Broad Specificity in Binding of NIPP-1, Nuclear Inhibitor of Protein Phosphatase-1, to PP1 Isoforms in Vivo

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KIM, S.-E., SHIMA, H., NAKAMURA, K. and KIKUCHI, K. *Broad Specificity in Binding of NIPP-1, Nuclear Inhibitor of Protein Phosphatase-1, to PP1 Isoforms in Vivo*. Tohoku J. Exp. Med., 2000, **191** (1), 39–45 — Protein phosphatase type-1 (PP1), one of the most abundant Ser/Thr protein phosphatases, plays a central role in the regulation of various cell functions. Almost all the PP1 molecules exist as holoenzymes in vivo consisting of a catalytic subunit (PP1C) and a variable regulatory subunit that regulates substrate specificity and/or subcellular localization. In order to clarify fine regulation of PP1, we overexpressed a nuclear inhibitor of PP1 (NIPP-1) in a Flag-tagged form in mammalian cells. The Flag-tagged NIPP-1 was found to be immunoprecipitated with three isoforms of PP1C, namely, PP1 α , PP1 γ 1, and PP1 δ with a similar efficiency, suggesting that NIPP-1 makes a complex with the PP1C through the region conserved among the three isoforms. These results suggested that NIPP-1 can be involved in the regulation of various PP1 holoenzymes in vivo. ——— PP1; binding affinity; co-immunoprecipitation; regulatory subunit; NIPP-1 © 2000 Tohoku University Medical Press

Type-1 protein phosphatase (PP1), one of the major types of serine/threonine protein phosphatases, is involved in various events in eukaryotic cells (Shenolikar 1994; Wera and Hemmings 1995). The functional diversity of PP1 is thought to be achieved by the presence of several isoforms of PP1 catalytic subunits (PP1Cs) and numerous regulatory subunits which target PP1C to specific locations, define substrate specificities, and regulate enzyme activities. There are four mammalian PP1C isoforms, namely PP1 α , PP1 γ 1, PP1 γ 2, and PP1 δ (Sasaki et al. 1990) which exhibit approximately 90% identity with a strong sequence diversity in their COOH-terminal 30 amino acid residues which might be involved in the

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isoform-specific functions (da Cruz e Silva et al. 1995; Villa-Moruzzi et al. 1996). PP1 α , PP1 γ 1, and PP1 δ are expressed in almost all the cell types, while PP1 γ 2 is specifically expressed in testis (Shima et al. 1993; Takizawa et al. 1994).

Biochemical (Jagiello et al. 1995) and immunohistochemical (Andreassen et al. 1998) studies showed that a large fraction of PP1 is present in the nucleus. The nuclear PP1 (PP1N) is implicated in the gene expression (Cardinali et al. 1994), pre mRNA splicing (Mermoud et al. 1994), and chromatin condensation (Bollen et al. 1993). However, it remains poorly understood how the PP1N is regulated. The major nuclear targeting subunits of PP1 have been identified as nuclear inhibitor of protein phosphatase-1 (NIPP-1)/p99 (Kreivi et al. 1997) and PP1 nuclear targeting subunit (Allen et al. 1998). The PP1 inhibition can be relieved by phosphorylation of NIPP-1 by protein kinase A and casein kinase 2, without disruption of the complex (Vulsteke et al. 1997). NIPP-1 was co-purified with PP1 δ as a holoenzyme from rat liver nuclear extracts (Jagiello et al. 1995). However it remains unclear whether NIPP-1 exists as a complex with PP1C in vivo.

For these two decades, we have studied neoplastic alterations of protein phosphatases with rat hepatomas, and reported the up-regulation of the nuclear PP1 α and PP1 δ (Imai et al. 1999). Recently, we also reported the up-regulation of NIPP-1 in ascites hepatomas (Kim et al. 2000). These results suggest some regulatory roles of NIPP-1 against PP1C isoforms. To elucidate these points further, we undertook to examine the specificity for the binding of NIPP-1 to PP1C. In the present study, we overexpressed NIPP-1 in mammalian cells and examined whether NIPP-1 makes a complex with PP1C in vivo and how specific its binding is towards the three isoforms of PP1C.

