PNUTS, a Protein Phosphatase 1 (PP1) Nuclear Targeting Subunit
CHARACTERIZATION OF ITS PP1- AND RNA-BINDING DOMAINS AND REGULATION BY PHOSPHORYLATION

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PNUTS, Phosphatase 1 NUncle Targeting Subunit, is a recently described protein that targets protein phosphatase 1 (PP1) to the nucleus. In the present study, we characterized the biochemical properties of PNUTS. A variety of truncation and site-directed mutants of PNUTS was prepared and expressed either as glutathione-S-transferase fusion proteins in Escherichia coli or as FLAG-tagged proteins in 293T cells. A 50-amino acid domain in the center of PNUTS mediated both high affinity PP1 binding and inhibition of PP1 activity. The PP1-binding domain is related to a motif found in several other PP1-binding proteins but is distinct in that Trp replaces Phe. Mutation of the Trp residue essentially abolished the ability of PNUTS to bind to and inhibit PP1. The central PP1-binding domain of PNUTS was an effective substrate for protein kinase A in vitro, and phosphorylation substantially reduced the ability of PNUTS to bind to PP1 in vitro and following stimulation of protein kinase A in intact cells. In vitro RNA binding experiments showed that a C-terminal region including several RGG motifs and a novel repeat domain rich in His and Gly interacted with mRNA and single-stranded DNA. PNUTS exhibited selective binding for poly(A) and poly(G) compared with poly(U) or poly(C) ribonucleotide homopolymers, with specificity being mediated by distinct regions within the domain rich in His and Gly and the domain containing the RGG motifs. Finally, a PNUTS-PP1 complex was isolated from mammalian cell lysates using RNA-conjugated beads. Together, these studies support a role for PNUTS in protein kinase A-regulated targeting of PP1 to specific RNA-associated complexes in the nucleus.

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1 The abbreviations used are: PP1, protein phosphatase-1; PNUTS, protein phosphatase-1 nuclear targeting subunit; DARP-S2, dopamine- and cAMP-regulated phosphoprotein, M, 32,000; PBS, phosphate-buffered saline; GST, glutathione S-transferase; ssDNA, single-stranded DNA; FLAG, epitope tag of sequence DYKDDDDK; PKA, protein kinase A; hNRNP, human nuclear ribonucleoprotein; ECL, enhanced chemiluminescence; PMSF, phenylmethylsulfonyl fluoride; ATP-S, adenosine 5’-(thiotriphosphate); pBR, retinoblastoma protein; CREB, cAMP-response element-binding protein.
for driving structural reorganization of the nuclear envelope, spindle apparatus, and chromosomal DNA (21–25). PP1 also interacts with other nuclear proteins including the p53-binding protein, p53BP (26), Box11 (27), and with sds22, a protein implicated in chromosome stability (28, 29).

We and others have reported recently (30, 31) the cloning and initial characterization of a novel nuclear PP1-binding protein named PNUTS (Phosphatase 1 NUclear Targeting Sub-unit) or p99. PNUTS exhibits a discrete nuclear compartmentalization and is found in a stable complex with PP1 in mammalian cell lysates. Recombinant PNUTS potently inhibits the catalytic activity of PP1 toward exogenous substrate in vitro. Purification and analysis indicate that the C terminus of PNUTS contains several closely spaced RGG sequences, motifs that are often found in RNA-binding proteins (32). PNUTS also contains a novel region of repetitive amino acid sequence that is rich in His and Gly, and a putative Zn2+ finger domain with the signature CXXCXXCXXH. In the present study, we have characterized further the biochemical properties of PNUTS. PNUTS contains a short ~50-amino acid central region that contains closely associated PP1-binding domains and inhibitory domains. Moreover, the interaction of PNUTS with PP1 is regulated by phosphorylation within the binding domain. We have also found that PNUTS binds to homopolymeric RNA with high selectivity for poly(A) and poly(G) via the RGG motifs and the novel region rich in His and Gly. These studies support the conclusion that PNUTS may mediate the reversible association of PP1 with specific RNAs in the nucleus of mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ribonucleotide homopolymer-agarose beads were obtained from Sigma. An in vitro transcription and translation kit was purchased from Promega. [35S]Methionine, ssDNA-digoxigenin–1–3 mg of denatured calf thymus DNA/ml gel), secondary antibodies, and enhanced chemiluminescence (ECL) reagents were obtained from Amersham Biosciences. Synthetic peptides (DARP-32 (1–39), with a Cys residue included at the C terminus), PNUTS-392–408, PNUTS-392–415, PNUTS-392–408, W401A), and PNUTS-392–415, PNUTS polyclonal PNUTS, PNPIs, and PP1 antibodies were prepared as described (30, 35). Anti-glutathione S-transferase (GST) monoclonal antibody and anti-hnRNP C monoclonal antibody were generous gifts from Drs. E.-Y. Choi and M.-Y. Choi, respectively. Full-length DARPP-32 was expressed in bacteria, purified, and phosphorylated at Thr34 by PKA and ATP essentially as described (36).

**pCdNA1Neo-FLAG Plasmid Construction and Co-immunoprecipitation**—DNA fragments of PNUTS were amplified by Pfu polymerase; PCR products were digested by SalI/NotI restriction enzymes and subcloned into pCdNA1Neo (Invitrogen) encoding a Flag epitope with a 5· SaI site in-frame with the Flag sequence. Internal deletion mutants were prepared from the ligation of two individual PCR fragments. HEK293T cells grown in Dulbecco’s modified Eagle’s culture medium (10% fetal bovine serum) were transiently transfected with different expression vectors. Plasmids were purified using a Qiagen kit according to the manufacturer’s instructions.

