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Alterations in Type-1 Serine/Threonine Protein Phosphatase PP1α in Response to B-Cell Receptor Stimulation[1]

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In response to stimulation of B-cells through cell surface IgM, the activity of the serine/threonine protein phosphatase PP1, but not PP2A, was transiently decreased and reached a minimum 10-20 min after the stimulation. The decrease was more profound in the immature B-cell line WEHI-231, than in the mature B-cell line BAL-17. Under these conditions, PP1α, an isoform of PP1, showed unique alterations in the patterns of several spots with distinct isoelectic points in the Western blot after two-dimensional electrophoresis, whereas another isoform, PP1δ, did not show any alteration. PP1γ1 and PP1γ2 were not detected in B-cells. Similar alterations in these spots were observed in B-cells stimulated by PMA. When partially purified PP1 consisting of PP1α and PP1δ was incubated with [γ-^{32}P]ATP and PKC, radioactive spots of PP1α could be detected, but no spot of PP1δ was detected. Because differences in sequence among PP1 isoforms are mostly restricted to their C-terminals, phosphorylation rates of the C-terminal peptides containing the PKC-phosphorylation motif were compared. The C-terminal peptide of PP1α is a better substrate for PKC than those of PP1γ1 and PP1γ2, and is phosphorylated at the serine residue corresponding to Ser-325 of PP1α. The corresponding C-terminal region of PP1δ does not contain the phosphorylation site. On the other hand, there was a large difference in subcellular distribution of PP1δ, but not PP1α, between immature and mature B-cells. From these results, it was strongly suggested that PP1α is involved, *via* phosphorylation by PKC, in the regulation of signal transduction in response to the stimulation of B-cells through cell surface IgM.

Key words: B-cell, B-cell receptor, PKC, PP1, protein phosphatase.

Signal transduction in B-cells is controlled by reversible phosphorylation of target molecules. B-cells show different responses at different maturational stages. Mature B220$^+$sIgM$^+$/sIgD$^+$ B-cells respond to antigenic stimuli positively by proliferation and differentiation, whereas immature B220$^+$sIgM$^+$/sIgD$^-$ B-cells respond negatively by apoptosis (*1-3*). Previous studies have demonstrated a variety of differences between immature B-cells and mature B-cells in various biochemical events including induction of *egar-1* and *c-fos* (*4*) genes, phosphorylation of PLC-γ1 (*5*), and expression of Fyn and Lck (*6*). There is accumulating evidence suggesting the involvement of serine/threonine protein phosphatases PP1 and PP2A in regulation of signal transduction of B-cells. Metcalfe and Milner reported that okadaic acid (OA), an inhibitor of protein phosphatases PP1 and PP2A, stimulated mitogenesis at early G1 of B-cells, but inhibited it at late G1 (*7*). OA activated MAP kinase in B-cells, but inhibited cellular proliferation (*8*). Xia *et al.* reported an increase in production of TNF in B-cells stimulated by OA (*9*). It is also reported that RAG-1 and RAG-2 gene expression and V(D)J recombinase activity in B-cells are enhanced by inhibition of PP1 and PP2A (*10*). These results suggest potential roles of serine/threonine protein phosphatases in immune response and gene expression of B-cells, but biochemical analyses of these protein phosphatases in B-cells have not been reported.

In this study, we investigated the responses of type-1 protein phosphatase (PP1) to stimulation through cell surface IgM (sIgM) of B-cells. PP1, one of the four major serine/threonine protein phosphatases, is widely distributed in the cells and associates with various regulatory proteins, including inhibitor-1 and inhibitor-2 in cytosol, NIPP-1 in the nucleus, G component in glycogen particles, and M in myofibrils (*11, 12*). PP1 has at least four isoforms, termed PP1α, PP1γ1, PP1γ2, and PP1δ (also called PP1β) (*13*). These isoforms show characteristic expression pat-

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Abbreviations: OA, okadaic acid; PP1, protein phosphatase 1; sIgM, cell surface IgM; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride.

terns in mammalian tissues, but their specific functions are not known (*14, 15*).

We have reported a selective elevation in the amount of PP1α in rat ascites hepatomas, suggesting roles of PP1α in regulating malignant phenotype and/or high proliferation rate (*16-18*). Also, we have reported that the amount of PP1α progressively decreased in livers of non-obese diabetic mice (IDDM model mice) as a function of increasing concentration of blood glucose, whereas the amounts of PP1γ1 and PP1δ were unchanged (*15*). These results suggest different roles for the different isoforms of PP1 and also the possible existence of characteristic regulatory mechanism(s) for each isoform.

Protein kinase C (PKC), a phospholipid-dependent serine/threonine kinase, plays key roles in cell proliferation, metabolism, and signal transduction of B-cells (*19*). Although PKC and PP1 may counteract each other in various cellular events, functional interactions between them remain uncertain.

