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Relationships of Subunits of Type-1 Serine/Threonine Protein Phosphatase to Morphology and Aggregation of B Cells[1]

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To elucidate the roles of serine/threonine protein phosphatases PP1 and PP2A in the morphological changes of B-lymphocytes during development and in immune responses, we investigated alterations of protein levels of catalytic subunits of PP1 and PP2A and regulatory subunits of PP1 including M130/M133, inhibitor-1 (I-1) and inhibitor-2 (I-2) in B-cell lines at different maturational stages and during their aggregation induced by phorbol myristate acetate (PMA). The protein levels of PP1δ and/or M130/M133 were significantly lower in B-cell lines without pseudopods, WEHI-231, BAL-17, Daudi, and CESS, than in those with pseudopods, Bcl.1, A20, M12, and SKW6.4, whereas the amounts of PP1α and PP2A were similar among them. During aggregation of A20 and CESS cells induced by PMA, an activator of PKC, the amount of PP1δ was progressively decreased, and this decrease was blocked by H7, an inhibitor of PKC. The amount of PP1α was constant under these conditions. Okadaic acid, an inhibitor of PP1 and PP2A, also induced aggregation of A20 cells at concentrations sufficient to inhibit PP1, but not at lower concentrations that inhibit PP2A alone. These results suggest that myosin light chain phosphatase composed of PP1δ and M130/M133 is involved in the maintenance and regulation of cytoskeletal structures in B-lymphocytes.

Key words: aggregation, B cell, morphology, PP1, protein phosphatase.

Protein phosphorylation and dephosphorylation are crucial mechanisms for regulation of a variety of biological events, such as activation, apoptosis, and differentiation of T- and B-lymphocytes. Morphological changes accompanying these biological events are regulated by reversible phosphorylation of cytoskeletal components such as ezrin (*1*) and paxillin (*2*). Hence, protein phosphatases are thought to play important roles in morphological alterations of lymphocytes in various immune responses and in development of the immune system (*3, 4*).

In this study, we examined protein levels of serine/threonine protein phosphatases PP1 and PP2A in various murine and human B-cell lines at different maturational stages and during aggregation of B-cells induced by PMA, a PKC activator (*5*), or by okadaic acid, a protein phosphatase inhibitor (*6, 7*).

Mammals have at least four isoforms of PP1 catalytic subunits, termed PP1α, PP1γ1, PP1γ2, and PP1δ (also called PP1β). Each catalytic subunit is associated with inhibitor-1 (I-1), inhibitor-2 (I-2), NIPP-1, G-component, or M130/M133. PP1 is thought to be located in nuclei in a form complexed with NIPP-1, and at myofibrils in a form complexed with M130/M133 (*8-10*). These isoforms of PP1 show characteristic patterns of tissue distribution (*11, 12*), but their distinctive functions are not well understood.

We have reported a selective elevation in amount of PP1α in rat ascites hepatomas, suggesting that PP1α is involved in malignant phenotypes and/or high proliferating rates (*13-15*). We have also reported that amounts of PP1α were progressively decreased in livers of non-obese diabetic mice (IDDM model mice) as a function of increasing concentrations of blood glucose, whereas the amounts of PP1γ1 and PP1δ were unchanged (*12*). These previous results suggest that the isoforms of PP1 have different roles and regulatory mechanisms. Recently, it was reported that PP1M, a holoenzyme of PP1 located at myofibrils, is composed of PP1δ and M130/M133 (*16*).

It is well-known that B-lymphocytes during development show morphological alterations accompanied with expression of various specific cell-surface markers, and that B-cells morphologically change in response to extracellular stimuli (*17*). To elucidate the roles of protein phosphatases in these immunological events, we have examined the correlation of protein levels of PP1 and PP2A with morphological features in various B-cell lines at different maturational stages and their alterations during aggrega-

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

tion induced by PMA or okadaic acid.