**Expression of GST Fusion Proteins in Bacteria**—The plasmids encoding various deletion mutants of PNUTS were amplified by PCR with primers containing 5′ EcoRI and 3′ NotI restriction sites. PCR fragments were digested with EcoRI and NotI and subcloned into the pGEX-5x-1 expression vector. Plasmids were transformed into Escherichia coli (BL21 DE3), and bacteria were cultured in LB medium in the presence of 50 mg/ml ampicillin to an A600 value of 0.6–0.8 at 37 °C. Expression of GST fusion proteins was induced by addition of 0.3 mg of isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 3 h. Cells were collected by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1 mM PMSF, 0.01% Brij 35, 0.3 mg/ml bovine serum albumin, 10% glycerol, 0.25 mM DTT, and 1% Triton X-100), and lysed by sonication on ice. Lysates were centrifuged at 12,000 × g for 20 min, and supernatants were loaded onto a column containing glutathione-agarose beads (Sigma) and washed extensively with PBS. Fusion proteins were eluted with 5 mM glutathione, 50 mM Tris-HCl, pH 8.0. Protein purity was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue, and protein concentration was determined using the BCA assay.

**Site-directed Mutagenesis of GST-PNUTS**—Point mutations were performed by Quick Change Mutation Kit using the manufacturer’s protocol (Stratagene). The W401A, V399A, and R396A mutant cDNAs were transformed into BL21 DE3 cells and bacteria were cultured in LB media containing 50 mg/ml ampicillin at 30 °C. Cells were collected by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100), and lysed by sonication on ice. Lysates were centrifuged at 12,000 × g for 20 min, and supernatants were loaded onto a column containing glutathione-agarose beads (Sigma) and washed extensively with PBS. Fusion proteins were eluted with 5 mM glutathione, 50 mM Tris-HCl, pH 8.0. Protein purity was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue, and protein concentration was determined using the BCA assay.

**Pull-down of PP1 Using GST-PNUTS Fusion Proteins**—Pull-down of PP1 was carried out essentially as described (37). Briefly, assays (final volume 30 μl) contained 50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 0.5% Brij 35, 0.3 mM bovine serum albumin, 10% glycerol, 0.25 mM DTT, and 1% Triton X-100. After centrifugation, beads were washed twice with lysis buffer, eluted with SDS sample buffer, and analyzed by SDS-PAGE (10% acrylamide). PP1 was detected by immunoblotting using an anti-PP1 antibody.

**PP1 Overlay Assay**—PP1 overlay assays were carried out essentially as described (37). Briefly, proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Filters were incubated with a buffer containing 10 mM Tris-HCl (pH 7.4), 2% (v/v) dried milk, and 0.1% Tween 20. Filters were washed with PBS containing 0.2% Nonidet P-40 and then incubated with PBS/Nonidet P-40 containing 0.1 μg/ml recombinant PP1 and 100 mM microcystin (to inhibit potential dephosphorylation of PNUTS) for 2 h at 4 °C. Filters were washed with PBS/Nonidet P-40, and bound PP1 was detected by immunoblotting using an anti-PP1 antibody.

**Metabolic Labeling**—PC12 cells were incubated in 200 μCi/ml of [32P]Orthophosphate (PerkinElmer Life Sciences) and phosphate-free, serum-free Dulbecco’s modified Eagle’s medium for 2 h. After metabolic labeling, cells were washed three times with PBS, harvested by lysis in an immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 20 mM 2-mercaptoethanol, and 1 mM PMSF), and centrifuged at 14,000 × g for 10 min. Unlabeled HEK293 cells, incubated in the absence of presence of 8-Br-CAMP, were lysed in the same way. Lysates were incubated with control IgG or anti-PNUTS antibody in the absence or presence of antigen peptide, followed by the addition of protein A-agarose beads. Immunoprecipitated proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and visualized by either autoradiography ([32P]-labeled samples) or by immunoblotting with PNUTS or PP1 antibodies.
Phosphorylation of GST-PNUTS Fusion Proteins by PKA—Phosphorylation reactions were performed using the protein of interest and the catalytic subunit of PKA (40 μg/ml) in an incubation mixture using 50 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM EGTA at 30 °C. Reactions were initiated by the addition of ATP (50 μM) in the absence or presence of [γ-32P]ATP. Reactions were terminated at various time points by dilution of the reaction mixture into SDS-PAGE sample buffer, and the stoichiometry of phosphorylation was assessed after SDS-PAGE and autoradiography.

Phosphopeptide Mapping and Phosphoamino Acid Analysis—After autoradiography, gel pieces containing 32P-labeled GST-PNUTS (382-433), GST-PNUTS (382-486), or PNUTS were re-swollen in destain buffer (total volume 40 μl) containing 10% methanol, and dried. Gel pieces were then incubated with 1:1 o-toluidine-2-phenylethyl chloromethyl ketone-treated trypsin (50 μg/ml, Worthington) in 50 mM NH₄HCO₃, pH 8.0 (1 ml) for 1 h at 37 °C. The supernatants containing the soluble phosphopeptides were recovered after centrifugation. The extraction efficiency (~85%) was quantified by Cerenkov counting of the gel pieces and supernatants before and after digestion. Two-dimensional phosphopeptide mapping was performed as described previously (38). For phosphopeptide mapping, electrophoretic separation was at pH 3.5 for 90 min at 400 V, and ascending chromatography was in pyridine/n-butyl alcohol/acetic acid/water (15:10:3:12).

