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A Major Myristylated Substrate of Protein Kinase C and Protein Kinase C Itself Are Differentially Regulated during Murine B- and T-Lymphocyte Development and Activation

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The regulation and expression of protein kinase C (PKC) and phosphomyristin C (PMC) (a principal substrate of PKC which is the major myristylated protein in lymphocyte and glioma lines that express it) in murine B and T lymphocytes were investigated. Both PMC and PKC are differentially regulated during T-cell development. The level of PMC expression is highest in $CD4^-8^-$, intermediate in $CD4^+8^+$, and lowest in $J11d^-$, CD4, or CD8 single-positive thymocytes. PKC is equally expressed by all three thymic populations. In striking contrast to thymocytes, resting peripheral lymph node T cells and T-cell clones express little if any PMC and reduced levels of PKC. Neither PKC nor PMC is significantly induced upon the activation of lymph node T cells: treatment with anti-CD3 antibodies or anti-CD3 and interleukin-2 fails to induce PKC, whereas PMC is not induced by anti-CD3 alone and is only slightly induced by anti-CD3 and interleukin-2. In contrast to the situation with T cells, PMC and PKC are constitutively expressed at moderate levels in mature B cells. PMC is greatly increased in B-cell blasts generated by cross-linking the antigen receptor with anti-immunoglobulin. These results demonstrate that PMC and PKC are differentially regulated during the development and activation of B and T cells, suggesting that cellular events that rely upon PKC and PMC may differ during ontogeny and activation of different lymphocyte subsets.

Protein kinase C (PKC) plays a central role in signal transduction through many different receptors and cell types (34), in which it is assumed to exert its effect through protein phosphorylation. One of the most strikingly phosphorylated proteins in murine B lymphocytes and neural cell lines treated with phorbol esters is phosphomyristin C (PMC). PMC, formerly known as protein 5 (16, 17), appears to be identical with the widely studied 80- to 87-kilodalton (kDa) PKC substrate in neural tissue (2, 38) and fibroblasts (5, 43, 46). The phosphorylation of PMC increases immediately after activation of PKC in lymphocytes (16), fibroblasts (43), and synaptosomes (26) and is stimulated by a number of physiological ligands (2, 5, 17, 22, 43, 46), as well as phorbol esters, potent pharmacological activators of PKC (34). The strength of the correlation between the phosphorylation of PMC and the physiological activation of PKC argues that PMC is an important mediator of PKC activity (2). Intriguingly, the macrophage equivalent of PMC has recently been shown to be myristylated (1), a posttranslational modification found on many transforming proteins (47). We have tentatively named this protein PMC because of its prominent phosphorylation after activating PKC and because, as shown below, it appears to be the major myristylated protein in cells which express it. A recently proposed alternative designation for this protein is myristoylated alanine-rich C-kinase substrate (MARCKS) (P. Blackshear, personal communication).

The involvement of protein kinase C in signal transduction through the antigen receptor in lymphocytes (9, 11, 17, 53) suggests that the expression of PKC and its substrates may influence cellular responses to antigen receptor cross-linking. The response of lymphocytes to antigen receptor occupancy is complex, depending upon the milieu and maturational state of the cells. Thus, antigen or anti-receptor antibodies can paradoxically inhibit or stimulate both growth and differentiation. The diverse responses of lymphocytes to receptor-mediated signaling may reflect alterations in PKC expression or activity, as well as alterations in substrate expression or availability at different stages of maturation and activation. We report here that both PMC and PKC are down-regulated during T-cell development and that neither molecule is substantially induced by treating mature, resting T cells with mitogenic forms of anti-CD3 antibodies, concanavalin A, or phorbol esters and various lymphokines. In contrast, PMC and PKC are expressed at significantly higher levels in B cells than in T cells. Furthermore, the expression of PMC is greatly increased upon cross-linking surface immunoglobulin in B cells, whereas the expression of PKC appears to moderately increase during B-cell mitogenesis. Collectively, the data suggest that the level of expression of PKC and PMC may be important factors in determining the diverse responses of lymphocytes to antigen receptor signaling during different stages of development or activation.

MATERIALS AND METHODS

Cell culture and lysis. Cells were cultured in complete RPMI 1640–10% heat-inactivated fetal calf serum (FCS) as described previously (17). Cell lines were kept in log-phase growth by being split 1:100 every 4 days. When indicated, cycloheximide was included at 0.5 mM. Cells analyzed directly by either two-dimensional (2-D) electrophoresis or immunoblotting were lysed at a density of 10^7 cells 0.25 ml^{-1}

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at 95°C in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris [pH 7.0], 0.4% SDS) and then treated with DNase and RNase (14). Prior to lysis, cell viability was determined by using trypan blue exclusion. If viability was less than 90%, dead cells were removed by Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) centrifugation. In most cases, viabilities were greater than 98%. The relative amount of protein was determined by measuring the A_{280} of cell lysates spectrophotometrically and corrected for absorption due to added nucleases. Cells analyzed by immunoprecipitation were lysed at 10^7 cells 0.25 ml^{-1} as described previously (17), with 0.5% Triton X-100 (no. 28314; Pierce Chemical Co., Rockford, Ill.) instead of 0.5% Nonidet P-40.

Production of antibodies against the 87-kDa PKC substrate from rat brain. The 87-kDa PKC substrate from rat brain was purified as described previously (38). Tryptic and chymotryptic peptides, which will be characterized in detail elsewhere (J. Patel and D. Kligman, manuscript in preparation), were isolated by using reverse-phase high-pressure liquid chromatography. An oligopeptide corresponding to 16 of the 21 amino acids of tryptic peptide 27, having the sequence NH_2-EAAEPEQPEQPEQPAA-COOH, was synthesized by solid-phase methods (32). The purity of the synthetic peptide was demonstrated by its elution as a single peak on reverse-phase high-pressure liquid chromatography and its amino acid composition. A portion of this peptide was coupled to keyhole limpet hemocyanin (H 2133; Sigma Chemical Co., St. Louis, Mo.) at a keyhole limpet hemocyanin-to-peptide weight ratio of 2:1. Rabbits were immunized at multiple sites intradermally with either holoprotein or keyhole limpet hemocyanin-peptide emulsified in complete Freund adjuvant and boosted with emulsions in incomplete Freund adjuvant. Antisera were collected at 10-day intervals following the boost.

