Molecular Cloning of NIPP-1, a Nuclear Inhibitor of Protein Phosphatase-1, Reveals Homology with Polypeptides Involved in RNA Processing

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NIPP-1 was originally isolated as a potent and specific nuclear inhibitory polypeptide (16–18 kDa) of protein phosphatase-1. We report here the cDNA cloning of NIPP-1 from bovine thymus and show that the native polypeptide consists of 351 residues and has a calculated mass of 38.5 kDa. The bacterially expressed central third of NIPP-1 completely inhibited the type-1 catalytic subunit, but displayed a reduced inhibitory potency after phosphorylation by protein kinase A and casein kinase 2. Translation of NIPP-1 mRNA in reticulocyte lysate phosphorylation by protein kinase A and casein kinase 2, but displayed a reduced inhibitory potency after phosphorylation by protein kinase A and casein kinase 2. Therefore, we have isolated polypeptides of 16–18 kDa from bovine thymus, termed NIPP-1, that are extremely potent and specific inhibitors of PP-1 (6). Recent data suggest that the isolated inhibitors are generated from a 41-kDa polypeptide by proteolysis (7). In nuclear extracts native NIPP-1 is present as an inactive heterodimeric complex with PP-1c, designated PP-1NC. Phosphorylation of NIPP-1 in PP-1NC by protein kinase A, both in vitro and in vivo, does not cause the dissociation or activation of the phosphatase but prevents the reassociation of PP-1c with NIPP-1, following the dissociation of the complex. In contrast, phosphorylation of the 16–18-kDa fragments of NIPP-1 by protein kinase A or casein kinase 2 causes the release of active catalytic subunit (8, 9).

We report here the cDNA cloning of NIPP-1 from bovine thymus and show that the inhibitory region is localized in the central third of the polypeptide, which also contains multiple phosphorylation sites for protein kinase A and casein kinase 2. Native NIPP-1 is a protein with a calculated mass of 38.5 kDa, but initial evidence suggests that fragments of NIPP-1 may also be generated by alternative initiation of translation and by alternative RNA splicing.

**EXPERIMENTAL PROCEDURES**

Materials—The source of purified protein kinases and protein phosphatases has been reported previously (9). Immobilid acid Sepharose 6B for Ni2+-affinity chromatography was purchased from Sigma. Oligo(dT)-cellulose and CNBr-activated Sepharose 4B were delivered by Pharmacia Biotech Inc. An RNA transcription kit and a rabbit reticulocyte lysate translation kit were obtained from Stratagene. UltradNA polymerase was purchased from Perkin-Elmer. [35S]Met(hi)

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1 The abbreviations used are: PP-1, protein phosphatase-1; PP-1c, catalytic subunit of PP-1; PP-2Aa, catalytic subunit of PP-2A; PP-IN, nuclear PP-1; NIPP-1, nuclear inhibitor of protein phosphatase 1; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).
molecular mass using 12% Tricine-SDS-PAGE (10). The NIPP-1 isoforms were localized by autoradiography (see Fig. 1) and the corresponding gel pieces were excised, pooled, and lyophilized. Subsequently, NIPP-1 was protelyzed as described by Kennedy et al. (11), using either S. aureus V8 protease or endoproteinase Lys-C. Finally, the separated peptides were transferred to a Problott membrane (Applied Biosystems) and visualized by Coomassie staining as well as by autoradiography. The peptides that could be localized were excised and sequenced, using an Applied Biosystems protein sequencer (model 477A).

Assays—The phosphorylase phosphatase activity was determined as described previously (6, 8). NIPP-1 was assayed as such, after phospho-rylation by protein kinase A and/or casein kinase 2, after dephosphorylation with PP-2A, or after trypsinolysis, as specified in Refs. 8 and 9. NIPP-1 was deglycosylated by incubation with N-glycosidase F (12) or by an alkaline borohydride treatment (13). Proteins were measured according to the method of Bradford (14).