MATERIALS AND METHODS

Materials—The rabbit anti-PP1 antibodies were prepared as described (*12*). The rabbit anti-PP2A antibody, which recognizes both PP2Aα and PP2Aβ, was obtained from UBI (Lake Placid, NY). The rabbit anti-M130/M133 antibody was prepared as described (*18, 19*). The rabbit anti-I-2 antibody which recognizes human I-2 was prepared as described (*20, 21*). The rabbit anti-I-1 antibody was prepared as described (*22*). Myosin light chain phosphatase, MLCP, was prepared as described (*23, 24*). Okadaic acid (OA) and phorbol 12-myristate 13-acetate (PMA) were obtained from Wako Pure Chemical Industries (Osaka). The protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) was from Seikagaku (Tokyo).

Cell Culture—The human B cell lines, Daudi (*25*), SKW6.4 (*26*), and CESS (*27*), were maintained in RPMI 1640 medium (GIBCO-BRL) supplemented with 2 mg/ml $NaHCO_3$, pH 7.2, 10% heat-inactivated fetal calf serum (FCS), and 2 mM L-glutamine, at 37°C in an atmosphere containing 5% CO_2. The murine B cell lines, WEHI-231 (*28*), Bcl.1 (*29*), BAL-17 (*30*), A20 (*30*), and M12 (*30*), were maintained in the human culture medium plus 50 μM 2-mercaptoethanol (2-ME).

Stimulation with PMA—Logarithmically growing A20 cells that adhered to the plate bottom (NIPRO, nontreated plate) were stimulated with various concentrations of PMA in a CO_2 incubator for 24 h. Logarithmically growing CESS cells were resuspended at 1×10^6 cells/ml in the culture medium, and then stimulated with 50 ng/ml PMA with or without 40 μM H7. After appropriate incubation in a CO_2 incubator, the culture was stopped with ice-cold 150 mM NaCl.

Preparation of Cell Extracts from B Cells—Cells (2×10^7) from logarithmically growing cultures were collected by centrifugation at $400\times g$ for 5 min, and washed with ice-cold 150 mM NaCl. The cell pellets were lysed by the addition of extraction buffer (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, and 1.0% Nonidet P-40 (NP-40). The lysates were centrifuged at $8,000\times g$ for 20 min at 4°C and the resulting supernatants were used as extracts.

Western Blot Analysis—Proteins (70 μg) of the extract were separated on a 12% SDS-PAGE gel under reducing conditions and electrophoretically transferred to a nitrocellulose membrane. The blot was blocked with skim milk, incubated for 3 h with the rabbit anti-protein phosphatase antibodies, washed with phosphate-buffered saline containing 0.1% Tween-20, and then incubated with 1 μg/ml horseradish peroxidase-labeled donkey anti-rabbit IgG antibody (Chemicon, Temecula, CA) for 1 h. Immunoreactive bands were detected with an ECL Western blotting detection kit (Amersham International plc, England).

RESULTS

Western Blot Analysis of PP1 and PP2A in Various Human and Murine B-Cell Lines—To examine the roles of serine/threonine protein phosphatases PP1 and PP2A in B-cells, we analyzed by Western blotting the amounts of catalytic subunits of PP1 and PP2A and regulatory subunits of PP1 including M130/M133, I-1, and I-2, in various murine and human B-cell lines.

In 5 murine cell lines, WEHI-231, Bcl.1, BAL-17, A20, and M12, the amounts of PP1α were at similar levels. PP1γ1, PP1γ2, and I-1 were undetectable in all the cell lines examined under similar conditions. In contrast, as shown in Fig. 1, PP1δ and M130/M133 showed a variety of levels, dependent on the cell lines. When compared with WEHI-231, a cell line at the earliest stage of maturation among the 5 cell lines, relative amounts of PP1δ in Bcl.1, BAL-17, A20, and M12 were 0.29, 1.14, 0.57, and 0.75, respectively, those of M130/M133 were 0.51, 0.95, 0.71, and 0.32, respectively, and those of PP2A were 1.20, 1.00, 1.20, and 1.20, respectively (Fig. 1A).

