BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 292–296 (1998)
ARTICLE NO. RC989126

The δ Isoform of Protein Phosphatase Type 1 Is Localized in Nucleolus and Dephosphorylates Nucleolar Phosphoproteins

Hirokazu Kotani,* Masaaki Ito,*,[1] Tetsuya Hamaguchi,* Kazuhito Ichikawa,* Takeshi Nakano,* Hiroshi Shima,‡ Minako Nagao,‡ Naoya Ohta,† Yukio Furuichi,† Takao Takahashi,† and Hayato Umekawa†

**First Department of Internal Medicine, Mie University School of Medicine and †Department of Agricultural Chemistry, Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, Japan; and ‡Carcinogenesis Division, National Cancer Center Research Institute, Chuuou-ku, Tokyo 104-0045, Japan*

Received July 3, 1998

The immunolocalization and substrates of protein phosphatases present in nucleolus were investigated using Swiss 3T3 cells and Novikoff hepatoma ascites cells. The protein phosphatase activity was detected in the extract of the isolated nucleoli and its activity was inhibited by okadaic acid with IC50 value of 160 nM. Immunoblotting assay indicated that PP1cδ but not PP1cα, PP1cγ1, and PP2Ac was localized in the isolated nucleoli. Confocal microscopy showed that PP1cδ was localized in nucleoli, nuclei, and cytosol, though the intensity of fluorescence at the nucleoli was stronger than that of the cytosol or nuclei. PP1cδ was co-localized with the major nucleolar phosphoprotein B23 at nucleoli. The phosphatase was capable of dephosphorylating several proteins in the nucleolus, including B23. The Km of PP1 for the recombinant B23.1, phosphorylated by endogenous kinase(s), was 3.5 μM. These results indicate that PP1cδ is the major serine/threonine phosphatase present in nucleolus and it dephosphorylates nucleolar phosphoproteins, including B23.

Protein phosphorylation plays a pivotal role in the execution and regulation of many cellular functions from metabolism to signal transduction, cell division and memory (1). The steady state level of phosphorylation of the proteins would be expected to be the result of the combined effects of the kinase and phosphatase system. The serine/threonine phosphatases are grouped into four categories, termed PP1, PP2A, PP2B and PP2C, based on substrate specificity, sensitivity to inhibitors, subunit structure and cation requirements (1, 2). More recent studies have identified four additional protein phosphatases, such as PP4, PP5, PP6 (1) and PP7 (3). They are distributed into multiple subcellular compartments, and the subcellular localization and functional specificity are thought to be directed by interaction of the catalytic subunit with a family of regulatory proteins termed targeting subunits (2, 4, 5). As for nucleolus, several phosphorylated proteins such as C23 (nucleolin), B23 (nucleophosmin, numatrin, NO38) and UBF (upstream binding factor) have been reported. Most of them are phosphorylated by casein kinase II which is enriched in the nucleolus and thought to play a major role in coordinating the events of ribosome biogenesis (6), although the reverse reaction of protein phosphatase is less characterized than the kinase. Using the isolated nucleoli from Novikoff hepatoma ascites cells and normal rat liver, the previous reports showed the biochemical characteristics of nucleolar protein phosphatase and that the phosphatase was capable of dephosphorylating C23, B23 and histone H1 (7, 8). However, these studies were performed more than twenty years ago, and many new insights of serine/threonine protein phosphatases, including classification, biochemical characteristics, and regulation and function, have since been developed (1, 2, 4). In this study, we examined the type and isoform of nucleolar protein phosphatase by immunoblotting and immunofluorescence microscopy, and investigated the substrates of the nucleolar phosphatase using Swiss 3T3 cells and Novikoff hepatoma ascites cells.

[1] To whom correspondence should be addressed: First Department of Internal Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Fax: 81-59-231-5201; E-mail: m-ito@clin.medic.mie-u.ac.jp.