Immunological Techniques—A synthetic decapeptide with a sequence corresponding to the 11 carboxyl-terminal residues of bovine NIPP-1 plus an additional NH₂-terminal cysteine, was coupled to keyhole limpet hemocyanin and to bovine serum albumin, using the Pierce immunogen conjugation kit. Rabbit polyclonal antibodies to the hemocyanin-coupled peptides were affinity-purified on albumin-coupled peptide that was linked to CNBr-activated Sepharose 4B. The antibodies were eluted with 3 M KSCN, extensively dialyzed against a buffer containing 20 mM Tris-HCl at pH 7.4 and 150 mM NaCl and stored at −20 °C.

Western analysis of a cytotoxic fraction and a nuclear salt extract from rat liver was essentially done as in Ref. 7, with the affinity-purified NIPP-1 antibodies at a final concentration of 2 μg/ml. The nuclear salt extract was prepared as described in Ref. 7. The cytotoxic fraction was separated, using an Applied Biosystems protein sequencer (model 477A), followed by 12% Tricine-SDS-PAGE (10). The NIPP-1 isoforms were localized by autoradiography (see Fig. 1) and the corresponding gel pieces were excised, pooled, and lyophilized. Subsequently, the filters were prehybridized during 3 h at 60 °C in 0.1 M Na2PO4, 10 mM EDTA, 1% SDS, and 100 μg/ml of denatured DNA from salmon sperm. Hybridization was performed overnight at 53 °C in the same solution containing in addition the NIPP-1 probe labeled (107 cpm/ml). Subsequently, the filters were washed for 10 min at room temperature in 6 × SSC, 0.1% SDS, followed by two washes for 30 min in 3 × SSC, 0.1% SDS at 53 °C. Positive clones were plaque-purified by four rounds of screening. Blueprints pS SK(−) phagemids were excised in vivo according to the manufacturer’s instructions (Stratagene).

Nucleotide sequencing on both strands was performed using the dyeinoxynucleotide chain termination method (16) in an automated laser fluorescent DNA sequencer. Both vector-specific and cDNA-specific oligonucleotide primers were used.

In Vitro Transcription and Translation—Two pBluescript (pBl) constructs were used for this purpose, i.e. pBl-2175 containing the full-length NIP-1 cDNA, and pBl-657 with an insert corresponding to nucleotides 432-1088 of the NIPP-1 cDNA. The latter 657 bp fragment was obtained by PCR, using GGAAGCTTGAAGAT-CTAGA(ACT/CCA)G/GA(A/T)CTT/CAAA corresponding to the peptide sequence DNLTEN. The filters were prehybridized for 3 h at 60 °C in 6 × SSC, 2 × Denhardt’s, 0.05% sodium pyrophosphate, 0.1% SDS, and 100 μg/ml denatured DNA from salmon sperm. Hybridization was performed overnight at 53 °C in the same solution containing in addition the NIPP-1 probe labeled (107 cpm/ml). Subsequently, the filters were washed for 10 min at room temperature in 6 × SSC, 0.1% SDS, followed by two washes for 30 min in 3 × SSC, 0.1% SDS at 53 °C. Positive clones were plaque-purified by four rounds of screening. Blueprints pS SK(−) phagemids were excised in vivo according to the manufacturer’s instructions (Stratagene).

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Microheterogeneity of NIPP-1—We have previously identified two heat-stable forms of NIPP-1, designated NIPP-1a and NIPP-1b, according to their order of elution from Mono Q (6). These forms could also be differentiated by their molecular mass during SDS-PAGE, which amounted to 18 and 16 kDa for NIPP-1a and five with a mass close to that of NIPP-1b, according to their order of elution from Mono Q (6).

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fected by hydrolysis of putative N-linked or O-linked oligosaccharides with an N-glycosidase F or alkaline borohydride treatment, respectively. Although we cannot rule out the possibility that the microheterogeneity of NIPP-1 results from other covalent modifications like deamidation or deacylation, the most likely explanation is that the various NIPP-1 forms are generated by differential proteolysis of the same native polypeptide. This explanation also fits with the similar phosphopeptide maps of NIPP-1a and NIPP-1b (8) and with recent observations that the generation of heat-stable inhibitory polypeptides of PP-1 in freshly isolated liver nuclei is mediated by endogenous proteases (7).

