Proteomic Characterization of Protein Phosphatase Complexes of the Mammalian Nucleus*

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Our knowledge of the serine/threonine protein phosphatases of the mammalian nucleus is limited compared with their cytosolic counterparts. Microcystin-Sepharose chromatography and mass spectrometry were utilized to affinity purify and identify protein phosphatase-associated proteins from isolated rat liver nuclei. Far Western analysis with labeled protein phosphatase 1 (PP1) showed that many more PP1 binding proteins exist in the nucleus than were previously demonstrated. Mass spectrometry confirmed the presence in the nucleus of the mammalian PP1 isoforms α1, α2, β, and γ1, plus the Aα and several of the B and B′ subunits that are complexed to PP2A. Other proteins enriched on the microcystin matrix include the spliceosomal proteins known as the U2 snRNPs SAP145 and SAP155 and the U5 snRNPs p116 and p200, myosin heavy chain, and a nuclear PP1 myosin-targeting subunit related to M_{110}. The putative RNA binding protein ZAP was also established as a nuclear PP1 binding protein using the criteria of co-purification with PP1 on microcystin-Sepharose, co-immunoprecipation, binding PP1 in an overlay assay, and presence of a putative PP1 binding site (KKRVRWAD). These results further support a key role for protein phosphatases in several nuclear functions, including the regulation of pre-mRNA splicing. ***Molecular & Cellular Proteomics 3:257–265, 2004.***

Protein phosphatase 1 (PP1)[1] and 2A (PP2A) are highly conserved serine/threonine-specific protein phosphatases that have been identified in all eukaryotic species examined (1, 2). Dephosphorylation by PP1 is controlled by targeting or regulatory subunits that take PP1 to specific locations in the cell, potentially alter its phosphatase activity, and allow regulation by intra- or extracellular-derived signals (3–6). Biochemistry has shown that PP1 activity is highly enriched in the nucleus, and recent fluorescence microscopy studies with tagged versions of PP1 have dramatically illustrated this (7). PP1γ1 resides primarily in the nucleolar compartment, PP1α in the nucleoplasmic fraction, and PP1β in both nuclear compartments (7). Several nuclear PP1-targeting subunits have now been identified (6–9). The two most-abundant nuclear PP1 binding subunits, p99 or PNUTS and nuclear inhibitor PP1 (NIPP-1), are RNA-binding proteins that likely play a role in pre-mRNA splicing (9–11). Both proteins contain the PP1 binding motif R/K-V/I-X-F/W that was originally identified from studies on the glycogen and myosin PP1-targeting subunits (12–16). This motif has been shown to be present in nearly all PP1-associating proteins. Due to the number of nuclear events controlled by phosphorylation/dephosphorylation, there undoubtedly exist many more, as yet unidentified proteins that target or localize nuclear protein phosphatases. Here, we have done an extensive examination of the mammalian nucleus for protein phosphatase-associated proteins by utilizing the protein phosphatase affinity matrix microcystin-Sepharose. This matrix has been used previously to successfully purify other protein phosphatase-targeting subunit complexes (12, 13). Using a combination of affinity chromatography and mass spectrometry, we have identified many new proteins localized to protein phosphatase complexes, and here we show many more PP1 binding proteins exist in the nucleus than have been previously demonstrated. This work implicates protein phosphatases as regulators of many nuclear events.

EXPERIMENTAL PROCEDURES

Materials—Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals and reagents were obtained from the companies indicated in parentheses. Human PP1γ1 was expressed and purified as described previously (17) and dialyzed into 25 mM $NaHCO_3$, pH 7.5, 200 mM KCl, 50% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol (2-ME) for storage. The human PP1α1 clone was kindly provided by S. Shenolikar (Duke University), transfected into *Escherichia coli* BL21 (DE3), and the protein expressed (18) and purified on microcystin-Sepharose (12).

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[1] The abbreviations used are: PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; snRNP, small nuclear ribonucleoprotein particle; SAP145, spliceosome-associated protein 145; SAP155, spliceosome-associated protein 155; NIPP-1, nuclear inhibitor protein phosphatase 1; hnRNA, heterogeneous nuclear RNA; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; NE, nuclear extract; DIG-PP1, digoxygenin-3-*O*-methylcarbonyl-aminocaproic-acid-*N*-hydroxy-succinamide ester.

Preparation of Nuclear Extracts from Rat Liver—Male Wistar rats were sacrificed, and livers were removed and Dounce homogenized in 2 volumes of TKM (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$), 0.25 M sucrose, 0.1% (v/v) 2-ME, and filtered through two layers of mira-cloth (Calbiochem, La Jolla, CA). Nuclei were pelleted by centrifugation at 2,400 × *g* for 10 min in a Sorval SLA-1500 rotor and resuspended in 3 ml TKM, 0.25 M sucrose/10 g tissue, and mixed with 2 volumes of TKM, 2.3 M sucrose at 4 °C. Nuclei were layered on 10 ml of TKM, 2.3 M sucrose, and centrifuged at 82,800 × *g* in a Beckman SW 27 rotor for 1 h at 4 °C. The isolated nuclei were resuspended in TKM, 0.25 M sucrose plus 0.5% (v/v) Triton X-100, and put on ice for 10 min to dissolve nuclear membranes. The nuclei were repelleted by centrifugation at 2,000 × *g* for 5 min at 4 °C in a Sorval SS-34 rotor and then resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1% (v/v) 2-ME, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 5 μg/ml leupeptin). After 10 min on ice, the nuclei were Dounce homogenized, and after centrifugation at 7,800 × *g* for 5 min at 4 °C in a Sorval SS-34 rotor the supernatant was retained and the nuclear pellet rehomogenized in buffer B (50 mM Tris-HCl, pH 7.5, 0.42 M NaCl, 0.1 mM EGTA, 5% (v/v) glycerol, 0.1% (v/v) 2-ME, 0.5 mM PMSF, 1 mM benzamidine, and 5 μg/ml leupeptin). After centrifugation at 7,800 × *g* for 10 min at 4 °C in a Sorval SS-34 rotor, this extracted protein was pooled with the first supernatant and the pellet re-extracted with buffer B, clarified as above, and all the supernatant fractions pooled and designated the nuclear extract (NE).