Abbreviations used: PP1 and PP2A, protein phosphatase type 1 and 2A, respectively; PP1c, catalytic subunit of PP1; PP1cα, δ, and γ1, α, δ, and γ1 isoform of PP1c, respectively; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/98 $25.00

MATERIALS AND METHODS

Materials and chemicals. Polyclonal antibodies were raised in rabbits against the chemically synthetic peptide of PP1cδ (9), γ1 and PP2A (10, 11). The anti-PP1cα was a generous gift from Dr. Villa-Moruzzi (Centro SERRA university of Pisa, Italy) (12). Mouse monoclonal B23 antibody was prepared as described (13). Thiophosphorylated myosin light chain-Sepharose was prepared as described by Mitsui et al. (14). Swiss 3T3 cells were obtained from Dainippon Pharmaceutical Co., Ltd. Chemicals and vendors were as follows: [γ-^{32}P] ATP (DuPont NEN); okadaic acid (Life Technologies, Inc.); peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad); rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG antibody (Chemicon International); recombinant PP1cα (Calbiochem-Novabiochem Corporation); and Heparin Sepharose CL-6B (Amersham Pharmacia Biotech). All other chemicals were of the highest grade commercially available.

Isolation of nucleoli. Isolation of nucleoli from Swiss 3T3 cells was performed as described (15). Novikoff hepatoma ascites cells were grown in male Sprague-Dawley rats, and the cells were harvested 6 days after transplantation. Nucleoli from Novikoff hepatoma ascites cells were prepared by the magnesium-sucrose sonication procedure as described previously (16).

Preparation of substrates for phosphatase. ^{32}P-labeled smooth muscle myosin light chain (P-MLC) was prepared as described (17). Recombinant B23.1 (rB23.1) expressed in *E. coli*, using the pET vector, was purified as described (13). Phosphorylation of rB23.1 by nucleolar protein kinase(s) was carried out at 30°C for 30 min in 30 mM Tris/Cl (pH7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM microcystin-LR and 0.1 mM [γ-^{32}P]ATP (approximately 400 cpm/pmol). The phosphorylated rB23.1 was dialyzed against 30 mM Tris/Cl (pH7.5), 0.1 mM dithiothreitol after incubation at 90°C for 10 min. After dialysis, the phosphorylation level was approximately 1.4 mole of phosphate/mole of rB23.1.

Phosphatase assay. The activities were measured as described (17). The substrates were incubated in 30 mM Tris/Cl (pH 7.5), 30 mM KCl, 1 mM EDTA, 1 mM EGTA with or without okadaic acid. The reactions were started by the addition of substrate and terminated by the addition of trichloroacetic acid to 12%. The precipitated protein was sedimented by centrifugation at 5,000 $\times$ g for 5 min, and the radioactivity of the supernatant was determined by Cerenkov counting. Phosphatase rates were estimated from the linear portions of time courses.

Preparation of nucleolar protein kinase and phosphatase. The isolated nucleoli were suspended with 10 ml of elution buffer (30 mM Tris/Cl (pH7.5), 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM dithiothreitol, 1 mM benzamidine, 0.1 mM leupeptin, 0.1 mM diisopropyl fluorophosphate, 0.1 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride) containing 10 μg/ml RNase and incubated for 30 min at room temperature. The suspension was centrifuged (10,000 $\times$ g) for 10 min, and the supernatant was dialyzed against 30 mM Tris/Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.2 mM dithiothreitol, 1 mM benzamidine and 0.1 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride, representing the nucleolar extract. The extract was applied to a column of Econo-Pac Q Cartridge (Bio-Rad) and the bound proteins were eluted with a 20-ml linear gradient of 25-500 mM NaCl. Each fraction was assayed for kinase activity in a reaction mixture (30 mM Tris/Cl (pH7.5), 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM dithiothreitol, 114 μg/ml rB23.1, 20 μM okadaic acid, 1 mM [γ-^{32}P]ATP) at 30°C for 25 min and terminated by addition of trichloroacetic acid to 5%. The precipitate was collected on a glass fiber filter and the radioactivity of the filter was determined by Cerenkov counting. The peak fractions were used as nucleolar protein kinase(s). The phosphatase activities of each fractions were also assayed as above and the peak fractions were further purified by chromatography using heparin Sepharose followed by thiophosphorylated myosin light chain-Sepharose as described previously (18).