The pattern of tryptic phosphopeptides was detected by autoradiography. For phosphoamino acid analysis, ~10% of the digested sample was hydrolyzed in HCl for 1 h at 110 °C. Lysates were lyophilized and resolved in pH 1.9 buffer containing standard phosphoamino acids (o-phospho-ct-serine, o-phospho-ct-threonine, and o-phospho-ct-tyrosine from Sigma). Phosphoamino acids were separated by one-dimensional thin layer electrophoresis. Standard amino acids were visualized by ninhydrin staining, and autoradiography was used to detect phosphorylated amino acids.

RNA Gel Retardation Assay—GST fusion proteins (10-40 ng) were incubated with ~5 ng of 32P-labeled β-globin mRNA in binding buffer (total volume 40 μl) containing 10 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM MgCl₂, and 40 ng of RNAsin (Roche Molecular Biochemicals). Samples were incubated for 10 min on ice. After addition of 5 μl of electrophoresis buffer containing 10% glycerol and 0.01% bromphenol blue, reaction mixtures were separated on a 4% native polyacrylamide gel for 2-3 h at 20 mA (about 6 V/cm) at room temperature. After electrophoresis, gels were dried and exposed to Hyperfilm MP.

In Vitro Transcription and Translation—cDNA encoding full-length PNUTS was subcloned into the pGEM T vector (Promega); the plasmid was linearized by digestion with ScaI and used as a template for RNA synthesis with T7 polymerase. The resulting RNAs were translated in rabbit reticulocyte lysate in the presence of [35S]methionine according to the manufacturer’s suggested conditions (Amersham Biosciences). Translated protein was analyzed by SDS-PAGE (10% acrylamide) and autoradiography.

Ribonucleotide Homopolymer and ssDNA Binding Assays—Assays were initiated by addition of 25 μl of ribonucleotide homopolymer-agarose or ssDNA-agarose into binding buffer with various GST fusion proteins (total volume of 125 μl of 10 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 0.1% Triton X-100, 3 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.05 mM EDTA, 0.1 mM NaCl, or other salt at the indicated concentrations, 1 mM PMSF) at 4 °C.

A 30 min of incubation, beads were centrifuged and washed 6 times with 0.5 ml of binding buffer, and proteins were eluted in 50 μl of SDS sample buffer. Samples were analyzed by SDS-PAGE (10% acrylamide) and immunoblotting using an anti-GST antibody.

Binding of in vitro translated protein to ribonucleotide homopolymer-agarose was performed essentially as described (39). An equivalent of 10° cpm of 32P-labeled in vitro translation product and 25 μl of homopolymer RNA beads were incubated at 4 °C for 10 min in a total volume of 0.25 ml of binding buffer (10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl₂, 0.5% Triton X-100, at the salt concentrations indicated). The beads were pelleted by a brief centrifugation and washed 5 times with 0.5 ml of binding buffer, and protein was eluted with SDS sample buffer. Samples were analyzed by SDS-PAGE (10% acrylamide) and autoradiography.

Ribonucleotide Homopolymer Pull-down Assay and Poly(G)-agarose Column Chromatography—HEK293T cells were lysed by brief sonication in buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP40, 10 μg/ml leupeptin and antipain, 10 μg/ml pepstatin A and chymostatin, 0.5% Nonidet P-40). Lysates were centrifuged at 15,000 × g for 20 min. The supernatant (400 μl, 500 μg of protein) was mixed with 50 μl of homopolymer RNA beads and incubated at 4 °C for 1 h. The beads were centrifuged and washed 5 times with 0.5 ml of lysis buffer containing 0.25 mM NaCl, and proteins were eluted in 50 μl of SDS sample buffer. Samples were analyzed by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membrane (Immobilon-P, Millipore). Proteins were detected by immunoblotting using antibodies against PNUTS, PP1α, and hnRNP C, and detection was by the ECL method.

For poly(G) column chromatography, 2 ml of the supernatants (2 mg of protein) were mixed with 200 μl of poly(G) and incubated at 4 °C for 1 h. Beads were washed once with 1 ml of lysis buffer and loaded onto a column, which was then washed extensively with lysis buffer. Bound proteins were eluted with a linear 0.1-2.0 M NaCl gradient in lysis buffer. Fractions (0.5 ml) were collected, and proteins in each fraction were analyzed by SDS-PAGE and immunoblotting with antibodies against PNUTS, PP1α, PP1γ, and hnRNP C.