Antibodies. Goat antibodies to PKC have been characterized elsewhere (18–20). The anti-CD3 antibody 2C11 (30) was provided by W. Yokoyama, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases. Goat antibodies to mouse immunoglobulin M (IgM) were affinity purified on mouse IgM-Sepharose columns from antiserum provided by Wayne Tsang, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases. Antibodies used in the purification of thymocyte populations include 3.155, a rat monoclonal antibody (MAb) to CD8 (45); LICR.LAU.RL172.3, a rat MAb against CD4 (10); Ly1.2, mouse antibodies to CD5.2 (no. NEI-017; Du Pont, NEN Research Products, Boston, Mass.) (13); goat anti-rat IgG (Organon-Teknika-Cappel); 53-6.72, a rat MAb to CD8 (29); H129.19, a rat MAb to CD4 (40); and J11d, a rat MAb against a surface epitope found on immature thymocyte subpopulations (7).

Cell lines and lymphocyte preparations. Cell lines used include WEHI-231, an $sIgM^+$ murine B lymphoma (41); A20, an $sIgG2a^+$ B lymphoma (25); 2B4, a T-cell hybridoma (44); 829M.1, a clone derived in our laboratory from the $CD4^-8^+$ $CD3^+$ AKR thymoma 829M (42); OE4, a cytotoxic T-lymphocyte clone (50); F1.A.2, a Th1 clone; D10, a Th2 clone (23); and C_6, a rat glioma (4).

Resting lymph node T cells were prepared from adult mesenteric nodes by nylon wool passage, complement-mediated cytolysis of B cells and macrophages, and Percoll gradient centrifugation as described previously (21). T cells were activated in vitro at a density of 10^6 ml^{-1} for 3 days on 2C11-coated plates, with phorbol myristic acetate (PMA) (10 ng ml^{-1}) and either recombinant human interleukin-2 (IL-2) (100 U ml^{-1}) or murine IL-4 (1,000 U ml^{-1}), or with concanavalin A (5 μg ml^{-1}). Plates were coated for 2 h with 2C11 (10 μg ml^{-1} in 0.1 M $NaHCO_3$) and blocked with 10% FCS.

Double-negative ($CD4^-8^-$), Ly1 (CD5)-low thymocytes were prepared from C57BL/6 thymocytes by two sequential eliminations with 3.155, LICR.LAU.RL172.3, and a 1:12 dilution of Lox-Tox-M rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Cells expressing high levels of CD5 were lysed with Ly1.2 antibodies and complement. Viable cells, isolated by centrifugation through Ficoll-Paque (Pharmacia), were greater than 98% pure when analyzed by flow cytometry with appropriate fluorescein isothiocyanate-labeled antibodies.

Double-positive ($CD4^+8^+$) thymocytes were enriched by three sequential positive selections on antibody-coated plates. $CD8^+$ thymocytes were isolated by adherence to plates coated with goat anti-rat IgG and 53-6.72. Cells were incubated on the coated plates for 30 min at 4°C. Nonadherent cells were removed by gentle washing and discarded. $CD4^+$ cells were enriched from the $CD8^+$ cells by two rounds of adherence to plates coated with anti-rat IgG and H129.19. After the third selection, more than 98% of the adherent cells were $CD4^+8^+$ by flow cytometry analysis.

$J11d^-$ thymocytes were prepared by negative selection with J11d and rabbit complement. More than 90% of the viable cells, collected after Ficoll-Paque centrifugation, were CD3 dense and either $CD4^+8^-$ or $CD4^-8^+$ by flow cytometry analysis.

Dense resting splenic B-cell populations were prepared by cytolytic elimination of T cells, plastic adherence or passage through G-10 columns to remove adherent cells, and Percoll (Pharmacia) gradient centrifugation as described previously (12). B cells were stimulated in vitro at a density of 10^6 cells ml^{-1} for 3 days with 25 μg of lipopolysaccharide (LPS) [no. 302; List Biological Laboratories, Campbell, Calif.; *Escherichia coli* K-12, D311m4 (Re)] ml^{-1} or 50 μg of affinity-purified goat anti-mouse IgM ml^{-1}.

Lymphocyte populations enriched for blasts were purified by discontinuous Percoll (no. 17-0891-01; Pharmacia) gradient centrifugation and collection of bands at the 50 to 60% interface.

Immunoprecipitation. [^{32}P]PMC was immunoprecipitated by incubating 20 μl of protein A-Sepharose (no. P-3391; Sigma) for 30 min with 20 μl of rabbit antiserum, washing the beads three times with rinse buffer (RB) (borate-buffered saline, 0.5% Triton X-100, 0.1% Tween-20), incubating the loaded beads with 0.1 ml of ^{32}P-labeled Triton X-100 lysate on an Eppendorf mixer (no. 5432) at 4°C for 2 to 4 h, rinsing them at least five times with RB, and desorbing them directly in isoelectric focusing sample buffer (35). When indicated, lysates were cleared by incubation for 4 h with protein A-Sepharose loaded with preimmune rabbit serum prior to specific immunoprecipitation.

Electrophoresis and immunoblotting. In experiments in which one-dimensional (1-D) SDS-polyacrylamide gel electrophoresis (27) was used, 0.07 optical density (280 nm) unit of cellular protein was electrophoresed per lane. Two-dimensional (2-D) electrophoresis was performed as described previously (17), except that isoelectric focusing was performed with a 3:1 ampholine mixture of pH 4 to 8 and 3 to 10 and that gels were equilibrated for 1.5 h before being subjected to SDS-polyacrylamide gel electrophoresis. The pH 4 to 8 ampholines (Resolyte; no. 44340) were from BDH Chemicals Ltd., Poole, England. The gels were dried under vacuum and autoradiographed by exposure to X-Omat AR film (no. 1651512; Eastman Kodak Co., Rochester, N.Y.)

with Cronex Lightning-Plus (Du Pont Co., Wilmington, Del.) screens. The gels were fluorographed by impregnation with Autofluor (no. LS-315; National Diagnostics Inc., Manville, N.J.) before being dried and subjected to autoradiography.

After 1-D SDS-polyacrylamide gel electrophoresis, proteins were blotted onto nitrocellulose (0.1-μm pore size; no. PH79; Schleicher & Schuell, Inc., Keene, N.H.) as described previously (16). Relative amounts of protein were estimated by densitometry of autoradiograms of experimental samples compared with standard dilution curves. ^{14}C-methylated protein molecular weight standards (no. CFA.626) were from Amersham Corp., Arlington Heights, Ill.).

