The C-terminus of NIPP1 (nuclear inhibitor of protein phosphatase-1) contains a novel binding site for protein phosphatase-1 that is controlled by tyrosine phosphorylation and RNA binding

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Nuclear inhibitor of protein phosphatase-1 (NIPP1; 351 residues) is a nuclear RNA-binding protein that also contains in its central domain two contiguous sites of interaction with the catalytic subunit of protein phosphatase-1 (PP1_C). We show here that mutation of these phosphatase-interaction sites did not completely abolish the ability of NIPP1 to bind and inhibit PP1_C. This could be accounted for by an additional inhibitory phosphatase-binding site in the C-terminal region (residues 311–351), with an inhibitory core corresponding to residues 331–337. Following mutation of all three PP1_C-binding sites in the central and C-terminal domains, NIPP1 no longer interacted with PP1_C. Remarkably, while both NIPP1 domains inhibited the phosphorylase phosphatase activity of PP1_C independently, mutation

of either domain completely abolished the ability of NIPP1 to inhibit the dephosphorylation of myelin basic protein. The inhibitory potency of the C-terminal site of NIPP1 was decreased by phosphorylation of Tyr-335 and by the addition of RNA. Tyr-335 could be phosphorylated by tyrosine kinase Lyn, but only in the presence of RNA. In conclusion, NIPP1 contains two phosphatase-binding domains that function co-operatively but which are controlled independently. Our data are in agreement with a shared-site model for the interaction of PP1_C with its regulatory subunits.

Key words: dephosphorylation, Lyn, mRNA splicing, substrate specificity, targeting.

INTRODUCTION

Type-1 protein phosphatases (PP1s) regulate numerous cellular processes by dephosphorylation of key proteins on specific serine or threonine residues [1–3]. They consist of a catalytic subunit of PP1 (PP1_C) and one or two non-catalytic subunits. While mammalian cells contain at most a few isoforms of PP1_C, they may express tens of structurally unrelated non-catalytic subunits [1–4]. Altogether, about 20 mammalian non-catalytic subunits of PP1 have already been characterized. They are endowed with substrate-specifying and regulatory roles and, in some instances, also mediate the anchoring of the holoenzymes to substrates or to subcellular structures to which the substrates are attached. For example, the G-subunits not only target PP1_C to glycogen particles but also increase its glycogen synthase-phosphatase activity [2]. In addition, the G-subunits mediate the hormonal and metabolic control of the holoenzyme by the binding of allosteric effectors or by the phosphorylation of sites that modulate their interaction with the catalytic subunit. Other regulatory subunits of PP1 seem to fulfil a more restricted role. Thus inhibitor-1 and the structurally related DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) are inhibitory to PP1_C, but only after phosphorylation by protein kinase A (PKA), and they have no known anchoring function [1–3,5].

A detailed understanding of how the regulatory subunits modulate the activity and substrate specificity of PP1_C requires an insight into the molecular determinants of their interaction. Nearly all regulatory subunits appear to have a short motif, often conforming to the sequence RVXF [6,7], which binds in a hydrophobic channel near the C-terminus of the catalytic subunit

[6]. On the other hand, it has been established that the β 12– β 13 loop near the active site of the catalytic subunit is essential for inhibition by various toxins and protein inhibitors [8]. While common or overlapping binding sites may explain why PP1_C does not interact directly with more than one regulator simultaneously, they do not account for the functional differences between the regulatory subunits. An emerging picture is that these differences stem from multiple sites of interaction between the regulatory and catalytic subunits. Thus inhibitor-1 and DARPP-32 contain, besides an RVXF-like sequence, a motif that inhibits the catalytic subunit by acting as a pseudosubstrate after phosphorylation by PKA [9]. Likewise, inhibitor-2 contains several sites of interaction with PP1_C, in addition to a sequence that may be equivalent to the RVXF sequence [9–11].

Nuclear inhibitor of PP1 (NIPP1; 39 kDa) forms an inactive complex with PP1_C, termed PP1N_{NIPP1} [12,13]. The holoenzyme can be activated by phosphorylation of up to four Ser/Thr residues in the central domain of NIPP1 by PKA and protein kinase CK2 [14–16]. This activation results from a disruption of the interaction between PP1_C and the RVXF sequence (residues 200–203) of NIPP1, and from a reduced inhibitory potency of an upstream polybasic inhibitory site (residues 191–200) [17]. Besides interacting with PP1_C, NIPP1 can also bind to RNA, preferentially A/U-rich sequences, via a lysine-rich motif in the C-terminal region [18,19]. Since the binding of PP1_C and RNA are not mutually exclusive, it has been proposed that NIPP1 may function as an RNA-targeting subunit of PP1 [18]. The N-terminus of NIPP1 consists largely of a ‘forkhead-associated’ protein-interaction domain that interacts with CDC5L, a regulator of pre-mRNA splicing and mitotic entry [20]. The association of NIPP1 with splicing factor(s) and/or RNA

Abbreviations used: EGFP, enhanced green fluorescent protein; MBP, myelin basic protein; PP1, protein phosphatase-1; NIPP1, nuclear inhibitor of PP1; PKA, protein kinase A; PP1_C, catalytic subunit of PP1; PP1N_{NIPP1}, complex of PP1_C and NIPP1; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa.