RESULTS

Characterization of Binding of PP1 to PNUTS and Effect on Cell Viability—Previous studies (30) have shown that full-length PNUTS and PNUTS(309–872) (the protein product of clone 14 originally isolated in the yeast two-hybrid screen) were able to interact with PP1α. To characterize further the interaction of PP1 and PNUTS protein, various FLAG-tagged PNUTS fragments were expressed in HEK293T cells, proteins were immunoprecipitated with anti-FLAG antibody, and PP1α was detected in the immunoprecipitates (Fig. 1). All the

FIG. 1. Characterization of binding of PP1 to PNUTS and effect on cell viability. A, the domain organization of PNUTS is shown at the top of the figure. The PP1-binding domain is localized approximately in the middle of the molecule (black shading), and a putative motif (KKKRK) for nuclear localization is at residues 157–161 (gray shading). The C terminus of PNUTS contains three distinct domains present in the mLSTM binding (detailed in Fig. 6A). 293T cells were transiently transfected with various FLAG-tagged PNUTS mutants (N- and C-terminal amino acid number is indicated within each rectangle). Cell toxicity was estimated by counting viable cell numbers 24 h after transfection. PP1 binding was measured as shown in B. For cell toxicity, – indicates no effect; + and ++ indicate slight and potent toxicity, respectively. B, cells were lysed and anti-FLAG antibody was used to immunoprecipitate each PNUTS mutant. Immunoprecipitated (IP) samples were analyzed by immunoblotting (WB) using a PP1α antibody. Lane 1 shows untransfected cells; lanes 2–15 correspond to the mutants shown in A. Qualitative analysis of the amount of co-precipitated PP1α is shown in the 1st column in A. Results are representative of three experiments.
PNUTS fragments were expressed at equivalent levels (data not shown). PP1 was bound to fragments containing residues 309–872, 309–589, 309–433, 357–537, 357–436, 357–433, 143–433, and a fragment between residues 143 and 872 with an internal deletion of residues 434–589. A low level of PP1 was found to bind to PNUTS-(309–401), but no binding was detected for PNUTS-(404–537), PNUTS-(589–872), and PNUTS-(724–872) or for two fragments with an internal deletion of residues 254–589. Together, these results indicate that PP1 binds to PNUTS between residues 357 and 433, with the C-terminal boundary of the binding site close to residue 401. The viability of the 293T cells was significantly affected by transfection with many of the PNUTS plasmids. Notably, all of the PNUTS fragments that bound strongly to PP1 caused cell death (Fig. 1). Neither a nuclear localization signal (a KKKRK motif at residues 157–161) nor the C-terminal region (residues 590–872) was required for cell toxicity. Expression of PNUTS-(357–468) caused cell toxicity more effectively than PNUTS-(357–433) or PNUTS-(309–433), suggesting that residues 434–486 contributed to regulation of cell viability but were not required for PP1 binding.

PNUTS Contains Closely Associated PP1 Binding and Inhibitory Domains—Our previous studies showed that a GST fusion protein containing residues 309–872 potently inhibited the phosphorylase phosphatase activity of PP1 in vitro (30). The results shown in Fig. 1 and other preliminary studies indicated that both PP1 binding and inhibition appeared to be contained within residues 382–537 of PNUTS. Various GST fusion proteins containing residues 382–537 of PNUTS were expressed in bacteria, purified, and incubated with a 293T cell extract. PP1 co-precipitated with GST-PNUTS fusion proteins containing residues 382–486, 382–450, 382–444, 382–438, 382–433, and 382–417 (Fig. 2). Further C-terminal deletion of amino acids 410–417 (GST-PNUTS-(382–409)) significantly reduced PP1 binding, and PP1 did not bind to GST-PNUTS-(404–537). Analysis of residues 382–409 of PNUTS identified a sequence, Arg-Lys-Thr-Val-Thr-Trp, that is similar to the (Arg/Lys)-(Arg/Lys)-X-(Val/Ile)-X-Phe docking motif found in many PP1-binding proteins. The interaction of PP1 with GST-PNUTS-(382–433) was completely abolished by mutation of Trp401, reduced by mutation of Val399, but not significantly affected by mutation of Lys397 or Arg396 (all mutations to alanine). These results indicated that both PP1 binding and inhibition appeared to be contained within residues 382–409 of PNUTS identified a sequence, Arg-Lys-Thr-Val-Thr-Trp, that is similar to the (Arg/Lys)-(Arg/Lys)-X-(Val/Ile)-X-Phe docking motif found in many PP1-binding proteins. The interaction of PP1 with GST-PNUTS-(382–433) was completely abolished by mutation of Trp401, reduced by mutation of Val399, but not significantly affected by mutation of Lys397 or Arg396 (all mutations to alanine). These results indicated that both PP1 binding and inhibition appeared to be contained within residues 382–409 of PNUTS identified a sequence, Arg-Lys-Thr-Val-Thr-Trp, that is similar to the (Arg/Lys)-(Arg/Lys)-X-(Val/Ile)-X-Phe docking motif found in many PP1-binding proteins.

Regulation of PP1 Activity by Peptides Encompassing the PP1-binding Site of PNUTS—We examined in more detail the features of PNUTS surrounding the PP1 docking motif. GST-PNUTS-(382–433) (at only 30 nM) antagonized the ability of thiophospho-DARPP-32-(1–39) to inhibit PP1 activity as demonstrated by an increase in the IC50 by more than 2 orders of magnitude (Fig. 3B and Table I). However, mutation of Trp101 (GST-PNUTS-(382–433;W401A)) resulted in a PNUTS fragment that was unable to antagonize the inhibitory action of thiophospho-DARPP-32-(1–39) (Fig. 3B). A shorter synthetic peptide encompassing residues 392–415 of PNUTS was also very effective at antagonizing the inhibitory action of phospho-DARPP-32 (Fig. 3C and Table I) (note here that full-length thiophospho-DARPP-32 and higher concentrations of competing peptide were used). However, a shorter peptide PNUTS-(392–408) was much less effective in antagonizing the actions of thiophospho-DARPP-32, suggesting that residues 409–415 (Glu-Tyr-Phe-Tyr-Phe-Glu-Leu) contribute to the binding of PNUTS to PP1. Similar results were obtained when PP1 was inhibited using various concentrations of spinophilin or inhibitor-2 (data not shown). As expected, mutation of Trp101 in PNUTS-(392–408) rendered the peptide completely ineffective in competing with thiophospho-DARPP-32 (Fig. 3C). However, surprisingly, PNUTS-(392–415;W401Y) was very effective in antagonizing the actions of thiophospho-DARPP-32.

