP2A, we performed indirect immunofluorescence analysis of asynchronously growing REF-52 fibroblasts. Both sets of anti-catalytic subunit antibodies gave similar results, qualitatively in agreement with the immunoblotting data in as much as the catalytic subunit ubiquitously localized to both the cytoplasm and nucleus (Fig. 2, A-D; Fig. 3). The nuclei appeared brighter than the cytoplasm which suggests a higher concentration of PP2Ac in the nuclei than in the cytoplasm. Fluorescence assessment revealed that PP2Ac is approximately twofold more concentrated in the nucleus compared to the cytoplasm (see below; Table II). This was significantly higher than found biochemically (Fig. 1 B). The recovery of PP2A in the nuclear fraction was very sensitive to salt and detergents in the lysis buffer. Also the nuclear immunofluorescence staining was lost when cells were fixed and extracted using methanol. This suggested that PP2A is present in a highly soluble form in the nuclear compartment and might have been partly lost during the hy-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Microinjection of antisense PP2Ac expression plasmid leads to loss of PP2Ac-specific immunoreactivity. REF-52 cells, grown on glass coverslips, were microinjected with the pECE-hCc\textalpha antisense expression plasmid and fixed 15 h later (at this time point only \textasciitilde20\% of the injected cells survived). The distribution of PP2Ac was determined by indirect immunofluorescence using AbC\textsuperscript{recomb} (A). The arrowheads indicate the microinjected cell as identified by costaining for the microinjection marker antibody shown in B. Bar, 5 \textmu m.
\end{figure}
potonic fractionation. The nuclear staining excluded the nucleoli (also confirmed by confocal laser scanning microscopy). It is noteworthy that AbC<sub>299/309</sub> did not react specifically with centrosomal structures. Since it has previously been shown that PPX is predominantly centrosomal (5), this observation confirms that the anti-carboxyl-terminal antibodies apparently did not recognize PPX in REF-52 cells. The staining pattern for PP2Ac was highly specific since it could be completely competed with the appropriate antigen (Fig. 2, B and D). Furthermore, injection of an antisense catalytic subunit expression construct, pECE-hCa, resulted in a decrease in the overall staining of AbC<sub>299/309</sub> (Fig. 3) and AbC<sub>299/309</sub> (data not shown) in a time-dependent manner. Diminution of staining occurred in both the nuclear and the cytoplasmic compartments within 8–10 h following injection of the antisense construct. Longer times ultimately resulted in death of the PP2A antisense-expressing cells. This confirms previous results in fission yeast where a decrease in the overall staining of AbC<sub>199/209</sub> was observed (12). Also our data suggests that the α-isoform of PP2Ac is the predominant isoform found in these cells which is in agreement with many other cell lines and tissues (24, 29).

Interestingly, the intensity of nuclear staining of PP2Ac varied considerably (an example is arrowed in Fig. 2 C). Indeed, 34% (n = 1151) and 38% (n = 1001) of nuclei of asynchronously dividing REF-52 cells exhibited reduced nuclear staining, when AbC<sub>299/309</sub> and AbC<sub>299/309</sub> were used in indirect immunofluorescence, respectively. Further results suggest these changes are related to cell cycle progression (see below).

Localization of the PR65 using Ab65<sub>171/176</sub> by indirect immunofluorescence correlated well with the results for the catalytic subunit. The PR65 protein was also ubiquitously found in both the cytoplasm and the nuclei. Again the nuclear staining was brighter than the cytoplasmic. As observed with anti-PP2Ac antibodies, nucleoli were not stained (Fig. 2 E). The immunofluorescence signal was completely abolished by preincubation with antigenic peptide (Fig. 2 F). Again this cyto-localization was qualitatively consistent with results obtained with immunoblots of fractionated cell extracts.

Similar results were also obtained with the human fibroblast cell line HS68. Both PP2Ac and PR65 were found at high levels in the cytoplasm and nuclei showed no pronounced intracellular restrictions except the nucleoli, which were not stained (data not shown).