Microcystin-Sepharose Chromatography—Extracted nuclear proteins (~1 mg protein per gram liver tissue) were incubated end over end with 1 ml of microcystin-Sepharose matrix prepared as described (12) and previously equilibrated in buffer C (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 5% (v/v) glycerol, 0.1% (v/v) 2-ME). After 1 h, the column was washed with buffer C and 0.3 M NaCl until essentially no protein was eluting off the column (250 column volumes). Proteins were eluted by incubation for 30 min with 3 M sodium isothiocyanate and then the first 20 ml was collected. Protein was dialyzed extensively in buffer C plus 0.5 mM PMSF and 1 mM benzamidine, concentrated in a centriprep 10 (Millipore, Bedford, MA) and centricon 10 (Millipore) to 50 μl, then boiled in SDS. A control matrix was prepared by blocking CH-Sepharose (Pharmacia, Piscataway, NJ)-reactive groups with Tris, as described by the manufacturer. In experiments where the control matrix was used, the pooled nuclear extract was divided in half and loaded separately onto 1-ml microcystin and control columns. Both matrices were washed and eluted identically and in parallel to allow direct comparison of eluted proteins. A typical preparation with 175 g of tissue resulted in the elution of 45 μg of protein from the microcystin-Sepharose matrix and 15 μg from the control matrix. In some cases, after elution with 3 M thiocyanate, the affinity matrix was washed with buffer C and warmed to room temperature, then incubated with 2% (w/v) SDS for 5 min and eluted with further SDS. The first 4 ml collected was concentrated with a speedivac concentrator to 500 μl and boiled in SDS.

Western Blotting and Immunoprecipitation—Monoclonal antibodies to PP1 and PP2A catalytic subunits were purchased from Transduction Laboratories (Lexington, KY) and were used at 0.25 μg/ml. The anti-talin antibody was obtained from Sigma and used at a 1,000-fold dilution. An anti-peptide antibody to ZAP was generated by coupling the ZAP peptide ^{1366}EKITDESGHLAERALNR^{1382} to KLH and performing injections as described in Tran *et al.* (19) and were affinity purified on a peptide affinity column (19). Affinity-purified antibodies to the smooth muscle myosin-targeting subunits M_{110} and M_{20} were provided by P. Cohen (University of Dundee). Other antibodies were kindly provided by the following individuals and used as described in the indicated references. NIPP-1 and p99 (L. Trinkle-Mulcahy and A. Lamond (7, 9)), U5 small nuclear ribonucleoprotein particles (snRNPs) p116 and p200 (P. Fabrizio and R. Lührmann, (20, 21)), U2 snRNPs spliceosome-associated protein (SAP) 145 and SAP155 (R. Reed (22, 23)), and myosin heavy chain (M. Walsh (24)).

Proteins were separated by SDS-PAGE, transferred for 300 Vh to nitrocellulose, blocked overnight in 5% (w/v) milk powder in 20 mM Tris-HCl, pH 7, and 500 mM NaCl. Blots were probed for 1 h, washed and incubated for 45 min with appropriate secondary antibody diluted 5,000-fold, and developed with the enhanced chemiluminescence system (Pharmacia). When necessary, blots were quantified using the ImageQuant 5.2 software.

For ZAP immunoprecipitation, 0.25 ml of ZAP crude immune sera or pre-immune sera was incubated with 0.2 ml of protein-A Sepharose, washed and coupled as described (25). Nuclear extract (10 mg) prepared as before plus 5 μg/ml pepstatin was mixed with 0.1 ml of anti-ZAP protein-A Sepharose beads for 2 h at 4 °C, washed three times with 1 ml of phosphate-buffered saline plus 0.1% (v/v) Tween-20, and proteins were solubolized with 100 μl of SDS mixture. For SAP155, 10 μl of crude immune sera was incubated with 0.03 ml of protein-A Sepharose and washed. Nuclear extract (3 mg) was mixed with 0.03 ml of anti-SAP155 protein-A Sepharose beads for 3 h at 4 °C, washed five times with 1 ml of phosphate-buffered saline plus 20 mM NaF, and proteins were solubolized with 50 μl of SDS mixture.

Far Western Analysis—For overlays, PP1γ1 was labeled with digoxygenin-3-*O*-methylcarbonyl-aminocaproic-acid-*N*-hydroxy-succinamide ester (DIG-PP1) as described (13) and used at the indicated concentration. Proteins were run on SDS-PAGE, transferred to nitrocellulose (300 Vh), and blocked overnight. Blots were probed for 3 h, washed and incubated for 45 min with anti-digoxygenin antibody diluted 10,000-fold (Roche, Indianapolis, IN), and developed with the enhanced chemiluminescence system. In experiments with peptides based on the PP1 binding motif, a peptide was synthesized surrounding the PP1 binding region of human NIPP-1 (26) and constituted residues 191–210 (RPKRKRKNSRVTFSEDDEII). The control peptide was identical, except Phe^{203} was replaced with an alanine, which abolishes PP1 binding to its targeting subunits (15, 26). The peptide GKKRVRWADLE based on the putative PP1 binding site of ZAP was used in PP1 overlays as indicated.

