Effects of Phorbol Esters on an Interleukin-3–Dependent Cell Line

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FDC-P1 is an interleukin-3 (IL-3)–dependent cell line that ceases to proliferate in the absence of IL-3. We have isolated variant cell lines from FDC-P1 that grow in response to phorbol myristate acetate (PMA). These variant cell lines (FD/PMA) have maintained their PMA-dependency for over 1 year. Lymphokine gene expression, which would support growth, was not detected in FD/PMA lines. FD/PMA lines had a different cell surface phenotype than the parental line. Mac-1, Mac-2, and Mac-3 were readily detected on the cell surface of FD/PMA lines; however, these antigens were not detected on FDC-P1. Because protein kinase C (PKC) activation may mediate PMA effects, we examined this kinase. PKC activity quantitated by 

\[ ^{32}P \]

incorporation into histone was increased in FDC-P1 as compared with FD/PMA cultured in IL-3. Moreover, PKC activity was undetectable in FD/PMA lines cultured in PMA. Using Western blotting, immunoreactive PKC was readily detected in cytosolic and solubilized particulate fractions of FDC-P1 cells, but not in FD/PMA cell extracts. Comparisons between the parental and FD/PMA lines should provide insight into IL-3– and PMA-mediated signal transduction. © 1990 by The American Society of Hematology.

Hematopoietic cell growth is regulated in part by low molecular weight growth factors. IL-3 (IL-3) is a multilineage growth factor normally produced by activated T cells. IL-3 supports the growth of early hematopoietic cells, including progenitor cells of the myeloid, erythroid, and lymphoid series.

IL-3–dependent cell lines have been isolated from murine fetal liver and bone marrow cells that are dependent on the presence of IL-3 for continuous growth in vitro. Abrogation of growth factor dependency occurs after infection with retrovirus containing certain oncopgenes, namely v-abl, v-src, v-myc, c-myc, v-fms, and v-erb-B. The resulting factor-independent cells are transformed by a nonautocrine mechanism since their growth is density-independent and not inhibited by antibodies to growth factors, and, in addition, growth factors supporting autocrine growth have not been detected. After infection of IL-3–dependent cell lines with recombinant retroviral constructs containing genes encoding either IL-3 or granulocyte macrophage colony-stimulating factor (GM-CSF), factor-independent cell lines have been isolated. These cell lines are transformed by an autocrine mechanism. Spontaneous factor-independent cells have been isolated infrequently (less than 10^{-7}) from factor-dependent cell lines; in those cases best documented, genetic rearrangements at lymphokine loci (IL-3 and GM-CSF) and expression of these growth factor genes were observed, suggesting an autocrine mechanism was responsible for the apparent growth factor-independent growth.

Phorbol 12-tetra-decanoate 13-acetate (PMA) is a potent phorbol ester that modulates growth and differentiation in a variety of hematopoietic cell lines. At least a portion of the cellular effects exerted by PMA are mediated by the ability of PMA to substitute for diacylglycerol and activate protein kinase C (PKC). A role for PKC in mediating IL-3 signal transduction has been proposed based on the ability of IL-3 to stimulate translocation of PKC from a cytosolic to a particulate fraction, and to mimic PMA-stimulated phosphorylation of several endogenous substrates. Addition of PMA to many IL-3–dependent cell lines results in H-thymidine incorporation, but usually will not replace IL-3 for continuous growth in vitro. To investigate the effects of IL-3 and PMA on the growth of an IL-3–dependent cell line, we have isolated variant cell lines from FDC-P1 that will grow continuously in the absence of exogenously supplied IL-3 when PMA is included in the culture medium. The resulting cell lines have remained PMA-dependent for over 1 year, do not produce detectable levels of lymphokines that would support growth, have different cell surface phenotypes than the parent cells, and display altered PKC activity.

Materials and Methods

Cell culture. The IL-3–dependent myeloid line, FDC-P1, was provided by Dr James Ihle (St Judes Hospital, Memphis, TN) via Dr Charles Kovacs (East Carolina University, Greenville, NC) and maintained in a humidified, 5% CO₂ incubator with Dulbecco's modified Eagle's medium (DMEM) containing fetal calf serum (FCS) and supernatant (10%) prepared from the WEHI-3B cell line as a source of IL-3. Reconstituted (r) murine IL-3 and GM-CSF were purchased from Genzyme (Boston, MA). The FD/PMA cell lines were maintained in DMEM containing 5% FCS and 32 mmol/L PMA (Sigma, St Louis, MO). Cellular proliferation assays were performed with H-thymidine (6.7 Ci/mmol, New England Nuclear [NEN], Boston, MA) as described.