In the studies of the various PNUTS peptides as antagonists...
of the actions of PP1 inhibitors, we noted an unusual property of PNUTS-(392–415). PNUTS-(392–415) alone was able to activate consistently PP1 activity by ~30–40% (Fig. 3D). PNUTS-(382–408) was much less effective as an activator, and PNUTS-(392–408;W401A) had no effect on PP1 activity. Consistent with the ability of the PNUTS peptide to activate PP1 via the C-terminal docking site where Trp401 binds, PNUTS-(392–415) had no effect on a PP1/PP2A chimeric enzyme in which the C terminus of PP1 (residues 274–330) was replaced by the equivalent residues of PP2A (data not shown) (see also Ref 40).

Phosphorylation of PNUTS Regulates Its Interaction with PP1—Examination of the amino acid sequence of residues 382–450 of PNUTS revealed the presence of several consensus sites for phosphorylation by PKA. In addition, in preliminary studies using PC12 cells metabolically labeled with [32P]phosphate, characterizations of PNUTS-(382–450) indicated concentrations of thiophospho-DARPP-32-(1–39) (P-D32-(1–39)), in the absence (CON, filled square) or presence of 30 nM GST-PNUTS-(382–433) (open square) or 30 nM GST-PNUTS-(382–433;W401A) (open circle). Results are representative of at least three separate experiments. The activity of PP1 was measured using [32P]phosphorylase a as substrate with the indicated concentrations of thiophospho-DARPP-32-(1–39) (P-D32-(1–39)), in the absence (CON, filled circle) or presence of GST-PNUTS-(392–415) (10 μM) (filled square), GST-PNUTS-(392–408) (10 μM) (filled diamond), PNUTS-(392–408;W401A) (10 μM) (open square), or PNUTS-(392–415;W401Y) (20 μM) (open circle). Results are representative of at least three separate experiments, and the error bars show S.D. Activity was normalized to that measured in the presence of 10 μM PNUTS. D, the activity of PP1 was measured using [32P]phosphorylase a as substrate with the indicated concentrations of PNUTS-(392–415) (filled square), PNUTS-(392–408) (filled diamond), or PNUTS-(392–408;W401Y) (open circle).

**TABLE I**

| Regulation of PP1 activity by GST-PNUTS fusion proteins and peptides | IC<sub>50</sub> values were obtained from curves showing PP1 activity in Fig. 3, A–C. | GST-PNUTS fusion proteins | IC<sub>50</sub> (M) |
|---|---|---|---|
| Inhibition of PP1 activity by GST-PNUTS fusion proteins | | 382–450 | 1 × 10<sup>−10</sup> |
| | | 382–450; W401A | >1 × 10<sup>−6</sup> |
| | | 382–450; V399A | 1 × 10<sup>−6</sup> |
| | | 382–450; R397A | 5 × 10<sup>−10</sup> |
| | | 382–450; R396A | 1 × 10<sup>−10</sup> |
| | | 382–444 | >1 × 10<sup>−6</sup> |
| Competition by GST-PNUTS (382–433) on inhibition by P-D32 (1–39) | | P-D32 (1–39) | 4 × 10<sup>−9</sup> |
| | | P-D32-(1–39) + PNUTS-(382–433) | 6 × 10<sup>−7</sup> |
| | | P-D32-(1–39) + PNUTS-(382–433;W401A) | 6 × 10<sup>−9</sup> |
| Competition by PNUTS peptides on inhibition by S-DARPP-32 | | S-DARPP | 1 × 10<sup>−9</sup> |
| | | S-DARPP + PNUTS-(392–415) | 1 × 10<sup>−6</sup> |
| | | S-DARPP + PNUTS-(392–408) | 6 × 10<sup>−8</sup> |
| | | S-DARPP + PNUTS-(392–408;W401A) | 2 × 10<sup>−9</sup> |
| | | S-DARPP + PNUTS-(392–415;W401Y) | 4 × 10<sup>−7</sup> |
Characterization of PNUTS

full-length PNUTS was found to be phosphorylated (Fig. 4A).

In vitro, GST-PNUTS-(382–433), -PNUTS-(382–450), and -PNUTS-(382–486) were all found to be excellent substrates for PKA (Fig. 4B). GST-PNUTS-(382–486) was more efficiently phosphorylated, and to a higher stoichiometry, than GST-PNUTS-(382–450). Moreover, tryptic phosphopeptide mapping studies indicated that GST-PNUTS-(382–486) contained two major phosphorylation sites, whereas GST-PNUTS-(382–433) contained only one major phosphorylation site (Fig. 4C, note the two phosphopeptides are likely derived from alternative tryptic digestion). Notably, GST-PNUTS-(382–486) contains two potential PKA consensus sites, Arg-Lys-Arg-Lys-Thr-Val-Thr-Trp (residues 394–401, either Thr residue might be phosphorylated) and Arg-Arg-Leu-Ser-His (residues 448–452), suggesting the possibility that phosphorylation of Ser651 might explain the increased level of phosphorylation of GST-PNUTS-(382–486). However, phosphoamino acid analysis of the [32P]labeled GST-PNUTS proteins indicated that phosphorylation occurred almost exclusively on threonine (Fig. 4D). These results indicate that Ser651 is not phosphorylated but that one or more threonine residues close to the PP1 docking motif are phosphorylated by PKA.

