NIPP-1, a Nuclear Inhibitory Subunit of Protein Phosphatase-1, Has RNA-binding Properties*

(Received for publication, June 10, 1997)

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NIPP-1 is a nuclear inhibitory subunit of protein phosphatase-1 with structural similarities to some proteins involved in RNA processing. We report here that baculovirus-expressed recombinant NIPP-1 displays RNA-binding properties, as revealed by North-Western analysis, by UV-mediated cross-linking, by RNA mobility-shift assays, and by chromatography on poly(U)-Sepharose. NIPP-1 preferentially bound to U-rich sequences, including RNA destabilizing AUUUA motifs. NIPP-1 also associated with single-stranded DNA, but had no affinity for double-stranded DNA. The binding of NIPP-1 to RNA was blocked by antibodies directed against the COOH terminus of NIPP-1, but was not affected by prior phosphorylation of NIPP-1 with protein kinase A or casein kinase-2, which decreases the affinity of NIPP-1 for protein phosphatase-1. The catalytic subunit of protein phosphatase-1 did not bind to poly(U)-Sepharose, but it bound very tightly after complexation with NIPP-1. These data are in agreement with a function of NIPP-1 in targeting protein phosphatase-1 to RNA.

The type 1 protein phosphatases (PP-1) represent a conserved family of Ser/Thr-specific protein phosphatases with functions in various cellular processes including metabolism, intracellular transport, and protein synthesis (1, 2). PP-1 is particularly abundant in the nucleus, where it is involved in the regulation of both transcription and mRNA processing. As for the latter, PP-1 has a demonstrated role in the subnuclear distribution of pre-mRNA splicing factors, spliceosome assembly, and (alternative) pre-mRNA processing, which has been explained by dephosphorylation of components of small nuclear ribonucleoproteins (snRNPs), such as the “SR” proteins, by PP-1 (3–5). The SR splicing factors share a common motif of Ser-Arg repeats that is reversibly phosphorylated in vivo and is essential for spliceosome assembly and function.

All known species of PP-1 are dimeric or trimeric enzymes (1, 2). They contain an isoform of the same catalytic subunit (PP-1C), but differ in the regulatory subunits. In addition to an activity-controlling and substrate-specifying function, the regulatory subunits also have a “targeting” role. This multifunctional role is best understood for the “G” subunit that anchors PP-1 to glycogen and promotes the dephosphorylation of glycogen-associated proteins like glycogen synthase (6, 7). Similarly, the “M” subunit targets PP-1 to myofibrils and enhances the dephosphorylation of myosin (6, 7). Other noncatalytic subunits of PP-1 (e.g. inhibitor-2, NIPP-1, and sds22) are known to affect the activity and/or substrate specificity of PP-1 in vitro, but it is unclear at present whether they also have a targeting role (1, 2, 8, 9).

NIPP-1 is a nuclear polypeptide that potently and specifically inhibits PP-1C (8). It can be extracted from the insoluble nuclear fraction as a heterodimeric complex with PP-1C-termed PP-1NIPP-1 (10). Phosphorylation of intact NIPP-1 (39 kDa) or its inhibitory core (16–18 kDa) by protein kinase A and/or casein kinase-2 reduces its affinity for PP-1C (10–13). The inhibitory site and all the phosphorylation sites for protein kinase A and casein kinase-2 reside in the central third of the polypeptide (13). The cDNA cloning of bovine NIPP-1 has revealed a limited structural similarity with the 70-kDa U1-RNA-associated protein (13), which is a component of the U1 snRNP complex in the spliceosome (14). Also, the COOH-terminal third of NIPP-1 (13) is identical to a human polypeptide of 13 kDa that can reverse the pleiotropic effects of deletion mutants of a 5′-segment of the rne gene in Escherichia coli. The latter protein was named Ard-1, for “activator of RNA decay,” since it restored deficient RNA processing in the bacterial rne mutants (15). The complementation by Ard-1 is not easily understood at the molecular level, since the rne gene encodes a multifunctional protein that not only displays an endoribonuclease E (RNase E) activity, but also contains domains that have been implicated in RNA binding, in macromolecular transport and in the maintenance of the cytoskeleton.

The established role of PP-1 in RNA-processing and the structural similarities between NIPP-1 and known RNA-processing enzymes have prompted us to investigate the potential RNA-binding properties of NIPP-1. We report here that NIPP-1 binds with high affinity to RNA, in particular to U-rich sequences. By analogy with the anchoring role of other regulatory subunits of PP-1, we propose that NIPP-1 targets PP-1 to RNA-associated substrates.

