Mapping of the RNA-binding and endoribonuclease domains of NIPP1, a nuclear targeting subunit of protein phosphatase 1

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NIPP1 (351 residues) is a major regulatory and RNA-anchoring subunit of protein phosphatase 1 in the nucleus. Using recombinant and synthetic fragments of NIPP1, the RNA-binding domain was mapped to the C-terminal residues 330–351. A synthetic peptide encompassing this sequence equalled intact NIPP1 in RNA-binding affinity and could be used to dissociate NIPP1 from the nuclear particulate fraction. An NIPP1 fragment consisting of residues 225–351 (Ard1/NIPP1 γ), that may be encoded by an alternatively spliced transcript in transformed B-lymphocytes, displayed a single-strand Mg²⁺-dependent endoribonuclease activity. However, full-length

NIPP1 and NIPP1^{143–351} were not able to cleave RNA, indicating that the endoribonuclease activity of NIPP1 is restrained by its central domain. The endoribonuclease activity was also recovered in the RNA-binding domain, NIPP1^{330–351}, but with a 30-fold lower specific activity. Thus, the endoribonuclease catalytic site and the RNA-binding site both reside in the C-terminal 22 residues of NIPP1. The latter domain does not conform to any known nucleic-acid binding motif.

Key words: Ard1, dephosphorylation, Rnase.

INTRODUCTION

Signalling by protein kinases and protein phosphatases is largely controlled by associated polypeptides that determine the activity and substrate specificity of the catalytic subunit and also bring the enzyme into close proximity to its substrates [1]. Often these various functions reside in a single polypeptide. A paradigm for this multilevel regulation are the type 1 Ser/Thr protein phosphatases. They consist of a catalytic subunit (PP1_C) and one or two regulatory subunits [2–4]. Close to 20 mammalian polypeptides have been described that can associate with PP1_C. The G-subunits anchor PP1_C to the glycogen particles and increase the activity of the holoenzyme (PP1G) towards the substrate glycogen synthase [2]. In striated muscle the G-subunit (G_M) is subject to phosphorylation by protein kinase A, which disrupts its interaction with PP1_C, and by p90^{RSK}, which increases the specific synthase phosphatase activity of PP1G. The hepatic G-subunit (G_L) is not controlled by phosphorylation, but rather by the allosteric binding of phosphorylase α , which prevents the dephosphorylation of glycogen synthase by PP1G. The myosin-phosphatase targeting subunits MYPT1 and MYPT2 promote the dephosphorylation of myosin by PP1_C in smooth muscle and striated muscle respectively [5,6]. MYPT1 is a substrate for phosphorylation by Rho-binding kinases, and such phosphorylation has been associated with an inhibition of the holoenzyme (PP1M). The myosin phosphatase activity of smooth muscle PP1M is also inhibited by the binding of acidic phospholipids. Nuclear inhibitor of protein phosphatase 1 (NIPP1) is a subunit that targets PP1_C to RNA in the nucleus [7,8]. The heterodimeric complex between NIPP1 and PP1_C (PP1N_{NIPP1}) is completely inactive, but can be activated by phosphorylation with protein kinases A and CK2, without disruption of the complex or its dissociation from RNA [9]. A second nuclear inhibitory and RNA-targeting subunit of PP1 is known as R111 [7], p99 [10] or

PNUTS [11]. Various other mammalian inhibitory polypeptides of PP1 have been described, including inhibitor-1 and the structurally related protein DARPP-32 [2,12], inhibitor-2 [2], inhibitor-3 [13], sds22 [14], RIPP1 [15] and p53BP2 [16], but these regulators have no established targeting function.