To determine whether phosphorylation of PNUTS by PKA might affect the interaction with PP1, GST-PNUTS-(382–433) was phosphorylated by PKA and [γ-32P]ATP for various times (Fig. 5A, top panel). Maximal phosphorylation was reached between 40 and 70 min (a maximal stoichiometry of ~1 mol/mol was determined). The phosphorylated samples were separated by SDS-PAGE, transferred to Immobilon-P membrane, and incubated with PP1α. The amount of PP1 bound to GST-PNUTS-(382–433) decreased in parallel to the increase in phosphorylation (Fig. 5A, middle panel). These results indicate that phosphorylation of PNUTS by PKA within the site of PP1 binding blocks the association of PNUTS with PP1. We further investigated whether PKA regulates interaction of PNUTS with PP1 in intact cells. In intact HEK293 cells incubated in the presence of forskolin, PNUTS was phosphorylated largely on threonine (Fig. 4D). In addition, phosphopeptide mapping studies indicated that PNUTS was phosphorylated at the same site as that phosphorylated by PKA within GST-PNUTS-(382–433) (Fig. 4C). In parallel studies, HEK293 cells were incubated in the absence or presence of 8-Br-cAMP (500 μM for 10 min), and the interaction between PP1α and PNUTS was examined following co-immunoprecipitation (Fig. 5B). By using antibodies specific for either PP1α or PNUTS, the interaction between PNUTS and PP1 was shown to be significantly reduced by activation of PKA with 8-Br-cAMP. Together these results suggest that phosphorylation of PNUTS by PKA negatively regulates the interaction of PNUTS with PP1.

The C Terminus of PNUTS Binds to mRNA and Single-stranded DNA—PNUTS contains multiple closely spaced repeats of the amino acid sequence, RGG, a motif often found in RNA-binding proteins (32) (Fig. 6A). The RGG motifs are followed by a region with several imperfect repeats of a sequence rich in histidine and glycine. The extreme C-terminal region then contains a putative zinc finger domain. These features together with its nuclear localization suggested that PNUTS might interact with nucleic acids. To initially examine the interaction with RNA, a gel retardation assay was performed with various GST fusion proteins and [32P]labeled β-globin mRNA. GST-PNUTS-(617–872) retarded the mobility of β-globin mRNA, but deletion of the putative zinc finger domain and most of the region rich in histidine and glycine (GST-PNUTS-(617–762)) resulted in a marked decrease in RNA binding (Fig. 6B). Moreover, partial or complete deletion of the RGG motif (GST-PNUTS-(617–726) or GST-PNUTS-(404–662)) led to an almost complete loss of RNA binding. We next examined the abilities of PNUTS fragments to bind to single-stranded DNA.
Characterization of PNUTS

The top panel, H9251 poly(U) and poly(C) (Fig. 7B) efficiently precipitated by poly(A) and poly(G) but not by binant GST-PNUTS fusion proteins, endogenous PNUTS was immunoblotting. Consistent with the properties of the recom-
agarose beads, and bound proteins were eluted and analyzed by

As a control, hnRNP C, one of the most abundant heterogeneous nuclear ribonucleoproteins (hnRNPs) and known to bind to pre-mRNA, was detected in all the precipitates.

Because PNUTS is able to interact directly with PP1 as well as with RNA, we hypothesized that the PNUTS-PP1 complex may bind to RNA. To examine this possibility, 293T cell lysates were incubated with poly(A), poly(C), poly(G), and poly(U)-agarose beads, and bound proteins were eluted and analyzed by immunoblotting. Consistent with the properties of the recombinant GST-PNUTS fusion proteins, endogenous PNUTS was efficiently precipitated by poly(A) and poly(G) but not by poly(U) and poly(C) (Fig. 7B). Moreover, PP1α was found only in the precipitates from poly(A)- or poly(G)-agarose beads. In addition, depletion of PNUTS from 293T cell extracts by immunoprecipitation using anti-PNUTS antibody significantly reduced the amount of PP1 bound to poly(G)-beads (data not shown). As a control, hnRNP C, one of the most abundant heterogeneous nuclear ribonucleoproteins (hnRNPs) and known to bind to pre-mRNA, was detected in all the precipitates.

Different Subdomains of PNUTS Bind to Poly(A) and Poly(U)—In order to characterize further the ribonucleotide-binding properties of PNUTS, GST-PNUTS fragments were incubated with poly(A), poly(C), poly(G), and poly(U) at 100 mM NaCl. GST-PNUTS-(617–837) bound very similarly to both poly(A) and poly(U) (Fig. 9). However, further deletion of the histidine/glycine-rich region (GST-PNUTS-(617–762)) resulted in a preferential decrease in binding to poly(A). Partial or complete deletion of the RGG motifs led to complete loss in binding to the poly(G) ribonucleic acid homopolymer. Increasing the bead volumes of poly(C) and poly(U) up to 4 times compared with poly(A) did not alter the inability of PNUTS to bind to poly(U) and poly(C) (data not shown).