EXPERIMENTAL PROCEDURES

Preparation and Assay of Proteins—NIPP-1 from bovine thymus was expressed in insect Sf9 cells under control of the polyhedrin promotor.
using the baculovirus expression system. The spontaneous and trypsin-revealed phosphorylase phosphatase activities were measured as described by Jagiello et al. (10). NIPP-1 was detected by Western blotting with polyclonal antibodies against the 11 carboxyl-terminal residues of NIPP-1, by an overlay with digoxigenin-labeled PPI1, and by the assay as an inhibitor of PP-1 (10). Protein concentrations were measured according to Bradford (16). En- doribonuclease activities, using AU4 and 9 S RNA as substrates, were measured as detailed in Wennborg et al. (17).

Preparation and Labeling of RNA and Oligo(deoxy)ribonucleotides—The homoribopolymers poly(A), poly(C), poly(G), and poly(U) were purchased from Sigma. The following oligoribonucleotides were obtained from Eurogentec (Liége, Belgium): AG1, 5'-CUCUGAGGAUGCAGG- UAGCCUGGUCCACG-3'; AU1, 5'-CUCUGAGGAUGCAUUAAACGU- CUUGGUACC-3'; AU2, 5'-CUAGAGGAGCAUUAUUAAAGCU- UGGGUAC-3'; AU4, 5'-AGGAUGCAAUUUAUUAUAAUAGGC- UUGG-3'; and AUMYC, 5'-CUUUAACGAUUGAUUAGA- AUUGUUAUAAUAAUGAGUUACA-3' (17). Oligo(deoxy)ribonucleotides were synthesized using a Cyclone DNA synthesizer (Perkin-Elmer Cetus) and D NTPs from Eurogentec (Liege, Belgium): AG1 or AU4 (0.4 Ci/ml) or bovine serum albumin (25 mg/ml) were incubated with 50 Ci/ml or 5 S RNA and mRNA encoding outer membrane protein A (ompA) were transcribed from 2 µg of the HaeIII-linearized plasmids pH90 and p106B-64, respectively, using the in vitro transcription kit from Stratagene (La Jolla, CA) in the presence of [γ-32P]ATP and 600 units/ml RNase-free DNase I (Pharmacia) in the presence of 0.1 ml of RNase inhibitor RNAsin (Promega). The labeled probes were extracted with a mixture of phenol/chloroform/isoamyloalcohol (25:24:1), precipitated with 70% (v/v) ethanol and 10 mM sodium acetate, and purified on 8% urea-PAGE. The labeled RNA was visualized by autoradiography and extracted with 10 ml Tris/HCl at pH 7.5 and 1 mM EDTA. The probes were once more extracted and precipitated as detailed above, lyophilized, and resuspended in 10 ml Tris/HCl at pH 7.5 and 1 mM EDTA.

The oligoribonucleotides AG1, AU1, AU2, AU4, and AUMYC were labeled at the 5'-end by phosphorylation with T4 polynucleotide kinase (Boehringer Mannheim) during 45 min at 37 °C, according to the manufacturer's instructions, in the presence of [γ-32P]ATP and 600 units/ml RNAsin. The labeled oligoribonucleotides were purified on 20% urea-PAGE and extracted as detailed above for RNA.

UV-mediated Cross-linking—NIPP-1 (5 µg/ml) or bovine serum albumin (25 µg/ml) were incubated in 10 ml Tris/HCl at pH 7.5, 0.05% (v/v) Triton X-100, 40 mM KCl, 3 mM dithiothreitol, 8.5% (v/v) glycerol, 2 mM MgCl2, and 5 mM EDTA with 5'-end-labeled AU4 (2 µCi/ml), or the 5'-untranslated region of the ompA mRNA (1 µCi/ml), or else 9 S RNA (1 µCi/ml) as described previously (17). Aliquots of 20 µl were exposed to UV light (254 nm at 250 mJ for 1 min) in a Stratallinker UV apparatus. Subsequently, the samples were subjected to 7.5% Tricine-SDS-PAGE, and the dried gels were exposed for autoradiography.