MATERIALS AND METHODS

Expression vector for NIPP-1 in mammalian cells

The mammalian expression vector for Flag-tagged NIPP-1 was constructed by ligation of the *Xba*I/*Hind* III fragment of rat NIPP-1 cDNA described elsewhere (Kim et al. 2000) into the *Xba*I/*Hind* III site of pFLAG-CMV-2 (Eastman Kodak, Rochester, NY, USA).

Expression of PP1 isoforms in E. coli and their purification

Plasmids for glutathione S-transferase (GST) fusion proteins for three kinds of PP1C isoforms were constructed. The primers were designed to introduce *Eco*RI/*Bam*HI restriction sites for PP1 α and PP1 γ 1, and *Bam*HI sites for PP1 δ based on the nucleotide sequence reported (Sasaki et al. 1990). Polymerase chain reaction (PCR) was carried out by using PP1 α cDNA (Kitamura et al. 1991) as a template for PP1 α or rat brain cDNA as templates for PP1 γ 1 and PP1 δ . Then the products were digested with the respective restriction enzymes and subcloned into pGEX-2T (Amersham Pharmacia Biotech., Little Chalfont, Buckinghamshire,

England). The nucleotide sequences of these inserts were confirmed to be free from any misincorporation during PCR reactions. The induction and purification of the GST fusion proteins in *E. coli* were performed according to the protocol provided by the manufacture (Amersham Pharmacia Biotech.).

Cell culture and transfection

COS7 cells and 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37°C in a 5% CO₂-95% air atmosphere.

The constructed plasmid was transiently transfected into COS7 cells and 293 cells in 10 cm dishes using a calcium phosphate-mediated transfection procedure with 2 µg/ml DNA/plate. The cells were incubated for 48 hours prior to cell lysis. For preparation of total cell lysates, cells were first washed twice with ice-cold phosphate buffered saline and then transferred to Eppendorf tubes. Then the cells from two dishes were sonicated in 0.5 ml of buffer A (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 10 µg/ml aprotinin) containing protease inhibitors (10 µg/ml leupeptin, 50 µM N-tosyl-L-phenylalanyl chloromethyl ketone, 50 µM N^α-tosyl-L-lysyl chloromethyl ketone, and 0.5 mM phenylmethanesulfonyl fluoride), and followed by centrifugation at 15 000 rpm for 15 minutes. The supernatants were collected as the lysate fraction.

Immunoprecipitation and Western blot analysis

To remove non-specific binding proteins to Protein G, 500 µg protein of the cell lysates were incubated for 1 hour with 30 µl of Protein G-Sepharose and centrifuged. To the supernatants, 100 µl of Protein G-Sepharose coupled to 3 µg of anti-Flag M2 antibody were added and incubated at 4°C overnight. After centrifugation at 16 000 rpm for 15 minutes, the pellets were washed 5 times in buffer B (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 10 µg/ml aprotinin) and subjected to Western blot analysis using anti-Flag antibody or three isoform-specific antibodies (Takizawa et al. 1994).

RESULTS AND DISCUSSION

To examine which isoform of PP1 α , γ 1, and δ can bind NIPP-1 in vivo, Flag- tagged NIPP-1 expression vector was transfected into 293 cells. As shown in Fig. 1A, the exogenously expressed NIPP-1 in the cell lysates was efficiently immunoprecipitated by anti-Flag M2 antibody. Next, each PP1 isoform in the co-immunoprecipitates with the exogenous NIPP-1 was analyzed by using three kinds of antibodies specific to each PP1C isoform. Amounts of the immunoprecipitated PP1C were quantified from the intensities of the bands of PP1C isoforms with each GST-PP1C isoform as a standard. Taking the difference of the molecular weight between 36–38 kDa PP1Cs and 62–64 kDa GST-PP1Cs into consideration, total amounts of PP1 α , γ 1, and δ were estimated to be 3, 6, 4.5 ng/10 µg protein of the cell extracts, respectively, whereas those of PP1 α , γ 1, and δ