**A Large Proportion of PP2Ac from Interphase Cells is Methylated at Its Carboxyl Terminus**

We have recently reported that a large portion of PP2Ac is carboxyl-terminally methylated in MCF-7 cells (13). Immunodetection using the carboxyl-terminal antibodies AbC<sub>299/309</sub> appears to be inhibited by this modification. Furthermore, the in vitro methylation reaction itself was found to be inhibited by AbC<sub>299/309</sub> (17). Treatment of cell-free extracts with dilute alkali leads to demethylation and a large increase in PP2Ac carboxyl-terminal immunoreactivity. This unmasking of the epitope for AbC<sub>299/309</sub> by alkali has been used to assess the methylation status of the enzyme (13). We therefore chose to examine whether alkali treatment (a) unmasks PP2Ac epitopes in non-transformed mammalian fibroblasts and (b) is also effective on fixed cells.

Alkali treatment (20–100 mM NaOH) after formalin fixation of REF-52 (or HS68) cells was sufficient to increase the PP2Ac signal with AbC<sub>299/309</sub> in both cytoplasmic and nuclear compartments approximately fivefold (Fig. 4). This effect was specific since the increased staining was blocked by competition with the antigenic peptide (data not shown) and no such increase was seen following alkali treatment using anti-recombinant PP2Ac antibodies or antibodies to proteins unrelated to PP2Ac (p67<sup>SRF</sup>, PP1c, CREF-binding protein, data not shown). Confirming these data, alkali treatment of cytoplasmic and nuclear extracts followed by immunoblotting with AbC<sub>299/309</sub> resulted in a significantly increased signal with both cellular compartments (Fig. 4 E). Phosphoimager quantitation showed that the increase was 5–10-fold for both compartments, suggesting that 80–90% of cellular PP2Ac is carboxyl methylated. As observed by immunofluorescence, no such increase in the intensity of the staining was observed with AbC<sub>299/309</sub> (data not shown), again confirming the specificity of this effect for the carboxyl-terminal epitope recognized by AbC<sub>299/309</sub>.

**Cell Cycle Changes of PP2A**

Since the staining pattern for nuclear PP2Ac, as detected with both classes of antibodies, appeared to be heterogeneous, we examined if this was temporally associated with passage through the cell division cycle. For this, REF-52 cells were made quiescent by serum starvation for 36–40 h as described previously (33). After serum addition, cells were assumed to be in G<sub>0</sub> for up to 15 h, in S between 15 and 20 h and in G<sub>1</sub> between 22 and 25 h. Alternatively cells were starved, refed, and further synchronized before S phase, using 2 mM hydroxyurea as described previously (14). Cells were extensively washed to release the hydroxyurea block and routinely fixed at 1–2 h to study cells in S phase, and after 5 h to obtain cells in G<sub>2</sub>. After fixation cells were stained with the antibodies described above.

In G<sub>0</sub> AbC<sub>299/309</sub> stained the nuclear compartment strongly (Fig. 5 A). Within 40–60 min of serum stimulation, staining for cytoplasmic PP2Ac increased approximately threefold whereas the nuclear staining remained constant (Fig. 5, A, B, G, and H). This staining pattern with its low ratio of nuclear to cytoplasmic staining (Table II) remained unchanged throughout G<sub>1</sub> until 12 to 15 h after serum refeeding. At this time a large number of cells showed an increased nuclear versus cytoplasmic signal (data not shown). After 15–16 h most if not all cells exhibited a predominant nuclear signal. A maximal plateau was reached 18 h after refeeding. A similar intense nuclear staining pattern was observed when S phase was studied in cells resynchronized by hydroxyurea. Throughout S and G<sub>2</sub> this pattern remained unchanged (Fig. 5 C). Quantitative analysis revealed that this apparent reaccumulation of nuclear stain at the onset of S phase was due to a threefold reduction in cytoplasmic staining, and an approximately twofold increase of nuclear stain (Fig. 5, G and H).