Peptides were synthesized by Denis McMaster (Peptide Synthesis Core Facility, University of Calgary) using *N*-9-fluorenylmethoxycarbonyl chemistry on an Applied Biosystems 431A peptide synthesizer (Applied Biosystems, Foster City, CA). The peptides were purified by Sephadex G-10 chromatography, and the peptide purity (>90%) and composition was verified by high-pressure liquid chromatography and amino acid analysis, respectively.

Mass Spectrometry—Proteins were electrophoresed on 4–12% gradient SDS-PAGE, lightly stained with Coomassie blue, and the appropriate band was excised. The protein was in-gel digested with trypsin after alkylation of cysteines with 4-vinyl-pyridine following the method described in Stubbs *et al.* (27). Tryptic peptides were analyzed on a Perseptive Biosystems (Framingham, MA) Elite STR matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer with saturated α-cyanocinnamic acid as the matrix. The mass spectrum was acquired in the reflector mode and was internally mass calibrated. The tryptic peptide ions obtained were scanned against the Swiss-Prot, NCBI, and GenPept databases using the MS-FIT program of ProteinProspector (prospector.ucsf.edu) run on a local server. In all cases, the mass tolerance was set at 40 ppm. In some cases, for confirmation of identity or where there was an ambiguity in the identity of a protein sample, liquid chromatography tandem mass spectroscopy (LC-MS/MS) was performed. The tryptic digest was injected on to a 0.075 × 100-mm PepMap C18 capillary column, equilibrated with 0.1% formic acid in water and attached to an LC-Packings Ultimate HPLC system (Dionex, Camberley, UK). The column was developed with a discontinuous acetonitrile gradient at

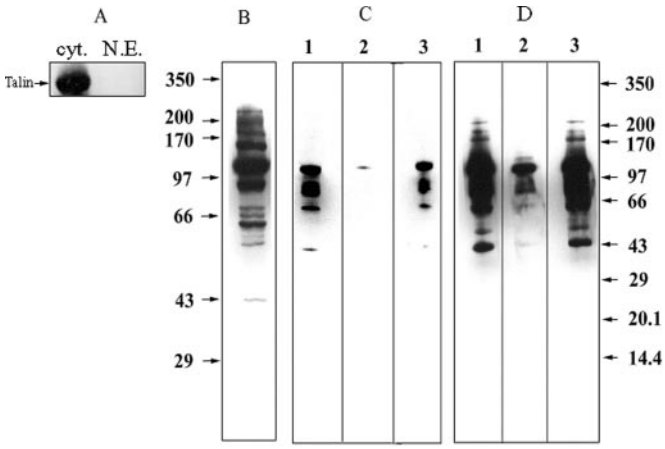

FIG. 1. **Far Western analysis of nuclear PP1 binding proteins.** *A*, Equal volumes of cytosolic (cyt) and nuclear extract (N.E.) proteins were electrophoresed on a 10% SDS gel, transferred to nitrocellulose, and probed with anti-talin antibody to show that the nuclear fraction was not contaminated with cytosolic proteins. *B*, Nuclear extract proteins (4.8 μg) were electrophoresed on a 10% SDS gel, transferred to nitrocellulose, and probed with 0.1 μg/ml DIG-PP1γ1 to reveal binding proteins. Proteins purified on the microcystin affinity matrix (0.20 μg/lane) were electrophoresed on a 4–12% gradient gel and transferred to nitrocellulose. *C* and *D*, Blots were probed with 0.1 μg/ml DIG-PP1γ1 plus the addition of the peptides indicated at 40-fold molar excess to PP1. *Lane 1*, Incubated with PP1 probe alone; *lane 2*, PP1 probe plus the PP1 binding peptide RPKRKRKNSRVTFSEDDEII; *lane 3*, PP1 probe plus the PP1 binding peptide with F203 changed to an alanine residue (RPKRKRKNSRVTASEDDEII). Blots in *C* and *D* are the same membranes exposed for different times to x-ray film to reveal both weak and strong signals (*C*, 10-s exposure; *D*, 60-s exposure). The positions of molecular mass marker proteins DNA-PKcs (350 kDa), myosin (200 kDa), α-2-macroglobulin (170), phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) are indicated.

0.2 μl/min, and the column was interfaced to a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The peptide ions generated by the electrospray interface were fragmented automatically using machine-defined collision voltages. The resultant peak lists were searched using the Sonar search engine (Genomic Solutions, Ann Arbor, MI) against the NCBI nr database.