The isoforms of PP1 have high sequence homology and migrate as single protein bands with similar mobilities on SDS-gel electrophoresis. Since their sequence differences are mainly in the C-terminal regions, only antibodies raised against peptides derived from these regions are isoform-specific. The C-terminal region also contains sites which can be phosphorylated by Cdc2-Cyclin B (*20*), v-Src (*21, 22*), and v-Abl (*23*).

Here, we show that sIgM cross-linking down-regulated PP1 activity rapidly and transiently in both mature and immature B cell lines, and that the phosphorylation of PP1α increases in response to sIgM or PMA stimulation. We also report that the catalytic subunit of PP1α, not PP1δ, is a possible substrate for PKC of B-cells, and that the C-terminal peptide of PP1α is a much better substrate for PKC than the C-terminal peptides of PP1γ1 and PP1γ2. We also show a large difference in subcellular distribution of PP1δ between immature B-cells and mature B-cells.

MATERIALS AND METHODS

Materials—PKC was prepared from rabbit brain according to Inagaki *et al.* (*24, 25*). Rabbit polyclonal anti-PP1 antibodies were prepared against the C-terminal peptides, RPITPPRNSAKAKK of PP1α, PPRGMITKQAKK of PP1γ1, QKASNYRNNTVLYE of PP1γ2, and SEKKAKYQYGGLNSG of PP1δ, as described (*17*). These antibodies were specific enough to allow determination of each isoform of PP1 (*15, 17*). Affinity-purified goat F(ab')$_2$ anti-mouse IgM (μ-chain specific) was purchased from Organon Teknika N.V. Cappel Products (West Chester, PA), and goat F(ab')$_2$ was from O.E.M. Concepts (Toms River, NJ). Phorbol 12-myristate 13-acetate (PMA) was from Wako Pure Chemical Industries (Osaka). Protein kinase C inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), was from Seikagaku (Tokyo).

Cell Culture—The murine immature B cell line WEHI-231 (*26*) and the mature B cell line BAL-17 (*27*) were obtained from Dr. H. Yakura (Tokyo Metropolitan Institute for Neuroscience). The cells were maintained in RPMI 1640 medium (GIBCO-BRL), pH 7.2, supplemented with 2 mg/ml $NaHCO_3$, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (2-ME), at 37°C in an atmosphere containing 5% CO_2.

Preparation of Cell Extracts from Stimulated B Cells—Cells were suspended at 1×10^7 cells/ml in the culture medium, and then stimulated with 10 μg/ml goat F(ab')$_2$ anti-mouse IgM or 50 ng/ml PMA. After appropriate times, the cells were collected by gentle centrifugation, washed with 150 mM ice-cold NaCl, and extracted with extraction buffer A (200 μl) containing 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 0.1 mM PMSF, 0.1 mM TPCK, 0.5 mM benzamidine, 1 μM leupeptin, 1 mM Na_3VO_4, and 1.0% Nonidet P-40 (NP-40). For Western blot analysis the extraction was carried out with the buffer A containing 20 mM NaF. The lysates were centrifuged at $8,000\times g$ for 20 min at 4°C and the resulting supernatants were used as extracts.

Fractionation of the Cell Extracts—The cells in 0.5 ml of extraction buffer A without NP-40 and sucrose were disrupted by passage through a narrow-gauge needle. The extracts were centrifuged at $800\times g$ for 10 min, and the pellets were disrupted again under similar conditions and centrifuged. The resulting supernatants were combined and centrifuged at $100,000\times g$ for 45 min at 4°C. The supernatants were used as cytosolic fraction. The pellets were solubilized with buffer A without sucrose for 30 min at 4°C, and then centrifuged at $12,000\times g$ for 10 min. The resulting supernatants were used as membrane fraction (*28*).

Preparation of ^{32}P-Labeled Proteins and Protein Phosphatase Assay—^{32}P-labeled phosphorylase *a* was prepared by using phosphorylase kinase (*29*). The activities of PP1 and PP2A were measured as previously described (*28*). One unit (U) of activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of phosphate per min.

Purification of PP1 from Muscle Glycogen Particle—Female New Zealand white rabbits were killed by intravenous injection of sodium pentobarbital (0.5 g/rabbit), and the muscle from the hind limbs and back of three animals (1,800 g) was rapidly excised and placed on ice. All subsequent procedures were carried out at 0-4°C. The muscle was minced and homogenized in 2.5 volumes of 2 mM EGTA, 2 mM EDTA, 0.1 mM PMSF, 0.1% 2-ME, pH 8.3, with a Waring Blender (25 s at the low speed setting). The homogenate was centrifuged at $4,200\times g$ for 35 min and the supernatant was decanted through glass wool and adjusted to pH 5.6 to precipitate the glycogen-protein particles. PP1 was solubilized by amylase treatment of the glycogen-protein particles and purified by four sequential column chromatographies with DEAE-cellulose, poly-Lys-Sepharose, aminohexyl-Sepharose, and heparin-Sepharose as described previously (*30, 31*). The PP1 isoforms were determined by using PP1 antibodies as described below.