Immunoblotting. We performed immunoblotting as described by Towbin et al. (19). Ten μg of isolated nucleoli was subjected to SDS-PAGE, and the separated proteins were electrophoretically transferred to a nitrocellulose membrane sheet. The sheet was processed to detect the protein phosphatases with the primary antibodies, followed by an incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. The immunocomplex was detected by the ECL detection kit (Amersham Pharmacia Biotech).

Cell culture and immunofluorescence microscopy. Swiss 3T3 cells were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air in DMEM containing 10% fetal calf serum (FCS) (Gibco Laboratories), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}), and used before the 5th week of thawing. For localization of protein phosphatases and B23, an immunofluorescence microscopy was performed as described previously (20).

Others. SDS-polyacrylamide gel electrophoresis in 7.5 to 20% gradient gels was carried out as described previously (21). Protein concentrations were estimated by the binchoninic acid protein assay reagent (Pierece Chemical Co.), using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Biochemical analysis of protein phosphatase in isolated nucleoli. To investigate the biochemical characteristics of nucleolar protein phosphatases, we, first, isolated nucleoli from Swiss 3T3 cells. The purity of nucleolar fraction was checked by electron microscopy and immunoblotting for major nucleolar phosphoprotein B23 with anti-B23 mouse monoclonal antibody. The electron microscopic image resembled to those shown in the previous reports (15, 22) (data not shown). The B23 was detected by immunoblotting in the nucleolar fraction (data not shown). From these results, we considered that the nucleolar fraction contained the isolated nucleoli exactly and the ultrastructual morphology of the nucleoli was almost maintained after isolation. Using the nucleolar extracts from the isolated nucleoli, we examined the activity of phosphatase and its sensitivity to okadaic acid. A relatively higher activity of phosphatase was detected in the extracts and its activity was almost completely inhibited by okadaic acid with an IC50 value (the concentration required for 50% inhibition) of 160 nM (Fig. 1). Similar results were obtained using the nucleoli from Novikoff hepatoma ascites cells (data not shown). This value of IC50 was almost identical to the sensitivity for PP1 (4). The PP2B or PP2C is known to require Ca^{2+}-calmodulin or Mg^{2+} for its phosphatase activity (4). In addition, the nucleolar phosphatase activity was affected by neither Ca^{2+}-calmodulin nor Mg^{2+} (data not shown), suggesting neither PP2B nor PP2C was present in the nucleolus. All these biochemical analysis indicate that the characteristics of protein phosphatase in nucleolus is consistent with those of PP1. We also measured PP1 activities in the isolated nucleoli as compared with those in the total cell lysate of Swiss 3T3 cells. As shown in Table 1, the protein phosphatase activity in nucleolus in the presence of 10 nM okadaic acid, which reflects

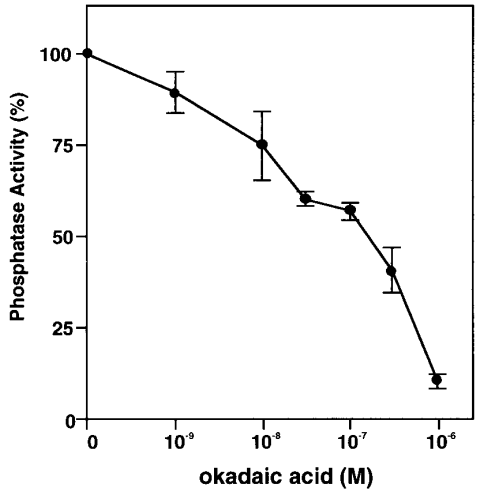

FIG. 1. Inhibition of protein phosphatase activity in isolated nucleoli by okadaic acid. Protein phosphatase in isolated nucleoli was assayed for 5 min with 5 μM ^{32}P-labeled myosin light chain as described in Materials and Methods.