Since we have shown that epitope masking can affect the immunofluorescence signal of AbC<sub>299/309</sub> (see Fig. 4), we pretreated synchronized and fixed REF-52 cells with dilute alkali as a means to demethylate the epitope recognized by the anti-carboxyl-terminal antibody. Following this treatment, the staining of AbC<sub>299/309</sub> was significantly increased in the cytoplasmic compartment of G<sub>0</sub> and S/G<sub>1</sub> cells and in
Methylation of PP2Ac from REF-52 cells as studied in situ and in fractionated extracts in vitro. (A–D) REF-52 cells were grown on glass coverslips, fixed with formalin, and treated with 100 mM NaOH for 10 min as described in Materials and Methods. Subsequently, the cells were neutralized and stained by indirect immunofluorescence using AbC3°2/3°9. Fluorescent micrographs of a representative cell after treatment with NaOH (A and B) and of a untreated control cell (C and D) are presented. The images in A and C were exposed and printed so that they are directly comparable. B and D show the corresponding DNA staining using Hoechst. (E) Western blots of cytoplasmic (lanes 1 and 2) or nuclear (lanes 3 and 4) extracts of growing cells. In lanes 2 and 4 the extracts were treated with alkali as described in Material and Methods. For electrophoresis 10 μg of each extract were loaded. Immunodetection was carried out using AbC3°2/3°9 followed by the enhanced chemiluminescence detection system (Amersham). Bar, 5 μm.

Comparison between Fig. 5, A–C and D–F reveals the extent of unmasking achieved by alkali treatment. It can be concluded that PP2Ac undergoes a cell cycle-specific demethylation in the cytoplasm as cells enter G1 and in the nucleus at the onset of S phase. In addition, PP2Ac is remethylated in the cytoplasm at the onset of S phase. Significantly, after alkali demethylation of the carboxyl-terminal epitope in PP2Ac, the ratio between the cytoplasmic and nuclear signal appeared to be constant during the cell cycle with an approximately twofold higher concentration in the nucleus than in the cytoplasm (Table II), indicating that the total amounts of PP2Ac do not change during cell cycle progression.

To further characterize PP2Ac during the cell cycle we used the antibody prepared using recombinant protein (AbCrecomb). Significantly cell cycle-specific changes were also observed when cells were stained with this antibody: the nuclear to cytoplasmic ratio of the signal was reduced during G1 just as observed with AbC3°2/3°9 (see Table II). However, in the case of this antibody the changes were restricted to the nuclear compartment. An approximately twofold reduction of the nuclear signal occurred at the G0/G1 boundary (Fig. 6, A, B, and E). The nuclear signal increased again approximately threefold as cells entered S phase (data not shown), and remained at this maximal value throughout S and G2 (Fig. 6, C and E). The cytoplasmic signal detected by AbCrecomb did not change during cell cycle progression (Fig. 6, A–D). Further, alkali treatment did not alter the immunofluorescence signal obtained with AbCrecomb (data not shown). This indicated that the immunofluorescence changes observed with this antibody were not directly linked to the methylation of PP2Ac.

The cell cycle distribution of the PR65 was analyzed in a similar manner to that described above for the catalytic...
subunit. Synchronized cells, fixed at different phases of the cell cycle, were stained with antibody Ab6557996. No detectable changes in the cytolocalization pattern of PR65 were observed during the cell cycle (Fig. 7). The cytoplasmic and nuclear signals remained high and constant throughout G0 (Fig. 7 A), immediately after refeeding (data not shown), 4 h after refeeding (Fig. 7 B), in S phase (Fig. 7 C) as well as in G1 (data not shown). In the light of PP2Ac and PR65 being always tightly complexed (10), this provided further evidence that the cell cycle related changes in the immunodetection of PP2Ac were brought about by epitope masking (i.e., methylation in the case of AbC299099), and were not due to physical translocation in and out of the nucleus. Similar results with PP2Ac and PR65 staining have been obtained using synchronized HS68 human fibroblasts (data not shown).