Phosphorylation of PP1 by Protein Kinase C—The PP1 (5 μg) purified as described above was incubated at 30°C for 2 h in a reaction mixture (50 μl) containing 100 μM [γ-^{32}P]ATP (4,000-5,000 cpm/pmol), 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM NaF, 1 μg/ml 1,2-dioctanoyl-*sn*-glycerol (Sigma Chemical, St. Louis, MO), and 50 μg/ml phosphatidylserine (Funakoshi, Tokyo), in the presence or absence of 5 μg/ml PKC. The reaction was stopped by adding urea until saturation.

Two-Dimensional Gel Electrophoresis—Isoelectric focusing (IEF) and SDS-PAGE were performed according to

the O'Farrell method (*32*). Briefly, IEF solution consisting of 4% acrylamide, 0.2% bisacrylamide, 9 M urea, 2% NP-40, and 2% ampholine (pH 3.5–10) was used to pour 16-cm-long, 3.0-mm diameter tubes. Samples were mixed with 2 volumes of IEF sample buffer [6 M urea, 3% NP-40, 7.5% 2-ME, 3% ampholine (pH 3.5–10), 15% glycerol] and urea was added until saturation. Samples were centrifuged at 800 × *g* at room temperature for 1 min, and the resulting supernatants (2.5 μg of PP1/tube) were applied to the tube gels. IEF was run at 200 V for 18 h, followed by 400 V for 2 h. After IEF, the gels were soaked in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME, 0.001% bromophenol blue) for 15 min before being overlaid onto 12% SDS-PAGE gels. After electrophoresis, the proteins were transferred onto nitrocellose membrane for Western blotting.

Western Blot—The blot was blocked with skim milk, incubated for 1 h with rabbit anti-PP1 antisera diluted 100- to 500-fold, washed with phosphate-buffered saline containing 0.1% Tween, and then incubated with 1 μg/ml horseradish peroxidase-labeled donkey anti-rabbit IgG antibody (Chemicon, Temecula, CA) for 1 h. Immunoreactive spots were detected with an ECL Western blotting detection kit (Amersham International plc, England) (*17*). The molecular mass and isoelectric point were estimated from the mobility of the spots relative to standard proteins (Bio-Rad, Hercules, CA).

Phosphorylation of PP1 C-Terminal Peptides by Protein Kinase—The PP1 C-terminal peptides (25 μg) were incubated at 30°C in the same reaction mixtures (50 μl) as described above for the phosphorylation of PP1, except that NaF was excluded. At the indicated times, a portion (3 μl) of the reaction mixture was spotted onto a square (2 cm × 2 cm) of P81 phosphocellulose paper, which was then washed 6 times with 75 mM H_3PO_4 and dried. The radioactivity was measured with a liquid scintillation counter.

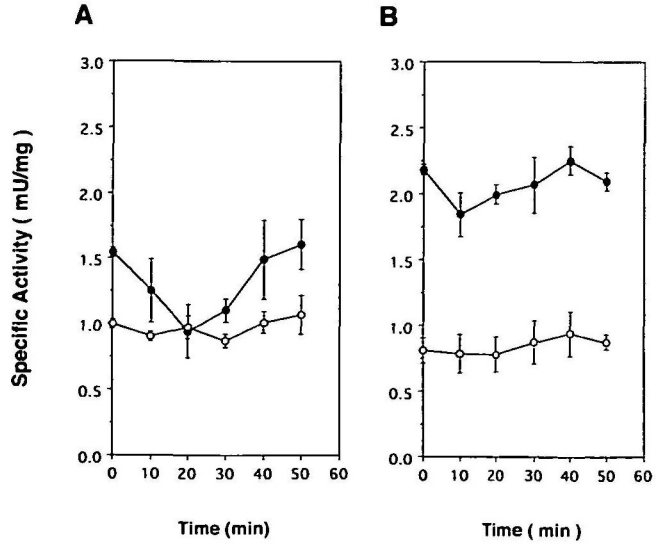

Fig. 1. **Alteration in PP1 and PP2A activities after sIgM stimulation of immature and mature B cell lines.** WEHI-231 cells (A) and BAL-17 cells (B) were stimulated with 10 μg/ml goat $F(ab')_2$ anti-mouse IgM for 0–50 min. PP1 (●) and PP2A (○) activities in the non-nuclear fractions were measured. Values are the means of four (A) or two (B) independent experiments.

RESULTS

Alteration of PP1 Activity after sIgM Stimulation of Immature and Mature B Cells—To investigate the roles of PP1 in sIgM-mediated signal transduction in immature and mature B cells, we examined the PP1 activity of two B lymphoma cell lines, the representative immature B cell line WEHI-231 expressing $B220^+sIgM^+sIgD^-$ and the mature B cell line BAL-17 expressing $B220^+sIgM^+sIgD^+$.