Northern analysis. Cellular RNA was isolated from 10⁶ cells after washing them two times in Ca²⁺-Mg²⁺ free phosphate-buffered saline (PBS). The cell pellet was lysed in guanidium-isothiocyanate (Bethesda Research Laboratories [BRL], Gaithersburg, MD) as described and then loaded onto a cushion of 5.7 mol/L CsCl containing 100 mmol/L EDTA. The RNA was collected by ultracentrifugation (30,000g, 24 hours). After ethanol precipitation, the RNA pellet was resuspended in Tris-EDTA buffer (10 mmol/L Tris, pH 7.6, 1 mmol/L EDTA). RNA concentrations were determined by UV spectrophotometry. Total RNAs (20 µg) were denatured with formaldehyde/formamide and electrophoresed in 1.2% agarose gels

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containing formaldehyde.\textsuperscript{79} Blotting of RNA gels was performed with Gene Screen Plus nylon membranes (NEN) as described.\textsuperscript{39} Filters were prehybridized for 4 hours at 42°C in a solution consisting of 50% formamide; 4X SSC (1X = 0.15 mol/L NaCl/0.015 mol/L sodium citrate); 5X Denhardt's solution; 50 mmol/L sodium phosphate buffer pH 7; 0.5 mg/mL sodium pyrophosphate; 100 μg/mL denatured salmon sperm DNA; and 1% sodium dodecyl sulfate (SDS). Hybridization was performed with 10\textsuperscript{6} cpm/mL of \textsuperscript{32}P-\alpha-dCTP (NEN, 3,000 Ci/mmol) random primed labeled probes\textsuperscript{40} in a solution the same as above, except that the concentration of Denhardt's was 1X, for 16 to 24 hours at 42°C. Filters were washed two times (30 min each) at room temperature in 3X SSC and 0.1% SDS followed by two washes (30 min each) at 65°C in 0.1X SSC and 0.1% SDS. The pellet was rinsed with buffer A, omitting the sucrose. The pellet was suspended in buffer A with 0.1% Triton X-100 (buffer B) to a 3 cm\textsuperscript{2} of Whatman P-81 (Whatman International Ltd., Maidstone, England). The murine IL-2 probe (pMIL2-20\textsuperscript{41}) was obtained from Dr Ian Young (Australian National University, Canberra). The murine IL-4 probe\textsuperscript{42} was provided by Dr William Paul (National Institutes of Health, Bethesda, MD). The murine IL-6 probe\textsuperscript{43} was obtained from Dr Jacques Van Snick (Ludwig Institute, Brussels, Belgium). The murine GM-CSF specific probe (gGM5'D19\textsuperscript{44}) was obtained from Dr Paula Pitt-Rowe (Johns Hopkins University, Baltimore, MD). The murine M-CSF and human G-CSF probes were obtained from Dr Steven Clark (Genetics Institute, Cambridge, MA). Purified DNA fragments specific for β-actin (p7000) were purchased from Oncor (Gaithersburg, MD). The bovine PKC α, β, and γ probes [phPKC-α-7, phPKC-β-15-Eco, and phPKC-γ-6\textsuperscript{17}]] were purchased from the ATCC (Rockville, MD). The PKC-ε probe (PMT-2-PKC-ε\textsuperscript{45}) was obtained from Dr Dick Schaa (Ludwig Institute for Cancer Research, London, England).

**Immunofluorescence.** Indirect immunofluorescence was performed as described.\textsuperscript{37} The rat anti-rat class I major histocompatibility complex (MHC) monoclonal antibody (MoAb) D468 served as a negative control.\textsuperscript{46} Fluorescence of stained viable cells was quantitated with a FACS Consort 30 Analyszer (Becton-Dickinson, Mountainview, CA). The following MoAbs were used: MS/114, anti-IA\textsuperscript{48}; AA4.1 and GFl.2, anti-lymphohematopoietic differentiation antigens; M3/38, anti-macrophage subpopulation-specific antigen Ma\textsuperscript{2-3}; 7D4, anti-IL-2 receptor.\textsuperscript{58} Fluorescence of stained viable cells was quantitated with a FACS Consort 30 Analyszer (Becton-Dickinson, Mountainview, CA). The following MoAbs were used: MS/114, anti-IA; AA4.1 and GFl.2, anti-lymphohematopoietic differentiation antigens; M3/38, anti-macrophage subpopulation-specific antigen Ma\textsuperscript{2-3}; 7D4, anti-IL-2 receptor.\textsuperscript{58}