RESULTS

Further Nuclear PP1 Binding Proteins Are Revealed—We have previously shown that the nuclear isolation technique used here results in highly pure nuclei that are not contaminated with cytosolic proteins (19). In addition, we have blotted the isolated nuclear and cytosolic fractions with the cytosolic marker protein talin and confirmed that the nuclear fraction is free of cytosolic contamination (Fig. 1*A*). To explore the possibility that additional nuclear PP1 regulatory subunits exist, proteins released from isolated nuclei by hypotonic lysis and salt extraction were run on SDS-PAGE, transferred to a membrane, and probed with labeled DIG-PP1γ1. This technique has previously been established as a sensitive method to identify putative PP1 binding proteins (13, 14). The result (Fig. 1*B*) indicated that many more nuclear PP1 binding proteins exist than have been previously detected (9). To explore this possibility further, protein phosphatases sensitive to microcystin were purified on a microcystin affinity matrix. Like Fig. 1*B* and previous work (9), the major PP1 binding proteins visualized (Fig. 1*C*) had molecular masses of 43 and 112 kDa and were later determined to be NIPP-1 and p99/PNUTS, respectively. A longer exposure of the same blot revealed numerous other putative PP1 binding proteins (Fig. 1*D*). The binding pattern is essentially identical to the crude nuclear extract, indicating that a majority of the nuclear PP1 binding proteins purifies on the microcystin matrix. The binding of PP1 to these proteins could be blocked by inclusion of a peptide based upon the PP1 binding site of the known PP1 regulatory subunit NIPP-1 (Fig. 1, *C* and *D*, *lane 2*). Changing the key binding residue of the peptide, F203 (26), to an alanine re-established this interaction (Fig. 1, *C* and *D*, *lane 3*), suggesting that these proteins are true nuclear PP1 binding proteins and encouraged us to pursue the identity of these proteins. Overlays performed with human PP1α1 gave identical results (data not shown).

Comparison of Microcystin-Sepharose and Control Matrix-bound Nuclear Proteins—Any affinity chromatography procedure results in a certain degree of nonspecific binding to the support matrix. As a control for this, we performed the chromatography procedure by dividing the nuclear extracted proteins into two identical fractions and running the microcystin and Tris-coupled "blank" matrix in parallel. Eluted proteins were concentrated separately to the same volume, and proteins were visualized by Coomassie blue staining after SDS-PAGE. A typical preparation resulted in three to four times more protein binding and eluting from the microcystin matrix compared with the control column. As shown in Fig. 2, several proteins are found in equal levels on both columns and can be excluded from further analysis. Note particularly the group of proteins migrating between the 14.4- and 20.1-kDa markers and the 135-kDa protein. These nonspecifically bound proteins were later identified as histones H2A, H2B, H3, and H4 and the putative DEAD/DEAH box helicase HELG.

Identification of Proteins Purified by Microcystin-Sepharose—The proteins purified on microcystin-Sepharose were run on a gradient gel and the pattern of Coomassie blue-stained proteins *versus* PP1 binding proteins is compared in Fig. 3, *A* and *B*. Two time points for exposure of the same blot are shown to demonstrate association of less-abundant binding proteins for PP1 (Fig. 3*B*, *lane 2*; 120-s exposure) and more-abundant and perhaps higher-affinity targets (Fig. 3*B*, *lane 3*; 10-s exposure). Comparing the blotted sample directly with the Coomassie-stained sample allowed particular protein bands that associate with PP1 to be identified unequivocally. This revealed that several proteins on the Coomassie-stained gel were PP1 binding proteins. Proteins that failed to bind PP1 may also be true PP1 binding proteins but fail to associate in an overlay assay. They could also be PP2A binding proteins or

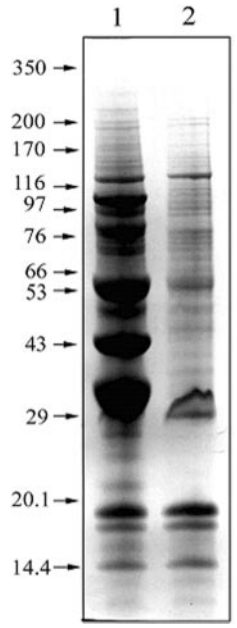

FIG. 2. **SDS-PAGE analysis of rat liver nuclear proteins purified with the microcystin affinity and control matrices.** Proteins extracted from isolated nuclei were chromatographed identically on 1 ml of microcystin or Tris-coupled CH-Sepharose columns. Eluted proteins were concentrated to 50 μl and equal volumes run on a 4–12% gradient gel and stained with Coomassie blue. *Lane 1* is 30 μl (48 μg) from the microcystin-Sepharose column and *lane 2* is 30 μl (15 μg) from the blank column. Molecular mass standards are as in Fig. 1 plus β-galactosidase (116 kDa), transferrin (76 kDa), and glutamate dehydrogenase (53 kDa).

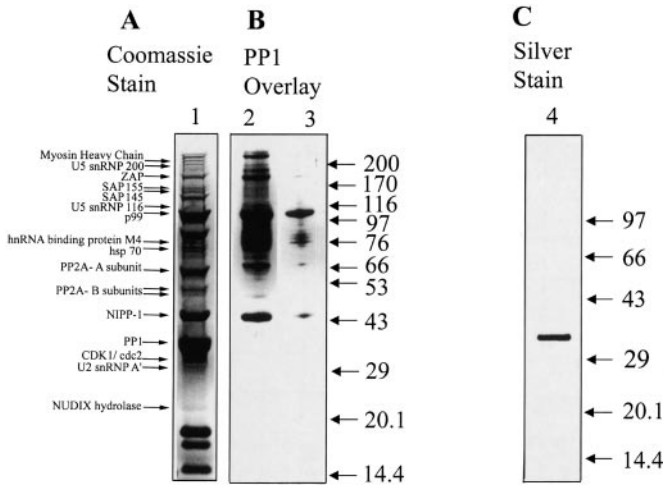

FIG. 3. **SDS-PAGE and PP1 overlays of purified nuclear protein phosphatase-associated proteins.** Proteins purified on microcystin-Sepharose were electrophoresed on a 4–12% gradient gel, and the gel was cut in half and either stained with Coomassie blue (*A*) or proteins transferred to nitrocellulose and probed with 0.5 μg/ml DIG-PP1γ1 (*B*). In *A*, the sample was run in *lane 1* (30 μg) and the gel was stained with Coomassie blue. *B* contains 2 μg of protein and *lanes 2* and *3* are the same membrane exposed for different times to x-ray film to reveal both weak and strong signals (*lane 2*, 120-s exposure; *lane 3*, 10-s exposure). In *C*, proteins were eluted from the microcystin-Sepharose column with 2% (w/v) SDS after thiocyanate elution, then run on SDS-PAGE (4–12%) and stained with silver (*lane 4*). Molecular mass standards are as in previous figures.