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may account for the localization of $PP1N_{NIPP1}$ in the nuclear speckles [21], which largely correspond to the interchromatin granule clusters [21].

A fragment encompassing the central domain of NIPP1 (residues 143–217) was found to be an equally potent inhibitor as the full-length protein [17]. However, two lines of indirect evidence pointed to the existence of additional phosphatase-binding site(s) in the N- and/or C-terminal thirds of NIPP1. First, activation of $PP1N_{NIPP1}$ by phosphorylation of NIPP1 [16] or by competition with the synthetic peptide NIPP1^{191–210} [17], which includes the polybasic and RVXF-binding sites for $PP1_c$, required the presence of salt. On the other hand, no salt was required for the activation of a complex with NIPP1^{143–217}. Second, while phosphorylation or the addition of NIPP1^{191–210} resulted in a disruption of the complex between $PP1_c$ and NIPP1^{143–217}, no dissociation was seen when the complex had been made with full-length NIPP1 [16,17]. In further agreement with the existence of additional phosphatase-interaction site(s), we report here that mutation of the $PP1_c$ -binding sites in the central domain of NIPP1 did not abolish its interaction with the catalytic subunit. Using various independent approaches, we have mapped an additional phosphatase-binding site to the C-terminus of NIPP1. Depending on the substrate used, the central and C-terminal binding sites acted independently or synergistically in the inhibition of $PP1_c$. We also show that the inhibitory potency of the C-terminal site is decreased by the binding of RNA and by phosphorylation of Tyr-335.

EXPERIMENTAL

The preparation of polyhistidine-tagged NIPP1^{225–351}, NIPP1^{225–310} and NIPP1^{311–351} has already been described [19]. The cDNAs encoding NIPP1^{1–142}, NIPP1^{143–224}, NIPP1^{1–329} and NIPP1^{1–351} were subcloned into the pET16b plasmid, using the *NdeI*–*XhoI* (NIPP1^{143–224}, NIPP1^{1–329}), *NdeI*–*BamHI*

(NIPP1^{1–142}) or *XhoI*–*XhoI* (NIPP1^{1–351}) restriction sites, for expression in BL21(DE3)pLysS cells as polyhistidine-tagged polypeptides. The latter proteins were purified on Ni^{2+} -pentadentate-chelator–Sephacolumns (Affilind). Constructs encoding NIPP1^{1–351} or NIPP1^{1–325} that were fused C-terminally to the enhanced green fluorescent protein (EGFP), for expression in COS-1 cells, were made by cloning of the bovine NIPP1 cDNAs into the *XhoI*–*SacII* sites of pEGFP-N1 (Clontech). All other NIPP1 fragments and phosphopeptides were made synthetically on a Milligen 9050 (Applied Biosystems), using the *N*-(9-fluorenyl)methoxycarbonyl method. Mutations of the RVTF and polybasic sequences in the central domain of NIPP1 (see Figure 1A) and of C-terminal phosphorylation sites were made according to the QuikChange site-directed mutagenesis protocol of Stratagene, using the appropriate primers and templates. The sequences of the DNA constructs were verified by cycle sequencing on an AlfiI sequencer (Amersham Pharmacia Biotech).

The indicated polyhistidine-tagged NIPP1 fragments (250 pmol) were applied to a column of 0.2 ml of Ni^{2+} -Sephacolumn, equilibrated in a buffer containing 50 mM Tris/HCl, pH 7.4, 5 mM imidazole, 0.5 mM benzimidazole, 0.5 mM PMSF and 50 mM NaCl. Subsequently, 120 pmol of rabbit muscle $PP1_c$ in equilibration buffer plus BSA (0.2 mg/ml) was applied to the columns. The retained catalytic subunit was eluted as a complex with the tagged NIPP1 fragment by elution with equilibration buffer supplemented with 0.4 M imidazole. The distribution of the catalytic subunit between the flow-through and eluate fractions was calculated from the trypsin-revealed phosphorylase phosphatase activity [22].

COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cells were transfected using the FuGene[®] 6 reagent (Roche Diagnostics). After transfection (36 h) the cells were washed twice with ice-cold PBS and lysed in 20 mM Tris/HCl, pH 7.5, 0.5 mM dithiothreitol, 0.2% Triton X-100, 0.3 M NaCl, 0.5 mM PMSF, 0.5 mM benzimidazole and 5 μ M leupeptin. Following centrifugation (5 min at 5000 g) the cell lysates were used for the immunoprecipitation of the NIPP1-EGFP fusion proteins with EGFP antibodies and Protein A–TSK-Sephacolumn[™] (Affilind). The immunoprecipitates were washed twice with Tris-buffered saline, resuspended in 20 mM glycylglycine, pH 7.4/1 mg/ml BSA, and used for the assay of the trypsin-revealed phosphorylase phosphatase activity and for Western-blot analysis with EGFP antibodies.

Unless indicated otherwise the protein phosphatase activities were measured with 4 nM catalytic subunit, and either phosphorylase *a* or myelin basic protein (MBP) as substrate [1]. The tyrosine kinase Lyn was purified from rat spleen as described in [23]. Phosphorylation reactions with the kinase Lyn were performed in 20 mM glycylglycine, pH 7.4, 0.5 mM dithiothreitol, 0.5 mM ATP, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM $CaCl_2$ and the indicated concentrations of poly(U) RNA. The tyrosine phosphorylation was assessed from Western-blot analysis with phosphotyrosine antibodies (Santa Cruz Biotechnology). All other materials and protocols were essentially as described by Beullens et al. [17,22].

RESULTS

Mapping of a novel $PP1_c$ -binding site to the C-terminus of NIPP1

Alanine mutation of either the polybasic or the RVTF-interaction sites for $PP1_c$ (Figure 1A) decreased the inhibitory potency of the central domain of NIPP1 (NIPP1^{143–224}) by two to three orders of magnitude (Figure 1B). The combined mutations were even more

Figure 1 Effects of mutation of the $PP1_c$ -binding sites in the central domain of NIPP1

(A) The sequence of the centrally located NIPP1 fragment (residues 191–210) that contains two established binding sites for $PP1_c$. Also indicated are the mutations that were introduced. (B) The inhibition of the phosphorylase phosphatase activity of $PP1_c$ by the indicated concentrations of wild-type and mutated NIPP1^{143–224} and NIPP1^{1–351}, expressed as means $\pm$ S.E.M. of four assays.

NIPP1 fragment (residues)	IC ₅₀ (nM)
1-351	1.8 ± 0.2
1-329	0.4 ± 0.1
1-142	> 1,000
143-224	0.27 ± 0.1
225-351	380 ± 35
225-342	760 ± 20
311-351	327 ± 38
311-330	> 500,000
330-351	3,300 ± 200
330-341	30,700 ± 290
341-351	> 500,000
331-337	53,300 ± 330

Figure 2 Mapping of an inhibitory PP1_C-binding site to the C-terminus of NIPP1

Shown are the concentrations of the indicated NIPP1 fragments (means ± S.E.M. from four experiments) that inhibited the phosphorylase phosphatase activity of 4 nM PP1_C by 50% (IC₅₀).

deleterious, resulting in an IC₅₀ of 11 μ M as compared with only 0.3 nM for the wild type. The pA plus RATA mutations (see Figure 1A) also abolished the binding of digoxigenin-labelled PP1_C to NIPP1^{143–224} in an overlay assay (results not shown). Thus the polybasic and RVTF sequences form essential sites for the binding and inhibition of PP1_C by NIPP1^{143–224}. By contrast, the pA-RATA mutations only moderately increased the IC₅₀ of full-length NIPP1, from 1.8 to 60 nM (Figure 1B). The pA-RATA mutant of NIPP1^{1–351} also retained some affinity for digoxigenin-labelled PP1_C in a far-Western assay (results not shown).

Paradoxically, with phosphorylase as substrate the central domain of NIPP1^{143–224} inhibited PP1_C with an IC₅₀ value (0.27 nM) even lower than that of intact NIPP1 (1.8 nM) and lower than expected for a stoichiometric inhibitor, i.e. 2 nM at 4 nM PP1_C (Figures 1 and 2). This sub-stoichiometric inhibition could indicate that the purified PP1_C from skeletal muscle was actually a mixture of an active and an inactive pool, which would both bind intact NIPP1, while NIPP1^{143–224} would only bind to the active pool of catalytic subunit. In support of this interpretation are observations that purified PP1_C is converted gradually into an inactive, metal-dependent, form during storage, which displays a severely decreased sensitivity to inhibitor-1 [24].