Partial Peptide Sequence of NIPP-1—Since the amount of purified NIPP-1 obtained was limited (6), and since our data suggested a close structural relationship between the various NIPP-1 species, we used a pool of all NIPP-1 forms for NH₂-terminal sequencing. It appeared that NIPP-1 was not susceptible to Edman degradation, indicating that the NH₂ terminus was blocked. Subsequently, the NH₂-terminal sequence of proteolytic fragments was determined. Following in-gel proteolysis of NIPP-1 with S. aureus V8, a 8-kDa peptide was obtained with the NH₂-terminal sequence LDNLTEFNT. A likely explanation for the absence of a signal at position 4 is discussed below.

cDNA Cloning of Bovine NIPP-1—Screening of 1.5 × 10⁶ plaques of a calf thymus cDNA library with a degenerated oligonucleotide based on the sequence DNLTEFN led to the isolation of four positives clones. The largest clone (pBl-2175) contained an insert of 2175 bp and was sequenced on both strands (Fig. 2A). The first ATG (bp 10–12) conformed to the Kozak consensus sequence for initiation of translation in eukaryotes (18). The 3'-untranslated region ended with a poly(A) tail of 21 nucleotides starting at position 2155 and contained three polyadenylation signals of a rare type, i.e. ATTAAA instead of the more common AATAAA (19).

Using the first ATG as start codon, pBl-2175 showed an open reading frame of 1053 bp, encoding a polypeptide of 351 residues with a calculated mass of 38.5 kDa (Fig. 2A). Both sequences obtained from peptide analysis were present in the central part of the predicted primary structure, except for the residue corresponding to Ser¹⁹⁹, which did not yield any signal during peptide sequencing. A likely explanation for this discrepancy is that Ser¹⁹⁹ was phosphorylated in the sequenced peptide fragment, which prevents detection by standard gas-phase and pulse-liquid sequencing procedures (20). The 5-kDa peptide that contained Ser¹⁹⁹ was indeed radioactively labeled. Moreover, the basic residues NH₂-terminal to Ser¹⁹⁹ make it a perfect site for phosphorylation by protein kinase A, which we used to phosphorylate NIPP-1 prior to proteolysis.

Analysis of the deduced amino acid sequence of NIPP-1 shows that 10% of the residues are proline, which is twice above average and suggests a relatively low abundance of α-helices and β-sheets. Although NIPP-1 is a neutral polypeptide (pl = 7.4), the charge is distributed unevenly (Fig. 2B). Indeed, while the NH₂-terminal and COOH-terminal thirds of NIPP-1 are rather basic, the central third of the polypeptide is very acidic. Consistent with the nuclear localization of NIPP-1, three putative nuclear localization signals are present (Fig. 2B). As expected from our previous work on purified NIPP-1 (8, 9), the primary structure also shows several putative phosphorylation sites for protein kinase A (Ser¹⁷⁸, Ser¹⁹⁹, Thr²⁴⁶) and casein kinase 2 (Thr¹²⁹, Thr¹⁶¹, Thr¹⁸¹, Thr²⁰², Ser²⁰⁴, Thr²⁷⁰), which are largely clustered in the central acidic domain (Fig. 2B).

Comparison with Known Polypeptides—No clear homology between NIPP-1 and other known regulatory polypeptides of PP-1 was evident. However, a database search using the basic local alignment search tool (BLAST) program (21) revealed that the COOH-terminal third of NIPP-1 (residues 225–351) was nearly identical to ard-1, a protein that recently has been implicated in RNA processing (22). At the amino acid level, the only difference between the COOH-terminal domain of bovine NIPP-1 and human ard-1 was at position 245 in NIPP-1, which is valine as opposed to glycine in ard-1 and which is likely to represent a species difference. Comparison of the cDNA-deduced mRNA sequences encoding ard-1 and NIPP-1 showed that the homology also extended to the noncoding regions (Fig. 3). Indeed, three regions in the NIPP-1 mRNA showed more than 90% identity with the corresponding domains in the ard-1 mRNA. However, each mRNA also contained two unique domains at their 5'-end, which account for the different length of the translation products. Taken together, our data suggest that the mRNAs encoding NIPP-1 and ard-1 are generated from the same pre-mRNA by alternative splicing.