protein components of larger macromolecular protein phosphatase complexes. It is unlikely that they are subunits of novel protein phosphatases because we did not find catalytic subunits of this group of phosphatases in the nuclear microcystin-Sepharose eluent. In an attempt to determine if further proteins were still bound to the matrix after isothiocyanate elution, we tried a more-stringent elution method: 2% (w/v) SDS. It was hoped that this technique would elute the PP2A catalytic subunit ($PP2A_c$) that is bound with very high affinity to this matrix. Indeed, we have been unable in the past to elute $PP2A_c$ from the column (12). The SDS-eluted proteins were stained with silver and are shown in Fig. 3*C*. The most prominent band is a protein of 36–37 kDa. Clearly, isothiocyanate had displaced all the regulatory subunits from the matrix, as the only SDS-eluted band is PP1 (as determined by MALDI-TOF MS). $PP2A_c$ could only be eluted by boiling in SDS (data not shown).

We adopted MALDI-TOF MS and LC-MS/MS as the techniques to identify the proteins eluted from the microcystin matrix, and the results are catalogued in Table I and indicated in Fig. 3*A*. MALDI-TOF MS confirmed that four of the five mammalian PP1 gene products are present in the nucleus. Peptides unique to PP1α1, α2, β, and γ1 were found in the mass ion data. It is possible that PP1γ2 is present in the nucleus, but our data is not conclusive; PP1γ1 and γ2 only differ in a short region in their C termini. The C-terminal tryptic peptide unique to PP1γ2 was not present in the mass ion data. Peptides unique to the PP2A Aα subunit were identified along with several PP2A-associated proteins belonging to the separate gene families called B and B′. Because of the number and relatedness of the different B and B′ subunits, we could not conclusively and unequivocally identify particular gene products. No peptides unique to PP2A Aβ were observed. Other protein bands identified include the myosin heavy chain, the U2 snRNPs A′, SAP145, SAP155, the U5 snRNPs p116 and p200, a putative RNA binding protein named ZAP, hnRNA binding protein M4, TPR and SH2 phosphoprotein p160, p99, NIPP-1, cdk1, and a putative nudix hydrolase related to syndesmos. We have used antibodies to confirm the specific association of some of these proteins to the microcystin matrix.

Western Blots of Putative Protein Phosphatase-associated Proteins—Some of the microcystin-Sepharose-bound proteins identified by mass spectrometry were of relatively low abundance in comparison to other Coomassie blue-stained bands (such as p99 or NIPP-1). To further validate a specific association with the microcystin matrix, Western blots were performed with identically prepared samples from control and affinity matrix (Fig. 4). Blots were first performed for PP1, p99, and NIPP-1 to affirm the specificity of the microcystin matrix. Results confirm that the splicing factors U5 snRNP p116 and

TABLE I
Nuclear proteins enriched on microcystin-Sepharose chromatography and identified by mass spectrometry

Identity	Database/accession number	Calculated/apparent mass (kDa)	MALDI-TOF protein coverage (%)	Q-TOF MS/MS sequence tags
Myosin heavy chain	Swiss-Prot/P02563	224/200	10	
U5snRNP 200	Swiss-Prot/075643	194/197	26	13
ZAP	NCBI/6016842	155/180	15	8
SAP155	NCBI/9885342	146/155		3
SAP145	NCBI/12653265	98/145		5
TPR-containing SH2 binding phosphoprotein	NCBI/6678441	133/155	14	1
U5snRNP 116	GenPept/15030278	136/116	12	4
p99	GenPept/2773341	93/112	21	23
p79	GenPept/24899184	103/79	25	
hnRNA binding protein M4	NCBI/479852	78/78	19	
Heat shock protein 71 kDa (hsp 70)	Swiss-Prot/P08109	71/70	36	
PP2A Aα subunit	GenPept/189428	65/60	60	
PP2A B/B′ subunit	GenPept/206299	52/50	33–45	
Heterogeneous ribonucleoprotein H	GenPept/6065880	49/48	24	
PP2A Bε subunit	GenPept/1418776	55/48	24	
NIPP-1	GenPept/19344095	39/44	58	
PP1 β isoform	NCBI/227436	37/37	60	
PP1 α1 isoform	NCBI/13928710	37/37	54	
PP1 α2 isoform	NCBI/542987	39/37	58	
PP1 γ1 isoform	NCBI/484316	37/37	57	
CDK1	NCBI/1345706	34/33	28	
U2 snRNP A′	Swiss-Prot/P57784	28/29	25	
NUDIX hydrolase	NCBI/13386394	24/22	36	