In 3 human cell lines, Daudi, SKW6.4, and CESS, amounts of PP1α were at similar levels. PP1γ1, PP1γ2, and I-1 were undetectable. However, the amounts of PP1δ and M130/M133 were 0.59 and 0.67 in SKW6.4, and 0.41 and 1.2 in CESS, respectively, when compared with those in Daudi (Fig. 1B). The amounts of I-2 and PP2A were 0.73 and 1.00 in SKW6.4 and 0.27 and 0.80 in CESS, compared with those in Daudi. Figure 2 shows the morphology of the B-cell lines examined. WEHI-231 and BAL-17 of the murine cell lines, and Daudi and CESS of the human cell lines were round without pseudopods. In contrast, murine Bcl.1, A20, M12, and human SKW6.4 possessed pseudopods. It should be noted that the protein levels of PP1δ and/or M130/M133 were relatively lower in the cells with pseudopods than in the cells without pseudopods. Taking into account that myosin light chain phosphatase, MLCP, is a holoenzyme form composed of PP1δ and M130/M133, the difference in amounts of PP1δ and/or M130/M133 may reflect the characteristic morphology of the B-cell lines examined.

Effect of Incubation with PMA on PP1 and PP2A—Previously, it was reported that in response to incubation with okadaic acid, platelets showed formation of pseudopods. Phorbol ester is known to induce aggregation of

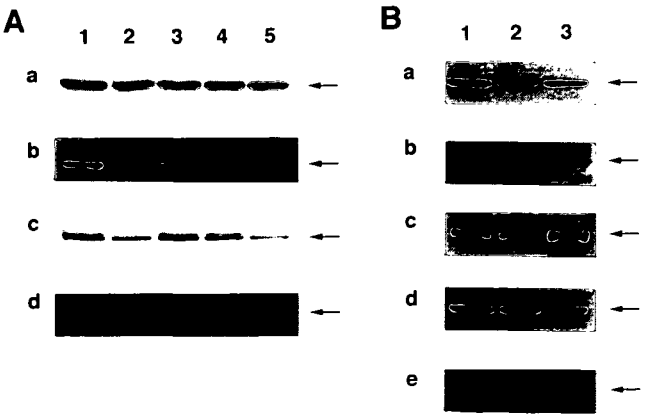

Fig. 1. **Western blot analysis of PP1 and PP2A in various human and murine B-cell lines.** Murine (A) and human (B) cell extracts (70 μg protein) were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Western blot analysis was carried out by using antibodies against PP1α (a), PP1δ (b), M130/M133 (c), PP2A (d), and I-2 (e). (A) Lanes: 1, WEHI-231; 2, Bcl.1; 3, BAL-17; 4, A20; 5, M12. (B) lanes: 1, Daudi; 2, SKW6.4; 3, CESS.