The effects of sIgM stimulation on PP1 and PP2A are shown in Fig. 1. In WEHI-231 cells, the effect on PP1 activity was rapid and transient; the activity reached a minimum 20 min after the stimulation and returned to the control level at 40 min, whereas no effect on PP2A activity was observed. The maximal PP1 inactivation was variable, ranging from 51 to 18% of the control value, with an average of 49%. BAL-17 cells showed very little alteration of PP1 activity, and no effect on PP2A activity was observed.

To clarify whether the inactivation of PP1 activity is due to a quantitative alteration of this enzyme, we carried out Western blotting of the PP1 catalytic subunit. As shown in Fig. 2, a major band was detected at 39.5 kDa for PP1α and at 39.0 kDa for PP1δ. However, no significant alteration in the amount of PP1α or PP1δ was observed during the sIgM stimulation. This observation suggests that the transient alteration of PP1 activity was not due to change in amount of PP1, but was due to secondary modification.

Two-Dimensional Gel Electrophoresis of PP1α and PP1δ—To clarify whether the inactivation of PP1 activity is due to modification, we carried out isoelectric focusing for PP1 catalytic subunits. In lysates of WEHI-231 cells cultured for 24 h, major spots of PP1α and PP1δ were detected at 39.5 and 39.0 kDa, respectively (Fig. 3). The center region of this two-dimensional Western blot is marked with a border to show the area that will be displayed for other experiments (*e.g.* Fig. 4). The major forms of PP1α and PP1δ were located at approximately pH 5.9 and corresponded to the form of PP1 purified from rabbit skeletal muscle.

Transient Modification of PP1 in Response to sIgM

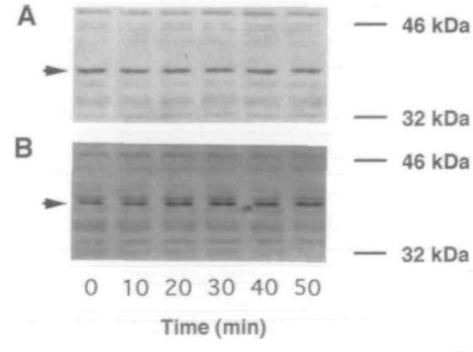

Fig. 2. **Western blot analysis of PP1α and PP1δ in WEHI-231 cells during sIgM stimulation.** Cells were stimulated with 10 μg/ml goat $F(ab')_2$ anti-mouse IgM for 0–50 min. Incubation was terminated at the indicated times and then non-nuclear fractions were prepared. A 70 μg aliquot of protein was subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Western blot analysis was carried out by using antibody against PP1α (A) and PP1δ (B).

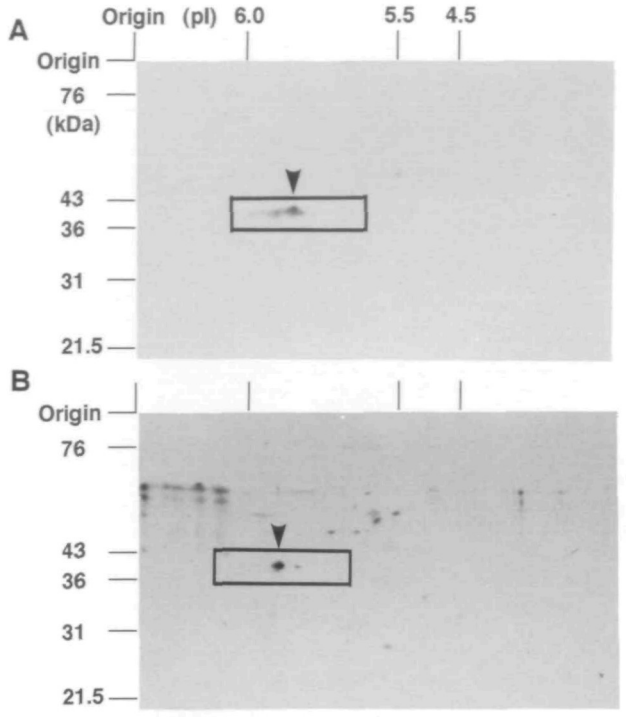

Fig. 3. **Two-dimensional gel electrophoresis and Western blotting of PP1α and PP1δ.** WEHI-231 cells (2×10^7) from logarithmically growing cultures were collected and the non-nuclear fraction containing 150 μg of protein was analyzed by two-dimensional Western blotting with anti-PP1α (A) and anti-PP1δ (B) antibodies. The entire two-dimensional Western blot is shown with the positions of molecular masses and isoelectric points of standards (Bio-Rad) on the left and the top, respectively: hen egg white conalbumin type 1 (76 kDa, pI 6.0-6.6), bovine muscle actin (43 kDa, pI 5.5), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36 kDa, pI 8.3-8.5), bovine carbonic anhydrase (31 kDa, pI 5.9-6.0), and soybean trypsin inhibitor (21.5 kDa, pI 4.5). The major spots of PP1α and PP1δ are indicated by arrowheads.

