Vol. 268, No. 27, Issue of September 25, pp. 20110-20115, 1993
Printed in U.S.A.

Phorbol Ester-induced Myeloid Differentiation Is Mediated by Protein Kinase C-α and -δ and Not by Protein Kinase C-βII, -ϵ, -ζ, and -η*

(Received for publication, March 25, 1993, and in revised form, May 22, 1993)

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It is generally accepted that the multiple, similar protein kinase C (PKC) isozymes are responsible for different specialized physiological processes, but evidence that directly assigns specific functions to specific isozymes is scarce. To test whether specific PKC isozymes are involved in myeloid differentiation, we have studied the effect of overexpression of PKC-α, -βII, -δ, -ϵ, -ζ and -η in 32D, a mouse myeloid progenitor cell line that does not differentiate in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA). No significant morphological or phenotypic changes could be observed in unstimulated cells that overexpress any of these isozymes. However, the cell lines that overexpressed PKC-α or -δ had acquired the ability to become mature macrophages 2–6 h after TPA stimulation. The overexpression of PKC-βII, -ϵ, -ζ, or -η, in contrast, did not permit TPA-induced differentiation. These results indicate that only these two members of the PKC gene family can participate in TPA-induced myeloid differentiation.

Several human promyelocytic cell lines, *e.g.* HL-60 (reviewed by Collins, 1987) or U937 (Ways *et al.*, 1987), are able to differentiate into mature macrophages upon stimulation with phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[1] TPA represents a potent activator of protein kinase C (PKC) (Castagna *et al.*, 1982), a family of serine/threonine protein kinases that appear to be involved in a pleiotropic set of processes such as growth, differentiation, and cytokine secretion (reviewed by Ohno *et al.*, 1991 and Nishizuka, 1992). To date, nine different PKC isozymes, encoded by eight different genes, have been identified and cloned (Knopf *et al.*, 1986; Ono *et al.*, 1988; Osada *et al.*, 1990; Osada *et al.*, 1992). As we described recently, seven of the known members of the PKC gene family are differentially expressed in mouse hemopoietic cells (Mischak *et al.*, 1991b). Unique characteristic patterns of PKC isozyme expression have been observed in virtually every tissue or cell line investigated (Sposi *et al.*, 1989; Borner *et al.*, 1992; Dlugosz *et al.*, 1992). The tissue-specific patterns of expression of the nine different PKC isozymes led us and others to hypothesize that the different isozymes might fulfill different biological functions. Direct evidence that assigns specific roles to specific isozymes is meager; PKC-α and -β have been shown to play a major role in the acquisition of differentiation competence and neural development of *Xenopus* (Otte and Moon, 1992), and PKC-β and -δ have been shown to be involved in the secretory response of antigen-stimulated rat basophilic RBL-2H3 cells (Ozawa *et al.*, 1993). To begin to dissect the roles of individual PKC isozymes in growth, development, and transformation of mouse hemopoietic cells, we decided to study the patterns of PKC isozyme expression in primary hemopoietic cells in comparison with cell lines and tumors (Mischak *et al.*, 1991b). Our studies indicated that most mouse myeloid cell lines express only low or undetectable levels of PKC-α and -β, whereas they usually express considerable amounts of PKC-δ and -η. Most of these cell lines also are unable to differentiate in response to TPA. In contrast, normal mouse peritoneal monocytes, like the human cell lines HL-60 and U937, are able to differentiate upon TPA treatment, and they all express easily detectable levels of PKC-α and -β (Hashimoto *et al.*, 1990; Mischak *et al.*, 1991b). Although it has been shown that prolonged activation of PKC by TPA is necessary for HL-60 differentiation (Aihara *et al.*, 1991), no insight has been obtained into whether specific isozymes of this family are involved in this process. The difference between the human and mouse myeloid cell lines in their response to TPA and in their expression of PKC-α and -β led us to suspect that PKC-α or -β might be the key isoform in TPA-induced myeloid differentiation.