p200 and U2 snRNPs SAP145 and SAP155 do specifically associate with a protein phosphatase or that a protein phosphatase associates with the splicing complex with high enough affinity to allow purification of the complex during chromatography. Longer exposures do reveal that these splicing factors associate weakly with the control matrix but are enriched on microcystin-Sepharose. The amount of each of these splicing factors that associate with the affinity matrix is ~0.1–0.2% of the total amount of splicing factor present in a nuclear extract. To confirm the interaction of the U2 snRNPs with a protein phosphatase, we performed a co-immunoprecipitation experiment using the SAP155 antibody. This demonstrated that PP1, but not PP2Ac, was in a complex with the U2 snRNP SAP155 (Fig. 5). The targeting of a protein phosphatase to the splicing machinery is consistent with previous results showing that particular steps of the splicing event are regulated by reversible phosphorylation (28–31). The ~200-kDa band was identified by LC-MS/MS as myosin. Although myosin does bind nonspecifically to the control matrix, this molecule was consistently enriched 2- to 3-fold on the microcystin matrix for several different preparations (Fig. 4). In light of this result, we then blotted the nuclear extract and microcystin and blank column eluents with antibodies generated against the smooth muscle myosin phosphatase regulatory proteins M_{110} and M_{20}. No immunoreactive bands for the M_{20} subunit were seen in the nuclear extract or column eluents. A doublet of ~120 kDa and a band of 55 kDa were noted in nuclear extracts and the microcystin-Sepharose eluent for the M_{110} antibody. Fig. 4*J* shows the ~120-kDa immunoreactive doublet. The 55-kDa band is not shown but is likely the classic M-subunit proteolytic degradation product, which retains the ability to bind PP1 (12). The putative RNA binding protein ZAP

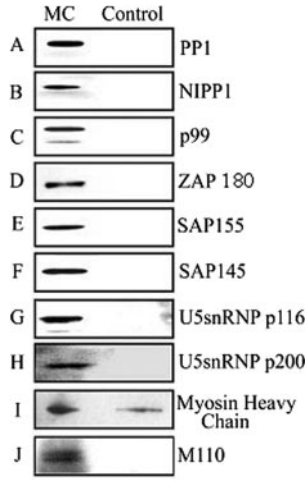

FIG. 4. **Validation of MS results.** MS results were confirmed by Western blot analyses of nuclear proteins eluted from microcystin-Sepharose or a control matrix. Proteins identified by mass spectrometry were found to be specifically associated with the microcystin affinity matrix by running control columns in parallel, eluting and concentrating to an identical volume. Equal volumes of either the microcystin (*MC*) or control column (*Control*) elution were run on SDS-PAGE, blotted to nitrocellulose, and probed with the indicated antibodies (see "Experimental Procedures"). All immunoreactive bands were at the expected molecular mass. *A*, PP1; *B*, NIPP-1; *C*, p99; *D*, putative RNA binding protein ZAP; *E*, SAP155; *F*, SAP145; *G*, U5 snRNP p116; *H*, U5 snRNP p200; *I*, myosin heavy chain; *J*, myosin phosphatase M_{110} subunit.

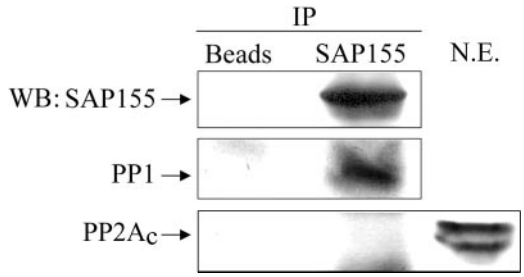

FIG. 5. **PP1 but not PP2A$_c$ associates with the U2 snRNP complex.** A nuclear extract sample was immunoprecipitated with anti-SAP155 antibodies or beads alone and equal volumes of immunoprecipitated proteins separated by SDS-PAGE, blotted to nitrocellulose, and probed with SAP155, PP1, and PP2A$_c$ antibodies. The lane labeled *NE* contains 40 μg of nuclear extract protein.

was also shown to associate specifically with the affinity matrix (Fig. 4). To further establish an interaction between PP1 and ZAP, we generated ZAP anti-peptide antibodies. Western analysis of nuclear and cytosolic fractions with this serum revealed an immunorecative band the same size as the band identified as ZAP in Fig. 3 (180 kDa) and an additional protein of $\sim$ 240 kDa. Both were localized exclusively to the nucleus (Fig 6*A*). After immunoprecipitation with the anti-ZAP sera, both the 180- and 240-kDa forms were visualized by Western blotting with the same antibody (data not shown). Blotting the same immunoprecipitation samples also confirmed that PP1, but not PP2A$_c$, forms a complex with ZAP (Fig. 6*B*). Far Western experiments show that the 180- and 240-kDa immunoprecipitated ZAP proteins also have the ability to bind PP1 in an overlay, suggesting they form a direct interaction with PP1, and this can be blocked with the ZAP peptide GKKRVRWADLE containing the putative PP1 binding site (Fig. 6*C*).