Radiolabeling of cells in culture. For labeling with [^{3}H]lysine, cells were grown at 10^7 cells ml^{-1} for 4 h at 37°C in lysine-deficient RPMI 1640 supplemented with 250 μCi of L-[4,5-^{3}H]lysine (no. TRK.752; Amersham) ml^{-1} and 5% dialyzed FCS. The cells were labeled with ^{32}P by being cultured at 10^7 cells ml^{-1} for 1 h at 37°C in PO_4^{3-}-deficient medium supplemented with 500 μCi of $^{32}P_i$ (ICN Pharmaceuticals Inc., Irvine, Calif.) ml^{-1} and 5% dialyzed FCS (17). Unless otherwise indicated, the cells were labeled with ^{3}H-labeled fatty acids by being grown at 10^7 cells ml^{-1} for 2 h at 37°C in RPMI 1640 supplemented with 250 μCi of [9,10-^{3}H]myristic acid or [9,10-^{3}H]palmitic acid (Dupont, NEN Research Products; NET-830 and NET-043, respectively) ml^{-1} and 1% FCS.

PKC enzymatic assay. PKC activity was determined by measuring histone IIIS phosphorylation as previously described (20), with the following modifications. Cells were lysed at 4°C by sonication in 0.5% Nonidet P-40–20 mM Tris hydrochloride (pH 7.5)–1 mM dithiothreitol–0.5 mM EDTA–0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–10% glycerol–2 μg of leupeptin, pepstatin, chymostatin, and aprotinin ml^{-1}–0.5 mM phenylmethylsulfonyl fluoride. Lysates were spun at 45,000 rpm for 1 h, and the supernatants were collected for the PKC assay. Because crude cellular protein fractions were assayed, the total amount of protein added to the reaction mixture was increased to either 8.15 or 10.75 μg. Additionally, the Nonidet P-40, phosphatidylserine, and dioleoylglycerol concentrations were increased to 1.11, 0.216, and 0.058 mM, respectively. The PKC activity was calculated by subtracting the basal kinase activity, measured with 1 mM EGTA and without added lipid, from the total activity, measured with 0.4 mM $CaCl_2$ and lipid. The PKC activity is expressed as the number of nanomoles of phosphate from ATP incorporated into substrate per milligram of protein per minute at 30°C. The assays were performed in triplicate, and the reported activities represent the arithmetic mean and standard deviation of two independent experiments.

Protein phosphorylation in cells treated with PMA or 2C11. Cells were treated with PMA (0.32 μM) at 37°C for the times indicated in the text. 829M.1 cells, at 10^7 cells ml^{-1} and preloaded with $^{32}P_i$, were incubated with 2C11 (10 μg ml^{-1}) at 37°C for 10 min, centrifuged, and suspended in goat anti-mouse IgG (50 μg ml^{-1}) at 37°C for an additional 20 min.

RESULTS

PMC cross-reacts with antibodies against the 87-kDa PKC substrate found in rat brains. The phosphorylation of PMC, an acidic phosphoprotein with an apparent molecular mass of 65 to 70 kDa, dramatically increases after B cells are treated with anti-immunoglobulin or with phorbol esters (17). The isoelectric point and pattern of phosphorylation of

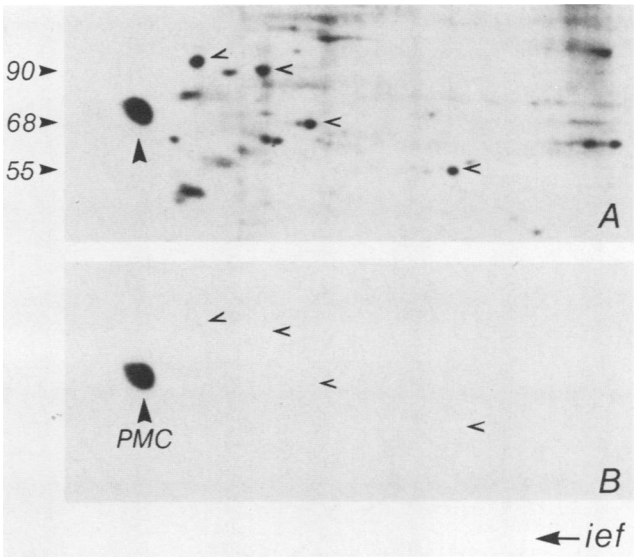

FIG. 1. Immunoprecipitation of [^{32}P]PMC from the mouse B-lymphoma line WEHI-231 with antibodies against the 87-kDa protein isolated from the rat brain. The molecular weight (in thousands), indicated on the left margin of panel A, was determined from the location of phosphoproteins of known molecular weights (17). PMC (▲) has an average isoelectric point of 4.7 to 5.4 (3, 5, 17). (A) WEHI-231 cells were labeled with $^{32}P_i$, treated with PMA for 20 min, and lysed in 0.5% Triton X-100. A sample of the lysate equivalent to 4 × 10^5 cells was analyzed by 2-D electrophoresis followed by autoradiography. (B) The lysate of ^{32}P-labeled WEHI-231 cells was precleared, immunoprecipitated with antibodies to the 87-kDa protein, and analyzed as in panel A. Abbreviation: ief, isoelectric focusing.

PMC suggested that it may be the lymphocytic equivalent of the 80- to 87-kDa protein kinase C (PKC) substrate found in brain and other tissues from many species (3, 5, 43, 46). Furthermore, we have previously shown that rat glioma and neuroblastoma cell lines express a phosphoprotein that behaves similarly to PMC from mouse lymphocytes (17). The relationship between the 87-kDa protein and PMC was examined immunologically. Antibodies against the rat 87-kDa holoprotein immunoprecipitated [^{32}P]PMC from the lysate of WEHI-231 cells (Fig. 1), as did antibodies to a peptide from the 87-kDa protein (see Fig. 8), strongly suggesting that PMC and the 80- to 87-kDa molecule are closely related if not identical. Control immunoprecipitations with the sera from preimmune rabbits failed to precipitate PMC (data not shown). Although we have consistently observed PMC to have an apparent molecular mass of 65 to 70 kDa (17) (see Fig. 6), estimates of the molecular mass reported in the literature for the 87-kDa protein vary from 65 to 87 kDa. Such variation has been previously explained as reflecting differences in electrophoretic conditions, an unusually elongated and asymmetric tertiary structure, post-translational modifications, and species differences (1, 2, 5).