Fig. 1. Identification of Flag-tagged NIPP-1 and its binding to PP1 isoforms in 293 cells. The soluble fractions from 293 cell extracts transfected with pCMV-2 or pCMV-2/NIPP-1 were immunoprecipitated using anti-Flag antibody. Ten μ g of the cell lysates (L), and the immune complexes (IP) were subjected to Western blot analysis with anti-Flag antibody (A), and 3 kinds of PP1 isoform-specific antibodies (B, C and D). A: The Flag-tagged NIPP-1 protein was detected by immunoblotting (about 45 kDa). B-D: GST-PP1 α , γ 1, and δ proteins at the indicated concentrations were loaded simultaneously with the immunoprecipitates. About 36–38 kDa of endogenous PP1Cs were detected by Western blot analysis (arrowhead), and the amounts of each PP1C isoform was estimated from the signal-intensities with the corresponding GST-PP1C as the standard.

Fig. 2. Identification of Flag-tagged NIPP-1 and PP1 isoforms bound to that in COS7 cells. Experimental conditions were same as described in the legend to Fig. 1. Except that COS7 cells were used instead of 293 cells.

co-immunoprecipitated with NIPP-1 were estimated to be 0.03, 0.06, and 0.06 ng from 10 μ g protein of the cell extracts (Figs. 1B, C and D). It is difficult to evaluate the amount of PP1C binding to NIPP-1 because only PP1C binding to exogenous NIPP-1 could be detected and the ratio of exogenous over endogenous NIPP-1 depends on the transfection efficiency. However, these results showed that the three PP1C isoforms were immunoprecipitated with similar efficiency, suggesting that the exogenous NIPP-1 bind to three kinds of PP1C with similar affinity in vivo. A reason for the low amounts of PP1C in the co-immunoprecipitation with NIPP-1 is discussed below. To confirm generality for the binding property, similar experiments were carried out using COS7 cells (Fig. 2). The efficiencies of co-immunoprecipitation of the three PP1Cs with NIPP-1 seemed to be similar. Therefore we concluded that NIPP-1 is bound to three isoforms of PP1C with similar affinity in vivo irrespective of cell types employed.

NIPP-1 was originally reported as a protein co-purified with PP1 δ (Jagiello et al. 1995). However, no information was available on specificity of associations between NIPP-1 and three isoforms of PP1C. Here we showed that NIPP-1 could bind not only PP1 δ but also the other two isoforms PP1 α and γ 1 with the similar efficiency. These results suggest a possibility that NIPP-1 binds to the region conserved in PP1C isoforms. Recently, the β 12- β 13 loop in PP1C was

identified as a site essential for associations of toxins and endogenous inhibitors with PP1C (Connor et al. 1999). It is important to analyze whether the associations of NIPP-1 with PP1Cs occur through interactions between the $\beta 12$ - $\beta 13$ loop in PP1C and the typical PP1 binding motif found also in the rat NIPP-1 cDNA (Kim et al. 2000). In these experiments we showed that exogenously expressed NIPP-1 has an ability to associate with PP1C. However it should be pointed out that PP1C molecules mostly exist as several holoenzymes complexed with various regulatory subunits in tissue-, localization- and isoform- specific manners. The low amounts of PP1C isoforms in the co-immunoprecipitations with the exogenous NIPP-1 might be due to this fact. PP1C molecules existing in tissues as various holoenzymes could be attenuated to bind exogenous NIPP-1. Further quantitative analyses are necessary to elucidate physiological significance of NIPP-1.

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