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Subcellular Localization of Protein Phosphatase Type 1 Isotypes in Mouse Osteoblastic Cells

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The cytolocalization of protein phosphatase type 1 catalytic subunits in exponentially growing mouse osteoblastic MC3T3-E1 cells was determined. Formaldehyde-fixed and alcohol-permeabilized cultured cells were reacted with the PP1α, PP1δ, PP1γ1, and PP1γ2 antibodies using immunohistochemical methods. With PP1α antibody intense staining occurred in the nuclei, while with PP1δ antibody nucleolus-like bodies were intensely stained. PP1γ1 localized in the perinuclear region and in the nucleus of the cultured cells, with the staining reaction of the former being much stronger than that in the latter. An immunoreaction did not occur in the cells interacted with PP1γ2 antibody or with the normal rabbit serum. Proteins were prepared from the exponentially growing cells and subconfluent cells. Cellular fractionation was also done with the exponentially growing cells and proteins were prepared from each fraction. Each protein preparation was subjected to SDS-PAGE followed by Western blot analysis with the antibodies. PP1α recognized the 38 kDa proteins mainly present in the nucleus, whereas PP1δ interacted with the proteins in the nucleolar fraction whose molecular weight was estimated as 37 kDa. PP1γ1 antibody recognized a band corresponding to an estimated molecular weight of 36 kDa mainly in the cytosolic fraction. PP1γ2 antibody and the normal rabbit serum did not interact with any proteins prepared from the cultured cells. Our observations show that four different isozymes of protein phosphatases occupy distinct compartments in MC3T3-E1 cells. This differential distribution suggests that these isozymes may play different roles in cellular functions.

Protein phosphorylation and dephosphorylation has been recognized as a key mechanism in the regulation of cellular metabolism and functions in various tissues (1, 2). Four major types of protein serine/threonine phosphatase (PP) catalytic subunit have been identified in eukaryotic cells by biochemical techniques and termed PP1, PP2A, PP2B, and PP2C (2, 3). The cDNAs encoding the catalytic subunit of PP1 were isolated from yeast, rabbit, and mouse (4). In rat, cDNA cloning revealed the existence of at least four isoforms of PP1 catalytic subunit termed PP1α, PP1γ1, PP1γ2, and PP1δ (5). Marked elevations of mRNA and activity of PP1 in nuclei are observed after partial hepatectomy (6). PP1 is required for rat fibroblasts to exit from mitosis (7). It also is evident that some isoform of PP1 is involved in carcinogenesis (8). These findings suggest that PP1s play important roles in cell growth and differentiation.

Antisera were developed in rabbits against the oligopeptides from the C-termini of the four types of PP1 catalytic subunit. We previously characterized the specificity of these antisera, and the expression of PP1s was investigated at the mRNA and protein levels in tissues and organs (9). Immunocytochemistry also was carried out in rat cerebellum and salivary glands (10, 11) and testis (12) with PP1γ1 and PP1γ2, respectively. However, the subcellular distribution of each PP1 isotype in mammalian cells are still unclear. In the present study, the antibodies were used to detect and localize proteins interacting with each antibody in cultured mouse osteoblastic MC3T3-E1 cells (13).

MATERIALS AND METHODS

Antisera. The antisera used were those described previously (9). Briefly, antibodies were raised against oligopeptides corresponding to the predicted amino acid sequence at the carboxy terminal domains (CNPGGRPITPPRNSAKA) for PP1α, (CTPPRTANPPKKR) for PP1δ, (CTPPRTGMITQAKK) for PP1γ1, and (CGLNPSIQKASNYRNNTVLYE) for PP1γ2. A cysteine residue was incorporated in each peptide for configuration.