The above data (Figure 1) reinforced earlier indications for the existence of additional inhibitory binding site(s) for PP1_C in the N- or C-terminal thirds of NIPP1 (see the Introduction). Assays of recombinant NIPP1 fragments indeed revealed that, besides the central domain, the C-terminal third of NIPP1 (residues 225–351) was also inhibitory to the phosphorylase phosphatase activity of PP1_C, with an IC₅₀ of 380 nM (Figure 2). Moreover, NIPP1^{225–351} also interacted with digoxigenin-PP1_C in an overlay assay (results not shown). Analysis of smaller C-terminal fragments showed that the full inhibitory power was retained in residues 311–351 (Figure 2). An N-terminally truncated peptide, NIPP1^{330–351}, showed a 10-fold higher IC₅₀ value (3.3 μ M). A further 16-fold increase in the IC₅₀ (53 μ M) was noted for NIPP1^{331–337}, which consists of the sequence KKKK-

Table 1 Effects of combined mutations of the PP1_C-binding sites in the central and C-terminal domains of NIPP1

(A) Purified PP1_C (120 pmol) was applied to Ni²⁺ columns saturated with 250 pmol of polyhistidine-tagged wild-type or mutated NIPP1. The distribution of the catalytic subunit between the flow-through and the 0.4 M imidazole eluate was assessed from the trypsin-revealed phosphorylase phosphatase activities. (A) Shows the proportion of retained catalytic subunit (mean of two experiments) as a percentage of the wild type. A Ni²⁺ column that was saturated with NIPP1 (wild-type) retained 94% of the applied activity, while a column that did not contain any NIPP1 retained only 7% of the applied PP1_C. (B) The indicated NIPP1 mutants were expressed as fusion proteins with EGFP in COS-1 cells. After transfection (36 h) the NIPP1-EGFP fusion proteins were immunoprecipitated from the cell lysates with EGFP antibodies. Listed are the trypsin-revealed phosphorylase phosphatase activities in the washed pellets (mean of two experiments) expressed as a percentage of the activities associated with the wild type. The values were normalized for the same amount of NIPP1-EGFP fusion proteins, as determined by Western analysis. For details on pA-RATA mutations, please see Figure 1(A).

(A)	
Purified NIPP1 mutant	Binding of PP1 _C (% retained)
NIPP1 ^{1–351}	100
NIPP1 ^{1–351} (pA-RATA)	38
NIPP1 ^{1–329}	60
NIPP1 ^{1–329} (pA-RATA)	8
(B)	
NIPP1 mutant expressed in COS-1 cells	Binding of PP1 _C (% retained)
NIPP1 ^{1–351} -EGFP	100
NIPP1 ^{1–351} -EGFP (pA-RATA)	23
NIPP1 ^{1–325} -EGFP	98
NIPP1 ^{1–325} -EGFP (pA-RATA)	5

YAK and is reminiscent of the polybasic inhibitory site in the central domain. Thus residues 331–337 appear to represent the core inhibitory sequence, but the N- and C-terminally flanking sequences, which are not inhibitory by themselves (Figure 2), are required for the high-affinity binding to PP1_C. Figure 2 also shows that deletion of the C-terminal inhibitory region (residues 330–351) in intact NIPP1 decreased its IC₅₀ from 1.8 nM to the sub-stoichiometric value of 0.4 nM. This indicates that it is the C-terminal region which binds to both inactive and active PP1_C (see above).

Relative importance of the PP1_C-binding sites

To delineate the extent to which the central and C-terminal domains of NIPP1 contribute to the binding of PP1_C, we have generated NIPP1 variants that are mutated in either one or both phosphatase-binding sites. Mutation of either the central sites (pA-RATA mutation) or deletion of the C-terminal site (residues 330–351) merely decreased the binding of the catalytic subunit to the corresponding NIPP1-Sepharose, whereas the combined mutation and deletion prevented the binding of PP1_C (Table 1). We also expressed wild-type and mutant NIPP1 as fusion proteins with EGFP in COS-1 cells. NIPP1-EGFP could be immunoprecipitated from the cell lysates as a complex with PP1_C, as shown by the trypsin-revealed phosphorylase phosphatase activity (Table 1). However, both the pA-RATA mutation and the deletion of the C-terminal binding site were required to abolish the co-immunoprecipitation with PP1_C.