**Cellular fractionation.** Cells were collected by centrifugation. The pellet was suspended in buffer A (20 mmol/L Tris-HCl pH 7.4, 10 mmol/L EDTA, 2 mmol/L EGTA, 100 mmol/L β-glycerophosphate, 5 mmol/L L-mercaptoethanol, 2 mmol/L aprotinin, 2 mmol/L pepstatin A, 50 μg/mL phenylmethylsulfonyl fluoride (PMSF), 100 μg/mL leupeptin, and 0.25 mol/L sucrose). The cells were disrupted by sonicating with a 10-second burst. The homogenate was centrifuged at 100,000 g for 60 minutes. The supernatant, termed cytosol, was removed. The pellet was rinsed with buffer A, omitting the sucrose, and resuspended in buffer A with 0.1% Triton X-100 (Sigma), omitting the sucrose. The pellet was suspended by sonicating as described above. The samples were incubated at 4°C for 45 minutes before centrifugation at 100,000 g for 60 minutes. The supernatant containing the solubilized particulate fraction was removed. In certain experiments, a total cell solubilized fraction was prepared by sonicating the cells in buffer A containing 0.1% Triton X-100. After a 45-minute incubation at 4°C, the sample was centrifuged at 100,000 g for 60 minutes, yielding in the supernatant fraction both cytosolic and solubilized particulate fractions. The protein content of the samples was determined as described.\textsuperscript{39}

**Determination of PKC activity.** PKC activity was quantitated by determining the transfer of \textsuperscript{32}P from [\textsuperscript{32}P]-γ-adenosine triphosphate (ATP) into histone as described previously.\textsuperscript{60} Cellular fractions, 40 μg, were incubated with 10 mmol/L MgCl\textsubscript{2}, 0.75 mmol/L CaCl\textsubscript{2}, 1.0 mmol/L EDTA, 0.2 mmol/L EGTA, 40 μg histone, Sigma Type (V-S), and 20 mmol/L Tris-HCl, pH 7.4, in a total volume of 100 μL. In certain tubes, 10 μg phosphatidylycerine and 2 μg dioxogen were added to activate PKC. The reaction was initiated by warming to 27°C and adding 10 μmol/L (\textsuperscript{32}P)-γ-ATP, 2 μCi/tube (NEN, 3,000 Ci/mmol). After an 8-minute incubation, the reaction was terminated with an equal volume of 60% acetic acid (vol/vol) containing 1 mmol/L ATP. Forty-microliter aliquots of the sample were spotted in duplicate onto 2 x 2 cm squares of Whatman P-81 (Fisher Scientific) papers. The papers were washed for 15 minutes in a 30% acetic acid solution, followed by two washes with 15% acetic acid. Radioactivity associated with histone was determined in a β-scintillation counter. PKC activity was defined as the difference in radioactivity between samples treated in the absence and presence of PKC activators.

**Preparation of PKC antisera.** Female New Zealand white rabbits were immunized with a peptide corresponding to amino acid residues 280 to 292 of the deduced sequence for PKC-α.\textsuperscript{61} A cysteine residue was added to the amino terminus to enable the peptide to be coupled to keyhole limpet hemocyanin using m-maleimido benzyl-n-hydroxyl succinimide ester.\textsuperscript{62} One milligram of the conjugate was injected intramuscularly in complete Freund’s adjuvant, followed by booster injections of 0.5 mg every 4 weeks. Phlebotomy was performed 7 to 10 days after the booster injections.

**Western blotting.** Western blotting was performed as described.\textsuperscript{63} Briefly, equal protein concentrations of cytosolic and solubilized particulate fractions were added to an equal volume of a buffer containing 2% SDS, 100 mmol/L dithiothreitol (DTT), 0.01% bromophenol blue, and 60 mmol/L Tris, pH 6.8, and heated to 110°C for 3 minutes. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% running gel. Prestained molecular weight markers (Sigma) were concomitantly electrophoresed. The proteins were transferred to nitrocellulose. After blocking, the filters were incubated with a 1:250 dilution of the antisera for 12 hours at 4°C. To determine specificity, the antisera was preabsorbed with the immunizing peptide, 4 μg, for 60 minutes at 23°C before incubation with the filter. After washing, the filters were incubated with 0.4 μCi/mL of \textsuperscript{12}I-protein A (NEN, 8.8 μCi/μg) for 60 minutes. The filters were washed and autoradiography was performed.