In addition to our analyses using indirect immunofluorescence, we performed immunoblot analyses of extracts prepared from synchronized REF-52 (and HS68) fibroblasts. PP2Ac was analyzed in the cytoplasmic and the nuclear fractions using AbC299099 or AbC299099. In accordance with the results obtained by immunofluorescence, the signal detected by AbC299099 varied significantly: a ~2.5-fold increase of the signal was observed when cytoplasmic extracts of G0
cells were compared with G₀ cells (Fig. 8 A, middle panel). As cells enter S phase the signal detected was again reduced approximately fivefold and remained low throughout S and G₂. These levels of cytoplasmic PP2Ac were constant, when the extracts were treated with 100 mM NaOH (Fig. 8 A, lower panel). Equal signals were detected with AbC299r3°9 in nuclear extracts of G₀ and G₁ cells (data not shown). As observed by immunofluorescence, the immunoblot signal increased 2.5-fold as cells entered S phase (Fig. 8 B, middle) and remained high throughout S and G₁ (data not shown). Again alkaline treatment of the blots revealed that this change was not due to different amounts of PP2Ac in the extracts, but to a demethylation as cells enter S phase (Fig. 8 B, bottom). In agreement, AbComb (Fig. 8, A and B, top) and Ab65comb (data not shown) did not show any consistent changes in the level of the PP2A core subunits in cytoplasmic or nuclear extracts at any time of the cell cycle. This correlated well with the immunolocalization of unmasked PP2Ac (as detected with AbC299/3°9 after NaOH treatment; Fig. 5, D–F) and PR65 (Fig. 7) and agrees with the previous work by Ruediger et al. (48) indicating that PP2A proteins do not change in their intracellular level during cell cycle progression. Significantly, the immunodetection with AbCcomb varied exclusively when used in immunofluorescence and not after SDS-denaturation of PP2A on Western blots. This indicates that the cell cycle changes detected with this antibody can be attributed to conformational changes of the enzyme.

**PP2A Is Excluded from the Condensed Chromatin during Mitosis**

To study the localization pattern of PP2A in cells at mitosis, REF-52 cells were synchronized by serum starvation and hydroxyurea block as described above and fixed 8 h thereafter. The staining pattern revealed that PP2Ac and PR65 were excluded from condensed chromatin. Similar results were obtained with all antisera to PP2A. To discount any possibility of epitope masking, we stained cells in mitosis with AbC299/3°9 after alkaline-mediated demethylation of PP2Ac (Fig. 9). As expected this increased the intensity of the staining, however, the distribution was identical to that observed with either AbCcomb or AbC299/3°9 (without alkali pretreatment) or Ab65/77/6°. As cells enter prophase (Fig. 9, A and B) we observed a significant loss of PP2A from the nucleus. In particular, the nuclear regions devoid of PP2A staining corresponded to those already containing condensed chromatin. PP2A remained excluded from the condensed chromatin throughout metaphase (Fig. 9, C and D), and anaphase (Fig. 9, E and F). However, as nuclei reformed in late telophase, PP2Ac clearly relocated into the nuclear compartment of the two daughter cells (Fig. 9, G and H).