While the above data indicate strongly that both the central and C-terminal domains of NIPP1 are required for an efficient binding of the catalytic subunit, they do not reveal the contribution of these domains to the inhibition of PP1_C. Actually, the C-terminal domain might contribute little to the inhibitory potency of NIPP1, since the central domain is at least as potent

Figure 3 Substrate specificity of the central and C-terminal inhibitory sites of NIPP1

The Figure shows the effect of the indicated NIPP1 fragments and mutants on the activity of 2 nM PP1_c using phosphorylase (○) or MBP (●) as substrates. The results are expressed as means ± S.E.M. of three observations.

an inhibitor of the phosphorylase phosphatase activity as is full-length NIPP1 (Figure 2). However, since the inhibition by some regulators of PP1 is substrate-dependent [12], we have explored whether this perhaps also applies to the central and C-terminal domains of NIPP1. Figure 3 compares the effects of NIPP1 fragments and mutants on the dephosphorylation of phosphorylase *a* and of MBP by PP1_c. Interestingly, while full-length NIPP1 (Figure 3A) and NIPP1²²⁵⁻³⁵¹ (Figure 3B) were equally inhibitory to the dephosphorylation of phosphorylase and MBP, NIPP1¹⁴³⁻²²⁴ only inhibited the dephosphorylation of phosphorylase (Figure 3C). Furthermore, mutation in intact NIPP1 of either the central or the C-terminal PP1_c-binding sites altered the inhibitory potency to that of the remaining intact site when phosphorylase was used as a substrate (Figures 3D and 3E). However, either mutation abolished the inhibition of the MBP dephosphorylation. Finally, mutation of both the central and C-terminal binding sites almost completely abolished the inhibitory potency towards the dephosphorylation of phosphorylase (Figure 3F). The latter mutations even converted NIPP1 into a mild activator of the MBP phosphatase activity of PP1_c (Figure 3F), as was also observed with NIPP1¹⁴³⁻²²⁴ (Figure 3C). Thus both inhibitory sites appear to act independently with phosphorylase as a substrate, but synergistically with MBP as a substrate. Surprisingly, with MBP as substrate the IC₅₀ of the pA-RATA mutant of full-length NIPP1 (10 μM) was even higher than

the IC₅₀ of NIPP1²²⁵⁻³⁵¹ (200 nM), which may indicate that the interdependency of the central and C-terminal inhibitory sites is enhanced further by sequences that are N-terminal to residue 225 but which are different from the polybasic and RVTF motifs.

NIPP1¹⁹¹⁻²¹⁰ and NIPP1³³⁰⁻³⁵¹ interact with different fragments of PP1_c

Synthetic peptides comprising either the central (NIPP1¹⁹¹⁻²¹⁰) or the C-terminal (NIPP1³³⁰⁻³⁵¹) phosphatase-binding domains were unable to disrupt a complex between PP1_c and NIPP1-Sepharose ([17] and results not shown). However, NIPP1¹⁹¹⁻²¹⁰ and NIPP1³³⁰⁻³⁵¹ were able to disrupt complexes between PP1_c and either NIPP1¹⁻³²⁹-Sepharose or pA-RATA-mutated NIPP1-Sepharose, respectively (results not shown). As to the phosphorylase phosphatase activity of PP1_c, we have shown that NIPP1¹⁹¹⁻²¹⁰ antagonized the inhibitory effects of NIPP1¹⁴³⁻²²⁴ as well as that of inhibitor-1 and inhibitor-2 [17]. In contrast, NIPP1³³⁰⁻³⁵¹ opposed only the inhibitory effect of inhibitor-2 (Figure 4), and not those of NIPP1¹⁴³⁻²²⁴ and of inhibitor-1 (results not shown). Overall, these data suggest that the central and C-terminal sites of NIPP1 interact with different fragments of the catalytic subunit and that the C-terminal binding site in NIPP1 is equivalent to a phosphatase-interaction site in inhibitor-2.

Figure 4 NIPP1³³⁰⁻³⁵¹ antagonizes the inhibition of PP1_C by inhibitor-2

The phosphorylase phosphatase activity of 1 nM PP1_C was assayed in the presence of the indicated concentrations of inhibitor-2 and NIPP1³³⁰⁻³⁵¹. The activities (means $\pm$ S.E.M. for three assays) are expressed as a percentage of the activity without inhibitor-2. The 100% value was affected only slightly by 1 μ M NIPP1³³⁰⁻³⁵¹.

Figure 5 Effect of ionic strength on the inhibition of PP1_C by NIPP1 fragments

The Figure shows the IC₅₀ values of the indicated NIPP1 fragments for the inhibition of the phosphorylase phosphatase activity displayed by 4 nM PP1_C in the absence (—, open bars) and presence (+, hatched bars) of 150 mM NaCl. The results are expressed as means $\pm$ S.E.M. of three assays. The numbers above the bars represent the IC₅₀ values in nM.

Regulation of the C-terminal site by ionic strength and RNA binding

The existence of an additional C-terminal interaction site has prompted us to reconsider some earlier observations [17]. We had noted that the phosphorylase phosphatase activity of the inactive complex between PP1_C and intact NIPP1 could be restored by phosphorylation or by competition with NIPP1¹⁹¹⁻²¹⁰, but only in the presence of salt, whereas no salt was required for the activation of a complex with NIPP1¹⁴³⁻²¹⁷ (see the Introduction).