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Cell culture and cellular fractionation. Mouse osteoblastic MC3T3-E1 cells were provided by Dr. Masayoshi Kumegawa. The cells were grown in plastic dishes containing the α modification of Eagle's Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were subcultured every 5 days using 0.25% trypsin together with 1 mM EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS). For cellular fractionation, MC3T3-E1 cells growing in 90 mm plastic dishes were washed twice with PBS, scraped into PBS, pelleted at 3,000 g, and resuspended in hypotonic buffer (20 mM Hepes, pH 7.2, 10 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 0.5 mM EDTA). Cells were allowed to swell for 10 min on ice before lysis by addition of 0.1% NP-40 and 100 mM potassium acetate. After 5 min in ice and vortexing, nuclei were pelleted by centrifugation for 10 min at 8,000 g, resuspended in lysate buffer containing 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 mM EGTA in PBS, and termed the nuclear fraction, whereas the supernatant was termed the soluble cytosolic fraction. The nucleolar fraction was prepared from purified nuclei according to the method of Muramatsu et al. (14). For immunocytochemistry, the cells were plated on 18-mm round coverslips in 60-mm plastic dishes and cultured for the indicated periods.

Immunocytochemistry. After cultivation for varying periods, the coverslips were washed three times with PBS and fixed with 4% formaldehyde for 10 min at ambient temperature followed by methanol-permeabilization for an additional 10 min at −20°C. After washing three times with cold PBS the coverslips were incubated with each antiserum diluted 1:50 to 1:800 in PBS containing 2% BSA for 45 min at ambient temperature. As a negative control, the same dilution of the normal rabbit serum was used. After three washes with 0.1% BSA in PBS containing 0.05% Tween-20 over a 15 min period at ambient temperature, the coverslips were incubated with a 1:100 dilution of FITC-conjugated goat anti-rabbit IgG (Cappel) in 2% BSA in PBS for another 45 min at ambient temperature. The coverslips were washed as described above and mounted while wet with PermaFluor aqueous mounting medium (Lipshaw, Pittsburgh, PA. USA). After the immunocytochemical staining the slides were examined under an Olympus BX50 microscope equipped with epi-fluorescence illumination (BX-FLA) and for photomicroscopy (PM-30). Immunofluorescent microphotographs were taken on Fuji Prest 400 film using the automatic exposure.

SDS-PAGE and immunoblot of proteins. After appropriate periods of cultivation, cells were washed twice with PBS and scraped into lysate buffer. The cells were sonicated for 10 second with a sonifier cell disrupter. The sonicates were centrifuged for 10 min at 15,000 rpm and the supernatants were analyzed by SDS-PAGE and immunoblot. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the basic procedure of Laemmli (15). The proteins were denatured in sample buffer and the mixtures were heated in boiling water for 3 min. Proteins and prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD, USA) were separated by SDS-PAGE and transferred electrophoretically from the gels to the polyvinylidene difluoride (PVDF) transfer membranes (Immobilon, Millipore, Bedford, MA, USA) according to the basic procedure of Towbin et al. (16). The membranes were placed in a blocking solution containing 4% BSA and 4% normal goat serum in PBS and incubated for 2 h at ambient temperature. The membranes were rinsed briefly in PBS and incubated overnight in the blocking solution containing the 1:200 diluted antisera at 4°C. The membranes were washed four times within 30 min in PBS containing 0.1% BSA and 0.05% Tween-20 using a rotary shaker at ambient temperature. The washed sheets were incubated in the blocking solution containing peroxidase-conjugated affinity purified $F(ab')_2$ fragment of goat anti-rabbit IgG (Cappel, diluted 1:200) for 2 h at ambient temperature. The membranes were washed as described above and were placed in solution containing 4-chloro-1-naphthol and hydrogen peroxide according to the method of Hawkes et al. (17). Positive bands were visualized as blue-purple reaction against a white background.