An understanding of how, when and where PP1 holoenzymes are activated requires a detailed insight into the modes of interaction of the catalytic subunit with its regulator(s). The emerging picture is that regulatory subunits of PP1 are modular proteins, with distinct and independently controlled binding sites for the catalytic subunit, substrate(s), anchoring molecules and allosteric effectors [5,17]. Preliminary evidence suggests that this may also be true for NIPP1. Thus, NIPP1 (39 kDa) consists of basic N-terminal and C-terminal thirds and an acidic central domain [18]. The N-terminus of NIPP1 contains a ‘forkhead-associated domain’ that is also present in many other nuclear proteins and is believed to function as a protein-binding motif [19]. The central domain of NIPP1 is essential for the binding and inhibition of PP1_C [20]. It contains two PP1 interaction sites, i.e. a polybasic inhibitory sequence and a downstream non-inhibitory RVXF-sequence [20], which represents a consensus PP1 binding-motif that is present in most regulators of PP1 [21,22]. Activation of PP1N_{NIPP1} by protein kinases A and CK2 results from phosphorylation of four residues in the central domain. Two of these residues flank the RVXF-motif and their phosphorylation causes a disruption of the interaction of the RVXF-sequence with the catalytic subunit and, in addition, decreases the inhibitory potency of the upstream polybasic inhibitory site [9,20]. In addition to interacting with the catalytic subunit, NIPP1 also binds *in vitro* with high affinity to RNA, preferentially to A/U-rich sequences such as the RNA-destabilizing AUUUA-motif [8]. The C-terminal third of NIPP1, which is known as Ard1 [23,24] or NIPP1 γ [25] and may be encoded by a rare, alternatively-spliced NIPP1 transcript in

Abbreviations used: HA, haemagglutinin; PP1, protein phosphatase 1; PP1_C, catalytic subunit of PP1; NIPP1, nuclear inhibitor of PP1; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanonehydrochloride; TPCK, L-1,4'-tosylamino-2-phenylethyl-chloromethyl ketone.

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transformed B-lymphocytes [25], has also been shown to bind to RNA [24]. Surprisingly, Ard1/NIPP1 γ could also complement mutations of the bacterial *rne* gene that encodes the multifunctional RNase E [23], and purified recombinant Ard1/NIPP1 γ was shown to display a single-strand endoribonuclease activity with a specificity similar to that of bacterial RNase E [24].

In an effort to understand the relationship between the binding and hydrolysis of RNA, we have mapped the RNA-binding site and the endoribonuclease catalytic site of NIPP1. Using a series of recombinant and synthetic NIPP1 fragments, we found that the ability to bind and hydrolyse RNA resides in the C-terminal 22 residues. Surprisingly, the endoribonuclease activity, unlike the ability to bind to RNA, is restrained by the central domain of NIPP1.

EXPERIMENTAL

Materials and buffer

Peptides were synthesized on a Milligen 9050 peptide synthesizer (Applied Biosystems), using the *N*-(9-fluorenyl)methoxycarbonyl method, and dissolved (10 mg/ml) in 10 mM Tris/HCl at pH 7.5. The AU4 ribonucleotide, 5'-AGGAUGCAUUUAUUUAUUUAUUUAAGCUUGG-3', was obtained from Eurogentec (Liège, Belgium). Poly(U)-Sephacrose (Amersham Pharmacia Biotech) was equilibrated in buffer A consisting of 10 mM Tris/HCl at pH 7.4, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 0.5 mM PMSF, 50 μ M 1-chloro-3-tosylamido-7-amino-2-heptanonehydrochloride (TLCK), 50 μ M L-1,4'-tosylamino-2-phenylethyl-chloromethyl ketone (TPCK) and 5 μ M leupeptin.