To address this question directly we used 32D, an IL-3-dependent murine myeloid cell line (Greenberger *et al.*, 1983) which is widely used as a model for mouse myeloid progenitor cells. This cell line does not differentiate when treated with TPA, but it is capable of differentiation into mature macrophages if transfected with CSF-1-receptor and subsequently stimulated with CSF-1 (Pierce *et al.*, 1990). We overexpressed six of the eight members of the PKC family in this cell line, hoping that one or more would enable the cells to differentiate upon TPA treatment, thereby proving which of the PKC isozymes are responsible for myeloid differentiation.

EXPERIMENTAL PROCEDURES

Construction and Transfection of Expression Vectors—pLTR is an expression vector based on the Harvey sarcoma virus long terminal repeat that contains the selectable marker xanthine-guanine phosphoribosyltransferase (Mulligan and Berg, 1981). This vector and the two PKC-α overexpressing vectors, pLTR-α and pLTR-B25, were obtained from Dr. N. Mazurek (Megidish and Mazurek, 1989). pLTR-ϵ and -η were constructed by inserting the complete mouse cDNAs of PKC-ϵ, cloned from a mouse brain cDNA library (Mischak *et al.*, 1993) and PKC-η, cloned from a mouse lymph node cDNA library,

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; IL-3, interleukin-3.

into the *Eco*RI site of pLTR. pMTH is an expression vector based on the mouse metallothionine promoter that contains the neomycin resistance gene as a selectable marker (McClinton *et al.*, 1990) and was kindly provided by Dr. G. Shen-Ong. pMTH-β, -δ, -ϵ, -ζ, and -η were constructed by inserting blunt-ended cDNAs into the blunt-ended *Bam*HI site of pMTH. Rat PKC-βII cDNA was a kind gift from Dr. J. Knopf (Knopf *et al.*, 1986), mouse PKC-δ cDNA was cloned from a mouse myeloid tumor cDNA library (Mischak *et al.*, 1991a), and mouse PKC-ζ cDNA was cloned from a mouse brain cDNA library (Goodnight *et al.*, 1992). All vectors were transfected using electroporation as described (Pierce *et al.*, 1990).

Western Blot Analysis—2 × 10^7 cells of each cell line were lysed with 400 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) by sonication for 10 s and spun at full speed in an Eppendorf centrifuge to remove any insoluble material. 10 μl each of these samples, diluted 1:1 with 2 × SDS sample buffer (60 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM 2-mercaptoethanol, 20% glycerol, and 2% SDS) were loaded in each lane of a 10% SDS-polyacrylamide gel, resolved by electrophoresis, and electrophoretically blotted onto nitrocellulose. Equal amounts of protein were loaded in each lane as verified by staining duplicate gels with Coomassie Brilliant Blue R-250 (Life Technologies, Inc.) (data not shown). The Western blots were probed with a monoclonal antibody against PKC-α (Upstate Biotechnology, Inc.), a rabbit antiserum raised against PKC-β, a kind gift of Dr. Denise Cooper (Ishizuka *et al.*, 1992), a rabbit antiserum against PKC-γ (Life Technologies, Inc.), a rabbit antiserum against the C terminus of PKC-δ (Mischak *et al.*, 1991a), affinity-purified rabbit antisera against the C terminus of PKC-ϵ or PKC-ζ (Life Technologies, Inc.), or a rabbit antiserum against the 18 C-terminal amino acids of PKC-η (Mischak *et al.*, 1993). Secondary antibodies were goat anti-mouse or anti-rabbit IgG coupled to alkaline phosphatase (Life Technologies, Inc.). The immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitro blue tetrazolium chloride (Life Technologies, Inc.).