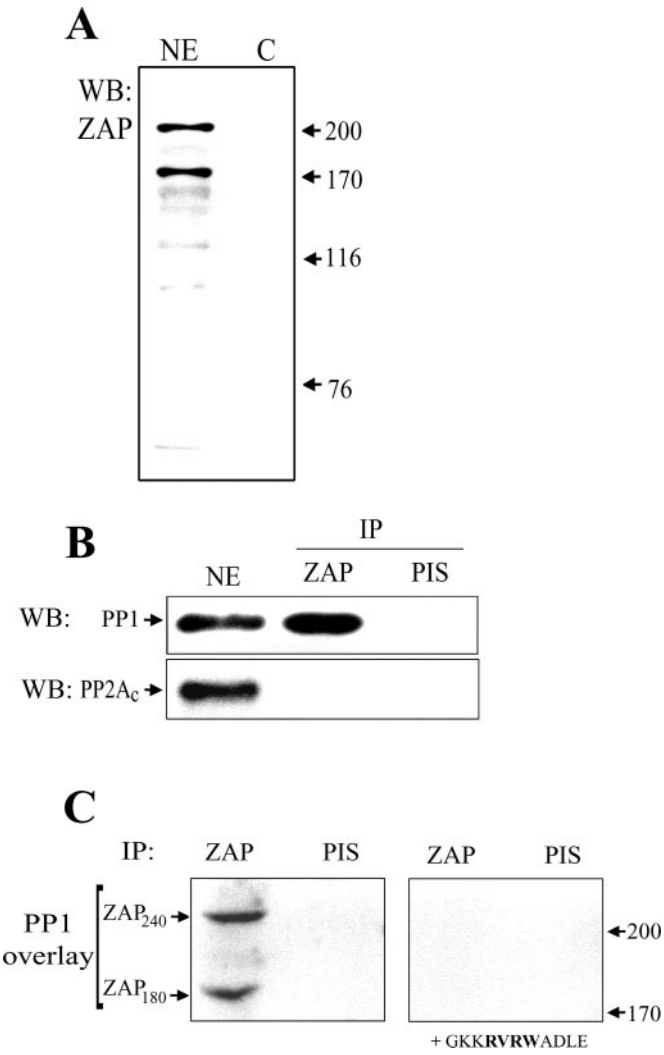

FIG. 6. **Characterization of the ZAP-PP1 complex.** ZAP anti-peptide antibodies were affinity purified and used at 1 μg/ml to probe cytosolic and nuclear extract (*NE*) fractions resolved by 7.5% SDS-PAGE and blotted to nitrocellulose (*A*). A nuclear extract sample was immunoprecipitated with anti-ZAP antibodies and immunoprecipitated proteins separated by SDS-PAGE, blotted to nitrocellulose, and probed with PP1 and PP2A$_c$ antibodies (*B*). The immunoprecipitated sample was also probed with labeled PP1 to demonstrate that the immunoprecipitated ZAP bands retained the ability to directly bind PP1 (*C*, *left panel*), and this can be blocked by inclusion of the GKKRVRWADLE peptide derived from ZAP.

DISCUSSION

The nucleus is a dynamic and highly regulated cellular environment. Reversible protein phosphorylation plays a key role in regulating a vast array of processes in the mammalian nucleus, yet little is known about the nuclear protein phosphatases. We have exploited the power of the protein phosphatase affinity matrix microcystin-Sepharose, the PP1 overlay technique, and mass spectrometry to identify novel PP1-associated proteins and phosphatase complexes of the mammalian nucleus. In this study, we have isolated rat liver nuclei, extracted proteins, and demonstrated that many more PP1 binding proteins are present in the nucleus then previously shown. These proteins copurify on microcystin-Sepharose and several were identified.

By utilizing immunofluorescence confocal microscopy and isoform-specific antipeptide antibodies, other studies have shown that the PP1 isoforms α, β, and γ1 are present in the nucleus (32), although their serum did not distinguish between PP1α1 and α2 or recognize PP1γ2. Trinkle-Mulcahy *et al.* (7) have shown PP1γ1 and α1 to be highly enriched in the nucleolus and nucleoplasm, respectively, using fluorescent PP1 fusion proteins. Our mass spectrometry data confirms that four of the five mammalian PP1 gene products are present in the nucleus. The sole difference between PP1γ1 and PP1γ2 is a short region on the C terminus that is generated by alternative splicing. The tryptic peptide unique to γ1 was identified in our ion mass data, but not the peptide unique to γ2. The PP2A Aα gene product and what is likely a mixture of B and B′ subunits of PP2A were also present in the column eluate.

We have identified a novel protein, previously annotated as ZAP, as a new potential PP1-targeting subunit. This protein purifies on microcystin-Sepharose, binds readily to PP1 in an

overlay assay (this association can be blocked by a peptide containing the PP1 binding region; Figs. 1 and 6*C*), and has a classic PP1 binding motif near its C terminus. This interaction was supported by immunoprecipitating ZAP from a nuclear extract and showing that PP1, and not $PP2A_c$, co-immunoprecipitates. Furthermore, the immunoprecipitated ZAP binds PP1 in the overlay assay. A compiled list of established PP1 binding proteins and their PP1-interacting sites show that a majority of binding sites conform to the K/R-V/I-X-F/W signature. This list, in conjunction with data from panning a random peptide library with PP1 (16), also reveals a preference for other amino acids at particular positions. Notable is an acidic residue two positions C-terminal to the F/W and a basic residue (usually R or H) between the V/I and F/W site. A string of basic residues often precedes the signature R/K position. The putative PP1 binding site in ZAP fits all this criteria perfectly, and we propose that PP1 interacts with ZAP at the site KKRVRWAD. Furthermore, the peptide GKKRVRWADLE based on this site blocks the binding of PP1 to ZAP (Fig. 6*C*). Consistent with the idea that this is the PP1 binding region, this motif resides near the C terminus of the protein and likely is positioned on the protein surface and not buried. The function of ZAP in the nucleus is not defined at the moment. Blast, Fasta, and motif searches with the protein sequence reveal that ZAP has regions that display homology to RNA helicases, the viral protein 2C which is an RNA/nucleotide binding protein, adenylate and uridine kinases, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase, and several pre-mRNA splicing factors. The ZAP protein contains an abundance of RG motifs and proline-rich domains followed by an arginine/aspartate (RD)-rich region. RG, Pro, and RD-rich motifs have been noted to be present in RNA binding proteins associated with pre-mRNA splicing (33, 34), including snRNP-B′, U1 snRNP 70 kDa, SAP49, SAP 62, SF3a 120K, and U2 snRNP A′, all of which were identified as having homologous regions in our database searches. Taken together, this information suggests a link of ZAP function to RNA binding/RNA metabolism. Although ZAP has motifs found in many splicing factors, a recent catalogue of the spliceosomal proteins did not identify ZAP (33, 34). Future work will examine the role of targeting PP1 to this nuclear protein.