RESULTS

Immunolocalization of PP1 Isotypes in MC3T3-E1 Cells

To examine the cytolocalization of PP 1 isozymes in mouse osteoblastic cells, exponentially growing MC3T3-E1 cells were fixed, permeabilized, and stained with the rabbit polyclonal anti-peptide antibodies against the catalytic subunits of PP1α, PP1δ, PP1γ1, and PP1γ2. The immunoreactivity of each isoform showed quite different cellular distributions. Although a weak staining reaction was observed in cytoplasm, the distribution of PP1α was mainly in nucleus (Fig. 1A). Intense staining occurred in the nucleus with the PP1δ antibody. PP1δ was specifically stained as dots in the nucleus that may represent nucleoli (Fig. 1B). PP1γ1 was localized in both the nucleus and the cytoplasm. In the nucleus, the staining pattern is similar to that of the PP1δ. The punctated distribution of PP1γ1 in cytoplasm was especially distinct in the perinuclear region. The staining reaction in the cytoplasm was much stronger than that that occurred in the nucleus (Fig. 1C). The immunoreaction was not observed with PP1γ2 antibody (Fig. 1D) and the normal rabbit serum (data not shown). That the distribution of PP1s is not limited to MC3T3-E1 cells was confirmed by the fact that it had an identical distribution in 3T3-L1 cells (data not shown).

Detection of PP1 Isozymes in Subcellular Fractions with Western Blotting

Twenty μg of proteins obtained from the exponentially growing MC3T3-E1 cells and cells at the subconfluent stage were subjected to SDS-PAGE and followed by immunoblotting. Figure 2 shows the reaction between the PP1 antibodies (PP1α, PP1δ, and PP1γ1) and whole cell lysate obtained from the cultured cells. The antibodies reacted with a major band corresponding to estimated molecular weights of 38 kDa and 37 kDa for PP1α and PP1δ, respectively, present in the whole cell lysates. PP1γ1 recognized a band corresponding to 36 kDa proteins. The intensity of immunoreaction was not different between the proteins prepared from the cultured cells at the different stages. PP1γ2 and the normal rabbit serum did not recognize any proteins present in the whole cell lysate (data not shown). The immunoreactivity was eliminated by preincubating the antibodies with the peptides used as the antigens.

Because each protein phosphatase isozyme showed specific localization in cultured cells by immunocytochemistry and because the immunoreactive proteins were detected in the whole cell lysates, we determined the subcellular localization of PP1 proteins by Western