**Discussion**

**PP2A Is Present in the Cytoplasm and the Nuclei of Interphase Fibroblasts**

Many different PP2A holoenzymes have been isolated and characterized biochemically. In the future it will be necessary to assign specific functions to the different oligomers, to gain a better understanding of the complex patterns of phosphorylation and dephosphorylation control in eukaryotic cells. Previous reports suggested that PP2A is largely cytoplasmic (reviewed in reference 51). Indeed, cytochemical studies with fission yeast (31), MRC-5 cells (5), and CV-1 cells (53) support this view. However, a few reports provide some biochemical evidence of PP2A in the nuclear compartment (26, 27, 59). In REF-52 and HS68 fibroblasts we found, using subcellular fractionation and Western blot analysis, that the core subunits of PP2A, the catalytic subunit and PR65, are found at high levels in the nuclear compartment. When equal amounts of protein from cytoplasm and nuclei were analyzed, the core subunits of PP2A was on average 1.5-fold more concentrated in the cytoplasm. In contrast, using cytolocalization techniques, PP2A was found to be approximately twofold more concentrated in the nuclear compartment. For immunofluorescence staining, we found it essential to fix cells in formalin, since methanol fixation

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**Table II. Ratios of Nuclear to Cytoplasmic Voxel Fluorescence during the Cell Cycle**

|       | AbC299/3°9 | AbC299/3°9 + NaOH | AbCcomb |
|-------|------------|------------------|---------|
| G₀    | 3.59       | 2.20             | 3.63    |
| G₁    | 1.10       | 2.12             | 1.63    |
| G₂    | 7.73       | 2.01             | 4.95    |

For calculation the values determined in Figs. 5 and 6 were used.
abolished nuclear staining with all anti-PP2A antibodies described here. Since methanol fixation simultaneously fixes and extracts cells, it can lead to loss of proteins. Together with the difficulties we had in obtaining PP2A in the nuclear fractions when using low concentrations of detergents or salt during cell fractionation, the data presented in this report suggest that PP2A is present in a highly soluble and extractable form in the nuclear compartment and may be lost easily during homogenization and/or fixation. This possibility has already been put forward by Jessus et al. (27). Therefore the ratio of the nuclear to cytoplasmic concentrations of PP2A deduced from our immunofluorescence data appears to be representative of the true distribution of PP2A in living cells, differing from that obtained by biochemical means. Also, the high solubility of nuclear PP2A is likely to explain why it has traditionally not been found in the nuclear compartment at the high levels we report now.

Interestingly, neither the catalytic subunit nor the PR65 appear to contain any putative nuclear localization signals (22, 29). As monomers their size would still allow passive diffusion into the nucleus. However, PP2Ac and PR65 appear to be tightly associated (reviewed in reference 10) and therefore it is more likely that a third or "variable" regulatory subunit confers nuclear translocation. In this respect it is noteworthy that the primary structure of the PR72 regulatory subunit contains a putative nuclear localization signal (23).

Figure 6. Cell cycle-dependent heterogeneity of AbC°st staining in the nuclei of synchronized REF-52 cells. An identical experiment as shown in Fig. 5 was performed except that cells were stained with AbC°st. Confocal scans recorded in parallel with the experiment shown in Fig. 5, with the same settings for laser power, pinhole size, and photomultiplier sensitivity, are presented. Therefore the images and values in Fig. 5 and 6 are directly comparable. The panels show representative images for quiescent, serum-starved cells (A), G1 cells 5 h after serum stimulation (B), and G2 cells 22 h after serum stimulation (C). D and E show the quantitation of this experiment. Cytoplasmic (D) and nuclear (E) voxel fluorescence has been calculated in the same manner to that described for Fig. 5. Bar, 5 μm.
Figure 7. Immunolocalization of the PR65 subunit does not change during the cell cycle. To determine the intracellular distribution of PR65 subunit during the mammalian cell cycle, REF-52 cells were synchronized by serum starvation. Subsequently, the cells were restimulated with 10% FCS and further resynchronized for S phase using hydroxyurea. Cells were stained by indirect immunofluorescence with affinity-purified Ab65. Shown are fluorescent micrographs of quiescent cells (A), of cells after 4 h serum stimulation (G1) (B) and S phase cells, fixed 1 h after releasing the hydroxyurea block (C). Bar, 5 μm.