Stimulation and to PMA—We next investigated the effects of stimulation of sIgM receptor on modifications of PP1α and PP1δ. As shown in Fig. 4, there were multiple spots of PP1α with several isoelectric points at 39.5 kDa, whereas PP1δ showed major and minor spots at 39.0 kDa. From 10 min to 30 min after sIgM stimulation, the density of spot a of PP1α was increased, and from 20 min to 40 min, the densities of spots b and c of PP1α were increased. On the other hand, the Western blot for PP1δ did not show any new spot under similar conditions. Since PKC is known as one of the downstream molecules of the sIgM-mediated

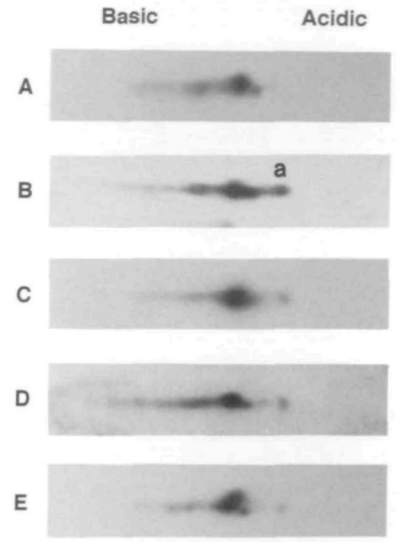

Fig. 5. **Transient modification of PP1 in response to PMA with and without H7.** WEHI-231 cells were stimulated with 50 ng/ml PMA with or without 40 μM H7, and lysed at different times after stimulation. The extracts containing 150 μg of protein were subjected to two-dimensional electrophoresis and Western blotting by anti-PP1α antibody. Panels: A, 0 min; B, 20 min with PMA; C, 40 min with PMA; D, 20 min with PMA plus H7; E, 40 min with PMA plus H7.

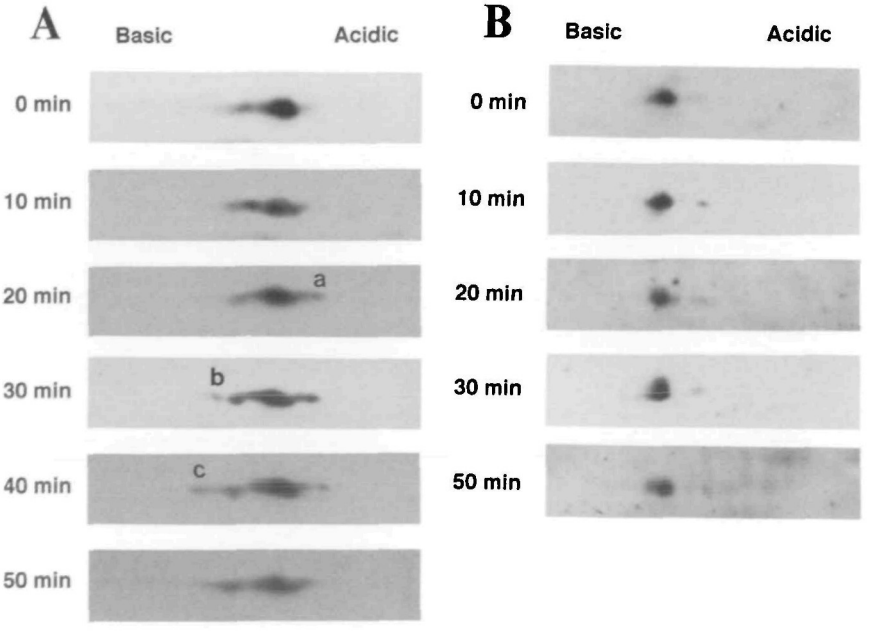

Fig. 4. **Transient modification of PP1α and PP1δ in response to sIgM stimulation.** WEHI-231 cells were stimulated with 10 μg/ml anti-IgM antibody, and lysed at different times after stimulation. The extracts containing 150 μg of protein were subjected to two-dimensional electrophoresis and Western blotting with anti-PP1α (A) and PP1δ (B) antibodies as described in Fig. 3.