Recombinant NIPP1 fragments

Full-length bovine thymus NIPP1 was expressed in Sf9 cells and purified as described by Vulsteke et al. [9]. NIPP1¹⁻²²⁴, NIPP1²²⁵⁻³⁵¹, NIPP1²²⁵⁻³¹⁰ and NIPP1³¹¹⁻³⁵¹ were expressed as polyhistidine-tagged proteins in BL21 (DE3) *Escherichia coli* cells, using the pET16b vector (Novagen), and purified on Ni²⁺-iminodiacetate-Sepharose (Sigma) as detailed elsewhere [20]. The NIPP1¹⁻²²⁴ cDNA was obtained by PCR using the oligonucleotides 5'-ATCGAACATATGGCGGCAGCCGCGAAC-TC-3' (*Nde*I site underlined) and 5'-CTGCACCTCGAGTC-AGTTCCGAAAGCGACCAACC-3' (*Xho*I site underlined) as sense and antisense primers respectively, and bovine NIPP1 cDNA as template. The PCR product was digested with *Nde*I/*Xho*I and subcloned in the pET16b vector. The pET16b vector expressing human NIPP1²²⁵⁻³⁵¹ (Ard1) was a gift from Dr. A. von Gabain (Institute of Microbiology and Genetics, Vienna Biocenter, Vienna, Austria). The cDNA encoding NIPP1²²⁵⁻³¹⁰ was obtained by PCR using 5'-TTCAGGCATATGGTGCAACTGCAGTGGTC-3' (*Nde*I site underlined) and 5'-AGG-GTTGGATCCTCATGCTGACGGCACAACAGGAGTC-3' (*Bam*H1 site underlined) as sense and antisense primers respectively and the NIPP1²²⁵⁻³⁵¹ expression vector as template. Likewise, the NIPP1³¹¹⁻³⁵¹ cDNA was obtained using 5'-GTGCCGCATATGGTGAACATGAACCCTGCACCA-3' (*Nde*I site underlined) and 5'-TCACGTGGATCCTCAAATC-AGCAAGGAAGGTGT-3' (*Bam*H1 site underlined) as sense and antisense primers respectively. The NIPP1²²⁵⁻³¹⁰ and NIPP1³¹¹⁻³⁵¹ PCR fragments were subcloned in the pET16b expression vector and the polyhistidine-tagged fusion proteins were purified by chromatography on Ni²⁺-iminodiacetate-Sepharose [20]. PCR was performed using standard conditions and *Pyrococcus woesei* DNA-polymerase (Roche). The constructs were verified by nucleotide sequencing.

Intact bovine thymus NIPP1 as well as various NIPP1 fragments (Figure 5) were subcloned into pACT2, in frame with the Gal4 activation domain and the haemagglutinin (HA) epitope, and expressed in yeast as described by Beullens et al. [20].

RNA-binding assays

The oligoribonucleotide AU4 was labelled at the 5'-end by phosphorylation with T4 polynucleotide kinase for a period of 45 min at 37 °C, according to the manufacturer's instructions (Roche), in the presence of [γ -³²P]ATP and 600 units/ml RNasin, an inhibitor of RNase A. The labelled oligonucleotide was extracted with a mixture of phenol/chloroform/isoamylalcohol (25:24:1, by vol.), precipitated with 70% (v/v) ethanol and 100 mM sodium acetate, purified on 8.3 M urea/20% PAGE and re-extracted. Synthetic NIPP1 fragments (1 nmol) were incubated with 5'-end-labelled AU4 (2 μ Ci/ml) in 20 μ l buffer B (20 mM Tris/HCl at pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1 M NH₄Cl, 0.2 mg/ml yeast tRNA and 3 units/ml RNasin) and exposed to UV light (254 nm, 250 mJ) in a Stratalinker UV apparatus. Subsequently, the samples were subjected to 12% Tricine-SDS/PAGE, and the dried gels were autoradiographed.

Poly(U)-Sephacrose (15 μ l), equilibrated in buffer A, was incubated for 30 min at 4 °C on a rotating wheel after addition of 50 μ l of buffer A with or without 20 nmol of the indicated NIPP1 peptides (Figure 1B). Subsequently, the supernatant was removed and the gel was washed first with 200 μ l of buffer A plus 1 M NaCl and then three times with 200 μ l of buffer A. The gel was then boiled in SDS/sample buffer and the supernatant was subjected to 12% Tricine-SDS/PAGE.

The indicated NIPP1 peptides (2.5 nmol) (Figures 1C and 7B) in 50 μ l buffer B plus 5'-end-labelled AU4 (approx. 20000 c.p.m.) were extracted with 50 μ l (water-saturated) phenol/chloroform/isoamylalcohol (25:24:1, by vol.). After centrifugation (4 min at 13000 g) the upper 40 μ l were considered as the aqueous phase and the next 10 μ l as the water/phenol interphase.