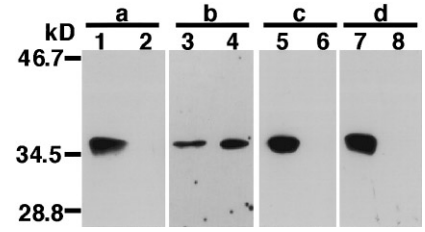

FIG. 2. Immunoblotting analysis of protein phosphatases in the isolated nucleoli from Swiss 3T3 cells. Using anti-PP1cα antibody (a), anti-PP1cδ antibody (b), anti-PP1cγ1 antibody (c) or anti-PP2Ac antibody (d), Western blotting was performed as described in Material and Methods. Lane 1, 100 ng of bacterially expressed, purified PP1cα as the standard; lane 3, 0.5 μg of purified PP1cδ from chicken gizzard as the standard; lane 5 and 7, 10 μg of rat brain homogenate as the standard; lanes 2, 4, 6 and 8, 10 μg of isolated nucleoli from Swiss 3T3 cells.

mainly the activity of PP1, was 1.5 folds higher than that of total cell lysate.

Immunoblotting for protein phosphatases in the isolated nucleoli. The PP1c has four isoforms, α, γ1, γ2 and δ, in mammalian cells (10). PP1cα, γ1 and δ were distributed in various tissues, though PP1cγ2 was only expressed in testis. We examined, by immunoblotting, which isoform(s) of protein phosphatases were detectable in the nucleoli isolated from Swiss 3T3 cells. The immunoblotting analysis showed the presence of PP1cδ in the nucleoli (Fig. 2, lanes 3 and 4), but not PP1cα or PP1cγ1 (Fig. 2, lanes 1, 2, 5-6). PP2A was again undetectable by immunoblot (Fig. 2, lanes 7 and 8). These results suggest that the PP1 is localized in nucleolus and PP1cδ is the major isoform of PP1c present in nucleolus. We obtained the same results of immunoblot using the isolated nucleoli from Novikoff hepatoma ascites cells (data not shown). The identification of the types of protein phosphatases in nucleolus was not established in the previous reports (7, 8). It is possible that their prepared nucleolar phosphatase is PP1, though we cannot make it clear at present because their results have only limited information to classify the phosphatase.

TABLE 1

PP1 Activity in Total Cell Lysate and Nucleolar Fraction from Swiss 3T3 Cells

	PP1 activity
Total cell lysate	85 ± 0.23 nmol/min/mg
Nucleolar fraction	120 ± 8.6 nmol/min/mg

Note. Protein phosphatases were assayed with 10 nM okadaic acid. Mean values ± SD.

Subcellular localization of PP1cδ in Swiss 3T3 cells. To confirm that PP1cδ is localized in nucleoli, we analyzed the subcellular localization of PP1c by immunofluorescence microscopy with anti-PP1cδ rabbit polyclonal antibody. Confocal microscopic images showed that PP1cδ was localized at cytoplasm, nuclei and nucleoli in Swiss 3T3 cells (Fig. 3, a). The intensity of fluorescence of PP1cδ at the nucleoli was stronger than that of cytoplasm or nuclei. The localization of other protein phosphatases, namely PP1cα, PP1cγ1 and PP2Ac, in nucleoli was not observed in Swiss 3T3 cells (data not shown). We compared the subcellular localization of PP1cδ with that of B23. B23 was co-localized with PP1cδ at nucleolus (Fig. 3, a and b). The same results of immunofluorescence microscopy were obtained using other cell lines, e.g., NIH 3T3 cells and MDCK cells (data not shown). Murata et al. (23) examined the subcellular localization of PP1cδ in rat aorta smooth muscle cells by immunofluorescence microscopy with anti-PP1cδ rabbit polyclonal antibody which showed that PP1cδ was localized at nucleus and the periphery of the cell, but the staining of PP1cδ in nucleoli was not pointed out. In another previous report, the immunolocalization of PP1c through the cell-cycle of mammalian fibroblasts (REF-52 cells) was examined (24). It showed that PP1c was mainly localized in the cytoplasm to G1 and S phase cells, accumulated in the nucleus during G2 phase and intensely co-localized with individual chromosomes at mitosis. However, it did not mention about the nucleolar localization of PP1c during the mammalian cell cycle. As to the localization of PP1c at cytoplasm and nucleus, it is consistent with our results, but there are discrepancies between these and our results with regard to the localization of PP1c in nucleolus. The reason for these discrepancies is not known but may be due to the differ-