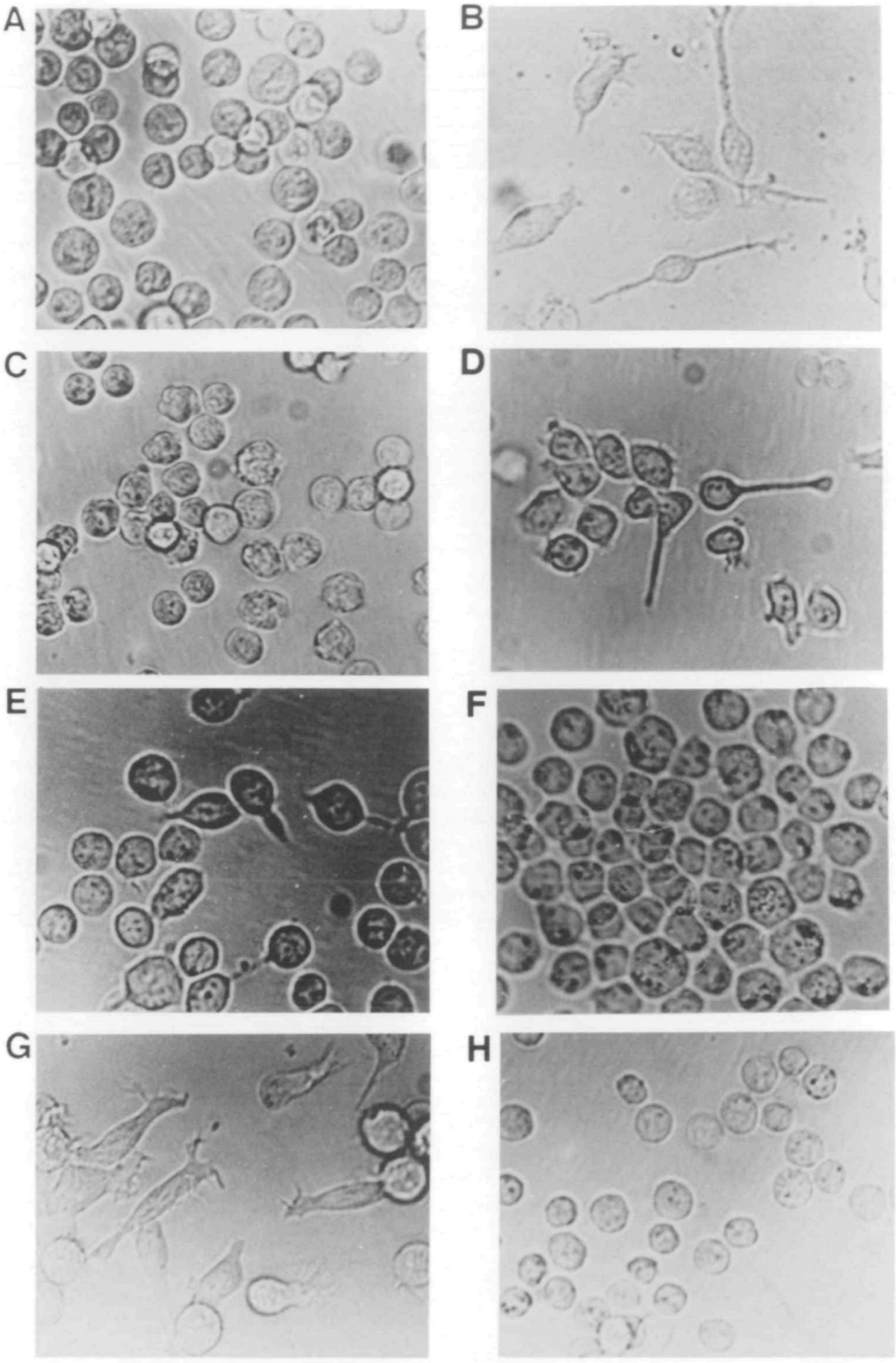

Fig. 2. **Morphology of human and murine B-cell lines.** Cells (2×10^5 cells/ml) were incubated for 48 h in culture medium. Panels: A, WEHI-231; B, Bcl.1; C, BAL-17; D, A20; E, M12; F, Daudi; G, SKW6.4; H, CESS.

platelets. Myosin light chain serves to maintain the morphology of cells, and myosin light chain phosphatase composed of PP1δ and M130/M133 is thought to be involved in the maintenance of cellular structure through dephosphorylation of myosin light chain. Hence we examined effects of incubation with PMA on aggregation of various B-cell lines. Of the 6 cell lines, WEHI-231, Bcl.1, A20, M12, SKW6.4, and CESS, only A20 and CESS aggregated in response to incubation with 50 ng/ml PMA. As shown in Fig. 3, the aggregation of A20 cells at 24 h of incubation became more marked with increasing concentrations of PMA. As shown in Fig. 4, under these conditions, the amounts of PP1δ and M130/M133 were decreased to 0.50 and 0.71 of the controls, respectively, whereas that of PP1α remained constant. Figure 5 shows the aggregation of CESS cells in response to incubation with 50 ng/ml PMA. The amounts of PP1δ and M130/M133 were decreased to 0.48 and 0.70, respectively, of the controls after 12-h