Using the BLAST program (21), we also found 40% identity of two fragments (residues 156–195 and 232–253) of NIPP-1 with domains of the heavy chain of rabbit myosin. Furthermore, residues 253–289 of NIPP-1 were 31% identical to a domain in the 70-kDa RNA-binding protein of the U1 small nuclear ribonucleoprotein complex.

In Vitro Translation of NIPP-1—The calculated molecular mass of NIPP-1 (38.5 kDa) was much higher than that of the purified inhibitor (16–18 kDa), indicating that purified NIPP-1 was a proteolytic degradation product. However, since the second (bp 436–438) and third ATG (bp 682–684) codons in the NIPP-1 cDNA also conformed to the Kozak sequence for initiation, it could not be excluded that smaller inhibitory polypeptides were generated by translational initiation at an internal ATG. To investigate the latter possibility, in vitro translation in reticulocyte lysates was performed. Translation of the full-length NIPP-1 mRNA indeed resulted in the accumulation of two polypeptides with apparent masses of 47 and 29 kDa, as visualized by autoradiography after Tricine-SDS-PAGE (Fig. 4). The masses of the synthesized polypeptides were somewhat higher than the 38.5 and 23 kDa expected from translational initiation at codons 10–12 and 436–438, respectively. However, such an anomalous electrophoretic migration has also been noted for other inhibitory polypeptides of PP-1 and has been explained by the low binding of SDS, due to the high content of hydrophilic residues (1).

Two further lines of evidence showed that the 29-kDa translation product resulted from initiation at the second ATG (bp 436–438), rather than from proteolysis of the 47-kDa species of NIPP-1. First, translation of an in vitro transcribed NIPP-1 mRNA that lacked 431 nucleotides at the 5'-end still resulted in the accumulation of the 29-kDa polypeptide (Fig. 4). Second, mutation of the first ATG codon in the full-length cDNA into an ATC completely abolished the synthesis of the 47-kDa product, but still yielded the 29-kDa polypeptide (not shown).

Bacterial Expression of an Active Fragment of NIPP-1—Initial trials to obtain intact NIPP-1 by expression in E. coli failed because of rapid proteolysis of the recombinant polypeptide. We therefore aimed at the expression of the 29-kDa fragment of NIPP-1 since this fragment, in contrast to intact NIPP-1, proved to be resistant to denaturing procedures like boiling and acid treatment (Fig. 4). This enabled us to quickly stop proteolysis by boiling the bacterial pellet. Following chromatography of the heat-stable bacterial fraction on Ni²⁺-Sepharose, a major polypeptide of 15 kDa was obtained (Fig. 5A). The mass of the recombinant polypeptide was about half the expected size (29 kDa), indicating that proteolysis had taken place before...
**Fig. 2. Nucleotide and predicted amino acid sequence of NIPP-1 from calf thymus.** In A the nucleotide sequence is presented in the 5' to 3' direction and is numbered on the left. The deduced protein sequence is given below the nucleotide sequence (one-letter code) and is numbered on the right. Also indicated are the three consensus initiator codons (bold), the stop codon (asterisk), the three putative polyadenylation signals (boxed), and the primary structure that was also obtained from NH2-terminal sequencing of peptides obtained after limited proteolysis (underlined). B schematically illustrates some basic features of the deduced primary structure of NIPP-1, represented by the bar. Indicated are potential nuclear localization signals (NLS), putative phosphorylation sites for protein kinase A (PKA) and casein kinase 2 (CK2), the occurrence of basic and acidic domains, and the pI of some of these domains.
and/or during the heat treatment of the bacteria. This is confirmed by the finding that the recombinant fragment, in contrast to the native inhibitor (see below), was not recognized by antibodies against the COOH terminus of NIPP-1 (not shown).

On the other hand, the recombinant NIPP-1 fragment was not susceptible to Edman degradation, indicating that the NH₂ terminus was blocked.