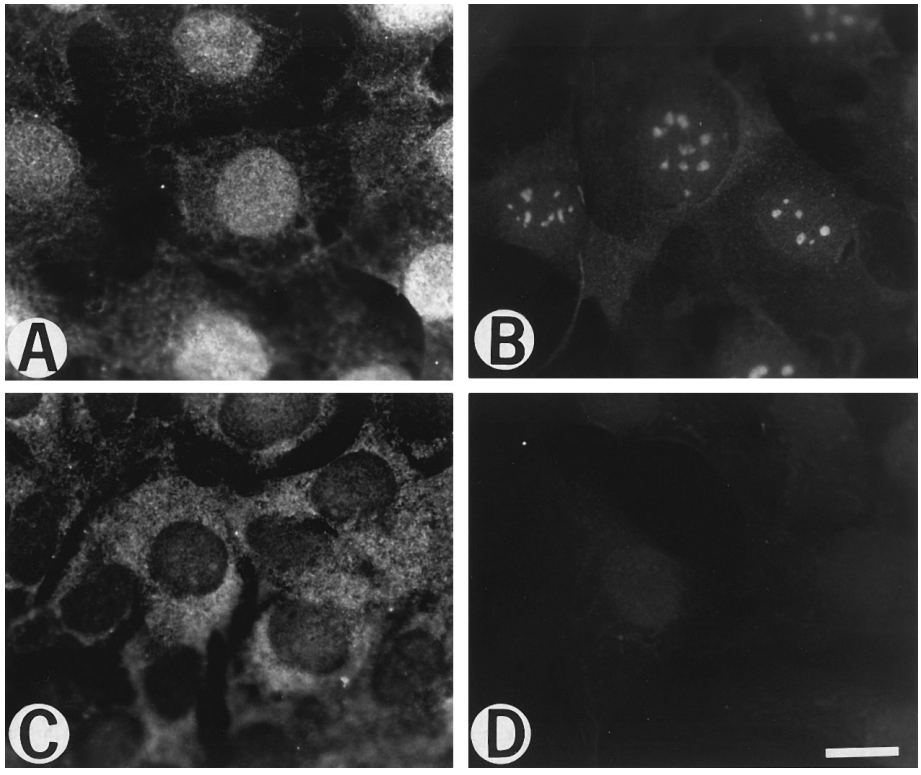

FIG. 1. Immunocytochemical identification of PP1 isotypes in cultured MC3T3-E1 cells. The staining method is described in the text. (A) Immunocytochemical staining of PP1α in MC3T3-E1 cells. Intense staining is present in the entire nucleus. (B) Immunocytochemical staining of PP1δ in MC3T3-E1 cells. Dot-like intense staining present in the nucleus appears to be associated with nuclei. (C) Immunocytochemical staining of PP1γ1 in MC3T3-E1 cells. Intense staining is present in the perinuclear region, whereas weak staining is observed in the nucleus. (D) Immunocytochemical staining of PP1γ2 in MC3T3-E1 cells. No staining occurred in the cells. Bar represents 10 m.

blot analysis. Figure 3 shows that the PP1α antibody interacted with a major band corresponding to the 38 kDa proteins present in the nuclear and nucleolar fractions; however, very little of the immunoreactive proteins were detected in the cytosolic fraction. PP1δ recognized the 37 kDa proteins present in the nuclear and nucleolar fractions, with the reaction in the latter being much stronger. Although weak staining occurred in nucleolar fraction PP1γ1 interacted with the 36 kDa pro-

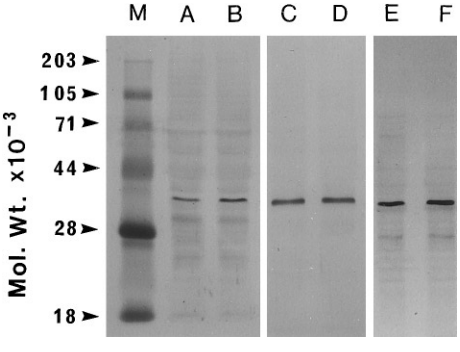

FIG. 2. Identification of PP1 isotypes in cultured MC3T3-E1 cells. Whole cell lysates were prepared from the exponentially growing cells (A, C, and E) and subconfluent cells (B, D, and F). Twenty μg of both preparations were separated on 12.5% SDS-PAGE, transferred to Immobilon membranes, and incubated with each antibody. A and B, PP1α; C and D, PP1δ; E and F, PP1γ1. Molecular weight markers are indicated (M).

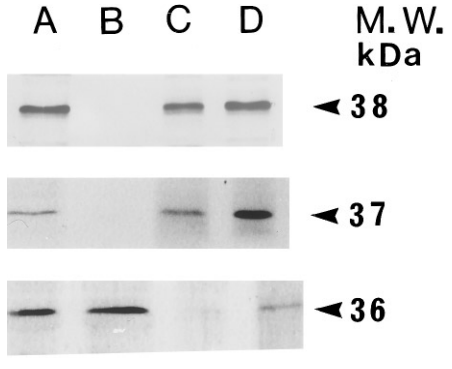

FIG. 3. Identification of PP1 isotypes in cellular fractionation in exponentially growing MC3T3-E1 cells. The cultured cells were fractionated as described under Materials and Methods and proteins obtained from each fraction. Twenty μg of each fraction were separated on 12.5% SDS-PAGE, transferred to Immobilon membranes, and incubated with each antibody. A, whole cell lysate; B, cytosolic fraction; C, nuclear fraction; and D, nucleolar fraction. Upper panel, PP1α; middle panel, PP1δ; and bottom panel, PP1γ1.

teins present mainly in the cytosolic fraction. PP1γ2 and the normal rabbit serum did not interact with the proteins prepared from the cultured cells (data not shown).