Cytostaining and Differentiation Assays—Cytospins of 2–5 × 10^5 cells were stained with Wright-Giemsa. Lysozyme activity was assayed as described (Osserman and Lawlor, 1966). The ability of wild-type 32D cells and the PKC overexpressers to phagocytize either before or after 16-h stimulation with 10 ng/ml TPA was determined by incubating 10^7 myeloid cells with 10^8 yeast particles in 10 ml of medium for 1 h at 37 °C. Cell populations were considered to be phagocytic if at least 30% of them contained intracellular yeast particles. Flow cytometry was performed on 10^4 viable cells for each sample using a Becton Dickinson FACScan. Monoclonal antibodies used were anti-Mac-1 (M1/70) and anti-Fc receptor (2.4G2) from CellTag (San Francisco, CA) and anti-Mac-2 (M3/38) and anti-Mac-3 (M3/84) from Boehringer Mannheim. Nonspecific FcR-mediated binding was ruled out by the lack of staining of the cells with an irrelevant isotype-matched antibody. A 1:40 dilution of fluorescein isothiocyanate-labeled goat anti-rat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used as a secondary antibody.

PKC Kinase Assays—PKC activity was determined on lysates of each of the PKC overexpressers as described (Kazanietz *et al.*, 1992) after partial purification. Briefly, 2 × 10^7 cells were lysed in 500 μl of lysis buffer that contained 30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 1% Triton X-100 and sonicated on ice for 10 s. The insoluble fraction was removed by centrifugation at 14,000 × *g* for 10 min, and each supernatant was applied to a DEAE-52 column. The PKC activity was eluted in one step with 300 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.3% 2-mercaptoethanol. 10 μl of eluate were added on ice to 40 μl of assay mix that contained 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 10 mM magnesium acetate, 1 μM TPA, 80 μg/ml phosphatidylcholine, 20 μg/ml phosphatidylserine, 0.1 mM ATP/[γ-^{32}P]ATP (specific activity 200 Ci/mmol), and 1 μM PKC-α substrate peptide. The reactions were incubated at 30 °C for 10 min, and subsequently 25 μl of each reaction was spotted onto Whatman PE-81 paper. The paper was washed, three times with 0.1 M H_3PO_4 and once with acetone, air-dried, and counted. Background (phospholipid-independent) kinase activity was assayed under identical conditions without TPA or phospholipid and subtracted from the total kinase activity measured in the presence of TPA and phospholipid. The PKC activity is expressed as counts/min of ^{32}P incorporated into the substrate/μg protein of the partially purified lysate.

RESULTS

Stable Overexpression of PKC Isozymes Does Not Abrogate IL-3 Dependence—As shown in Fig. 1, we obtained cell lines that stably overexpress PKC-α, -βII, -δ, -ϵ, -ζ, and -η. Unless otherwise indicated, all further characterizations of the overexpressers were performed using the highest expressing cell lines as judged by the Western blot analysis. The isozymes excluded from our studies were PKC-γ and -θ for which we have not yet successfully made expression vectors. Since PKC-γ is expressed exclusively in brain (Ohno *et al.*, 1991), and PKC-θ seems to be the principal isozyme in skeletal muscle (Osada *et al.*, 1992), even if either of these were shown to induce differentiation in TPA-treated 32D, their relevance to normal macrophage differentiation is questionable. To prove that the cell lines overexpressed functional PKCs, we performed kinase assays on DEAE-purified lysates of 32D that overexpressed the different isozymes. As shown in Table I, all of these lines revealed increased kinase activity. We also used immunohistochemistry to observe the TPA-induced translocation of the overexpressed PKC isozymes (data not shown), a commonly accepted parameter for activation. With the exception of PKC-ζ, which does not bind TPA (Goodnight *et al.*, 1992), all PKCs revealed translocation within 15 min.

In the absence of TPA, none of the cell lines that overexpressed PKC isozymes revealed any significant morphological difference from the parental 32D cell line. Furthermore, as has been reported previously for PKC-β in FDCP-1, an IL-3-dependent myeloid progenitor cell line (Kraft *et al.*, 1990), no abrogation of the IL-3 dependence of 32D could be observed in any of the overexpressers in the absence or presence of TPA.