**DEAE-chromatography.** Cellular extracts were applied at a protein concentration of 1 mg/mL in a buffer containing 20 mmol/L Tris-HCl, pH 7.4; 5 mmol/L L-mercaptoethanol; and 0.01% Triton X-100 (vol/vol) (buffer B) to a 3 x 0.9 cm column containing DEAE cellulose previously equilibrated with buffer B. After sample application, the column was sequentially washed with 20 mL of buffer B and 20 mL of buffer B containing 20 mL NaCl. Elution of PKC was performed isocratically with 20 mL of 120 mmol/L NaCl. One-milliliter fractions were collected, and 30-μL aliquots of each fraction were analyzed for PKC activity in an identical manner as previously described.
RESULTS

Isolation of PMA-dependent cell lines. FDC-P1 is a myeloid IL-3-dependent cell line that ceases proliferation in the absence of IL-3. FDC-P1 cells incorporated \(^{3}H\)-thymidine after IL-3 or PMA addition (Fig 1A). However, a nonselected population of FDC-P1 did not double within 48 hours in the presence of PMA (Fig 1B). We observed that a minority (10%) of FDC-P1 cells were viable in the presence of 32 nmol/L PMA. To determine whether these variants could be selected from the population of FDC-P1, which would grow continuously in the presence of PMA but in the absence of IL-3, FDC-P1 was plated in 96-well plates at cell concentrations ranging from 0.03 to 100 cells per well in media containing IL-3 (10% WEHI-3B supernatant), PMA (32 nmol/L), or no additive (DMEM + 5% FCS) (Fig 1C). The concentrations of WEHI-3B supernatant and PMA chosen represent optimal doses for \(^{3}H\)-thymidine incorporation (Fig 1A). After approximately 2 weeks of culture in the presence of IL-3 or PMA, microtiter wells positive for growth were enumerated; however, no wells were positive for growth in the plates that received nonsupplemented media (Fig 1C). At a cell concentration of 1 cell per well, 66% of the wells should receive 1 or more cells, and 33% of the wells should receive less than 1 cell per well according to the Poisson distribution. The data presented in Fig 1C indicated that FDC-P1 had a plating efficiency of 1 in the presence of IL-3. However, approximately 1 of 10 FDC-P1 cells grew continuously in the presence of PMA. Therefore, the number of wells that grew into microtiter well colonies in the presence of IL-3 were approximately 10-fold higher than those observed from plates fed PMA.

Cells were isolated from four microtiter wells containing PMA that were plated at 1 cell/well and grown into individual cultures in PMA. These cultures were subcloned by limiting dilution at 0.3 cells/well in the presence of PMA, and four subclones (one from each initial culture) were isolated. The plating efficiencies and \(^{3}H\)-thymidine incorporation in response to IL-3, PMA, and media were determined for the subclones (Fig 2). Limiting dilution analysis indicated that cell growth was observed when cells were plated in either IL-3 or PMA; however, no cell growth was observed in wells that received media lacking either IL-3 or PMA. All lines incorporated \(^{3}H\)-thymidine after either IL-3 or PMA addition but not after nonsupplemented media addition (Fig 2). All subclones divided approximately 24 hours after addition of either IL-3 or PMA (data not presented). These lines have been maintained in culture with PMA for over 1 year and have retained their PMA dependency. These cell lines continue to display similar IL and PMA growth responses as they did when they were initially isolated. Therefore, continuous growth in PMA has not abrogated the growth factor requirements of FD/PMA lines.

Synergy of IL-3 and PMA in stimulating \(^{3}H\)-thymidine uptake in FD/PMA lines. To investigate the role of PKC in IL-3-induced signal transduction, FDC-P1 and FD/PMA lines were plated with suboptimal doses of PMA and different concentrations of IL-3. Surprisingly, it was observed that the responses of FD/PMA lines, at low concentrations of IL-3 and suboptimal doses of PMA, were greater than the predicted value of either growth stimuli alone (Fig 3). However, amounts of \(^{3}H\)-thymidine uptake lower than the predicted values were observed when parallel experiments were performed with the parental line. These experiments were repeated three times, and synergy between IL-3 and PMA was always observed with all four FD/PMA lines with all concentrations of PMA tested (0.01 to 16 nmol/L), while no synergy was observed with the FDC-P1 line. Moreover, identical results were observed when either rIL-3 or rGM-CSF was used in place of conditioned media from WEHI-3B. Therefore, IL-3 and PMA can synergize for the growth of the FD/PMA lines; thus, it is likely that PMA acts through a different pathway from that mediating growth induced by IL-3 in the FD/PMA lines.