The splicing of pre-mRNAs into mature mRNA is carried out by a large nuclear protein/RNA complex referred to as the spliceosome, which is composed of at least 145 proteins and five small nuclear RNAs (U1, U2, U4, U5, and U6) (33, 34). There is a stepwise assembly of spliceosomal components onto pre-mRNAs through a recruitment of factors from a nuclear "reservoir" known as speckles (29, 30). The purification of spliceosomes has revealed that the complex is sensitive to salt, and many protein components dissociate in even 200 mM NaCl (35). Considerable evidence has implicated protein (de)phosphorylation with assembly, catalysis, and disassembly of the spliceosome (28–31), with particular steps dependent upon PP2C and the okadaic acid-sensitive protein phosphatases (PP1 and PP2A). Western analysis confirmed the MS results that U5 snRNPs p116 and p200 and U2 snRNPs SAP145 and SAP155 are selectively retained on the microcystin affinity matrix. The U2 snRNP A′ also appears to be enriched compared with the control matrix. Only ~0.1–0.2% of the nuclear pool of these factors was bound to the matrix, consistent with the idea that only a portion of these factors are actively involved in splicing and that microcystin-sensitive protein phosphatases may only interact transiently with the spliceosome. We performed a co-immunoprecipitation experiment with SAP155 antibodies and demonstrated that PP1 is retained in this complex. This is consistent with a recent report where it was demonstrated that the FHA domain of the nuclear PP1-targeting subunit NIPP-1 associates with SAP155 in a phosphorylation-dependent manner (31). SAP155 is only phosphorylated in functional spliceosomes, and this modification takes place concomitant with or just after the first splicing step of the U2 snRNP (23). This implies that a dephosphorylation event must take place. It is likely that the U2 snRNPs and possibly a larger part of the splicing complex were retained on our affinity matrix due to the association of NIPP-1 with SAP155. The fact that only a selection of splicing factors appear to be enriched on the phosphatase affinity matrix may reflect the association of protein phosphatases with the complex only during specific steps of assembly, catalysis, and disassembly. It is also likely that this is partially due to the salt sensitivity of the splicing machinery complex (35). Our work supports the idea that protein phosphatases are important components of the splicing machinery. To date, mass spectrometric analysis of splicing components and even 145 distinct spliceosomal proteins from the whole complex surprisingly failed to find the association of any protein phosphatase subunits, with the exception of PP2C (33). This is likely due to the fact that protein phosphatase inhibitors have not been used during isolation of the complex. Here, we have incubated the nuclear extracts with a potent phosphatase inhibitor coupled to a matrix. This should abolish all microcystin-sensitive protein phosphatase activity and lock the phospho-status of the extract. No PP1 or PP2A has been isolated with spliceosomal preparations, yet we have purified several splicing components with protein phosphatases, which suggests that these phosphatases may interact transiently with the splicing machinery in a phosphorylation-dependent event. Further work is necessary to define specific roles for protein phosphatases in splicing events and to determine if protein phosphatase regulatory subunits other than NIPP-1 target to the splicing complex.

The nudix hydrolases or nudix pyrophosphatases are a group of enzymes currently thought to play a role in cleansing the cell of nucleoside diphosphate derivatives (36). To date there is no information suggesting the interaction of one of these enzymes with a protein phosphatase (or phosphatase complex) or the specific localization to the nucleus. It is interesting that this enzyme appears to enrich on our matrix (Figs.

2 and 3), and determining if it is associated with a protein phosphatase specifically or if it exists in a phosphatase complex will be an intriguing endeavor.

Myosin, in conjunction with actin, functions as a molecular motor in cells. Myosin has been localized to the nucleus (37), but to our knowledge, this is the first demonstration of a myosin PP1-targeting subunit in the nucleus of the cell and most likely explains the enrichment of myosin on our matrix. Further work is necessary to determine the role of PP1 bound to myosin in the nucleus. Antibodies were not available to determine if all proteins that appeared to be enriched on the microcystin matrix (Table I) are specifically associated or not. These proteins await further studies.

Our overlay method revealed several more PP1 binding proteins with molecular masses between 185 and 250 kDa that are present in the column elution (Fig. 3*B*). Although mass spectrometry did not determine the ~240-kDa protein to be ZAP, Western analysis indicated that this PP1 binding protein is ZAP_{240}. To date, we have been unable to identify these other high-molecular-mass proteins unequivocally, and further work is needed to reveal their identity. We speculate that these, and likely more, lower-abundance PP1 binding proteins are present in the nucleus. Due to the nonspecific binding of protein and likely protein/nucleic acid complexes to Sepharose beads, we have probably reached the limit of the power of microcystin-Sepharose to purify PP1 complexes (Fig. 2) from nuclear extracts. An additional refinement of the technique may be necessary to proceed and further enrich these complexes to identify additional PP1-targeting subunits.

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