North-Western blot Analysis—NIPP-1 was subjected to 7.5% Tricine-SDS-PAGE and blotted onto nitrocellulose membranes (Hybond-C extra, Amersham Corp.) Prehybridization was performed for 90 min at 44 °C in a buffer containing 10 mM Tris/HCl at pH 8.0, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 0.02% Ficol, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 150 µg/ml preheated (10 min at 100 °C) followed by an incubation at 0 °C) salmon sperm DNA (Sigma). The hybridization was done for 2 h at 44 °C in the same buffer supplemented with in vitro transcribed 9 S RNA (0.2 µCi/ml) or 5'-end-labeled AG1 or AU4 (0.4 µCi/ml). Subsequently, the membranes were washed four times for 10 min in 10 mM Tris/HCl at pH 8.0, 1 mM EDTA, 50 mM NaCl, and 1 mM dithiothreitol, and the radioactive bands were visualized by autoradiography.

RNA Mobility Assays—Various proteins (1–25 µg/ml) were incubated for 30 min at 0 °C in a buffer containing 10 mM Heps at pH 7.5, 40 mM KCl, 3 mM dithiothreitol, 8.5% (v/v) glycerol, 2 mM MgCl2, 0.05 mM EDTA, 0.05% (v/v) Triton X-100, and one of the 5'-end-labeled oligoni

FIG. 1. Identification of NIPP-1 as a RNA-binding protein by North-Western analysis and by UV-mediated cross-linking. Left panel, NIPP-1 (5 µg/ml) was subjected to 7.5% Tricine-SDS-PAGE and blotted onto nitrocellulose membranes. The lanes were either stained with Amido Black or subjected to autoradiography after hybridization with 32P-labeled 9 S RNA, AU4, or AG1 as indicated. Right panel, 5'-end-labeled AU2 (50 ng/ml) was incubated either as such or in the presence of native NIPP-1 (5 µg/ml) or bovine serum albumin (25 µg/ml) and subjected to UV-mediated cross-linking prior to electrophoresis and autoradiography.

RESULTS

Identification of NIPP-1 as an RNA-binding Protein—The present study was performed with bovine thymus NIPP-1 that was expressed in insect Sf9 cells, using the baculovirus expression system, and purified until homogeneity (Fig. 1, left panel). The properties of the isolated recombinant inhibitor were similar to those of native NIPP-1 (10) and of the NIPP-1 fragments that were previously isolated from bovine thymus (8, 11–12), with respect to the specificity and potency of its inhibition of PP-1, and the decreased inhibitory potency after its phosphorylation by protein kinase A and casein kinase-2 (not illustrated).

Since it has been reported that the COOH-terminal third of NIPP-1 can substitute for some functions of the bacterial RNase E (see Introduction), we have initially focused on known substrates of this endoribonuclease to investigate putative RNA-binding properties of NIPP-1. These substrates include 9 S RNA (the bacterial precursor of 5 S ribosomal RNA), the ompA mRNA, and the oligoribonucleotides AG1, AU1, AU2, and AU4, containing 0, 1, 2, or 4 copies, respectively, of the destabilizing AUUUA motif. Using these RNAs, NIPP-1 was identified as an RNA-binding protein by several independent criteria. Thus, North-Western analysis showed a binding of denatured NIPP-1 to bacterial 9 S RNA and to the oligoribonucleotides AU4 and AG1 (Fig. 1, left panel). We were also able to obtain a UV-mediated cross-linking of native NIPP-1 to AU4 (Fig. 1, right panel), and to ompA and 9 S RNA (not shown).

Following cross-linking of NIPP-1 to AU4, a radioactive band of 50 ± 1 kDa (n = 4) was detected, which corresponds to the combined masses of NIPP-1 (38.5 kDa) and AU4 (10 kDa). In addition, a radioactive band of 130 ± 4 kDa (n = 4) was

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detected, which might represent a cross-linked AU4-NIPP-1 dimer. No cross-linking was obtained under identical conditions when bovine serum albumin was used instead of NIPP-1 (Fig. 1, right panel).

A binding of NIPP-1 to RNA could also be reproducibly demonstrated by assays of RNA-mobility shifts in native gels, as is illustrated for ompA, 9 S RNA, and AU4 in Fig. 2. Under the same conditions, no RNA band shifts were seen with the control proteins casein, myelin basic protein, bovine serum albumin, and PP-1C (not illustrated). The specificity of the interaction between NIPP-1 and AU4 is also illustrated by the effects of NIPP-1 antibodies and of the antigenic peptide on the mobility shift (Fig. 2). Phosphorylation of NIPP-1 by protein kinase A or casein kinase-2, which decreases its potency as an inhibitor of PP-1C (10–13), had no effect on its ability to induce RNA-mobility shifts (not shown).