Overexpression of Only PKC-α and -δ Enables TPA-induced Myeloid Differentiation—Cell lines that showed overexpression of PKC isozymes on Western blots were stimulated with different doses of TPA, and their responses were compared with that of normal 32D cells and those containing vector alone. Although 32D did not reveal any morphological or phenotypic changes upon stimulation with 1–1000 ng/ml TPA, 32D-LTR-α and 32D-LTR-B25, the cell lines that overexpress PKC-α, responded dramatically to the addition of TPA at 10 ng/ml. The cells became adherent within 30 min and revealed gross changes in morphology within 1 h. After 4 h the vast majority of the cells showed the morphology of mature macrophages, *i.e.* large irregular adherent cells with abundant vacuolated cytoplasm (Fig. 2). All PKC-α overexpressing 32D cells respond essentially identically to TPA, although the amount of PKC-α expressed in the different lines varies (Fig. 1 and data not shown).

Cells that overexpress PKC-δ, 32D-MTH-δ, also differentiated upon treatment with 10 ng/ml TPA. The kinetics of TPA-induced differentiation of these cells, however, differed from that of 32D-LTR-α. The response was slower, leading to adherence after 1–2 h and clear morphological changes after 4 h. After 12–18 h most of the cells displayed the morphology of mature macrophages. Low amounts of additional PKC-δ, as in 32D-MTH-δ2 (Fig. 1, *lane 7*), did not enable the cells to differentiate upon TPA treatment.

We performed additional transfection experiments of 32D with pLTR-α and pMTH-δ to confirm the results reported above. These transfections resulted in two more cell lines each of which expressed levels of the respective PKC isozyme comparable with 32D-LTR-α and 32D-MTH-δ, and they responded to TPA treatment identically to 32D-LTR-α and 32D-MTH-δ, respectively.

In contrast to the PKC-α and PKC-δ overexpressers, the cells that overexpress PKC-βII (32D-MTH-β and 32D-MTH-