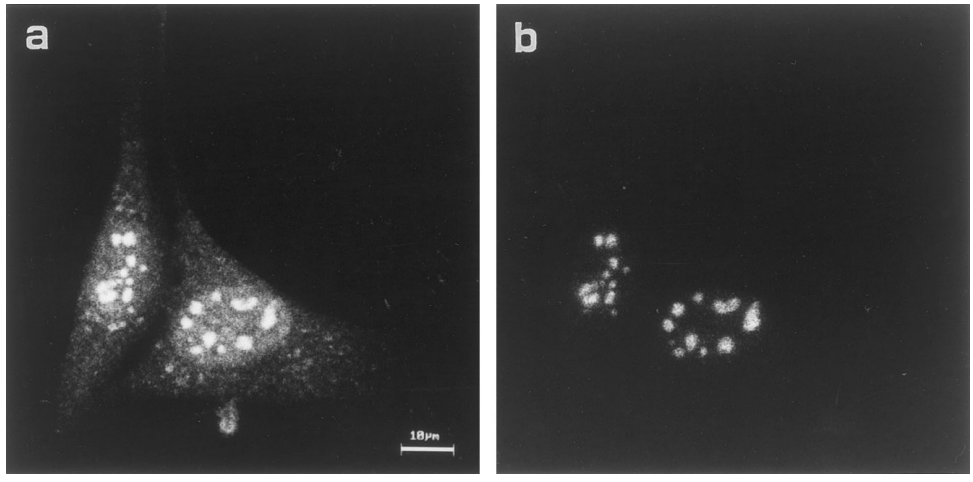

FIG. 3. Localization of PP1cδ in Swiss 3T3 cells. Swiss 3T3 cells were double-stained with anti-PP1cδ antibody (a) or anti-B23 antibody (b) and analyzed by confocal microscopy as described in Material and Methods.

ence in the cell types and/or the difference in the antibodies.

Dephosphorylation of nucleolar proteins. The localization of PP1cδ in nucleolus suggests a possible function of PP1 to dephosphorylate nucleolar phosphoproteins. To investigate whether the dephosphorylation of nucleolar proteins is catalized by PP1, the nucleolar proteins from Novikoff hepatoma ascites cells were incubated in the presence of MgATP and examined the sensitivity on the phosphorylation of the nucleolar proteins to okadaic acid. In the presence of MgATP, several nucleolar proteins were phosphorylated by endogenous kinase. As shown in Fig. 4 (lane 1), the molecular masses of these major phosphoproteins on SDS-PAGE were 95, 38, 34 and 19 kD. The labeling intensities of these bands increased according to the increase of okadaic acid concentrations (Fig. 4, lanes 1-7). We confirmed that 38 kD band was B23 by Western blotting with anti-B23 antibody (data not shown). A 50% inhibition of phosphorylation of B23 was achieved at about 100-300 nM okadaic acid, when the intensity at 10,000 nM okadaic acid was taken as 100% activity. This sensitivity to okadaic acid corresponded to that of PP1 (4). This result suggests that the phosphorylation level of B23 may be regulated by intrinsic PP1 in nucleolus. The 95 and 19 kD proteins seemed to be C23 and histone H1, respectively, though the 34 kD band was not identified at present. The labeling intensities of these proteins were also affected by okadaic acid, though the effect of okadaic acid on the phosphorylation of 95 kD protein was not observed dramatically like that on B23, 34 kD and 19 kD proteins. The reason for the less-sensitivity of okadaic acid on 95 kD protein is not known but it may be due to their phosphatase and/or targeting subunits being lost while the procedure for preparation of the nucleolar proteins was performed. These results are consistent with the previous report showing that the crude extract of nucleolar protein phosphatase from Novikoff hepatoma ascites cells was capable of dephosphorylating the major phosphorylated nucleolar proteins, namely B23, C23 and histone H1 (8).