Since NIPP-1 is associated with PP-1C in the nucleus (10), it appeared important to determine whether the complex (PP-1NNIPP-1) also displayed RNA-binding properties. In Fig. 3A, it is shown that PP-1C did not bind to poly(U)-Sepharose. In contrast, free NIPP-1 (Fig. 3B) as well as an in vitro reconstituted heterodimeric complex of NIPP-1 and PP-1C (Fig. 3C) was completely retained by the affinity column. The binding of NIPP-1 and PP-1NNIPP-1 to poly(U)-Sepharose was very strong, since they were not eluted at NaCl concentrations up to 1.5 M. NIPP-1 and PP-1NNIPP-1 could subsequently be eluted, however, with 3 M KSCN.

Specificity for Binding of NIPP-1 to Nucleic Acids—The non-labeled ribohomopolymers showed a different potency in competing for the binding of NIPP-1 to 5'-end-labeled AU4 at 0.1 \( \mu \)g/ml (Fig. 4). Indeed, while poly(G) was not a competitor at concentrations up to 2.5 \( \mu \)g/ml, poly(U) at only 0.02 \( \mu \)g/ml nearly completely prevented the binding of NIPP-1 to AU4. Poly(A) and poly(C) were less potent.

At the same concentrations and specific radioactivities, the AUUU-containing oligoribonucleotides AU1, AU2, and AU4 bound more NIPP-1 than did AG1, where the AUUA motif is altered into AGGUA (Fig. 5, upper panel). We did not observe a difference in the affinities for AU1, AU2, and AU4, indicating that their binding to NIPP-1 was not affected by the number of the AUUA motifs. These findings were corroborated by competition assays with nonlabeled probes, showing that AU2 and AU4 were 5–10 times better than AG1 as competitors for binding of NIPP-1 to AU4 (Fig. 5, lower panel). Combined with the data of Fig. 4, this suggests that NIPP-1 preferentially binds to U-rich RNA.

We have also compared RNA and DNA as competitors for...
binding of NIPP-1 to AU2 at 0.15 μg/ml. In Fig. 6 it is shown that the oligoribonucleotide AU4 and the corresponding deoxyribonucleotide dAT4 were equally efficient competitors. However, the complementary strand of dAT4, i.e. dTA4, was 5–10 times less efficient and the double-stranded dAT4/dTA4 was completely inefficient at concentrations up 2.5 μg/ml.

The above experiments were all performed with bacterial RNAs or with designed synthetic oligonucleotides. However, we found that NIPP-1 bound with similar affinity to AU4 and to AUMYC, which comprises the destabilizing AUUUA-repeat motif of the 3'-untranslated region of c-myc mRNA (17), as determined by UV-mediated cross-linking, by AUMYC-mobility shift assays, and by competition for the binding of NIPP-1 to AU4 (not shown).

**DISCUSSION**

**RNA-binding Specificity of NIPP-1**—We report here several independent lines of evidence indicating that NIPP-1 is an RNA-binding protein. Thus, NIPP-1 was able to induce RNA-mobility shifts (Figs. 2, 4–6) and could be covalently bound to RNA by UV-mediated cross-linking (Fig. 1). An interaction of NIPP-1 with RNA could also be demonstrated by North-Western analysis (Fig. 1) and by affinity chromatography on poly(U)-Sepharose (Fig. 3). Moreover, the RNA binding was shown to be due to a protein with the same size as NIPP-1 (Fig. 1) and was blocked by NIPP-1-specific antibodies (Fig. 2).

AU4 and dAT4 were equally efficient competitors for the binding of NIPP-1 to AU2 (Fig. 6), suggesting that NIPP-1 has the same affinity for RNA and single-stranded DNA. On the other hand, NIPP-1 showed a clear preference for U-rich oligo-
nucleotides and displayed no affinity for double-stranded nucleic acids (Figs. 4 and 6). Analysis of the primary structure of NIPP-1 did not reveal the presence of known RNA-binding motifs such as the “RNP” motif, the arginine-rich motif, the RGG box, or the KH motif (18). However, it is striking that the amino- and carboxyl-terminal thirds of NIPP-1 are very basic (pI 9–10) and are not needed for the interaction with PP-1C (13). It is therefore tempting to speculate on an electrostatic interaction between nucleic acids and the basic extremities of NIPP-1. However, it is clear that additional binding determinants must exist to account for the specificity of the interaction of NIPP-1 with nucleic acids. It is also important to note that the binding of NIPP-1 to both PP-1C (10) and to RNA (Fig. 1) could still be observed after SDS-PAGE and blotting of NIPP-1, indicating that the RNA- and PP-1C-binding domains resist denaturing procedures.