Absence of lymphokine gene expression in cell lines growing continuously in the presence of PMA. To determine whether the growth of the FD/PMA cell lines was due to activation of lymphokine gene expression, we examined...
the expression of certain IL and CSF genes by Northern blot analysis. While mRNAs encoding these genes were detected in control cell lines, we were unable to detect mRNAs encoding IL-2, IL-3, GM-CSF, IL-4, IL-6 (Fig 4), or IL-1β, G-CSF, or M-CSF (data not presented) in the FD/PMA cell lines. Moreover, we have not detected rearrangement of these lymphokine genes in the FD/PMA cell lines by Southern blot analysis (data not presented), whereas rearrangement was detected at the IL-3 locus in the WEHI-3B cell line, previously shown to be rearranged at IL-3.64 In addition, FD/PMA lines did not have a significantly higher plating efficiency when plated in IL-3 rather than PMA (Fig 2A). Therefore, the continuous growth of these cell lines in the presence of PMA does not appear to be due to PMA-induced lymphokine gene expression.

Identification of genes whose expression change in cell lines permanently cultured in PMA. To examine the phenotypic consequences of continuous growth in PMA, we determined the expression of genes that are detected at early stages of hematopoietic differentiation. The expression of 6 out of 10 cell surface antigens was identical in FDC-P1 and FD/PMA cell lines (MHC class I, MHC class II, AA4, GFI, IL-2 receptor, and B220). However, differences were detected in the expression of LFA-1, Mac-1, Mac-II, and Mac-III (Table 1).

Mac-I, Mac-II, and Mac-III were not detected on the cell surface of FDC-P1. These molecules were detected in FD/PMA lines, with Mac-I showing the greatest increase in expression compared with FDC-P1. Moreover, while LFA-1 was detected at low levels on the FDC-P1 parent, the amount detected on FD/PMA lines was increased. Culturing either FDC-P1 or FD/PMA lines in the presence of either IL-3 or PMA for 24 hours did not appear to significantly change the expression of these cell surface antigens (Table 1), although longer culture periods may be required to reverse the effects of PMA.

Alteration of PKC in FD/PMA cells. Given the role of PKC in PMA-mediated signal transduction, we examined the levels of PKC activity in the FDC-P1 and FD/PMA cells. As defined by histone phosphotransferase activity stimulated by calcium, phosphatidylserine, and diolein, PKC activity was readily definable in crude cytosolic and solubilized particulate fractions derived from FDC-P1 cells (Table 2). In contrast, negligible PKC activity was present in either cellular fraction in FD/PMA cells.

The decrease of PKC activity in the FD/PMA cells was not due to an increase in basal histone phosphorylation, which would diminish activity stimulated further by the addition of phosphatidyl serine and diolein. Histone phosphorylation in the absence of PKC activators was similar in the cellular fractions of both cell lines (FD/PMA cytosol = 94% ± 14% of FDC-P1 [mean ± SEM, N = 4]; FD/PMA solubilized particulate fraction = 99% ± 13% of FDC-P1 [mean ± SEM, N = 4]).

The absence of PKC activity in FD/PMA cells was not unexpected since enhanced degradation of PKC leading to downregulation occurs to varying degrees in a variety of cell types.65 To ensure that the decrease in PKC activity in the FD/PMA cells was not secondary to altered substrate specificity, we used other histones, protamine, and casein as phosphate acceptors. PKC-dependent phosphorylation of these substrates was also negligible in FD/PMA cells (data not shown). Production of endogenous inhibitors of PKC could cause an apparent reduction in the PKC activity in FD/PMA cells. To test this hypothesis, cellular extracts were subjected to anion exchange chromatography that serves to separate PKC from the bulk of possible endogenous cellular inhibitors of this kinase activity. Using an extract from FDC-P1 cells, a peak of PKC activity eluted from the DEAE resin during a isocratic elution with 120 mmol/L NaCl (Fig 5). However, no PKC activity eluted under identical conditions when extracts from FD/PMA cells were examined.