Another possibility is that PP2A is specifically retained as nuclei reform in telophase: PP2A was found to translocate out of the nucleus as cells enter mitosis and did not colocalize specifically to the condensed chromatin. As cells were released from mitosis and nuclei reformed, PP2A was again very abundant in the nuclear compartment. This observation contrasts with previous results where PPlc was found tightly associated with chromatin throughout the mitotic period (14). This again confirms that the two major serine/threonine protein phosphatases, despite a broad, overlapping substrate specificity in vitro (reviewed in reference 10), do not necessarily share substrates in vivo. From its localization it also appears that the function of PP2A during mitosis is much broader than that of PPl. Indeed many aspects of how PP2A may negatively regulate p34<sup>Cdc2</sup> have been put forward in the past (1, 8, 15, 16, 35). Moreover, PP2A has been shown to be essential for the metaphase–anaphase transition in <i>Drosophila</i> (40, 41) and for cytokinesis in budding yeast (21, 47, 57).

Thus the intracellular distribution of PP2A is distinct from PPlc (14), but also from PPX, which has been reported to be predominantly centrosomal (5).

**Figure 8.** Immunoblot analyses of cytoplasmic and nuclear extracts from synchronized cells reveals differential methylation. REF-52 cells were synchronized for Go, G1, and S phase as described in Materials and Methods and immunoblot analyses performed with anti-PP2Ac–specific antibodies. For all immunobots shown primary antibodies (AbC<sub>noemb</sub> or AbC<sub>502/509</sub> as indicated) were detected using <sup>125</sup>I-coupled donkey anti–rabbit IgG and quantified using a PhosphoImager. (A) 5 μg of cytoplasmic protein extracts (untreated or treated with 100 mM NaOH as indicated) were resolved by SDS-PAGE. Cytoplasmic extract of quiescent, serum-starved cells (G0), early G1 cells that had been serum stimulated for 6 h (G1), and cells harvested 1 h after release of the hydroxyurea block (S) are presented. (B) Nuclear protein extracts (10 μg) were resolved by SDS-PAGE. Data shown are nuclear extracts of early G1 cells that had been serum stimulated for 4 h following starvation (G1), and of cells harvested 1 h after release of the hydroxyurea block (S). The immunoblot was treated with 200 mM NaOH at 30°C for 20 min before immunodecoration as indicated. In similar experiments comparison of nuclear extracts from G0 and G1 cells revealed no changes (data not shown).

**Differential Methylation of PP2A during the Mammalian Cell Cycle**

PP2Ac has previously been shown to be carboxyl-terminally methylated in vitro and in cultured cells (13, 34, 61). We show here that PP2Ac is also methylated to a high extent in nontransformed mammalian fibroblasts. Alkaline treatment of cytoplasmic and nuclear extracts increased the signal of the methylation-sensitive antibody AbC<sup>502/509</sup> 5–10-fold, in agreement with our previous data from MCF-7 cells (13). In addition, we developed a novel method to study the extent of the methylation of PP2Ac in situ. This enabled us to determine precisely when and where carboxyl-terminal methylation of PP2Ac occurs in the cell cycle. That reversible carboxyl methylation of PP2Ac is ubiquitously used, from yeast to man and plants (13, 17, 34, 60, 61), suggests that it is a significant regulatory mechanism. Although it is thought that PP2A exerts specific functions at restricted time points during the cell division cycle, previous studies have failed to identify any cell cycle-specific changes in the total amount of PP2A subunits (48). Thus it appears feasible that cell cycle
regulation of PP2A is conferred by posttranslational events such as covalent modifications, differential subunit association, intracellular localization, or a combination of these. In this manuscript we demonstrate that methylation of PP2Ac is regulated in a cell cycle-specific manner: we observed a two- to threefold increase of cytoplasmic PP2Ac-specific immunoreactivity at the G0/G1 boundary when the methylation sensitive AbC299/309 was used. At the onset of S phase the cytoplasmic immunoreactivity returned to the level detected in resting cells. Concomitantly, the nuclear signal increased ~2.5-fold. However, after alkaline demethylation in vitro or in situ, no more cell cycle-specific changes of the carboxyl-terminal immunoreactivity of PP2Ac were observed. In agreement the PR65 did not show any consistent changes during cell cycle progression. Therefore, a fraction of PP2Ac appears to undergo demethylation specifically in the cytoplasm as cells enter S phase. To our knowledge, this is the first report demonstrating that PP2Ac undergoes differential posttranslational modification during cell cycle progression and also that reversible protein methylation is regulated in a cell cycle-specific manner. In the future it will be important to elucidate how this differential methylation of PP2A is functionally controlled. In this respect it is noteworthy, that the carboxyl methylation of PP2Ac is stimulated by cAMP and inhibited by okadaic acid in cell-free extracts of Xenopus eggs (17).