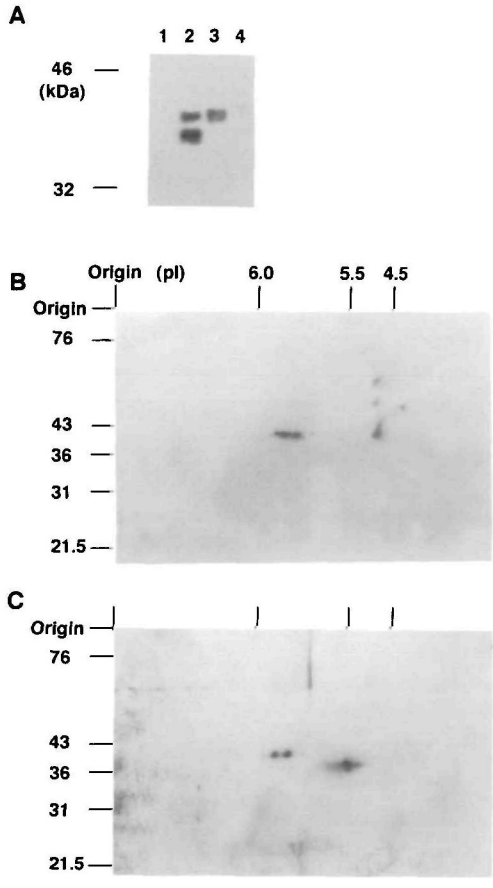

Fig. 6. **Western blot analysis of glycogen PP1.** (A) The PP1 partially purified from glycogen particles (1 μg/4-mm lane) was subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. The blot was incubated with antisera against PP1γ1 (lane 1), PP1δ (lane 2), PP1α (lane 3), and PP1γ2 (lane 4). Immunoreactive bands were visualized as described in the text. The positions of the molecular-mass markers ovalbumin (46 kDa) and carbonic anhydrase (32 kDa) are indicated. (B and C) Purified PP1 (2.5 μg) was subjected to 2-dimensional electrophoresis (IEF pH 3.5-10 and 12% SDS-PAGE). The proteins were transferred onto nitrocellulose membranes. The blots were incubated with rabbit antisera against PP1α (B) and PP1δ (C).

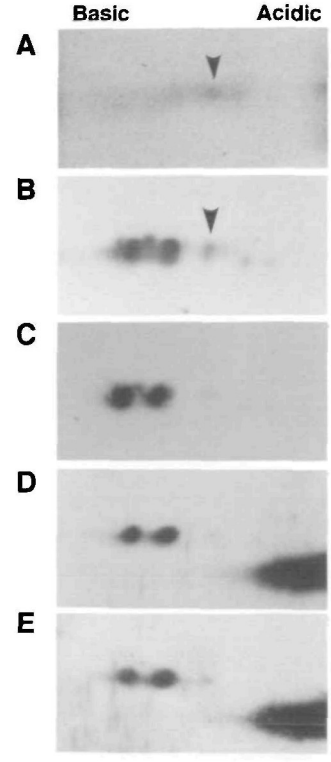

Fig. 7. **Phosphorylation of partially purified PP1 by protein kinase C.** The purified PP1 (5 μg) was incubated at 30°C for 2 h in reaction mixtures (50 μl) containing 100 μM [γ-^{32}P]ATP (4,000-5,000 cpm/pmol), 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM NaF, 1 μg/ml 1,2-dioctanoyl-*sn*-glycerol, and 50 μg/ml phosphatidylserine, in the presence (A, B, D) or absence (C, E) of 5 μg/ml PKC. After 2-dimensional electrophoresis (IEF pH 3.5-10, 12% SDS-PAGE), the proteins (2.5 μg of PP1/membrane) were transferred onto nitrocellose membranes for autoradiography (A) and Western blotting with the rabbit antisera against PP1α (B, C) and PP1δ (D, E).

signal transduction, WEHI-231 cells were then stimulated with 50 ng/ml PMA. At 20 min after the PMA stimulation, the density of spot a of PP1α was increased and at 40 min, it was decreased (Fig. 5, A-C). This PKC-induced increase of spot a of PP1α was blocked by H7 (Fig. 5D). On the other hand, the Western blot for PP1δ did not show any change in two-dimensional electrophoresis under similar conditions (data not shown).

Partial Purification, Two-Dimensional Electrophoresis, and Western Blot of PP1—We have partially purified PP1 from rabbit skeletal muscle according to a previous method with a slight modification and detected a band for PP1α at 39.5 kDa and two bands for PP1δ at 39 and 37 kDa by Western blotting (Fig. 6A). Bands of PP1γ1 and PP1γ2 were not detected. The band of PP1δ at 37 kDa, is thought to be a proteolytic product of the 39 kDa band (*15, 17*). The catalytic subunit of PP1 in muscle glycogen particles is reported to be PP1δ (*33*). The reason for this discrepancy is dealt with under "DISCUSSION." Western blotting after two-dimensional electrophoresis showed multiple spots with anti-PP1α antibody (Fig. 6B) and two spots with anti-PP1δ antibody (Fig. 6C). The difference in the electrophoresis patterns between this partially purified PP1 from rabbit skeletal muscle (Fig. 6) and PP1 in WEHI-231 cells (Fig. 3) may reflect different modification states.