The presence of factors inhibiting PKC activity was also
Fig 3. (A) \(^{3}\)H-thymidine incorporation of FDC-P1 in different amounts of IL-3 (WEHI-3B conditioned media) and either 0.8 nmol/L or 0.4 nmol/L PMA. (□), Levels of \(^{3}\)H-thymidine uptake observed in the presence of IL-3 alone. (■), The amount of \(^{3}\)H-thymidine uptake observed when PMA and IL-3 were added together (add [□] and [■]) to obtain value observed with PMA and IL-3. (□), The \(^{3}\)H-thymidine uptake observed when only PMA was added. NA, no growth factor addition. (B) Same as panel A except the FD/PMA.4 cell line was used. (C) The observed counts per minute (cpm) were subtracted from the expected cpm and plotted as a function of IL-3 concentration. The expected cpm was calculated by adding the cpm observed with PMA alone to the cpm observed at the varying doses of IL-3 alone. (□), FD/PMA.4 + 0.8 nmol/L PMA; (□), FD/PMA.4 + 0.4 nmol/L PMA; (■), FDC-P1 + 0.8 nmol/L PMA; (◇), FDC-P1 + 0.4 nmol/L PMA. The SEM in these experiments was less than ±5%.

examined by mixing crude extracts of FDC-P1 and FD/PMA cells to purified PKC. PKC from rat brain cytosol was purified by DEAE and hydroxapatite chromatography. Fractions from the peaks of activity eluting from the hydroxap-

tite column representing the \(\gamma\), \(\beta\), and \(\alpha\) isoforms were pooled and used as a source of PKC. Cellular extracts from the FDC-P1 and FD/PMA lines, containing the cytosolic and solubilized particulate fractions, were prepared. Addition of the FDC-P1 extract to the PKC preparation inhibited activity of the latter by 30% in two separate experiments. In the same experiments, the FD/PMA extract decreased PKC activity by 49%. While the FD/PMA extract was slightly more potent than FDC-P1 extract in inhibiting PKC activity,

Fig 4. Growth conditions were a 24-hour incubation period with either IL-3 (10% WEHI-3B supernatent) or PMA (32 nmol/L). EL4 is a T-cell thymoma, which is induced after PMA addition to express many lymphokine genes. FL5.12 is a lymphoid IL-3-dependent cell line that expresses IL-6 mRNA and growth factor. FD/PMA is the FD/PMA.4 clone. The same Northern blot was hybridized with the indicated probes. Molecular weights of RNAs were determined by linear regression analysis with the sizes of rRNAs and a BRL RNA ladder serving as internal standards. Northern blot analysis was performed with the other FD/PMA lines and identical results were observed.
this difference does not seem to be of sufficient magnitude to explain the total abolition of PKC activity observed in the FD/PMA line.

Using antisera produced by a rabbit immunized with a peptide corresponding to amino acids 280 to 292 of the deduced PKC-α sequence. Western blotting was performed (Fig 6). This antiserum recognizes the α, β and γ isoforms but not the ε isoform (R.S.R. and D.K.W., unpublished observations, September 1989). The antiserum detected bands with approximate molecular weights of 50 and 80 Kd in FDC-P1 cells. Preabsorption of the antiserum with the peptide used to immunize the rabbit blocked the appearance of the 80-Kd band, indicating that this band was specifically recognized by the PKC antisera. However, preabsorption of the antiserum with the peptide did not alter the intensity of the 50-Kd band, suggesting that this band was not specifically recognized by the antiserum. The inability to readily detect the 80-Kd band in FD/PMA cellular fractions indicates a significant reduction of immunoreactive PKC in this line as compared with FDC-P1. While the 80-Kd band was markedly diminished in the FD/PMA line, traces of this protein could be detected in the cytosol and solubilized particulate fraction of this line when the blots were exposed for extended periods of time (data not shown). Enhanced destruction of PKC could decrease the 80-Kd band and cause accumulation of the proteolytically derived 35-Kd regulatory domain that is detected by this antiserum (reference 61, and R.S.R. and D.K.W., unpublished observations, October 1989). The 35-Kd regulatory domain was not detected in either the FDC-P1 or FD/PMA line (Fig 6). These results argue strongly that the decrease in PKC activity in FD/PMA cells is due to a primary decrease in the amount of PKC.