AU4 endoribonuclease assay

Approx. 5000 c.p.m. of 5'-end-labelled AU4 was incubated with the indicated NIPP1 fragments in buffer B. After 30 min at 37 °C, the reaction was arrested by extraction with phenol/chloroform/isoamylalcohol (see previous paragraph). For this purpose water-saturated phenol (acidic phenol) was used, except for the experiment illustrated in Figure 7(A), where the phenol was first saturated with 10 mM Tris/HCl at pH 8.0, to prevent a shift of AU4 to the water/phenol interphase (see Results section). The RNA in the aqueous phase was precipitated with 70% ethanol in the presence of 0.1 M sodium acetate. The pellet, obtained by centrifugation for 20 min at 13000 g, was lyophilized and dissolved in 10 μ l of loading buffer containing 98% deionized formamide, 10 mM EDTA at pH 8, 0.1% Xylene Cyanol and 0.1% Bromophenol Blue. After a denaturing procedure (4 min incubation at 65 °C), aliquots were loaded on 8.3 M urea/20% PAGE. The gel was dried and the radioactive bands were visualized by autoradiography using the PhosphorImager (Molecular Dynamics).

Immunological procedures

A peptide with the sequence of residues 341–351 of bovine NIPP1, plus an additional C-terminal cysteine, was coupled to keyhole limpet haemocyanin and to BSA, using the Pierce immunogen conjugation kit. Rabbit polyclonal antibodies against the haemocyanin-coupled peptide were affinity purified

A. UV cross-linking

B. Poly(U)-Sephadex

C. Extraction with phenol/chloroform

Figure 1 Mapping of the RNA-binding site to the C-terminus of NIPP1

(A) 32 P-labelled AU4 was incubated under UV cross-linking conditions, as such (control) or with the indicated NIPP1 peptides (see Experimental section). The cross-linked samples were subjected to 12% Tricine-SDS/PAGE and the AU4 in the dried gel was visualized by autoradiography. The arrowhead indicates the position of free AU4 and the arrow indicates the migration of the AU4 that was cross-linked to peptide. (B) Poly(U)-Sephadex was incubated with buffer A (control) or with buffer A plus the indicated NIPP1 peptides. Subsequently, the gel was washed with buffer A plus 1 M NaCl, boiled in SDS/sample buffer and the supernatant was subjected to 12% Tricine-SDS/PAGE. The Coomassie-stained gel is shown and the arrow indicates the position of the retained peptide. (C) The NIPP1 peptides were extracted from a mixture with AU4 with acidic phenol/chloroform/isoamylalcohol, as detailed in the Experimental section. The amount of AU4 that was extracted in the interphase is shown as a percentage of the total radioactivity recovered in the interphase plus the aqueous phase.

on albumin-coupled peptide linked to CNBr-activated Sepharose 4B. The antibodies were eluted first with 0.1 M glycine at pH 3.0 and then with 0.1 M triethanolamine at pH 10.0. The eluate was collected in 0.1 volume of Tris/HCl at pH 8.0. The pool was dialysed against a buffer containing 20 mM Tris/HCl at pH 7.4 and 150 mM NaCl, and stored at -80°C .

Western analysis was done with NIPP1 antibodies ($2\ \mu\text{g/ml}$) or with monoclonal antibodies against the Gal4 activation domain (Clontech, $0.4\ \mu\text{g/ml}$), and the peroxidase-labelled secondary antibodies were detected by enhanced immunofluorescence. For immunoprecipitation, the samples were incu-

bated with the NIPP1 antibodies ($30\ \mu\text{g/ml}$) or with monoclonal anti-HA antibodies (clone 12CA5, $30\ \mu\text{g/ml}$) for 1 h at 4°C . Subsequently, protein-A-TSK[®] (Affiland, Liège, Belgium) equilibrated in buffer A was added, and the mixture was rotated for 1 h at 4°C . After centrifugation (1 min at $10000\ g$), the pellets were washed in a buffer containing 10 mM Tris/HCl at pH 7.5, 1 mM dithiothreitol and 0.25 M LiCl, and then washed twice in the same buffer without LiCl. The amount of the immunoprecipitated NIPP1 fusion proteins was estimated by Western blotting with antibodies against the Gal4 activation domain. Aliquots with the same amounts of NIPP1 fusion proteins were assayed for AU4 endoribonuclease activity.