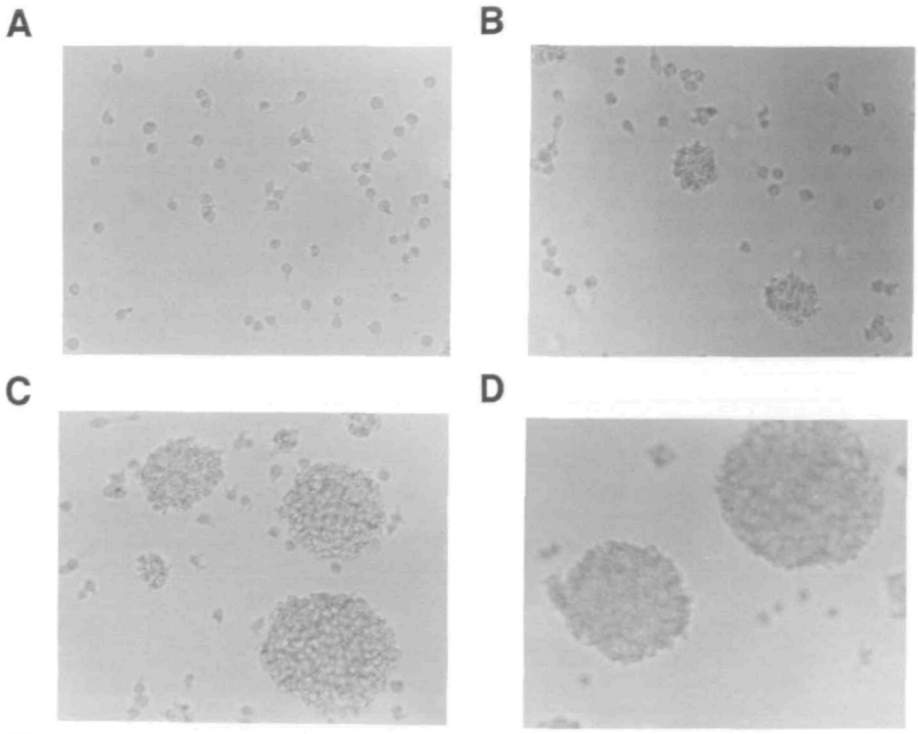

Fig. 3. **Aggregation of A20 cells with PMA.** Cells were incubated for 24 h with various concentrations of PMA. Panels: A, none; B, 0.8 ng/ml; C, 8.0 ng/ml; D, 80 ng/ml; E, 800 ng/ml.

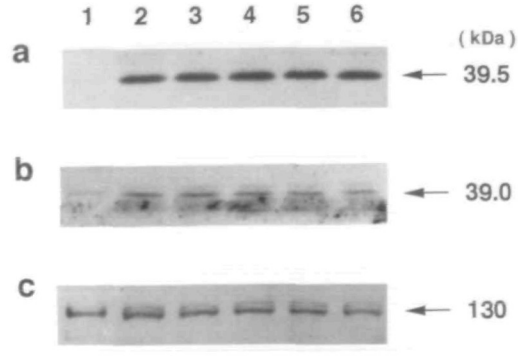

Fig. 4. **Effects of incubation with PMA on PP1 and PP2A in A20 cells.** Cells were incubated for 24 h with various concentrations of PMA. Cell extracts (70 μg protein) were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Western blot analysis was carried out by using antibodies against PP1α (a), PP1δ (b), and M130/M133 (c). Lane 1, myosin light chain phosphatase (0.2 μg); lanes 2-6, cells incubated without (lane 2) or with 0.8 ng/ml (lane 3), 8 ng/ml (lane 4), 80 ng/ml (lane 5), and 800 ng/ml (lane 6) PMA.

incubation, but under identical conditions, the amounts of PP1α, PP2A, and I-2 were at the control levels (Fig. 6). As shown in Fig. 7 and Fig. 8, 40 μM H7, a PKC inhibitor, blocked the PMA-induced aggregation of CESS cells at 24 h, as well as the decrease in amount of PP1δ with PMA, 55%, and with PMA plus H7, 77% of the control.