PMC is the dominant myristylated protein in lymphocytes and the glioma line C_6. Since it has recently been reported that the macrophage equivalent of PMC is myristylated (1), the myristylation of PMC in two lymphocyte lines and a glioma line was investigated. WEHI-231, 829M.1, and C_6 cells were biosynthetically labeled with [^{3}H]myristic acid, lysed, and analyzed by 2-D electrophoresis. PMC is the dominant labeled species in all three cell types (Fig. 2A to C). In contrast, labeling 829M.1 cells with [^{3}H]lysine for 4 h

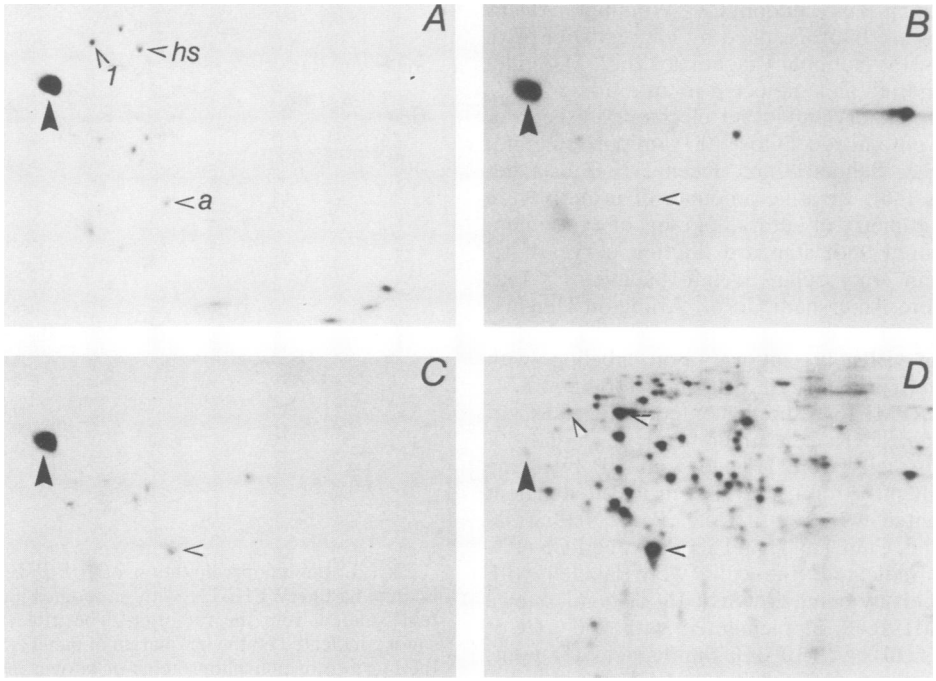

FIG. 2. Myristylation of PMC in WEHI-231, 829M.1, and C6 cells. Cells were labeled with [^{3}H]myristic acid (A to C) or [^{3}H]lysine (D) and lysed in SDS. Then, 2×10^6 cell equivalents of [^{3}H]myristate-labeled cells and 4×10^5 equivalents of [^{3}H]lysine labeled cells were subjected to 2-D electrophoresis. Fluorography of [^{3}H]myristate- and [^{3}H]lysine-labeled samples was performed for 12 and 3 days, respectively. The electrophoretic position of PMC is indicated (▲). The range of isoelectric points varies from approximately 4.7 on the left to 7.9 on the right of each gel. Cellular proteins which serve as internal molecular mass markers include the 90-kDa heat shock protein (M_r 90×10^3), marked *hs* in panel A, and actin (M_r 43×10^3), marked *a* in panel A. Cell lines include WEHI-231 (A), 829M.1 (B), C6 (C), and 829M.1 (D).

revealed that relatively little PMC was synthesized during this period (compare Fig. 2B and D). Since myristylation is tightly coupled to translation (8, 31), these results together suggest that PMC is an extraordinarily effective substrate for myristyl transferase. Its [^{3}H]myristate-derived label is insensitive to hydroxylamine hydrolysis, supporting the conclusion that PMC is myristylated (data not shown). A number of proteins are poorly and variably labeled after being cultured with [^{3}H]myristic acid, including the 90-kDa heat shock protein, tubulin isomers, and actin (Fig. 2A). Although the nature of the labeling of these minor species was not proven, preliminary evidence suggests that they are labeled via radioactive amino acids, a suggestion consistent with previous reports of the interconversion of [^{3}H]myristate and amino acids (51).

It has been proposed that after PMC is phosphorylated in the phorbol response of macrophages, much of it may be demyristylated within 30 min (1). To examine the relationship between myristylation and phosphorylation in lymphocytes, we labeled WEHI-231 cells with [^{3}H]myristate. After the labeling had continued for 1.5 h, cycloheximide was added to the culture, followed by PMA for 45 min. 2-D electrophoresis followed by autoradiography revealed that [^{3}H]myristate-labeled PMC migrated with a more acidic isoelectric point following treatment with PMA (compare Fig. 3A and B). To compare [^{32}P]PMC with [^{3}H]myristate-labeled PMC, WEHI-231 cells were labeled with $^{32}P_i$, treated with PMA, and analyzed by 2-D electrophoresis. Treatment with PMA increases the amount of ^{32}P associated with PMC and induces PMC to migrate with a more acidic isoelectric point (compare Fig. 3C and D). Since PMC has been shown to be phosphorylated on additional sites after treatment with

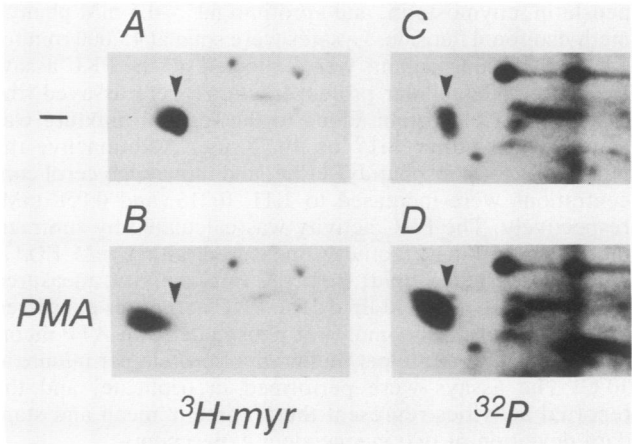

FIG. 3. Effect of phorbol ester treatment upon the isoelectric behavior of myristylated and phosphorylated forms of PMC in WEHI-231 cells. Cellular lysates were analyzed by 2-D electrophoresis, with more acidic proteins focusing on the left. The electrophoretic position of PMC is indicated (▼). (A and B) Cells were labeled with [^{3}H]myristic acid and treated with cycloheximide. Five minutes later, PMA was added to half the cells for 45 min, which were lysed in hot SDS, electrophoresed, and subjected to fluorography for 12 days. Densitometry revealed that the label associated with PMC in the PMA-treated cells (B) was equivalent to that in untreated cells (A) and that more than 90% of the labeled PMC migrated with a more acidic isoelectric point after treatment with PMA. (C and D) Cells were labeled with $^{32}P_i$ and treated with cycloheximide. Five minutes later, PMA was added to half of the cells for 45 min, which were lysed in hot SDS, electrophoresed, and subjected to autoradiography. (C) Untreated cells. (D) PMA-treated cells.