**DISCUSSION**

In the present study, we have characterized the properties of the interactions between PNUTS and both PP1 and RNA. The
Characterization of PNUTS

results obtained suggest that PNUTS may bind to specific types of RNA implicating PP1 in specific functions within the nucleus. Studies of the interaction with PP1 revealed that PNUTS contains two closely associated subdomains in the center of the protein within −50 amino acids (residues 400–450). These include a high affinity PP1-binding domain located within residues 397–401 of PNUTS and a distinct inhibitory region located within residues 445–450. The binding domain, Lys397-Thr-Val-Thr-Trp401, resembles the consensus motif (Arg/Lys-Arg/Lys-Val/Ile-Phe) found in many other PP1 regulatory subunits except for the replacement of Phe with Trp.

Consistent with residues 397–401 of PNUTS binding to a common docking site in PP1, competition studies indicated that PNUTS peptides as short as residues 392–408 were potent antagonists of the inhibitory actions of thionphospho-DARPP-32. Site-directed mutagenesis studies of PNUTS indicated that the order of importance of amino acids contributing to the association with PP1 is Trp401 > Val399 > Lys397, a pattern consistent with our previous studies of DARPP-32, where Phe111 and Ile8 play critical roles, with Lys7 playing a lesser role in the interaction with PP1 (8, 41). Therefore, it is likely that the conserved docking motif of PNUTS interacts with the exposed hydrophobic docking site in PP1 in a similar manner to DARPP-32 and other PP1-binding proteins.

A notable feature of the PP1 docking motif in PNUTS is the presence of Trp instead of Phe as the most important binding residue (30). In an analysis of a random peptide library that bound to PP1, Phe and Trp were identified with equal frequency in the interacting peptides (42). However, PNUTS appears to be the first well characterized PP1-binding protein that contains Trp within the PP1-docking motif. The affinity of PNUTS for PP1 is suggested from the PP1 inhibition and competition assays to be very high, presumably being in the low nM range. It is possible that the presence of Trp contributes to the high affinity, but it is clear that parts of PNUTS outside of the minimal PP1-docking motif also play a role. PNUTS-(392–408) was very effective in competing against the inhibitory action of thionphospho-DARPP-32, but addition of residues 409–415 made PNUTS-(392–415) a much more effective antagonist. In addition, mutation of Trp401 to Tyr had only a small effect on the antagonist properties of PNUTS-(392–415), and mutation of Phe11 in DARPP-32 to Trp did not affect its inhibitory potency significantly, consistent with the idea that the presence of Phe or Trp as the key docking residue is not critical. The results with PNUTS also support the idea that like DARPP-32, inhibitor-1 and inhibitor-2, targeting proteins are likely to bind to PP1 via multiple subdomains (5, 36, 41, 43, 44).

PNUTS is a highly potent inhibitor of PP1, with an IC50 value of ~10−11 nM using phosphorylase a as substrate. Deletion mutagenesis studies identified a short sequence, ETARRL (residues 445–450), that was responsible for a greater than 104-fold factor in inhibitory potency. Like DARPP-32 and inhibitor-1, the PP1 docking and inhibitory subdomains are contained in a short stretch of amino acids, but unlike phospho-DARPP-32 (phosphorylated at Thr34 by PKA), PNUTS does not have to be phosphorylated in this subdomain to be an effective inhibitor. Possibly, residues 445–450 of PNUTS interact with the active site of PP1 in the manner of a pseudosubstrate, or these residues may act to block interactions of phosphorylase at a substrate-binding site that is situated close to but not within the active site of the phosphatase. Surprisingly, in contrast to the highly potent inhibitory actions of residues 382–450 of PNUTS, addition of peptides encompassing just the docking motif resulted in activation of PP1. This effect required the presence of Trp401 but was more robust when residues 409–415 were included. The molecular basis for this is not currently known, but may reflect an allosteric effect that stabilizes the substrate-binding site or alters the active site.

The PP1-binding domain of PNUTS contains a number of
potential consensus sites for phosphorylation. These include a site at Thr^{359} within the PP1-docking motif and at Ser^{451} close to the inhibitory subdomain. Our results show that a polypeptide encompassing residues 382–433 of PNUTS was efficiently phosphorylated by PKA in vitro, and the resulting phosphorylation decreased its affinity for PP1 in vitro and also in intact cells following stimulation of PKA. Based on analysis of phosphorylation of different PNUTS fragments, and phospho-amino acid analysis, Thr^{359} is a likely candidate for phosphorylation by PKA; notably Ser^{451} was not phosphorylated in vitro. In the human homologue of PNUTS, Thr^{359} is replaced by serine (31). Therefore, these results suggest that the interaction of PNUTS with PP1 may be negatively regulated by phosphorylation in an analogous manner to that observed in studies of a few other PP1-binding proteins. For example, phosphorylation of Ser^{497} in the glycogen-binding G_{s} subunit results in the dissociation of PP1-G_{s} complex (5, 45). Similarly, NIPP1 has phosphorylation sites for PKA (Ser^{193}) and CK2 (Ser^{204}), that flank the PP1-binding motif, and the phosphorylation of either serine impairs PP1 binding and reduces the activity of NIPP1 as a PP1 inhibitor (46, 47). We have also shown that phosphorylation of the brain-specific actin-binding protein, neurarbin, at Ser^{461} by PKA significantly reduces its binding to PP1 (48). These observations together with our present data suggest that reversible phosphorylation of a site at or near the Arg-Lys-Arg/Lys-Val/Ile-X-Phe/Trp motif of PP1 regulatory proteins may be a common control mechanism that adds to the complexity of PP1 regulation in the nucleus and other cellular compartments.