Effects of Okadaic Acid on Aggregation of A20 Cells—We examined the effects of okadaic acid on aggregation of A20 cells. During incubation of A20 cells with various concentrations of okadaic acid for 24 h in the absence or presence of 8 ng/ml PMA, 1 nM okadaic acid showed no effect on aggregation, irrespective of the presence of PMA, but at concentrations higher than 10 nM it increased the aggregation (Fig. 9). CESS cells did not show any aggregation up to 10 ng/ml okadaic acid, and died *via* an apoptotic process on incubation with 50 ng/ml okadaic acid (data not shown). From these results, it was concluded that cell aggregation was induced by okadaic acid at concentrations sufficiently high to inhibit both PP1 and PP2A, but not at low concentrations that inhibit PP2A alone (*31*).

DISCUSSION

In the present study, we have measured the protein levels of catalytic subunits of PP1 and PP2A and regulatory subunits of PP1 including M130/M133, I-1, and I-2 by Western blotting in 5 murine and 3 human B-cell lines and examined their changes during aggregation induced by an activator of PKC, PMA, or an inhibitor of PP1 and PP2A, okadaic acid. However, PP1γ1, PP1γ2, or I-1 was not detected in any of the 8 cell lines examined. The amounts of PP1α and PP2A were at similar levels among the 8 B-cell lines, whereas the amounts of PP1δ and M130/M133 showed a variety of levels. The B-cell lines used in the

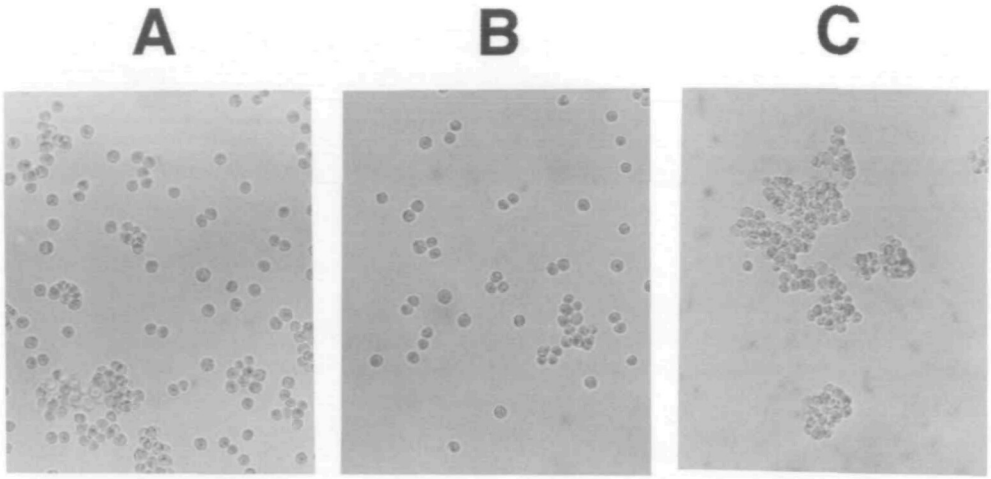

Fig. 5. **Aggregation of CESS cells with PMA.** Cells were incubated for 6 h without (panel A), with 0.4% DMSO (panel B), or with 0.4% DMSO plus 50 ng/ml PMA (panel C).

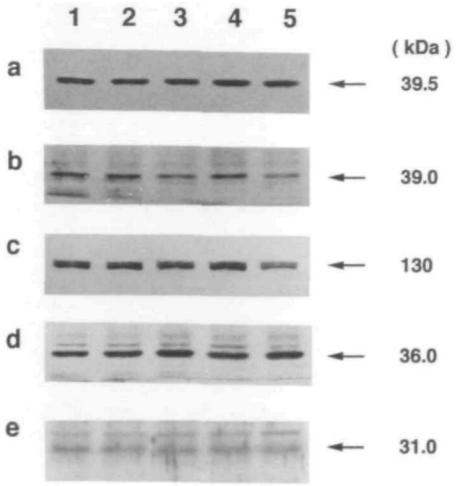

Fig. 6. **Effects of incubation with PMA on PP1 and PP2A in CESS cells.** Cells were incubated with or without 50 ng/ml PMA for various times. Lanes: 1, 0 h; 2, 6 h without PMA; 3, 6 h with PMA; 4, 12 h without PMA; 5, 12 h with PMA. Cell extracts (70 μg protein) were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Western blot analysis was carried out by using antibodies against PP1α (a), PP1δ (b), M130/M133 (c), PP2A (d), and I-2 (e).