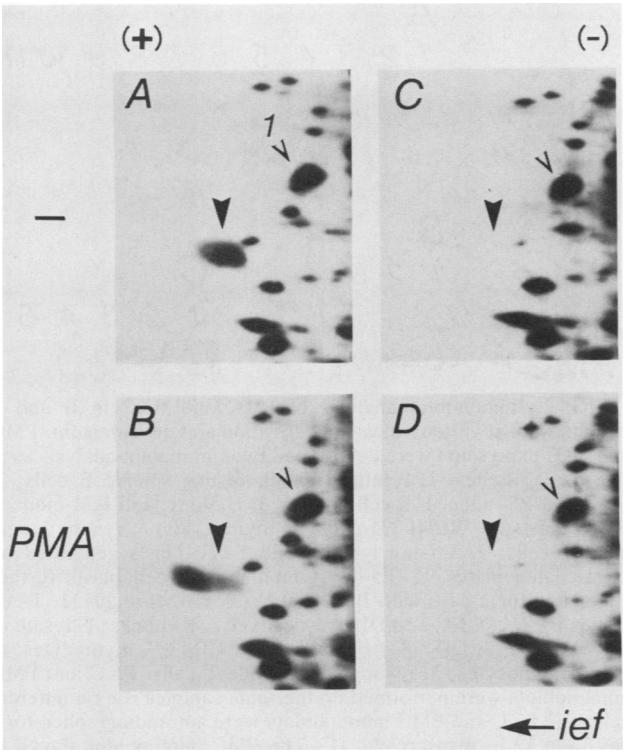

FIG. 4. Comparison of the expression of [^{3}H]lysine-labeled PMC in WEHI-231 B-lymphoma cells and in the T-hybridoma line 2B4. Cells were labeled with [^{3}H]lysine and split into two aliquots. One aliquot was untreated and the other was treated with PMA for 20 min before being subjected to lysis, 2-D electrophoresis, and autoradiography. (A) WEHI-231 without PMA. (B) WEHI-231 with PMA. (C) 2B4 without PMA. (D) 2B4 with PMA. The position of PMC (▼) and the location of the constitutive phosphoprotein 1 (V) are shown. The apparent molecular weight of phosphoprotein 1 is 95 $\times 10^3$, whereas that of PMC is 65 $\times 10^3$ to 70 $\times 10^3$ (17) (see Fig. 6). Abbreviation: ief, isoelectric focusing.

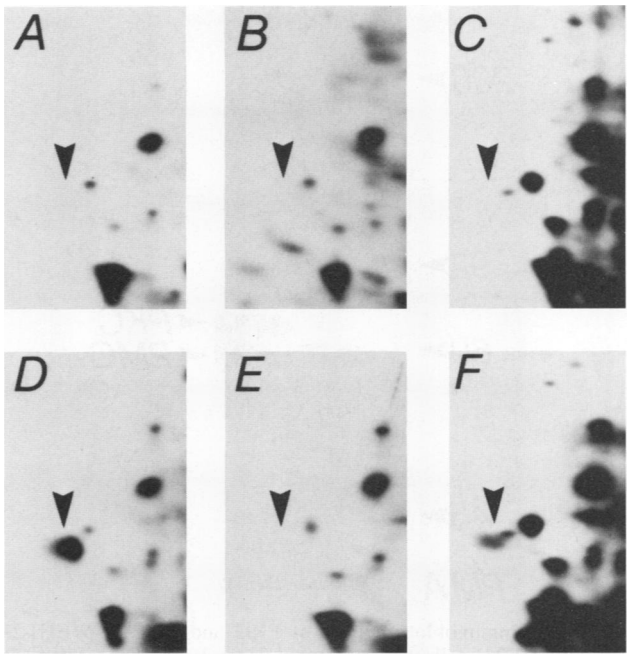

FIG. 5. Expression of PMC following cross-linking of the antigen receptor in splenic B cells (A and D), lymph node T cells (B and E), and A20 cells (C and F). Cells were biosynthetically labeled with [^{3}H]lysine for 4 h, lysed, and analyzed by 2-D electrophoresis. The electrophoretic position of PMC is indicated (▼). (A) Resting splenic B cells; (D) B-cell blasts generated by anti-IgM (50 μg ml^{-1}) treatment for 3 days; (B) resting lymph node T cells; (E) T-cell blasts generated by treatment with plate-bound 2C11 for 3 days; (C) A20 B lymphoma cells; (F) A20 cells treated with soluble anti-IgG (50 μg ml^{-1}) for 24 h.

PMA (17), the mobility shifts observed for both [^{32}P]PMC and [^{3}H]PMC appear to be due to the increased net negative charge donated by the newly phosphorylated sites. We conclude that virtually all of the myristylated protein is phosphorylated by PKC. Additionally, since densitometric analysis reveals that little of the [^{3}H]PMC is lost by up to 45 min after the addition of PMA and that near-stoichiometric phosphorylation of PMC is complete after 20 min of treatment with PMA (16), we conclude that the phosphorylation of PMC apparently does not lead to its demyristylation in WEHI-231 cells.

PMC is expressed in the B-lymphoma line WEHI-231 but not in the T-cell hybridoma 2B4. The phosphorylation of PMC dramatically increases after the activation of PKC in B cells but not in lymph node T cells or in T-cell hybridomas (17). The lack of phosphorylated PMC in T cells could be due to either a deficiency of expression or, if it is expressed, a deficiency in its phosphorylation. To distinguish between these possibilities, we investigated the synthesis of PMC by biosynthetic labeling with radioactive amino acids. The B-cell line WEHI-231 and the T-cell line 2B4 were labeled with [^{3}H]lysine. Lysine was chosen because it is unusually abundant in the amino acid composition of the 87-kDa PKC substrate from the rat brain (38). Half of the cells were treated with PMA before being subjected to lysis in SDS, 2-D electrophoresis, and autoradiography. Comparison of the autoradiograms reveals that, in contrast to WEHI-231, 2B4 does not express PMC (Fig. 4). Additionally, treatment of WEHI-231 with PMA causes an acidic shift in the isoelectric point of PMC, suggesting that most of the protein is phosphorylated by PKC.