Figure 6 The inhibition by NIPP1²²⁵⁻³⁵¹ is abolished by RNA

The phosphorylase phosphatase activity of PP1_C (1 nM) was measured in the presence of the indicated concentrations of NIPP1²²⁵⁻³⁵¹ and either buffer (○) or 25 μ g/ml poly(U) RNA (●), and is expressed as means $\pm$ S.E.M. of four observations.

These data suggested that the PP1-binding site in the C-terminus could be sensitive to the ionic strength. In accordance with this interpretation we found that the IC₅₀ of NIPP1²²⁵⁻³⁵¹ was increased to the micromolar range by the addition of 150 mM NaCl, while the inhibitory potency of NIPP1¹⁴³⁻²²⁴ was barely affected by the ionic strength (Figure 5). Likewise, the inhibitory potency of full-length NIPP1 became dramatically dependent on the ionic strength after mutation of the central binding sites. By contrast, the C-terminally deleted NIPP1¹⁻³²⁹ was not salt-sensitive.

The C-terminal phosphatase-interaction site was mapped to the same NIPP1 fragment that also contains the lysine-rich RNA-binding motif [19]. In Figure 6 it is shown that the addition of RNA nearly abolished the inhibitory effect of NIPP1²²⁵⁻³⁵¹ on the phosphorylase phosphatase activity of PP1_C. Similar data were obtained with the pA-RATA mutant of full-length NIPP1 (results not shown). However, the binding of NIPP1²²⁵⁻³⁵¹ or of the pA-RATA mutant of NIPP1¹⁻³⁵¹ to poly(U)-Sephacel did not affect their ability to bind PP1_C (results not shown). This indicated that RNA interfered with the inhibitory potency of the C-terminal site but not with its ability to bind PP1_C.

Effects of phosphorylations in the C-terminus

The interaction between PP1_C and the central domain of NIPP1 is weakened by phosphorylation with PKA and protein kinase CK2 [14-16]. We wondered whether the interaction with the C-terminal inhibition site was also affected by phosphorylation. For that purpose we prepared NIPP1³³⁰⁻³⁵¹ with its three Ser/Thr/Tyr residues variously phosphorylated. In Figure 7(A) it is shown that phosphorylation of Tyr-335 decreased the inhibitory potency of the peptide on the phosphorylase phosphatase activity about 10-fold, whereas the phosphorylation of Ser-348 or Thr-346 had little or no effect. However, the triple-phosphorylated peptide was clearly less inhibitory than the Tyr-335-phosphorylated peptide. We also found that the Tyr-335 to Asp mutation in NIPP1²²⁵⁻³⁵¹ mimicked the effect of phosphorylation, in that it increased the IC₅₀ from 115 $\pm$ 32 nM to 2267 $\pm$ 370 nM (Figure 7B). By contrast, the Ser-348 to Asp or Thr-346 to Asp mutations had no clear effect on the inhibitory potency of NIPP1²²⁵⁻³⁵¹. We also found that the Tyr-335 to Asp mutation abolished the binding of digoxigenin-labelled PP1_C to NIPP1²²⁵⁻³⁵¹ in an overlay assay (Figure 7B, inset). Furthermore,

Figure 7 The inhibitory potency of NIPP1³³⁰⁻³⁵¹ is decreased by phosphorylation of Tyr-335

(A) The phosphatase activity of PP1_c in the presence of the indicated concentrations of unphosphorylated NIPP1³³⁰⁻³⁵¹ (○, wt), or after phosphorylation of Tyr-335 (■), Thr-346 (□), Ser-348 (●) or all three (▲). (B) The activity of PP1_c in the presence of the indicated concentrations of NIPP1²²⁵⁻³⁵¹ (○, wt), NIPP1²²⁵⁻³⁵¹ Y335D (■), NIPP1²²⁵⁻³⁵¹ T346D (□) and NIPP1²²⁵⁻³⁵¹ S348D (●). The results are expressed as means ± S.E.M. of three or four observations. Inset: overlay of the NIPP1²²⁵⁻³⁵¹ mutants with digoxigenin-labelled PP1_c.

the pA-RATA mutant of NIPP1¹⁻³⁵¹-EGFP from COS-1 cell lysates, which still co-immunoprecipitated with PP1_c (Table 1), no longer did so following the Tyr-335 to Asp mutation (results not shown). On the other hand, a Ni²⁺-Sepharose column that was saturated with the Tyr-335 to Asp mutant of NIPP1²²⁵⁻³⁵¹ was still able to bind some PP1_c (results not shown). Collectively, these data suggest that the Tyr-335 to Asp mutation severely decreases, but does not entirely abolish, the affinity of NIPP1²²⁵⁻³⁵¹ for PP1_c.