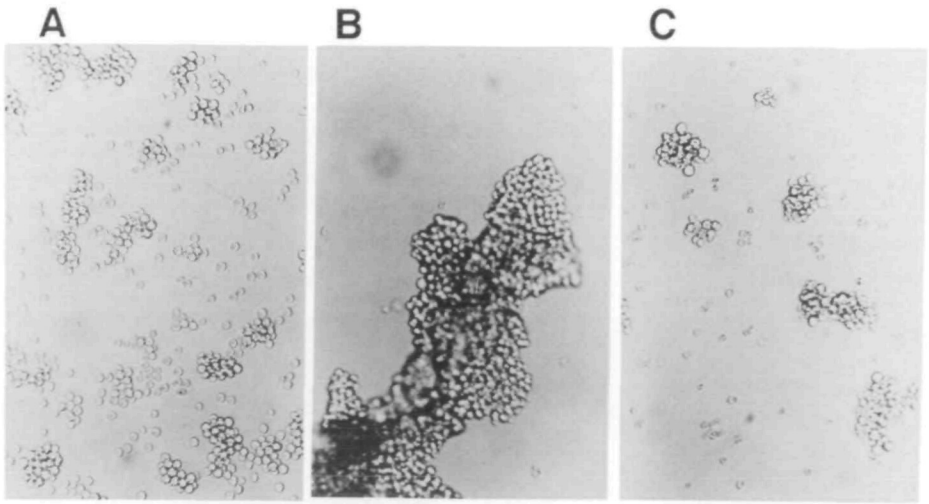

Fig. 7. **Effects of H7 on aggregation of CESS cells induced with PMA.** Cells were incubated for 24 h without (A), with 50 ng/ml PMA (B), or with 50 ng/ml PMA plus 40 μM H7 (C).

present study express different surface markers, demonstrating that they are frozen at different maturational stages during B-cell development (*32*). Of the 8 B-cell lines used, WEHI-231 expresses the earliest surface markers (*28*), and BAL-17 and Bcl.1 express mature markers such as IgM (*29, 30*). A20 cells represent the intermediate markers. Bcl.1 can be activated by LPS to secrete IgM (*33*). M12 lacks the ability to express surface Ig (*30*). SKW6.4 is an Epstein-Barr virus (EB virus)-positive clone of Daudi (*26*). SKW6.4 and CESS respond to IL-6 by increasing the

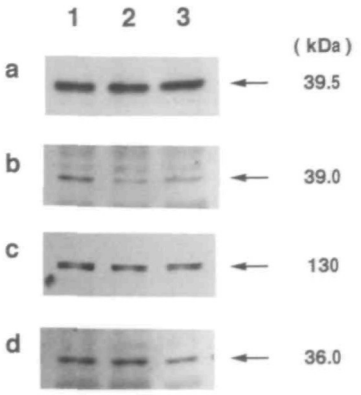

Fig. 8. **Effects of H7 on the suppression of PP1 and PP2A with PMA in CESS cells.** Cells were incubated without (lane 1), with 50 ng/ml PMA (lane 2), or with 50 ng/ml PMA plus 40 μM H7 (lane 3). Cell extracts (70 μg protein) were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Western blot analysis was carried out by using antibodies against PP1α (a), PP1δ (b), M130/M133 (c), and PP2A (d).

number of IgM- and IgG-secreting cells (*34, 35*). These B-cell lines showed a variety of morphological features (Fig. 2). WEHI-231, BAL-17, Daudi and CESS cells were round and did not possess any pseudopod, whereas Bcl.1, A20, M12, and SKW6.4 possessed pseudopods of various shapes. It should be noted that the protein levels of PP1δ and M130/M133 in the B-cells with pseudopods were specifically lower than those in the B-cells without pseudopods. In thrombin-activated platelets, both of two potent inhibitors of PP1 and PP2A, okadaic acid and calyculin A, were previously reported to induce extremely long pseudopods which contained an array of microtubules and actin filaments (*36*). Taking these previous results into account, our present data suggest that PP1δ and M130/M133 are involved in the formation of pseudopods.