PMC and PKC are differentially expressed in B and T cells. Cross-linking of lymphocyte antigen receptors can induce important changes in cellular physiology, including differentiation, effector functions, and growth regulation. Because PMC may play an important role in signal transduction through receptors which are linked to PKC, the influence of antigen-receptor cross-linking upon PMC expression was investigated. Resting splenic B cells and lymph node T cells were stimulated with anti-IgM and anti-CD3, respectively. Resting cells at time zero and blasts at 3.5 days were biosynthetically labeled with [^{3}H]lysine, lysed, and analyzed by 2-D electrophoresis. PMC was greatly enhanced in B-cell blasts generated with anti-IgM (Fig. 5A and D), whereas it was undetectable in T-cell blasts generated with anti-CD3 (Fig. 5B and E).

It could be argued that the apparent induction of PMC in splenic B cells by treatment with anti-immunoglobulin may be due to selection either for PMC$^+$ or against PMC$^-$ subsets rather than up-regulation of PMC expression. To investigate the influence of anti-immunoglobulin on PMC expression at the clonal level, we investigated PMC expression in the sIgG2a$^+$ B-lymphoma line A20 (Fig. 5C and F). PMC became detectable after 24 h of treating A20 with anti-IgG, demonstrating that cross-linking surface IgG in B cells can induce the expression of PMC at the clonal level.

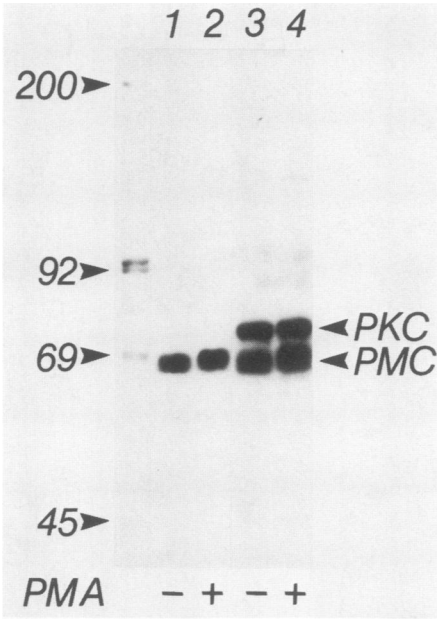

FIG. 6. Immunoblot analysis of PKC and PMC in WEHI-231 cells. WEHI-231 cells were untreated (lanes 1 and 3) or treated with 200 ng of PMA ml^{-1} (lanes 2 and 4) 20 min prior to lysis in hot SDS. PMC and PKC were determined by sequential immunoblot analysis with anti-PMC peptide antibodies (lanes 1 and 2) followed by anti-PKC antibodies (lanes 3 and 4). The electrophoretic positions and molecular weights (in thousands) of ^{14}C-methylated myosin (200 × 10^3), phosphorylase *b* (92 × 10^3), bovine serum albumin (69 × 10^3), and ovalbumin (45 × 10^3) are marked on the left margin.

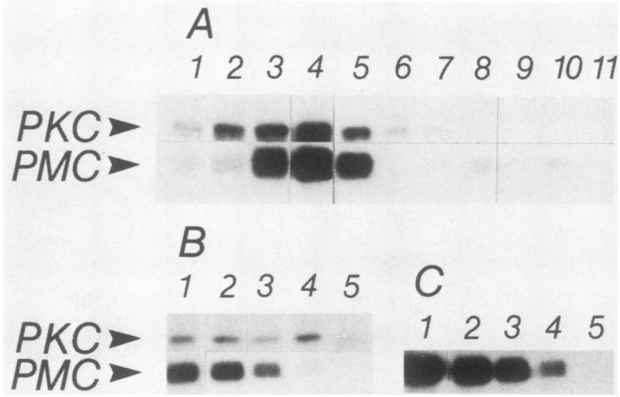

FIG. 7. Immunoblot analysis of PMC and PKC in B and T lymphocytes at various stages of activation and development. PMC and PKC expression were determined by immunoblot analysis as in Fig. 5. (A) Lanes: 1, lysate of small, resting splenic B cells; 2, 3.5-day LPS-induced B-cell blasts; 3, 3.5-day anti-IgM-induced B-cell blasts; 4, WEHI-231 cells; 5, thymocytes; 6, resting lymph node T cells; 7, 3.5-day lymph node T-cell blasts generated on 2C11-coated plates; 8, 3.5-day lymph node T-cell blasts further stimulated for 2 days with IL-2 (100 U ml^{-1}); 9 through 11, T-cell clones F1.A.2, OE4, and D10, respectively. (B) Lanes: 1, lysate of 829M.1 cells; 2, $CD4^-8^-$ thymocytes; 3, $CD4^+8^+$ thymocytes; 4, $J11d^-$ thymocytes; 5, resting lymph node T cells. PKC and PMC immunoblots were performed on the same samples run on different gels. The PKC and PMC immunoblots were autoradiographed for 3 days and 12 h, respectively. (C) The PMC immunoblot shown in panel B was reexposed for 2 days.

The coexpression of PMC and PKC was examined by using immunoblot analysis. PMC was visualized with rabbit anti-peptide antibodies, and PKC was visualized with a polyvalent goat antiserum which recognizes PKC types I, II, and III (18, 19). The immunoblot assay for PMC in WEHI-231 cells is specific for a single species with an apparent molecular mass of 65 to 70 kDa (Fig. 6). Treatment of WEHI-231 with PMA (0.32 μM) 20 min prior to lysis, a protocol known to induce near-stoichiometric phosphorylation of PMC (17) (Fig. 4), causes a slight increase in the apparent molecular mass of PMC, presumably owing to phosphorylation. The PMC immunoblot was subsequently probed for PKC, revealing a major band at 77 kDa with little detectable difference after treatment with PMA (Fig. 6, lanes 3 and 4).