Is NIPP-1 an RNA-targeting Subunit of PP-1?—Since both NIPP-1 and PP-1NINIPP-1 (but not PP-1C) bind to RNA (Fig. 3), we propose that NIPP-1 functions as an RNA-anchoring subunit of PP-1 and enables the phosphatase to dephosphorylate RNA-associated substrates. The activity of PP-1NINIPP-1 would be regulated through phosphorylation of NIPP-1 by protein kinase A and casein kinase-2, which decrease its affinity for RNA-associated substrates. The activity of PP-1NINIPP-1 with RNA agrees with our findings that the holoenzyme is associated with the nuclear insoluble fraction (8, 10) and can be solubilized by the mere incubation of this fraction with ribopolymers.\(^2\) The targeting model of PP-1NINIPP-1 is also strikingly similar to that of glycosgen-associated PP-1 (PP-1G). Indeed, the latter holoenzyme consists of PP-1C and a glycogen-anchoring G-subunit and the interaction between both polypeptides is controlled by reversible phosphorylation of the G subunit (1, 2, 6, 7).

The RNA-anchoring hypothesis implies that PP-1NINIPP-1 is a physiologically active enzyme. At first glance, this contrasts with findings that NIPP-1 is inhibitory to PP-1C (8, 10). However, it is well known that the effects of the regulatory subunits of PP-1 are substrate-dependent. Thus, the G subunit endows PP-1C with synthase phosphatase activity but decreases its phosphorylase phosphatase activity (1, 6, 7). It has also been reported that inhibitor-2 blocks the dephosphorylation of most, but not all substrates of PP-1C (19). Similarly, we have found that intact NIPP-1 inhibits the dephosphorylation of all five tested substrates of PP-1C, albeit to a different extent (10). Also, a 16-kDa fragment of NIPP-1 was inhibitory to the dephosphorylation of phosphorylase, but actually enhanced the dephosphorylation of histone H1A (8). In conclusion, it is possible that NIPP-1 is not a true inhibitor of PP-1, but rather acts as a “substrate-specifying” subunit in vivo. The effects of phosphorylation of NIPP-1 by protein kinase A or casein kinase-2 may have to be reinterpreted along the same lines. Indeed, phosphorylation of NIPP-1 reduces its affinity for PP-1C but, depending on the physiological role of NIPP-1, such phosphorylation may cause an activation or inactivation of PP-1NINIPP-1.

Among the most likely substrates of PP-1NINIPP-1 are the SR-splicing factors, which have been shown to be dephosphorylated by PP-1C \(\text{in vitro}\) and to reverse the inhibition of spliceosome assembly by PP-1C (3, 4). Interestingly, the described effects of PP-1C on spliceosome assembly and splicing in cell extracts and in permeabilized cells were all obtained at rather high concentrations of the phosphatase (3, 4), which may indicate a requirement for a substrate-specifying and -anchoring subunit. It will be interesting to investigate whether NIPP-1 might promote the dephosphorylation of SR proteins by PP-1C.

NIPP-1 displayed a particularly high affinity for AUUU sequences (Fig. 5). These are destabilizing motifs in the 3’-end of short-lived mRNAs encoding human (proto)-oncogenes and growth factors (20). The same sequences have recently also been identified as destabilizing motifs in snRNAs (21). The AUUU motif is specifically cleaved by RNase E from \(E.\) \textit{coli} as well as from mammalian cells (17). In \(E.\) \textit{coli}, RNase E is part of a large RNA-degradation complex, called the degradosome (22). We speculate therefore that PP-1NINIPP-1 takes part in the control of RNA processing in mammalian cells by the dephosphorylation of polypeptides, like RNase E, that are part of a putative mammalian degradosome. It is also important to point out here that, while NIPP-1 is highly enriched in the nucleus, both cell fractionation (13) and immunofluorescence studies\(^2\) show the existence of a cytoplasmic pool of NIPP-1. Thus, NIPP-1 could control RNA-processing in the cytoplasm as well as the nuclear cell compartment.

Acknowledgments—V. Feytons, N. Sente and P. Vermaelen are acknowledged for expert technical assistance. We thank Drs. D. Angerer and Dr. J. Kaszuba (Vienna) for helpful advice in setting up the initial RNA-binding experiments.

Note added in proof—It was recently reported by Claverie-Martin et al. (Claverie-Martin, F., Wang, M., and Cohen, S. N. (1997) \textit{J. Biol. Chem.} 272, 13823–13828) that Ard-1 is an RNA-binding protein but, in contrast to NIPP-1 (this work), also displays a Mg\(^{2+}\)-dependent endoribonuclease activity.

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