The aggregation and adhesion are thought to be due to activation of LFA-1, a lymphocyte integrin (*37*). Recently, it was reported that Rho is positively involved in the PMA-induced aggregation of JY cells, a B-lymphoblastoid cell line (*38*). Rho is thought to exert this action by increasing phosphorylation of 20 kDa myosin light chain, MLC (*39*). Conversely, the exotoxin C3 caused *in situ* ADP ribosylation of Rho (*40, 41*) and inactivated it, resulting in a block of the PMA-induced cell aggregation (*42*). It was also reported that GTPγS enhances Ca^{2+}-induced, wortmannin-sensitive phosphorylation of MLC through inhibition of its dephosphorylation. The exotoxin C3 completely abolished the effects of GTPγS. These results indicated that Rho is positively involved in the GTPγS-induced phosphorylation of MLC (*43*). Myosin light chain phosphatase, MLCP, is composed of PP1δ and M130/M133 and is involved in regulation of phosphorylation of MLC (*16*). Our present results demonstrate that PP1δ and M130/M133 are specifically decreased during aggregation of B-cells induced by PMA and that okadaic acid at sufficiently high concentration to inhibit PP1 activity induces cell aggregation. Taken together, our results strongly suggest that PP1δ and M130/M133 are involved in the regulation of pseudopod formation and aggregation of B-cells, both accompanied with re-organization of microtubules and actin filaments.

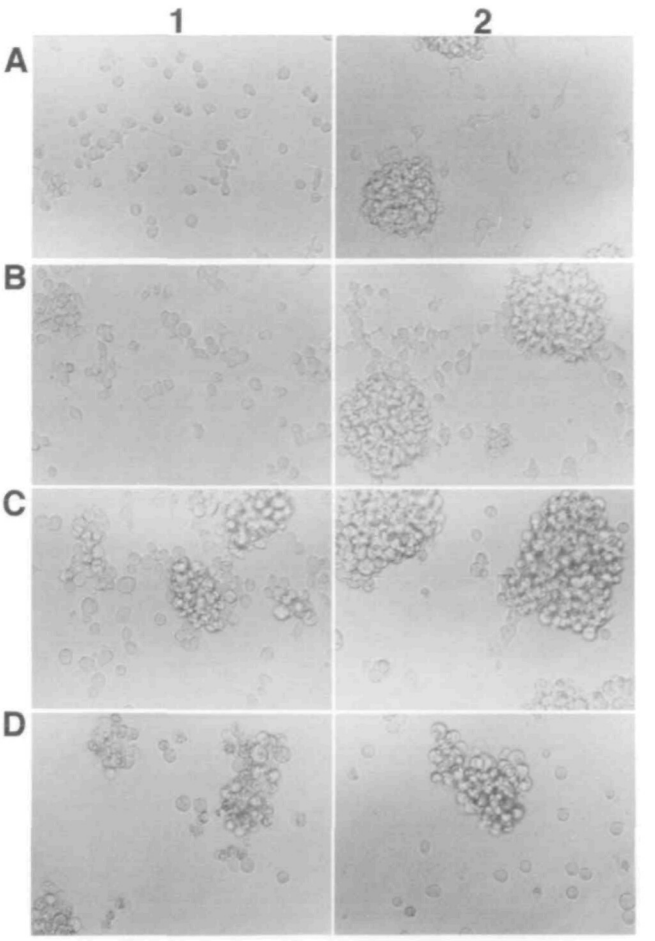

Fig. 9. **Effects of okadaic acid on aggregation of A20 cells.** A20 cells were stimulated by various concentrations of OA without (1) or with (2) 8 ng/ml PMA. Panels: A, 0 ng/ml OA; B, 1 ng/ml OA; C, 10 ng/ml OA; D, 50 ng/ml OA.

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