The possibility that PMC is developmentally regulated in lymphocytes was investigated by using stimulated and unstimulated splenic B cells and lymph node T cells, B- and T-cell lines representative of different developmental stages, and a series of thymic T cells at various stages of maturation. PMC is expressed at modest levels in resting splenic B cells, is slightly increased in LPS blasts, and is greatly increased in anti-IgM blasts (Fig. 7A, lanes 1 to 3). WEHI-231 cells constitutively express very high levels of PMC (lane 4), similar to that seen in anti-immunoglobulin-induced B-cell blasts. In contrast to B cells, neither resting lymph node T cells nor T-cell blasts induced by 2C11 expressed detectable PMC (lanes 6 and 7), confirming the observation from 2-D analysis of [^3H]lysine-labeled lysates that 2C11 fails to induce the expression of PMC in T-cell blasts (Fig. 5). Additionally, PMC was not induced in lymph node T cells by strongly mitogenic doses of concanavalin A or PMA plus IL-2, IL-4, or soluble 2C11 (data not shown). Modest levels of PMC were induced by treatment with 2C11 and IL-2 (Fig. 7A, lane 8). Of three T-cell clones examined, two expressed undetectable levels of PMC (lanes 9 and 11) and one (lane 10) expressed levels equivalent to that seen in resting B cells and T-cell blasts induced by 2C11 and IL-2. In striking contrast to lymph node T cells, thymocytes express elevated levels of PMC (lanes 5 and 6).

To determine whether PMC is developmentally regulated during thymocyte development, we examined the level of expression of PMC in thymic subpopulations. PMC expression appears to be inversely proportional to the stage of maturation within the thymus, with its order of expression being $CD4^-8^- > CD4^+8^+ > J11d^-$ (Fig. 7B, lanes 2, 3, and 4, respectively). The thymoma line 829M.1 expresses substantial amounts of PMC. Although no PMC was evident in $J11d^-$ thymocytes after a short fluorographic exposure (Fig. 7B, lane 4), a longer exposure revealed that PMC was detectable in this subpopulation (Fig. 7C, lane 4). It cannot be ruled out that this represents contamination with cells of less mature phenotypes. By contrast, no PMC was detected in lymph node T cells, even after the longer exposure (Fig. 7C, lane 5).

As with PMC, PKC appears to be developmentally regulated in T cells. Immunoblotting analysis revealed that whole thymocytes and thymocyte subsets expressed significantly higher levels of PKC than lymph node T cells did (Fig. 7A and B). 829M.1 thymoma cells express levels of PKC similar to those observed in normal thymocytes (Fig. 7B, lane 1 versus lanes 2 to 4), consistent with its derivation from a thymic precursor. All T-cell clones and hybridomas examined express even less PKC than lymph node T cells (Fig. 7A, lanes 9 to 11 versus lane 6).

The regulation of PKC expression during activation differs between B and T cells. Mitogenic doses of either LPS or anti-immunoglobulin can induce PKC in resting B cells (Fig.

7A, lanes 2 and 3), whereas mitogenic doses of 2C11 or 2C11 plus IL-2 consistently fail to up-regulate PKC expression in lymph node T cells (lanes 7 and 8). Additionally, concanavalin A failed to induce PKC in T cells (data not shown). It should be noted that the background level of PKC in dense splenic B cells can vary among experiments, presumably owing to preactivation of the B cells in vivo. In B cells with high PKC backgrounds, treatment with anti-IgM or LPS has little effect on PKC expression (data not shown).

The apparent difference in the expression of PKC between B and T cells was unexpected. To determine whether these differences in PKC expression as measured by immunoblotting reflect differences in PKC activity, PKC enzyme assays were performed on B-cell blasts generated by culture with anti-IgM, T-cell blasts generated by culture on 2C11-coated plates, and the T-cell hybridoma 2B4. The B-cell blasts expressed the most PKC activity (4.15 ± 0.33 nmol mg^{-1} min^{-1}), the T-cell blasts expressed 3.1-fold less than the B-cell blasts (1.33 ± 0.13 nmol mg^{-1} min^{-1}), and the 2B4 cells expressed 17-fold less than the B-cell blasts (0.24 ± 0.11 nmol mg^{-1} min^{-1}). These data support the observations from immunoblotting (Fig. 7) that murine T cells, especially T-cell lines and hybridomas, appear to express significantly less PKC than murine B cells do.

PMC is an apparent substrate of PKC in the thymoma line 829M.1. Since we have previously shown that the phosphorylation of PMC increases after cross-linking of the antigen receptor and in the phorbol response of B cells (17), we examined the phosphorylation status of PMC in a T-lineage cell line that expresses it. 829M.1 cells were labeled with $^{32}P_i$ and treated with either PMA or anti-CD3. PMC was immunoprecipitated with antipeptide antibodies and then analyzed by 2-D electrophoresis. PMA stimulates a 6-fold increase in the amount of ^{32}P associated with PMC, whereas cross-linking the CD3 complex with 2C11 stimulates a small increase, of approximately 1.4-fold, in the ^{32}P associated with PMC (Fig. 8C). The phosphorylation of PMC in the response to phorbol esters suggests that PMC is a cellular substrate of PKC in T cells which express it. Furthermore, the small but reproducible increase in the phosphorylation of PMC following treatment with 2C11 suggests that cross-linking of the antigen receptor leads to a limited activation of PKC. The low level of phosphorylation induced by 2C11 relative to PMA is consistent with the observation that anti-CD3 antibodies induce a transient, reversible activation of PKC (28, 33), whereas PMA is a potent, irreversible activator of this enzyme.

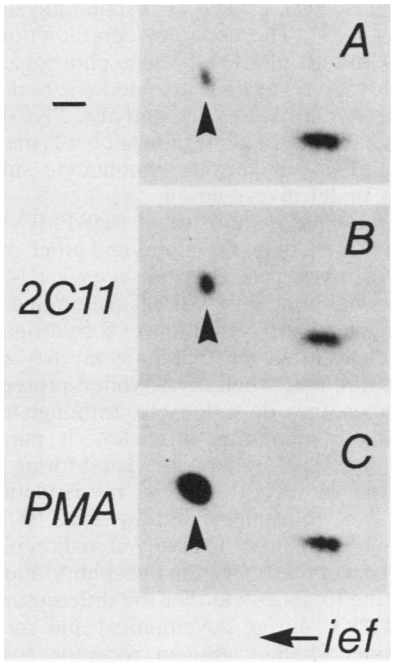

FIG. 8. Phosphorylation of PMC in the thymoma line 829M.1 following treatment with PMA or 2C11. 829M.1 cells were labeled with $^{32}P_i$ and then left untreated (A), or treated with 2C11 (B) or PMA (C), and lysed in 0.5% Triton X-100. PMC was immunoprecipitated with antipeptide antibodies, and the immunoprecipitate was analyzed by 2-D electrophoresis. The location of PMC is indicated (▲). A nonspecific, uninduced phosphoprotein which was coprecipitated in all groups (lower right corner of each frame) was used as a reference spot during densitometric analysis. The relative densitometric signals from PMC were 1.0, 1.4, and 6.2 for the untreated, anti-CD3 treated, and phorbol-treated groups, respectively.