The dephosphorylation of MBP by PP1_c was only inhibited by low concentrations of full-length NIPP1 when the phosphatase-binding sites in both the central and C-terminal domains were intact (Figure 3). Since the Tyr-335 to Asp mutation decreased the inhibitory potency of the C-terminal domain (Figure 7B), we speculated that this mutation would also decrease the inhibitory potency of full-length NIPP1 towards the dephosphorylation of MBP. In Figure 8 it is shown that the Tyr-335 to Asp mutant was indeed a much less potent inhibitor of the MBP phosphatase activity. By contrast, the dephosphorylation of phosphorylase was not affected by this mutation, in keeping with the observation that the inhibition of the phosphorylase phosphatase activity by NIPP1 is largely mediated by its central domain (Figure 3).

Figure 8 The Tyr-335 to Asp mutation selectively decreases the inhibitory potency of NIPP1 towards MBP

The phosphorylase phosphatase (○) and MBP phosphatase (●) activities of PP1_c were measured in the presence of the indicated concentrations of either NIPP1¹⁻³⁵¹ or the Y335D mutant of NIPP1¹⁻³⁵¹. The results are expressed as a percentage (mean ± S.E.M. of three observations) of the activities without NIPP1.

Figure 9 The phosphorylation of NIPP1²²⁵⁻³⁵¹ by the tyrosine kinase Lyn

NIPP1²²⁵⁻³⁵¹ and the indicated mutants thereof were incubated for 60 min at 30 °C under phosphorylating conditions with protein kinase Lyn. Phosphorylation of Tyr-264 without (A) and with (B) the indicated concentrations of poly(U) RNA, as described in the Experimental section. The phosphorylation of the mutants was visualized by Western blotting with phosphotyrosine antibodies.

We have also explored whether Tyr-335 of NIPP1 is a substrate for tyrosine protein kinases. We found that NIPP1²²⁵⁻³⁵¹ is a substrate for tyrosine phosphorylation by Lyn, a member of the Src kinase family (Figure 9). However, further analysis using recombinant and synthetic NIPP1 fragments showed that this phosphorylation involved Tyr-264. Thus the tyrosine phosphorylation of NIPP1²²⁵⁻³⁵¹ by Lyn was abolished by the Tyr-264 to Asp mutation. We found, however, that the Tyr-264 to Asp mutant of NIPP1²²⁵⁻³⁵¹ could be tyrosine-phosphorylated by Lyn in the presence of poly(U). The latter phosphorylation was abolished by the Tyr-335 to Asp mutation. Thus these data indicate that Lyn phosphorylates both Tyr-264 and Tyr-335, but that the phosphorylation of Tyr-335 is dependent on the association of NIPP1²²⁵⁻³⁵¹ with RNA.

DISCUSSION

Diversity of PP1_c-interaction sites in NIPP1

The present results confirm and extend our previous findings on the existence of two PP1_c-binding sites in the central domain of

NIPP1 [17]. While studies with synthetic peptides suggested that the RVTF motif as such was not inhibitory [17], we report here that mutation of this motif decreased the inhibitory potency of the central domain of NIPP1 by more than 1000-fold (Figure 1B). Thus the RVTF sequence, although not inhibitory as such, may be required for an efficient inhibition by the polybasic site. This view accounts also for observations that $PP1N_{NIPP1}$ is activated by disruption of the RVTF-mediated interaction site by phosphorylation of its flanking Ser residues [16,17]. We have mapped a distinct interaction domain for $PP1_c$ to the C-terminal site of NIPP1 (Figure 2). Following mutation of the phosphatase-binding sites in both the central and C-terminal domains, NIPP1 was no longer able to bind and inhibit $PP1_c$ (Table 1 and Figure 3F). This suggests that NIPP1 does not contain other sites that by themselves are sufficient to bind the catalytic subunit, but it does not rule out the possibility that NIPP1 contains additional sites that modulate the interaction with $PP1_c$.

The binding of $PP1_c$ to the C-terminus of NIPP1 accounts for our failure to disrupt a complex between $PP1_c$ and full-length NIPP1 under conditions (phosphorylation of NIPP1 by PKA plus protein kinase CK2 or competition with NIPP1^{191–210}) that disrupted a complex with NIPP1^{143–217} [17]. Likewise, the salt requirement for the activation of $PP1N_{NIPP1}$ [16,17] can be explained by the sensitivity of the C-terminal site to ionic strength (Figure 5). Finally, the requirement of both the central and the C-terminal sites for NIPP1 to inhibit the dephosphorylation of MBP by $PP1_c$ (Figure 3) clarifies why a proteolytic fragment of NIPP1, roughly corresponding to the central domain of NIPP1, was only a poor inhibitor of MBP dephosphorylation [12].