Phosphorylation of PP1α by Protein Kinase C—The above preparation consisting of PP1α and PP1δ as main constituents was incubated with PKC and [γ-^{32}P]ATP. After two-dimensional electrophoresis, Western blotting was carried out with anti-PP1α and anti-PP1δ antibodies (Fig. 7). In the Western blot for PP1α after incubation with PKC, the density of the lower-pI spot indicated by the arrowhead was markedly increased, which exactly corresponded with the major radioactive spot. On the other hand, Western blotting for PP1δ did not show any new spot under similar conditions.

Phosphorylation of C-Terminal Peptides of PP1 by PKC—Catalytic subunits of PP1 isoforms have high sequence homology, and differences in sequence among them are mostly restricted to their C-termini. The phosphorylation motif for PKC (R/K-X_{0-2}-S/T-X_{0-2}-R/K) is present in the sequences 317-320(Thr)-323 and 323-325(Ser)-327 of PP1α and 325-327(Ser)-330 of PP1γ2. The corresponding C-terminal of PP1δ does not contain the PKC motif. Figure

TABLE I. **Subcellular distribution of PP1 activity and PP1 isoforms.** PP1 and PP2A activities were assayed with phosphorylase *a* as a substrate and amounts of PP1α and PP1δ were measured by Western blotting. ($\pm$SD where shown, $n=3$)

	Specific activities (mU/mg)				Ratio of PP1 amounts (membrane/cytosol)	
	Cytosol		Membrane			
	PP1	PP2A	PP1	PP2A	α	δ
WEHI-231	1.40±0.49	0.56±0.09	0.50±0.20	0.05±0.03	0.68	0.10
BAL-17	0.70±0.09	0.39±0.05	1.26±0.21	0.10±0.01	0.62	0.85

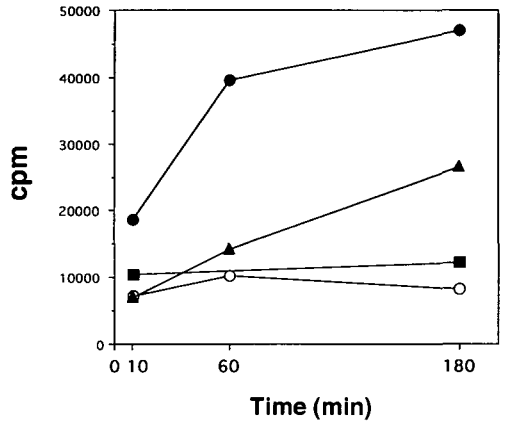

Fig. 8. **Phosphorylation of PP1 C-terminal peptides by protein kinase C.** The PP1 C-terminal peptides (25 μg) of PP1α (●), PP1γ1 (■), and PP1γ2 (▲) were incubated at 30°C in reaction mixtures containing [γ-^{32}P]ATP and PKC as described in the text. At the indicated times, a portion (3 μl) of the reaction mixture was spotted onto a square (2 cm × 2 cm) of P81 phosphocellulose paper, which was then dried. The radioactivity was measured with a liquid scintillation counter. The open circles indicate background incorporation by the reaction mixture without peptide.

8 shows that the C-terminal peptide of PP1α is a better substrate for PKC compared with the peptides of PP1γ1 and PP1γ2. The phosphoamino acid of the C-terminal peptide of PP1α was serine (data not shown). These results strongly suggest that Ser-325 of PP1α is at least one of the sites phosphorylated by PKC.

Subcellular Fractionation of PP1 in B Cells—We have reported that the protein amounts of the catalytic subunits of PP1α, PP1δ, and PP2A were at similar levels in WEHI-231 and BAL-17 (data not shown) (Takizawa, N. *et al.*, manuscript submitted for publication). PP1γ1 and PP1γ2 have not been detected in these cells. However, phosphorylase phosphatase activity of PP1 in BAL-17 was 1.4 times higher than that in WEHI-231, whereas the activity of PP2A was similar in the two cell lines (Fig. 1). After Co^{2+}-trypsin treatment of the cell extracts (conditions which liberate the PP1 catalytic subunits from various regulatory subunits) the PP1 activities in BAL-17 and WEHI-231 became similar (data not shown). These results suggest that the difference in PP1 activity between the two cell lines can be attributed to differences in the association with various regulatory subunits and/or cellular distribution. Therefore we determined the PP1 activity in cytosolic and membrane fractions prepared from WEHI-231 and BAL-17 cells. In WEHI-231, the cytosolic PP1 activity was higher than the membrane PP1 activity, whereas in BAL-17, the cytosolic PP1 activity was lower than membrane PP1 activity (Table I). It is known that several isoforms of PP1 show broad subcellular distributions (*11, 12*). So we measured the amounts of PP1α and PP1δ in the cytosolic and membrane fractions prepared from WEHI-231 and BAL-17 by Western blot analysis. In WEHI-231, the relative amounts of membrane PP1α and PP1δ were 0.68 and 0.10, respectively, of those in the cytosol. In BAL-17, relative amounts of membrane PP1α and PP1δ were 0.62 and 0.85, respectively, of those in cytosol (Table I).