Preparation of nuclear pellets

Rat liver nuclei were prepared as described by Jagiello et al. [7]. The nuclei were resuspended in a buffer containing 20 mM Tris/HCl at pH 7.4, 1 mM MgCl_2 , 0.25 M sucrose and 0.5% Triton X-100 to solubilize the outer nuclear membrane. The nuclear suspension was centrifuged for 5 min at $3500\ g$. The nuclear pellet was resuspended in a hypotonic solution containing 50 mM glycylglycine at pH 7.4, 5 mM 2-mercaptoethanol, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.5 mM benzamidin and $5\ \mu\text{M}$ leupeptin, and the lysed nuclei were submitted to centrifugation for 5 min at $13000\ g$. The nuclear pellet was washed twice in the same buffer. The resuspended pellet was incubated for 30 min at 4°C on a rotating wheel, as such (control) or in the presence of either 0.5 mM NIPP1³³⁰⁻³⁵¹ or 0.3 mg/ml of poly(U). After centrifugation (5 min at $13000\ g$) the supernatant was used for Western analysis with NIPP1 antibodies.

Figure 2 Full-length NIPP1 and NIPP1³³⁰⁻³⁵¹ bind with the same affinity to RNA

A mixture of 0.6 nmol of intact NIPP1, 1.2 nmol of NIPP1²²⁵⁻³⁵¹ and $0.2\ \mu\text{mol}$ of NIPP1³³⁰⁻³⁵¹ in 1 ml of buffer A was loaded on a column of 1 ml poly(U)-Sephadex, equilibrated in buffer A. The column was eluted with 20 ml of a linear gradient of KSCN (0–1.5 M). Fractions of 1 ml were collected and analysed by Western analysis with antibodies against the C-terminus of NIPP1.

Figure 3 NIPP1³³⁰⁻³⁵¹ and poly(U) dissociate NIPP1 from nuclear pellets

The insoluble nuclear fraction from rat liver was incubated with buffer (control) or 0.5 mM NIPP1³³⁰⁻³⁵¹ or 0.3 mg/ml poly(U), as detailed in the Experimental section. After centrifugation, the supernatant was analysed for the presence of NIPP1 and NIPP1³³⁰⁻³⁵¹ by Western analysis with antibodies against residues 341-351.

RESULTS

Mapping of the RNA-binding domain of NIPP1

Both full-length NIPP1 and NIPP1²²⁵⁻³⁵¹ (Ard1/NIPP1 γ) have been shown to bind to A/U-rich RNA [8,24]. We have further localized the RNA-binding sequence in NIPP1²²⁵⁻³⁵¹ by using a series of overlapping 22-mer peptides covering this entire domain (Figure 1). Only one of these peptides, NIPP1³³⁰⁻³⁵¹, could be UV cross-linked to the oligoribonucleotide AU4 (Figure 1A), which contains four copies of the AUUUA-motif that was shown to bind with high affinity to NIPP1 [8]. NIPP1³³⁰⁻³⁵¹ was also the only peptide that bound to poly(U)-Sepharose (Figure 1B). Finally, whereas AU4 was largely recovered in the aqueous phase following an (acidic) phenol/chloroform extraction, a major fraction (80%) of the oligoribonucleotide was recovered in the water/phenol interphase, when extracted in the presence of

Figure 5 Endoribonuclease activity of NIPP1 fragments expressed in yeast

The indicated NIPP1 fragments were expressed as Gal4-HA fusion proteins in yeast (see Experimental section). The control refers to the empty expression vector. The NIPP1 fusion proteins were immunoprecipitated from yeast extracts with anti-HA-tag antibodies and the washed immunoprecipitates were tested for endoribonuclease activity on AU4 in the absence or presence of 5 mM MgCl₂. During the endoribonuclease assays all NIPP1 fusion proteins were present at the same concentration, as determined by Western analysis with anti-Gal4 antibodies (results not shown).

an excess of NIPP1³³⁰⁻³⁵¹ (Figure 1C). None of the other NIPP1 peptides had a remotely similar effect on the phase distribution of AU4. In the absence of MgCl₂ only about 40% of the AU4 was shifted to the water/phenol interphase by NIPP1³³⁰⁻³⁵¹ and no such shift was seen when the extraction was done with phenol at pH 8 (results not shown). The co-extraction of AU4 and NIPP1³³⁰⁻³⁵¹ again points to an interaction between both components and also suggests that this interaction is not destroyed by denaturing procedures at acidic pH values.