DISCUSSION

MC3T3-E1 cells derived from newborn mouse calvaria are widely used as an osteoblastic model because they retain many osteoblastic properties (13, 18-20). In the present study, we examined the cytolocalization of PP1 isozymes in cultured MC3T3-E1 cells by immunofluorescent techniques. The subcellular distribution of PP1s also was carried out by Western blot analysis of proteins obtained from cellular fractionations. The present results revealed that PP1s are differentially localized in the cultured cells. PP1α and PP1δ antibodies recognized proteins with estimated molecular weight of 38 kDa and 37 kDa, respectively, present in the nuclear regions of cultured MC3T3-E1 cells, whereas PP1γ1 interacted with the 36 kDa proteins in cytoplasm. The estimated molecular weight of the interacted proteins is in good agreement with that calculated from the amino acid sequences (5, 9, 21). It is quite interesting that PP1δ was specifically distributed in the nucleolus-like bodies of cultured MC3T3-E1 cells. This finding was confirmed by the fact that the PP1δ antibody recognized the 37 kDa proteins present preferentially in nucleolar fraction. In our preliminary results, the dot-like bodies disappeared from nucleus when the cells were synchronized at the G1/S phase with a thymidine block (unpublished data). The 38 kDa immunoreactive PP1α isotype probably present in both nucleoplasm and nucleolus because immunocytochemistry demonstrated in Figure 1 shows that PP1α localized entire nucleus including nucleolus. PP1γ2 did not stain any proteins in MC3T3-E1 cells, probably because this isozyme is testis specific (12).

Our present results suggest that preferential sites for enzymatic actions of PP1s are compartmentalized either in the cytoplasm or in the nucleus. The punctate appearance of PP1γ1 suggests that PP1γ1 may be associated with cytoplasmic organelles. The specific localization of PP1α and PP1δ in the nuclei indicates that these are the sites where these proteins function. The significance of the PP1δ localization in nucleolus should be elucidated. The noncatalytic subunits of PP1s have a targeting function, enabling the phosphatases to associate with a particular cellular fraction. This targeting role explains the broad subcellular distribution of PP1γ1 noted in the present study. However, the highest concentration of PP1 is found in the nucleus (PP-IN), where it is in both the nucleoplasm and associated with heterochromatin (22). PP1 activity increased in the nuclei of hepatocyte primary cultures stimulated by EGF, whereas no isoforms showed any changes in concentration under these conditions (23). PP1α and PP1γ1 activity and immunoreactivity in the nuclear and membrane fractions were increased in the 1, 25 $(OH)_2$ D_3-stimulated HL60 cells, whereas those in cytosolic fraction were decreased (24).

The processes regulating nuclear location of PP1s are quite intriguing. It should be noted that PP1s do not exhibit any evidence of a nuclear localization signal in their sequences (5, 9, 21). However, proteins the size of catalytic subunit of PP1s (36-38 kDa) have been shown to freely diffuse into the nucleus without the necessity for specific translocation sequences. Once in the nuclei, they bind to an immobile component to maintain their nuclear localization. PP1δ is reported to be associated with Rb proteins and responsible phosphatase activity has been detected in the Rb-PP1δ complexes (25). This finding indicates that PP1δ may be the enzyme which dephosphorylates Rb proteins at the exit from mitosis (26, 27). The relocation mechanism might involve the reversible phosphorylation of the PP1 catalytic subunits or specific binding subunits in the nucleus utilizing cell cycle-dependent kinase activity. It was recently reported that PP1α and PP1γ1 were phosphorylated in vivo by cdc2 kinase (28).

Our present study shows that different isozymes of PP1s occupy distinct compartments in cultured cells. This differential distribution suggests that these isozymes may act in a sequential fashion on modulating the activity of this enzyme in mammalian cells.

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