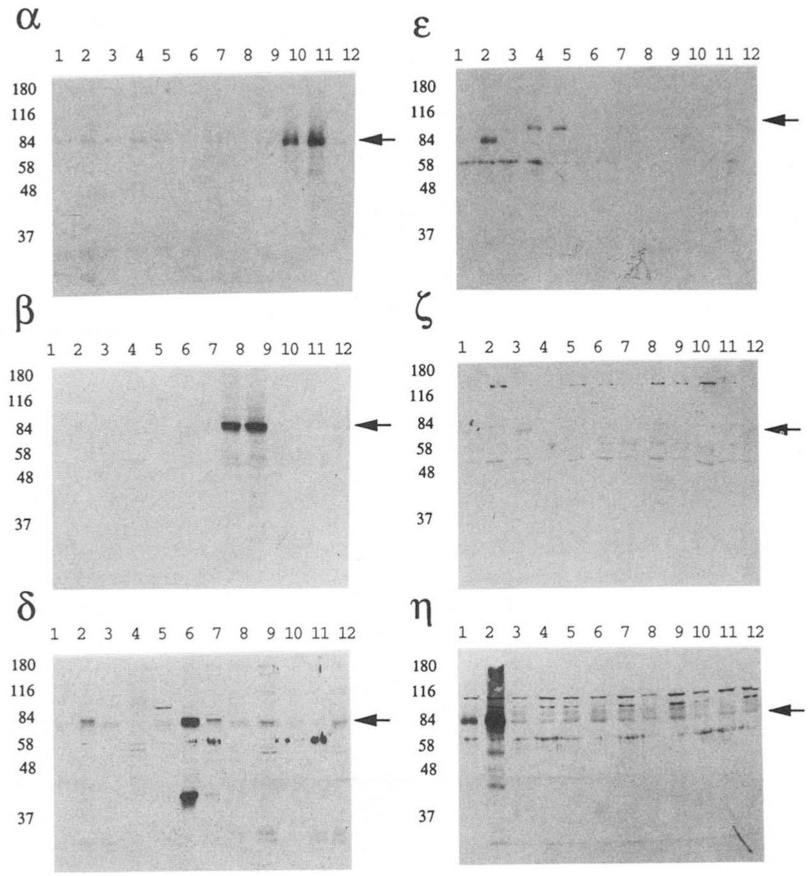

FIG. 1. **Western blots of wild-type 32D cells and lines that overexpress the different PKC isozymes.** The six blots were prepared with identical samples and probed with the antibodies that are specific for the PKC isozymes indicated on the *upper left. Lanes 1–12* contained lysates from the following cell lines: *lane 1,* 32D-LTR-η; *lane 2,* 32D-MTH-η; *lane 3,* 32D-MTH-ζ; *lane 4,* 32D-LTR-ε; *lane 5,* 32D-MTH-ε; *lane 6,* 32D-MTH-δ; *lane 7,* 32D-MTH-δ2; *lane 8,* 32D-MTH-β; *lane 9,* 32D-MTH-β2; *lane 10,* 32D-LTR-B25; *lane 11,* 32D-LTR-α; *lane 12,* 32D. The size and positions of prestained molecular weight markers are indicated on the *left. Arrows* indicate PKC protein bands.

TABLE I

PKC kinase assays of 32D wild-type and lines that overexpress PKC isozymes

The kinase activity was measured in cell lysates partially purified by stepwise elution of DEAE-52 columns as described under "Experimental Procedures" and is expressed as counts/min of ^{32}P incorporation in the substrate/μg of protein of the partially purified lysate.

Cell line	PKC activity
	cpm/μg protein
32D	14,000
32D-LTR-α	143,700
32D-MTH-β	63,700
32D-MTH-δ	33,800
32D-LTR-ε	52,300
32D-MTH-ζ	26,900
32D-MTH-η	38,400

β2), PKC-ε (32D-MTH-ε and 32D-LTR-ε), PKC-ζ (32D-MTH-ζ), or PKC-η (32D-LTR-η and 32D-MTH-η) did not reveal drastic morphological changes after treatment with 1–1000 ng/ml TPA. The cell lines that overexpressed PKC-ε and -η showed an increase in the fraction of adherent cells from 10% to up to 70% after treatment with 10–1000 ng/ml TPA. This increased adherence started 1–2 h after TPA treatment and persisted for up to 12 h. 32D-MTH-β also displayed an increased adherent fraction (up to 90% of the cells) as soon as 30 min after exposure to 10–1000 ng/ml TPA, but no signs of significant morphological changes could be observed. Adherence of these cells persisted for only 1–2 h. TPA treatment of 32D-MTH-ζ led to a slight and transient increase in adherence, identical to the effect observed on the parental 32D cells.

The lack of macrophage differentiation following TPA treatment of these cell lines is not due to increased down-regulation of the PKC isozymes. As shown in Fig. 3, all six isozymes are present in abundant amounts 6 h after TPA treatment, indicating that significant differences in down-regulation following TPA stimulation are not responsible for the differences observed in macrophage differentiation.

In order to further quantitate the effect of stimulation of the different PKC isozymes we performed flow cytometry (fluorescence-activated cell sorting analysis) on all cell lines before and after overnight stimulation with 10 ng/ml TPA. As shown in Fig. 4 for 32D-LTR-α, TPA treatment leads to the expression of the macrophage-specific surface markers Mac1, Mac2, and Mac3 as well as an increase in Fc-receptor expression. A similar pattern of TPA-induced surface antigen changes could be observed in 32D-MTH-δ (data not shown). In contrast, no significant change in these markers was observed in the cells that overexpress PKC-βII, -ε, -ζ, and -η (data not shown).

Further macrophage-specific characteristics were observed in 32D-LTR-α and 32D-MTH-δ that had been treated with TPA for 16 h: 1) both lines were able to phagocytize yeast (Fig. 5); and 2) both cell lines increased their lysozyme production 10–50-fold (Table II). In contrast, 32D cells that overexpress the other members of the PKC family did not reveal any of these characteristics of mature macrophages (Fig. 5 and Table II).