The above results defined an RNA-binding site in the C-terminal 22 residues of NIPP1. We have subsequently compared the RNA-binding affinity of intact NIPP1, NIPP1²²⁵⁻³⁵¹ and NIPP1³³⁰⁻³⁵¹. For that purpose a mixture of these polypeptides

Figure 4 Co-elution of NIPP1²²⁵⁻³⁵¹ and endoribonuclease activity during gel filtration

Purified NIPP1²²⁵⁻³⁵¹ (0.01 mg; left panel) and purified full-length NIPP1 (0.02 mg; right panel) were applied to a Superdex-75 column of 25 ml (Amersham Pharmacia Biotech), equilibrated with a buffer containing 20 mM Tris/HCl at pH 7.2, 0.1 M NaCl, 0.1% Brij, 0.2 mM PMSF, 20 μ M TLCK, 20 μ M TPCK and 2 μ M leupeptin. Fractions of 0.25 ml were collected and analysed by Western analysis with NIPP1 antibodies (upper panel). The fractions were also assayed for AU4 endoribonuclease activity. The lower panels show the fraction of AU4 that was not degraded, as detected by autoradiography.

Table 1 Effect of Mg^{2+} on the AU4-endoribonuclease activities of NIPP1 fragments

The indicated NIPP1 fragments were assayed for AU4 endoribonuclease activity in the absence or presence of 5 mM $MgCl_2$. The endoribonuclease activities were calculated from the amount of the AU4 that was not hydrolysed, as determined by scanning of autoradiograms. The activities (means $\pm$ S.E.M. for 3–4 observations) are expressed relative to the activity of NIPP1^{225–351} in the absence of Mg^{2+} .

NIPP1 fragment	Endoribonuclease activity (arbitrary units)	
	No Mg^{2+}	Plus Mg^{2+}
NIPP1 ^{225–351}	100	315 $\pm$ 49
NIPP1 ^{311–351}	28 $\pm$ 4	75 $\pm$ 13
NIPP1 ^{330–351}	3 $\pm$ 1	1 $\pm$ 1

A. AU4 endoribonuclease **B. Co-immunoprecipitation** **Figure 6** Mapping of the endoribonuclease catalytic site to the C-terminus of NIPP1

(A) The indicated NIPP1 peptides (0.1 nmol each) were assayed for AU4 endoribonuclease activity. The arrow indicates the position of the non-degraded AU4. The control condition represents AU4 that was incubated without peptide. (B) NIPP1^{311–351} was assayed for AU4 endoribonuclease activity before and after immunoprecipitation (IP) with antibodies against residues 341–351. The control represents the AU4 endoribonuclease activity in immunoprecipitates that were obtained without addition of the primary antibody.

was applied to a column of poly(U)-Sepharose. All three polypeptides were quantitatively retained by the resin (Figure 2) and remained bound to the column in the presence of 1.5 M NaCl (results not shown). However, the polypeptides could be eluted with a gradient of the chaotropic salt KSCN and all three eluted at the same concentration (0.3 M) of KSCN (Figure 2). On the other hand, NIPP1^{1–224} and NIPP1^{1–330} did not bind to the resin at NaCl concentrations above 0.3 M (results not shown). Collectively, these results showed that residues 330–351 represent the only RNA-binding site of NIPP1 and that NIPP1^{330–351} and the intact protein bind with equal affinity to RNA.

We have previously shown that NIPP1 is largely associated with the nuclear insoluble fraction and have suggested that this is due to the association of NIPP1 with RNA [7,8]. In further agreement with this view, we found that NIPP1 could be translocated to the soluble compartment by the addition of either exogenous soluble RNA or NIPP1^{330–351} (Figure 3), or by treatment with RNase A (results not shown).

A. AU4 endoribonuclease activity **B. RNA binding** **Figure 7** NIPP1^{330–351} represents the minimal endoribonuclease and RNA-binding domain

(A) AU4 endoribonuclease activity of 1 μ M NIPP1^{225–351} or 50 μ M of the other NIPP1 peptides. The control represents AU4 incubated without peptide. The arrow indicates the position of the undegraded AU4. (B) The ability of the same peptides to shift AU4 to the phenol interphase during an extraction with phenol/chloroform.