DISCUSSION

PKC is activated upon cross-linking of the antigen receptor of both B and T cells and appears to be important in the receptor-driven regulation of lymphocyte activation, differentiation, and clonal expansion (9, 11, 53). Paradoxically, cross-linking of the antigen receptor of lymphocytes can also suppress the target cells, depending upon environmental factors and the maturational state of the cell. During T-cell development, immature thymocytes expressing newly rearranged antigen receptors are thought to undergo both positive and negative selection driven by the interaction of the antigen receptor with nominal antigen, accessory surface molecules, and soluble factors (52). It has recently been demonstrated that anti-CD3 antibodies induce programmed cell death in a subpopulation of immature thymocytes (49), whereas the same antibodies activate mature T lymphocytes, including lymphokine production and cell division (6). B-lymphocyte responses to receptor-mediated signaling also vary during maturation and activation. Although a fraction of B cells from adult animals are polyclonally activated by anti-immunoglobulin (37), B-cell counterparts from young animals respond poorly to the cross-linking of surface immunoglobulin (48). Antibodies to IgM inhibit the differentiation of neonatal and adult LPS-stimulated B cells into plasma cells, but are 300-fold more effective at inhibiting neonatal B cells than adult B cells (24). The in vitro response of mature B cells to anti-IgM includes both negative and positive components. It is estimated that 50% of the surface IgM^+ cells are eliminated during the first 2 days in culture. Of the remaining cells, many appear to undergo one round of division followed by growth arrest and cell death. Although the details of these diverse lymphocyte responses to antigen receptor cross-linking are not known, it is possible that the differential expression of PKC and its substrates helps to determine the nature of these responses.

The level of PKC expression unexpectedly differs among B cells, thymocytes, and T cells. Thymocytes and B-cell blasts contain more PKC than T-cell blasts do, whereas PKC appears to increase during B-cell but not T-cell mitogenesis. The unexpected differences in PKC expression by B cells, thymocytes, and T cells suggest that this class of enzymes may play distinctive roles in these cell types.

Although many of the T-cell clones and hybridomas which we have examined express relatively little PKC (Fig. 7), they can acutely phosphorylate apparent PKC substrates in the

phorbol response (17), as well as respond physiologically to phorbol esters (53). The apparent contradiction that these cells express little PKC yet respond to phorbol esters may be explained if low levels of PKC are adequate to produce these responses or if phorbol esters activate PKC-independent effector mechanisms. The significance of the differential expression of PKC in various lymphocyte subsets is unknown but is under investigation.

Although we have demonstrated that PMC is the major myristylated protein in lymphocytes and other cell types, the significance of myristylation is not yet clear (47). Initially, myristylation was thought to direct membrane insertion of the modified proteins by addition of a hydrophobic tail. It has become clear, however, that many myristylated proteins are cytosolic and that when myristylated proteins associate with membranes, they do so loosely. Although myristylation does not dictate membrane insertion, it may determine intracellular targeting. Only myristylated forms of $p60^{src}$ are phosphorylated by PKC, whereas myristylation-defective mutants are not phosphorylated by PKC (8). Thus, the myristylation of PMC may be involved in directing it into the proper cellular compartment for phosphorylation by PKC.

It is tempting to speculate that the differential expression of PMC and PKC during development and activation may help determine whether antigen receptor engagement is stimulatory or inhibitory. During T-cell development in the thymus, PMC is expressed at high levels in immature thymocytes and at low levels in thymocytes with mature phenotypes. Thus, PMC appears to be enriched in thymic subpopulations in which positive and negative selection are thought to occur. In contrast to the differential expression of PMC, PKC appears to be expressed at equivalent levels throughout all stages of thymic development. Although we have not yet determined whether natural populations of pre- or pro-B cells have enhanced the expression of PMC or PKC, we have observed that B-cell blasts induced by cross-linking of the antigen receptor have little proliferative potential and express greatly elevated levels of PMC. Such cells undergo one or possibly two rounds of cell division before growth arrest and cell death ensue. In contrast, LPS blasts do not express elevated levels of PMC, and they have the potential to undergo many cycles of cell division. Thus, the induction of PMC in B cells correlates with the inhibition of growth produced by treatment with anti-immunoglobulin. This correlation is strengthened by the observations that IL-4 both reduces the level of PMC induced by treatment of resting B cells with anti-IgM (P. V. Hornbeck, unpublished observation) and relieves the inhibition induced by anti-immunoglobulin (36, 39). The possibility that PMC is associated with growth regulation in nonlymphoid cells has been raised by the observation that the loss of PMC expression correlates with tumor progression in JB-6 epidermal cells (15). Although the function of PMC remains a mystery, these data collectively suggest that the differential expression of PKC and its substrates at different stages of development and activation may contribute to the diverse responses of lymphocytes to antigen receptor cross-linking. Furthermore, the striking phosphorylation of PMC by PKC, as well as its unique regulation and distribution, argues that PMC plays an important role in determining the effects of PKC activation in lymphocytes.

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ADDENDUM IN PROOF

The complete amino acid sequence of the MARCKS protein, the putative bovine homolog of murine PMC, has recently been published (D. J. Stumpo, J. M. Graff, K. A. Albert, P. Greengard, and P. J. Blackshear, Proc. Natl. Acad. Sci. USA **86**:4012–4016, 1989). The relatedness of rat PMC to the bovine MARCKS was examined by comparing the sequences of PMC peptides prepared by J. Patel and D. Kligman with the sequence of the MARCKS protein. The sequence of PMC peptide 33, NH_2-FSFK-COOH, is present in the MARCKS protein. The sequence of tryptic peptide 21 from rat PMC (NH_2-GEAAAERPGEAAVAS?PSK?Y-COOH) is identical at 17 out of 19 residues found in the MARCKS protein between positions 12 and 32. Although the sequences of other peptides from rat PMC are not found within the MARCKS protein, the strong sequence homologies observed between certain regions of rat PMC and the bovine MARCKS protein argue that these proteins are closely related.

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