DISCUSSION

In this study, we have analyzed qualitatively and quantitatively the alterations of serine/threonine protein phosphatase PP1 in response to the stimulation of B-cells through sIgM using the immature B-cell line WEHI-231 and the mature B-cell line BAL-17. PP1 activity was significantly higher in BAL-17 than in WEHI-231, demonstrating that PP1 activity is increased during the maturational process of B-cells. PP1 activity showed a transient decrease in response to the sIgM stimulation. Under these conditions, there was no change in PP2A activity. The PP1 activity reached the minimum 20 min after stimulation, but recovered to the control level at 40 min. The decrease of PP1 activity, together with the increase of PKC activity (*34*), might play a role in accelerating the signal transduction of B-cells. The decrease was significantly larger in the immature WEHI-231 cells than in the mature BAL-17 cells. It is well-known that mature B220$^+$sIgM$^+$/sIgD$^+$ B-cells respond to antigenic stimuli positively by proliferation and differentiation, whereas immature B220$^+$sIgM$^+$/sIgD$^-$ B-cells respond negatively by apoptosis (*1-3*). Therefore, the difference in the response of PP1 activity between mature and immature B-cells may be relevant to the difference in response to antigenic stimuli between them (*4-7*).

To get insight into molecular mechanisms of the PP1 response, we analyzed further the properties and nature of PP1 by two-dimensional electrophoresis and subcellular fractionation of PP1. There were multiple spots with several isoelectic points of PP1α at 39.5 kDa, whereas PP1δ showed one major spot and a small spot at 39.0 kDa. After sIgM stimulation, the spots of PP1α showed unique alterations, whereas those of PP1δ did not. These results strongly suggest that only PP1α, not PP1δ, was modified under these conditions. PKC is known to be one of the downstream molecules in sIgM-mediated signal transduction. The fact that similar alterations only in the spots of PP1α were induced by PMA stimulation suggests that PKC is involved in the alterations in the spots of PP1α in response to the sIgM stimulation. We then analyzed the alterations in the spots after phosphorylation with PKC using PP1 partially purified from rabbit muscle glycogen fraction. This PP1 preparation contained PP1δ and PP1α as main constituents (Fig. 6A). It was previously reported

that only PP1δ among several PP1 isoforms is present in rabbit muscle glycogen particles (*33*). In the present study, we precipitated glycogen-protein particles at pH 5.6 instead of pH 6.1 to increase the recovery. Therefore, the large amount of PP1α in the present preparation might be due to contamination with other fractions including microsomes. However, it should be noted that PP1 purified from rat liver glycogen fraction, $PP1G_L$, was reported to contain both PP1α and PP1δ as main constituents (*35*). The Western blots either before or after incubation with PKC showed multiple spots (Fig. 7, B and C, and Fig. 8, D and E), which may at least in part reflect different modification states due to multiple phosphorylation of PP1.

It seems to be of considerable interest to elucidate how PP1α is regulated by its phosphorylation. It was previously reported that PP1 activity is decreased *via* tyrosine phosphorylation by src-family kinase. Recently, Kwon *et al.* reported that PP1α is phosphorylated by CDC2 at its C-terminal in a cell-cycle dependent manner. It is possible that the phosphorylation of holoenzymes of PP1 is involved in regulation of PP1 through alterations in its activity, substrate specificity, and subcellular translocation (*36*). It appears that multiple phosphorylations of PP1α and their differential time courses are implicated in the regulatory mechanisms of the response of B-cells through sIgM, one of which is the decrease in PP1 activity. The difference in subcellular distribution between WEHI-231 cells and BAL-17 cells also suggests a translocation of PP1δ during the maturation of B-cells.

We have demonstrated here that PP1α is phosphorylated *in vitro* by PKC. Phosphorylation motifs for PKC (R/K-X_{0-2}-S/T-X_{0-2}-R/K) are present in the sequences of 317-320(Thr)-323, 323-325(Ser)-327 of PP1α and 325-327(Ser)-330 of PP1γ2. Of these sequences, the C-terminal peptide of PP1α was shown here to be a better substrate for PKC compared with the C-terminal peptides of PP1γ1 and PP1γ2 (Fig. 8). The phosphoamino acid of the C-terminal peptide of PP1α was serine. These results strongly suggest that Ser-325 of PP1α is preferentially phosphorylated by PKC.

There was a large difference in subcellular distribution of PP1 activity between immature and mature B-cells. It should be noted that PP1δ in the membrane fraction is specifically increased during the maturational process of B-cells. These results may also be relevant to the difference of signal transduction between immature and mature B-cells.

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