Endoribonuclease activity of NIPP1 fragments

Claverie-Martin et al. [24] have reported that bacterially expressed NIPP1^{225–351} (Ard1/NIPP1 γ) displays an endoribonuclease activity with a specificity like that of the bacterial RNase E. Similarly, we found that purified Ard1/NIPP1 γ (13 kDa) was able to hydrolyse the established RNase E substrate AU4. Moreover, the latter activity co-eluted with Ard1/NIPP1 γ during gel filtration (Figure 4), indicating that it was not derived from contaminating bacterial RNase E (118 kDa). On the other hand, no endoribonuclease activity was found to be associated with full-length NIPP1 (Figure 4). We have also expressed NIPP1 and various NIPP1 fragments as Gal4-HA-tagged fusion proteins in yeast, which is not known to contain an RNase E activity. After immunoprecipitation of the fusion proteins with antibodies against the HA-tag, the immunoprecipitates were analysed for endoribonuclease activity using AU4 as a substrate (Figure 5). These assays confirmed that NIPP1^{225–351} was able to hydrolyse AU4, and this endoribonuclease activity was found to be Mg^{2+} -stimulated (see also Table 1), in agreement with the report by Claverie-Martin et al. [24]. In contrast, the other NIPP1 fragments, including intact NIPP1 and NIPP1^{143–351}, did not show any endoribonuclease activity. Thus, the central domain

of NIPP1 restrains the RNase activity that is expressed by the free C-terminal third of NIPP1.

The endoribonuclease catalytic site was further mapped by analysing smaller recombinant and synthetic fragments of NIPP1^{225–351}. Bacterially expressed NIPP1^{225–310} did not show any endoribonuclease activity (Figure 6A). On the other hand NIPP1^{311–351} was able to hydrolyse AU4 with a specific activity that was only 3–4-fold lower than that of NIPP1^{225–351} and this activity was stimulated several-fold by Mg²⁺ (Figure 6A and Table 1). It is also shown in Figure 6A that NIPP1^{225–351} and NIPP1^{311–351} cleaved the AU4 oligoribonucleotide into four products, in accordance with the presence of four AUUA-motifs in the substrate and with the established hydrolysis by RNase E-like enzymes within these sequences [26]. The RNase activity of NIPP1^{311–351} could be immunoprecipitated with antibodies directed against the C-terminal 11 residues of NIPP1 (Figure 6B), providing further evidence that the endoribonuclease activity was not due to a contaminant.

NIPP1^{330–351} represents the minimal endoribonuclease and RNA-binding domain

The above results showed that the endoribonuclease catalytic site is confined to the C-terminal 40 residues of NIPP1. The synthetic peptide NIPP1^{330–351} also displayed an AU4 endoribonuclease activity, albeit with a 30-fold lower specific activity (Figure 7A), and this activity was, if anything, inhibited by Mg²⁺ (Table 1). Smaller synthetic fragments, i.e. NIPP1^{331–337} and NIPP1^{341–351}, did not show any detectable endoribonuclease activity. We also found that the latter two peptides did not bind to RNA either, as revealed by UV cross-linking (results not shown), by chromatography on poly(U)-Sephadex (results not shown), and by their inability to shift AU4 to the water/phenol interphase (Figure 7B).

DISCUSSION

We have shown here by various independent approaches that the high-affinity binding of NIPP1 to RNA is mediated by its C-terminal 22 residues (Figure 1). Moreover, the synthetic C-terminus, NIPP1^{330–351}, bound with the same affinity to RNA as did intact NIPP1, whereas NIPP1^{1–330} did not bind tightly to RNA (Figure 2), suggesting that there are no additional binding determinants outside this domain. Thus, similar to what is known for most other RNA-binding proteins [27], the RNA-binding domain of NIPP1 is confined to a small fragment of the protein. This finding also explains why the RNA-binding of NIPP1 was not destroyed by denaturing procedures such as SDS/PAGE [8] or phenol/chloroform extraction (Figure 1C). The RNA-binding domain of NIPP1 does not conform to established RNA-binding motifs, but shows some similarity with so-called 'arginine-rich motifs' [27]. The latter motifs typically consist of 10–20 residues that include several arginines. It has been proposed that the arginines promote the RNA-binding in two ways [27]. First, the positive charges of the arginines increase, non-specifically, the affinity for RNA, thereby facilitating the search for high-affinity binding sites. A second function of the arginines is to make hydrogen-bonding networks with the RNA sugar-phosphate backbone and bases. One could envisage a similar interaction between RNA and the positively charged residues in the C-terminus of NIPP1 which, however, consist exclusively of lysines. Thus, the RNA-binding domain of NIPP1 should rather be denoted as a 'lysine-rich motif'. It is worthy of note that NIPP1^{331–337}, which contains five out of the seven lysines in the RNA-binding domain, was not able to bind to

RNA (Figure 7B). This suggests that the positive charges *per se* are not sufficient to enable RNA-binding and that C-terminally located residues are also essential for efficient interaction with RNA.