Several lines of evidence suggest that the recombinant 15-kDa fragment of NIPP-1, which probably roughly corresponds to the central third of NIPP-1, contains the inhibitory domain. First, in agreement with our previous findings for purified NIPP-1, the recombinant fragment inhibited the phosphorylase phosphatase activity of PP-1C, but was destroyed by trypsin (Fig. 5B). Second, the fragment was phosphorylated by protein kinase A and casein kinase 2 (Fig. 5A), and its inhibitory potency was decreased severalfold by such phosphorylation (Fig. 5B). When tested separately, protein kinase A turned out to be more efficient than casein kinase 2 in inactivating the recombinant NIPP-1 fragment (not illustrated).

Subcellular Distribution of NIPP-1—Antibodies raised against a synthetic peptide with a sequence encompassing the COOH-terminal part of NIPP-1, were used for Western blotting of liver fractions. NIPP-1 was easily detected in a nuclear salt extract, while at equal protein loading, no signal was detected in the cytosolic fraction (Fig. 6). The antibodies recognized a polypeptide of 47 ± 1 (n = 3) kDa after Tricine-SDS-PAGE, which is the same mass as that of the largest polypeptide that was synthesized during in vitro translation of NIPP-1 mRNA.

**Fig. 3.** Schematic comparison of the mRNAs encoding NIPP-1 and ard-1. The large bars represent the mRNAs encoding bovine NIPP-1 (2175 bp) and human ard-1 (2401 bp). The open, numbered bars represent fragments that have more than 90% sequence identity. The hatched boxes represent domains that are specific for the mRNAs encoding NIPP-1 or ard-1. The small solid bars delineate the coding regions.

**Fig. 4.** In vitro translation of NIPP-1 mRNAs encoding full-length NIPP-1 or a NIPP-1 fragment (residues 143–351) were prepared by in vitro transcription from pBl-2175 or pBl-657, respectively. In vitro translation was performed in reticulocyte lysates in the presence of [³⁵S]methionine. After translation the lysates were either directly boiled in SDS-sample buffer or first boiled (5 min) and centrifuged (1 min at 10,000 × g) before addition of the heat-stable fraction to the SDS-sample buffer. Following 30% Tricine-SDS-PAGE the translation products were visualized by autoradiography. Lanes 1 and 2 show the translation products of the full-length NIPP-1 mRNA in total lysates and in the heat-stable lysate fraction, respectively. Also shown are the translation products of the mRNA encoding residues 143–351 of NIPP-1 in total lysates (lane 3) and in the heat-stable lysate fraction (lane 4). Lane 5 shows a control translation with no exogenous mRNA added.

**Fig. 5.** Bacterial expression of an active fragment of NIPP-1. Bacteria were transformed with the pBl-657 plasmid, which contains an insert encoding residues 143–351 of NIPP-1. A shows a Coomassie staining of the purified recombinant polypeptide after 12% Tricine-SDS-PAGE (lane 1). Lanes 2 and 3 show an autoradiogram of the purified recombinant fragment after incubation with γ-³²P-labeled MgATP in the absence (lane 2) or presence (lane 3) of protein kinase A plus casein kinase 2. B shows the effect of the indicated concentrations of the recombinant NIPP-1 fragment on the phosphorylase phosphatase activity of PP-1C. The fragment was added as such (●), after trypsinolysis (○), or after phosphorylation by protein kinase A plus casein kinase 2 (■). The results represent the means ± S.E. for three experiments.
previously shown that this polypeptide is present as a complex depending on the adopted electrophoresis system. We have NIPP-1 with an apparent mass of 41 kDa (7) or 47 kDa (Fig. 6), central domain (Fig. 2).

Remarkably, the migration of NIPP-1 also depended on the adopted electrophoresis system. Thus, we have noted that NIPP-1 rather migrates as a polypeptide of 41 kDa when glycine instead of Tricine is used as the trailing ion during SDS-PAGE (7).