Regulation of the NIPP1– $PP1_c$ interaction

While the central $PP1_c$ -binding domain of NIPP1 is controlled by Ser/Thr phosphorylation [16,17], the C-terminal interaction site was found to be modulated by tyrosine phosphorylation (Figures 7–9), by the binding of RNA (Figure 6) and by ionic strength (Figure 5). Thus far we have not yet been able to demonstrate that NIPP1, when expressed as a fusion protein with EGFP in COS-1 cells, is phosphorylated on tyrosine. However, this is not really surprising, since the phosphorylation of Tyr-335 appears to be RNA-dependent (Figure 9) and we do not yet know what determines the association of NIPP1 with RNA *in vivo*. It is also possible that the phosphorylation of NIPP1 on Tyr-335 *in vivo* is very transient (e.g. during pre-mRNA splicing) and/or involves only a minor portion of NIPP1.

We previously speculated that NIPP1 acts as an RNA-targeting subunit of PP1, since it can bind simultaneously to $PP1_c$ [13]. Our present observations that the binding of RNA directly modulates the activity of $PP1N_{NIPP1}$ (Figure 6) and promotes the phosphorylation of NIPP1 on Tyr-335 (Figure 9) are also in agreement with a role of RNA as an allosteric effector. Such a role would make sense, since $PP1N_{NIPP1}$ is presumed to be involved in the regulation of pre-mRNA processing [18,21]. Moreover, our recent data suggest that the subnuclear targeting of NIPP1 may be assured by the forkhead-associated domain in the N-terminus, which associates with the splicing factor CDC5L [20].

Competition experiments with NIPP1^{330–351} suggested that the C-terminal domain of NIPP1 binds to a site on the catalytic subunit that is different from the RVXF-binding site, but which is similar to a binding site for inhibitor-2 (Figure 4). Interestingly, it has been demonstrated recently that inhibitor-2, besides an RVXF-like motif, also contains an N-terminal IKGI

Figure 10 Shared-site model for the binding of regulatory proteins to $PP1_c$

It is proposed that $PP1_c$ contains only a limited number of interaction sites for its regulatory subunits ($PP1_R$), and that the latter share binding sites. The diversity and specificity in the effects of the regulators is achieved by their interaction with a unique set of $PP1_c$ -binding sites. In addition, some R-subunits may contain targeting domains (hatched segments) that anchor the catalytic subunit in close proximity to specific substrates.

phosphatase-binding site [9]. We speculate that the IKGI site of inhibitor-2 and the C-terminal site of NIPP1 bind to the same domain or overlapping domains in $PP1_c$.

A shared-site model for the interaction of regulatory subunits with $PP1_c$

For a profound insight into PP1-mediated regulation of cellular processes it is essential to understand why so many different regulatory proteins can interact with the same catalytic subunit, and yet each have a specific effect on its activity and substrate specificity. Given its relatively small size, it seems likely that the catalytic subunit only has a limited number of binding sites for its regulators. They include the RVXF-binding site and the $\beta 12$ – $\beta 13$ loop in the C-terminal region of the catalytic subunit [6–8], the catalytic site that binds the pseudosubstrate domain of inhibitor-1 and DARPP-32 [9], and a putative site that interacts with the N-terminus of inhibitor-2 and the C-terminus of NIPP1 ([9] and this study). Work from various groups indicates that most regulatory subunits have multiple sites of interaction with the catalytic subunit and that they can share several binding sites [5,8,11,17]. We suggest that the binding to specific combinations of sites on the catalytic subunit may account for the specific effects of the regulators on its activity and substrate specificity (Figure 10). A simple calculation shows that, with only five binding sites for the regulatory subunits, these could theoretically interact in 31 different ways with the catalytic subunit if the number of interactions varies between 1 and 5. This model may also provide a basis for understanding some types of hormonal and metabolic regulation of PP1 holoenzymes, which may simply involve a control on the number and identity of interaction sites between its subunits. Examples of regulation of this type are the disruption of the RVXF-mediated interaction of NIPP1 and of the muscle G-subunit with $PP1_c$ by phosphorylation [2,17] and the binding of a fragment of inhibitor-1 and DARPP-32 as a pseudosubstrate to the catalytic site following phosphorylation [9]. An additional level of regulation of the PP1 holoenzymes may be provided by the anchoring of the regulatory subunits to specific substrates or subcellular structures (Figure 10).

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