A surprising outcome of this study was the finding that fragments of the C-terminus of NIPP1 displayed an endoribonuclease activity, whereas the full-length protein did not. We could exclude that this RNase activity stems from a contaminating protein, since the activity co-eluted with NIPP1^{225–351} during gel filtration (Figure 4) and co-immunoprecipitated with NIPP1^{311–351} (Figure 6B). Moreover, an endoribonuclease activity was also associated with the synthetic peptide NIPP1^{330–351} (Figure 7). It is not clear from the available data whether these NIPP1 fragments act as real enzymes or rather promote certain RNA sequences to adopt a conformation that enables autocatalytic cleavage.

We have recently found that mammalian cells express various NIPP1 transcripts that are generated by alternative splicing and polyadenylation [25]. In addition to the transcript that encodes NIPP1^{1–351} (NIPP1 α), these cells also express low concentrations of transcripts that are expected to encode NIPP1^{143–351} (NIPP1 β) and NIPP1^{225–351} (NIPP1 γ). Therefore our observation that only NIPP1 γ shows an endoribonuclease activity (Figure 5) raises the intriguing possibility that the splice-variants of NIPP1 have different functions. However, since it still remains an open question whether the splice-variants yield any NIPP1 β or NIPP1 γ protein, one could also envisage the possibility that NIPP1 α is a latent endoribonuclease, whose activity may be turned on by a specific co-factor or covalent modification. Such a co-factor or modification may oppose the inhibition of the endoribonuclease activity that is exerted by the central domain of NIPP1 (Figure 5).

The bacterial RNase E possesses an endoribonuclease catalytic site and a separate RNA-binding site [28]. There are several reasons to believe that the same may be true for NIPP1, even though the RNA-binding and endoribonuclease domains of NIPP1 were mapped to the same protein fragment of 22 residues and may thus be partially overlapping. First, intact NIPP1 and NIPP1^{225–351} bound with the same affinity to RNA (Figure 2), but only NIPP1^{225–351} displayed an RNase activity. Secondly, antibodies directed against the C-terminus of NIPP1 blocked the RNA binding [8] but did not affect the endoribonuclease activity (Figure 6B). Thirdly, the binding of NIPP1^{330–351} to RNA was stimulated by MgCl₂ whereas the endoribonuclease activity was inhibited.

Since NIPP1 can simultaneously bind PP1_c and RNA, we have proposed that NIPP1 functions as an RNA-anchoring subunit of PP1 [8]. In further agreement with this view we have shown here that the synthetic RNA-binding domain, as well as poly(U), were able to dissociate NIPP1 from the nuclear particulate fraction (Figure 3). The binding of NIPP1 to RNA in the nuclear particulate fraction seems less strong, however, than the binding of purified NIPP1 to poly(U), since only the disruption of the latter complex required the use of chaotropic salts such as KSCN. This may indicate that the binding of NIPP1 to RNA is subject to regulation, e.g. by phosphorylation of the RNA-binding domain or by interaction with allosteric effectors. Along the same lines, it has been reported that the glycogen-synthase phosphatase activity of partially purified hepatic PP1G was blocked by nanomolar concentrations of phosphorylase *a*, whereas, *in vivo*, inhibition required 100- to 1000-fold higher concentrations of phosphorylase *a*, owing to the presence of counteracting molecules [2].

In conclusion, we have provided here results indicating that the binding and degradation of RNA by NIPP1 are conferred by

sites in the C-terminal 22 residues that are phylogenetically conserved. It is furthermore reported that the endoribonuclease activity of NIPP1 is latent, due to inhibition of the catalytic site by the central domain of NIPP1.

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