The present study shows that the C-terminal region, including the seven closely repeated RGG boxes and particularly the histidine/glycine-rich domain, mediates the interaction of PNUTS with RNA and ssDNA. However, the zinc finger region did not appear to play any role in the interaction with RNA or ssDNA. The RGG box was first described as an RNA-binding domain in hnRNP U (32, 49). Typically, multiple RGG boxes are found, with as few as 6 and as many as 18 being present in an RNA-binding protein. The RGG boxes are also frequently found adjacent to or interspersed with other RNA-binding motifs such as KH and RBDDomain (32). In some cases such as the fragile X mental retardation protein (FMRP) or the TLS protein, the RGG boxes have intrinsic RNA-binding properties (50–52), whereas in other cases such as nucleolin, the RGG boxes complement the specificity of additional RNA-binding domains (53).

Together, the histidine/glycine region, and to a lesser extent the RGG boxes, appear to be responsible for the high affinity interaction of PNUTS with RNA and may be responsible for specifying the interaction of PNUTS with specific mRNAs in the nucleus. Our studies with RNA homopolymers indicated that PNUTS has high selectivity for binding to poly(A) or poly(G) but not for poly(U) or poly(C). The RGG boxes of PNUTS appeared to be involved in the preferential binding to poly(G). In other cases, the RGG boxes of hnRNP U exhibited highest binding to poly(G) and intermediate binding to poly(U) (49); Nopp44/46 has been shown to bind preferentially to poly(U) (54), and FMRP showed selective binding to poly(G) and poly(U) (54). Interestingly, in the splicing factor, TLS, an N-terminal group of RGG boxes showed selective binding to poly(U), whereas a C-terminal group of RGG boxes showed selective binding to poly(G) (52). The amino acid sequences surrounding the RGG boxes are often rich in aromatic amino acids. However, in PNUTS, the amino acid composition of the RGG domain differs from that of other proteins by the abundance of proline. Therefore, it is likely that the specific amino acid residues surrounding the RGG boxes influence the specificity and avidity of RNA interaction (54). The histidine/glycine region of PNUTS was also necessary for the interaction with native mRNA and ssDNA and perhaps is involved in the preferential binding to poly(A). The histidine/glycine-rich domain is unique to PNUTS, and presumably the repetitive feature of the domain plays some role in the specific interaction with RNA and ssDNA.

The results from our studies indicate that PP1 associates indirectly with RNA through its interaction with PNUTS and suggests a role for PNUTS in anchoring PP1 to RNA-associated complexes. Several lines of evidence indicate that PP1 plays a number of roles in mRNA splicing by reversing the actions of multiple protein kinases (9, 55). PP1 can regulate initial steps in splicing by dephosphorylating factors necessary for spliceosome assembly (17, 18). PP1 interacts with NIPP1 in nuclear speckles, and NIPP1 appears to play a role in the late stage of spliceosome assembly. However, the spliceosome function of NIPP1 seems not to be related to PP1 binding (16, 56), suggesting the possibility that NIPP1 plays an additional role to target PP1 to the splicing machinery. The PP1β isoform has also been found to interact with the poly pyrimidine tract-binding protein-associated splicing factor, although the function of this interaction is not known (57). Finally, in a recent study PP1 has been implicated in the control of alternative splicing of caspase 9 and bcl-x genes in lung adenocarcinoma cells (58).

Initial studies of the localization of PNUTS indicate that the protein exhibits a discrete punctate nucleoplasmic staining pattern with some accumulation in the nucleolus (30, 31). This pattern of localization and the fact that PNUTS interacts with RNA is consistent with a specific function for the protein in the nucleus. Most likely this would involve some aspect of RNA processing or transport of RNA within the nucleus and probably would involve the action of PP1 that is bound to PNUTS at these sites.

It is also possible that PNUTS serves to target PP1 to the nucleus for functions of the phosphatase in addition to, or instead of, regulation of RNA processing. Recent studies (59) have highlighted the close physical association of the transcriptional and splicing machinery. Moreover, although a subpopulation of PP1 is localized in the nucleus, recent studies (60) indicate that this localization is dynamic, and PP1 can rapidly move between subnuclear compartments. Within the nucleus, PP1 plays an important role in the dephosphorylation of transcription factors such as CREB and Sp1 (9, 12, 61). PP1 dephosphorylates Ser^{133} of CREB which is phosphorylated by a number of kinases, including PKA and multiple Ca^{2+}-dependent kinases (12). During de-differentiation associated with liver
regeneration, PP1 was also identified as the enzyme that dephosphorylated Sp1, reversing the action of CK2 (61). PP1 is also an important phosphatase involved in regulation of the retinoblastoma protein, pRb (62). PP1 can bind to pRb and selectively dephosphorylate specific sites of pRb and appears to be the phosphatase responsible for dephosphorylation of pRb at the time of mitotic exit. Moreover, biochemical studies have identified a high molecular complex that dephosphorylates pRb (63) and that contains PP1 and a 110-kDa protein that appears to be identical to PUNTS (62). Thus, PUNTS may not only serve to target PP1 to the nucleus but to influence its specificity toward nuclear substrates.

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PNUTS, a Protein Phosphatase 1 (PP1) Nuclear Targeting Subunit: 
CHARACTERIZATION OF ITS PP1- AND RNA-BINDING DOMAINS AND
REGULATION BY PHOSPHORYLATION

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