What is the functional role of the differential methylation during the cell cycle? A multitude of possibilities appear feasible. Since PP2Ac has been reported to be phosphorylated at Tyr307 (6, 7) these two posttranslational modifications may be interdependent. In a similar manner carboxy methyltransferases of ras and related proteins by methyltransferase type III has been shown to be dependent on isoprenylation (reviewed in reference 9). Methylation brings about a moderate increase of the in vitro activity of PP2Ac (13). Alternatively, differential cell cycle methylation of PP2Ac may alter the substrate specificity. Considering the plethora of PP2A subunit configurations, yet another possibility is that the methylation of PP2Ac induces a specific holoenzyme switch, i.e., dissociation/association of variable subunits. In partial support of this, we found a reduced staining of nuclei with AbC recomp during G1. However, no changes in the cytoplasmic staining, as seen with AbC299/309 during GI, were observed with AbC recomp. This reduced staining of nuclei during G1 is suggestive of epitope masking occurring also with AbC recomp. This was somewhat surprising since AbC recomp was raised against the full-length recombinant protein. Since we did not observe such epitope masking in immunoblots after SDS denaturation, it is likely to be the result of the association of distinct, regulatory proteins to the catalytic subunit in nuclei of G1 cells.

Considering the changes we observed at the G1/S boundary it is interesting to note that okadaic acid, when used at concentrations specific for PP2A, arrests fibroblasts and hepatocytes at the G1/S boundary (44, 52). The role of PP2A during DNA replication is well established. The dephosphorylation of SV40 large T by PP2A has been shown to be necessary for the replication of the virus genome (reviewed...
in reference 58). Enigmatic cell cycle-specific forms of PP2A are described where only an S phase-specific form is capable to dephosphorylate large T (37). Thus the G-specific form of PP2A that prevents the onset of DNA replication may structurally correlate to the nuclear form that exhibited low in situ immunoreactivity with AbC and AbC in mammalian fibroblasts. A possible endogenous substrate for PP2A at the G/S boundary may be the p53 tumor suppressor protein. It has been shown to be involved in the initiation and control of DNA replication (reviewed in reference 36). In addition, phosphorylated p53 is efficiently dephosphorylated by PP2A in vitro (50).

In conclusion we have demonstrated that a considerable portion of PP2A is present in the cytoplasm and the nuclei of mammalian fibroblasts. We find that PP2Ac undergoes differential, cell cycle-specific methylation. Regulated convergence of two different posttranslational modification systems, namely protein phosphorylation and methylation imposes another degree of complexity to intracellular signal transduction. In addition our immuno localization data are suggestive of cell cycle-specific holoenzyme rearrangements. It is now apparent that PP2Ac is not a "pac-man" phosphatase, since in addition to regulation by a sophisticated set of enzymes, since in addition to regulation by a sophisticated set of transcription factors, it is also controlled by posttranslational modifications. Further studies on the localization of the other PP2A regulatory subunits will be important to gain a complete understanding of the control of this ubiquitous enzyme by external and internal signals.

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