The absence of PKC in FD/PMA cells could be secondary to continuous downregulation of PKC stimulated by PMA or due to a primary intrinsic alteration in PKC expression in FD/PMA. To examine these possibilities, PKC activity was determined in FD/PMA cultured in IL-3 rather than PMA. Although PKC activity increased in a linear fashion from 50% to 72-hour exposure to IL-3 (Fig 7), the maximal levels obtained in FD/PMA were only 50% of that observed in the FDC-P1. Incubation of FD/PMA cells with IL-3 beyond the 72-hour period did not produce further increases in PKC activity (data not shown). Thus, the reduction of PKC activity in the FD/PMA lines was due to both PMA-induced downregulation and an intrinsic difference in the expression of PKC activity in the FD/PMA lines.

mRNA levels of PKC isoforms in FDC-P1 and FD/PMA lines. To determine whether the lack of detectable PKC activity might be due to PMA-induced downregulation of PKC gene expression, we performed RNA blotting experiments with FDC-P1 and FD/PMA cells grown in either IL-3 or PMA. mRNA transcripts encoding PKC-α, PKC-β, and PKC-γ were detected in low levels in the two cell lines in both growth conditions (data not presented). However, the levels of these transcripts were not significantly different when either cell line was cultured with IL-3 or PMA. mRNA transcripts encoding PKC-γ were not reproducibly detected in these cell lines under either growth condition. Moreover, to determine if the PKC isoforms were expressed in certain phases of the cell cycle, both FDC-P1 and FD/PMA lines were factor-deprived for 24 hours and either IL-3 or PMA

![Fig 5. FDC-P1 cells, 10^9, cultured in IL-3 and 10^9 FD/PMA.4 cells cultured in PMA were homogenized in the standard homogenization buffer containing 0.25 mmol/L sucrose and 0.1% Triton. After a 45-minute incubation at 4°C, the samples were centrifuged at 100,000g, yielding both cytosolic and solubilized particulate fractions in the supernatant. This total cell solubilized extract was applied to the DEAE column, and PKC activity was measured in the eluate derived from the FDC-P1 extract (–––) and FD/PMA.4 extract (–––). This experiment was repeated with the other FD/PMA lines and similar results were observed.]
EFFECTS OF PMA ON IL-3-DEPENDENT CELLS

Fig 6. Cytosolic and solubilized particulate fractions were derived from $5 \times 10^7$ FDC-P1 cells cultured in IL-3 or FD/PMA.4 cells cultured in PMA. The samples were separated using SDS-PAGE, transferred to nitrocellulose paper, and incubated with PKC antisera. Immune complexes were detected with $^{125}$I-protein A and visualized by autoradiography. The band recognized specifically by the PKC antisera is indicated by a line.

was added. Although mRNA transcripts encoding PCK-$\alpha$, PKC-$\beta$, and PKC-$\epsilon$ were detected, their levels did not change at different time points after IL-3 or PMA addition (data not presented).

DISCUSSION

We have established stable murine cell lines that respond to PMA as a signal for proliferation. Proliferation in response to PMA appears to be a direct effect of this agent rather than secondary to PMA-induced secretion of growth factors, which would support proliferation since we have not detected lymphokine gene expression by Northern blot analysis. More sensitive detection methods, eg, the polymerase chain reaction, might detect expression of these genes. However, the plating efficiencies of FD/PMA lines was less than twofold higher in the presence of exogenously supplied IL-3 than in PMA, whereas we have observed that autocrine transformed cell lines have a 20- to 50-fold higher plating efficiency when cultured in the presence of growth factors.

The FD/PMA cell lines differ in cell surface antigen expression from the FDC-P1 line. While the expression of certain antigens is similar in both cell types, the expression of Mac-1, Mac-2, Mac-3, and LFA-1 antigens is increased in the FD/PMA cell lines. The differential expression of these antigens appears to be due to an intrinsic alteration in gene expression rather than a specific PMA effect, since FD/PMA cells grown in IL-3 displayed a similar antigen profile. Despite the appearance of various parameters indicative of a more mature phenotype, the FD/PMA cell lines have not progressed to a point in the differentiation process where the capacity for proliferation ceases. Indeed, a dichotomy exists between the ability of phorbol esters to enhance several parameters of differentiation while maintaining cellular proliferation. Given the differentiation-inducing ability of PMA in many leukemic cell lines, this system displays both enhanced differentiation and maintenance of proliferation, and as such provides a unique opportunity to study the mechanisms by which phorbol esters alter growth and differentiation.