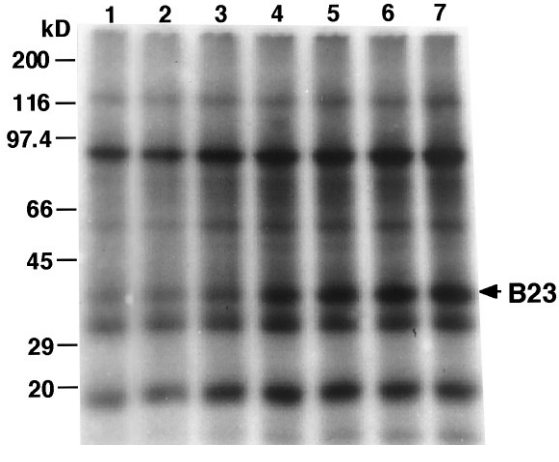

FIG. 4. Effect of okadaic acid on the dephosphorylation of nucleolar proteins from Novikoff hepatoma ascites cells. Lanes 1–7, autoradiograms from phosphorylation of nucleolar extracts with various concentrations of okadaic acid corresponding to 0, 1, 10, 100, 300, 1,000, 10,000 nM, respectively. The phosphorylation reaction was carried out at 30°C for 20 min in 30 mM Tris/Cl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM dithiothreitol and 50 μM [γ-^{32}P] ATP with the indicated concentrations of okadaic acid. The arrow indicates the position of B23.

To confirm the dephosphorylation of B23 by PP1, we examined whether rB23.1, phosphorylated by endogenous kinase(s) in nucleoli, was dephosphorylated by PP1, separated from the nucleoli from Novikoff hepatoma ascites cells. As shown in Fig. 5, the nucleolar phosphatase dephosphorylated the phosphorylated rB23.1 completely and the Km value for ^{32}P-labeled rB23.1 substrates was about 3.5 μM (Fig. 5).

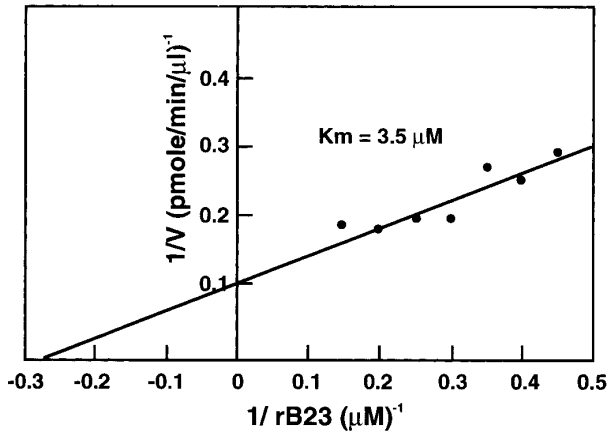

FIG. 5. Lineweaver-Bürk plots of nucleolar phosphatase toward phosphorylated rB23.1. PP1 activity was measured using 0.5 μg of nucleolar phosphatase with the various concentrations of phosphorylated rB23.1.

We showed here that the δ isoform of PP1 is localized in nucleolus and B23 is a candidate for one of the major substrates of PP1, using Swiss 3T3 cells and Novikoff hepatoma ascites cells. The PP1 consists of multimeric structures composed of a catalytic subunit complexed to a number of accessory subunits, termed targeting subunits (2, 4). Some nuclear targeting subunits have been identified, such as nuclear inhibitor of PP1 (NIPP1), (25), 111 kD protein (26), p99 (27) and phosphatase 1 nuclear targeting subunits (PNUTS), (28). It is possible that the nucleolar phosphatase has a targeting subunit (s) which determines the nucleolar localization and substrate specificity. In fact, two peaks of phosphatase activity with apparent molecular masses of about 66 kD and 38 kD were obtained by gel filtration of the nucleolar phosphatase on Superdex 200 (data not shown). In both peaks, the presence of PP1cδ was detected by Western blotting. The first peak may represent a heterodimer of catalytic subunit and targeting subunits or binding proteins. Studies are underway to define the structure of these subunits.

ACKNOWLEDGMENTS

We thank Dr. Villa-Moruzzi (Centro SERRA university of Pisa, Italy) for providing the anti-PP1cα antibody and Dr Jianhua Feng, Masaki Fujioka, Kazushi Sugimoto in our laboratory for their expert technical assistance. We thank Dr. Satoru Ogawa (Electron microscope laboratory, Mie university, Mie, Japan) for electron microscopy. This investigation was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture (to M.I.), Japan, by grants from Naito Foundation (to M.I.) and Kanae Foundation of Research for New Medicine (to K.I.).

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