NIPP-1 mRNA is Expressed in Different Tissues—In calf thymus a single NIPP-1 mRNA transcript of 2.2 kb was detected (Fig. 7A), which is nearly identical to the size of the isolated cDNA clone (2175 bp). Northern blot analysis revealed a slightly larger transcript (2.4 kb) in various human tissues (Fig. 7B), indicating a ubiquitous expression of NIPP-1. At equal poly(A) − RNA loading the highest expression of NIPP-1 messenger was noted in heart and skeletal muscle. However, very clear signals were also obtained for brain, placenta, lung, liver, and pancreas. NIPP-1 mRNA was relatively less abundant in kidney.

**DISCUSSION**

Functional Domains of NIPP-1—The NIPP-1 polypeptide chain contains three domains of about equal size, based upon the charge distribution and the localization of residues that delineate variants generated by alternative splicing or by alternative translational initiation (Fig. 2B). Analysis of a bacterially expressed fragment of NIPP-1 has shown that the PP-1c inhibitory domain is likely to reside in the central acidic third of the polypeptide (Fig. 5). This conclusion is corroborated by findings that the previously purified active fragments of NIPP-1 have an acidic pl (6) and that the two peptide sequences obtained from these fragments are both located in the central domain (Fig. 2).

Western analysis only revealed a single nuclear species of NIPP-1 with an apparent mass of 41 kDa (7) or 47 kDa (Fig. 6), depending on the adopted electrophoresis system. We have previously shown that this polypeptide is present as a complex with about half of all PP-1c that is present in a nuclear extract (7). Antibodies directed against the COOH terminus of NIPP-1 did not detect fragments corresponding to ard-1 or to alternative translational initiation products. This could mean that such fragments are not expressed in vivo or that they are much less abundant than the 41–47-kDa fragments. Alternatively, the generation of initiation or splice variants may be cell type- or cell cycle-dependent. It cannot be excluded either that other NIPP-1 species were not detected due to their rapid proteolysis or posttranslational modification at the COOH terminus.

Does NIPP-1 Play a Role in mRNA Processing?—The finding of homology of NIPP-1 domains with polypeptides that have been implicated in mRNA processing was unexpected. Most striking is the identity between the COOH-terminal third of NIPP-1 and the ard-1 polypeptide. The latter polypeptide was discovered by its ability to complement mutations in the rne gene of E. coli, hence its name “activator of RNA decay” or “ard-1” (22). The rne gene product is a multifunctional Rnase E that not only converts bacterial 9S pre-rRNA into 5 S rRNA, but also promotes general mRNA turnover. In addition, Rnase E has a structural role, since a loss-of-function mutation in the rne gene causes the cells to elongate and to accumulate long filamentous structures. The molecular basis for the complementation of rne mutations by ard-1 is not understood. It has been proposed that ard-1, similar to what has previously been suggested for Rnase E, somehow facilitates the formation or transport of multicomponent complexes of RNA and ribonucleases that are involved in RNA processing (22). A role in a transport process agrees with the (limited) homology of domains of Rnase E, ard-1, and NIPP-1 with cytoskeletal proteins like the myosin heavy chain.

Rnase E, ard-1, and NIPP-1 also show a limited homology to the 70-kDa U1 RNA-associated protein (Ref. 22; this work), which is a component of the U1 small nuclear ribonucleoprotein complex and is involved in RNA splicing in eukaryotes (23). Finally, a role for NIPP-1 in pre-mRNA splicing is also indicated by observations that NIPP-1 is physically associated with PP-1c (7), which has recently been shown to modulate both spliceosome assembly as well as the splicing process itself (24).

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**FIG. 6. Identification of NIPP-1 in subcellular liver fractions by Western analysis.** A cytoplasmic fraction and a nuclear salt extract were prepared from rat liver as indicated in the “Experimental Procedures.” About 15 μg of protein of each fraction was subjected to 10% Tricine-SDS-PAGE, transferred to a membrane, and probed with antibodies against the COOH terminus of NIPP-1.

**FIG. 7. Northern blot analysis in calf thymus and in various human tissues.** Each lane contains 2 μg of poly(A) − RNA isolated from the indicated tissue. The blots were hybridized with a probe corresponding to bp 432-1088 of the full-length NIPP-1 cDNA clone.
Primary Structure of NIPP-1

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