The mechanism by which phorbol esters support proliferation in the FD/PMA lines has yet to be elucidated. However, several possibilities exist that could explain this phenomenon. The absence of steady-state PKC activity or protein detected by anti-PKC antiserum does not necessarily imply that PMA signal transduction occurs independent of PKC activation in the FD/PMA lines. In several cell types, phorbol esters enhance degradation of PKC resulting in downregulation of kinase activity. However, the level of PKC synthesis and PKC mRNA levels are not altered. These findings suggest that a much higher turnover rate of PKC occurs in certain PMA-treated cells. In such a system, even in the absence of detectable PKC levels, PKC activation is constantly occurring, followed by rapid degradation, thus providing a continuous mechanism of PKC-dependent signal transduction. The similar levels of PKC mRNA transcripts in FDC-P1 and
FD/PMA lines suggests that a posttranscriptional mechanism is responsible for the decrease in PKC activity in the FD/PMA line. Another possible explanation for our findings is that the system used to determine PKC activity lacks the sensitivity required to detect a small but important subset of PKC that is integral to PMA-stimulated signal transduction (eg, a specific PKC isozyme). For instance, one member of the PKC gene family, PKC-ε, is not readily detectable using histone as a substrate when compared with PKC isozymes α, β, and γ. Thus, preferential downregulation of the majority of PKC isozymes, but the sparing of a PKC isozyme such as PKC-ε that is not downregulated, could provide continued signal transduction necessary for PMA-induced proliferation in the FD/PMA cell lines. PMA-signal transduction can also be modulated by the postactivational processing of PKC. Exposure of certain cells to PMA stimulates the proteolytic cleavage of PKC into a constitutively activated, phosphatidylserine, diolein-independent kinase activity termed M kinase. This cytosolic kinase, which has an approximate molecular weight of 50 Kd, differs in substrate specificity and intracellular localization from reversibly activated PKC that is membrane-associated. In some cellular models, it has been postulated that M kinase mediates events not mimicked by the reversible activation of PKC. Thus, postactivational processing of PKC provides another mechanism potentially mediating certain cellular responses to PMA. The inability of the antisera used in this study to detect a 50-Kd band on Western blotting does not preclude the existence of M kinase. The peptide sequence used for immunization is contained in the regulatory domain of PKC and does not recognize the antisera used in this study to detect a 50-Kd band on Western blotting does not preclude the existence of M kinase. Thus, preferential downregulation of the majority of PKC isozymes, but the sparing of a PKC isozyme such as PKC-ε that is not downregulated, could provide continued signal transduction necessary for PMA-induced proliferation in the FD/PMA cell lines. PMA-signal transduction can also be modulated by the postactivational processing of PKC. Exposure of certain cells to PMA stimulates the proteolytic cleavage of PKC into a constitutively activated, phosphatidylserine, diolein-independent kinase activity termed M kinase. This cytosolic kinase, which has an approximate molecular weight of 50 Kd, differs in substrate specificity and intracellular localization from reversibly activated PKC that is membrane-associated. In some cellular models, it has been postulated that M kinase mediates events not mimicked by the reversible activation of PKC. Thus, postactivational processing of PKC provides another mechanism potentially mediating certain cellular responses to PMA. The inability of the antisera used in this study to detect a 50-Kd band on Western blotting does not preclude the existence of M kinase. The peptide sequence used for immunization is contained in the regulatory domain of PKC and does not recognize the catalytic domain of PKC (eg, M kinase). However, the absence of an increase in basal, phosphatidylserine, and diolein-independent histone phosphorylation in the cytosol of FD/PMA cells, and the lack of a 35-Kd regulatory domain as determined by Western blot analysis cells ague against enhanced proteolytic processing of PKC and generation of M kinase in FD/PMA cells grown in PMA. Lastly, PMA-induced signal transduction in the FD/PMA lines could be mediated through mechanisms independent of PKC activation or the postactivational processing of PKC. Obviously, further studies will be required to discriminate among these multiple possibilities.

The mechanism by which IL-3 stimulates growth remains to be fully elucidated. The ability of PMA and IL-3 to synergistically enhance growth in the FD/PMA line suggests that these agents may act through separate signal transduction pathways. However, in the FDC-P1 line these agents did not act in a synergistic fashion to enhance growth, and thus may act through a single intracellular pathway. These data indicate that IL-3 and PMA signal transduction may vary depending on the cell line examined. Therefore, these findings demonstrate the complexity of IL-3 and PMA signal transduction and indicate that results obtained in one cell may not be generally extrapolated to other cell types.

In summary, we have isolated stable murine cell lines that proliferate in response to phorbol esters. The proliferation is a direct effect of PMA rather than being secondary to PMA-induced secretion of growth factors. The mechanism(s) by which phorbol esters support the growth of the FD/PMA lines is unknown. However, the FD/PMA lines provide an excellent system to gain insight into the biochemical processes responsible for PMA-stimulated proliferation and, in a more general sense, the mechanisms governing cellular growth and differentiation.

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