TPA-induced Differentiation Is Transient and Requires Continuous Activation of PKC—Differentiation of both 32D-LTR-α and 32D-MTH-δ is transient. The cells maintain their mature phenotype only in the continuous presence of TPA. If TPA is added only once to the cells, both lines start to dedifferentiate after 18–36 h. No mature macrophages are left in the cultures 2 days or 4–5 days after TPA stimulation of the PKC-α overexpressers and 32D-MTH-δ, respectively. If

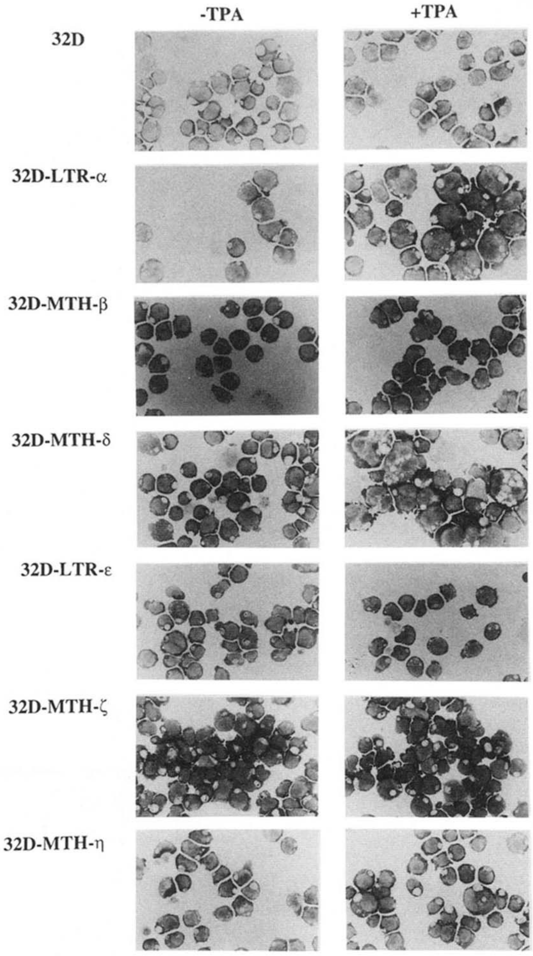

FIG. 2. **Photomicrographs of wild-type 32D cells and cell lines that overexpress the different PKC isozymes.** *Right panels* had been treated with 10 ng/ml TPA for 4 h, and *left panels* were untreated. The cells were applied to microscopic slides using a cytofuge and stained with Wright-Giemsa.

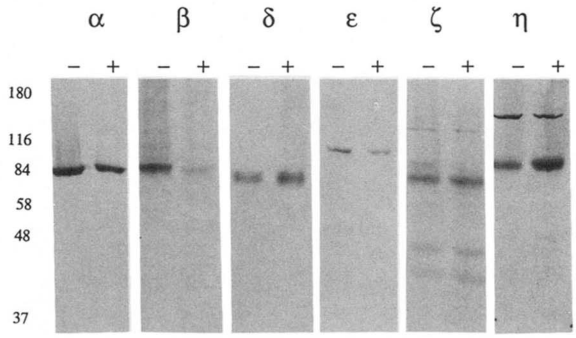

FIG. 3. **Western blots of overexpressing cell lines.** Cell lysates of 32D-LTR-α, 32D-MTH-β, 32D-MTH-δ, 32D-MTH-ϵ, 32D-MTH-ζ, and 32D-MTH-η were prepared as described in the legend to Fig. 1 from parallel cultures of either untreated cells (−) or cells that had been treated with 10 ng/ml TPA (+) for 6 h.

additional doses of TPA are added every 24 h, both 32D-LTR-α and 32D-MTH-δ retain their macrophage phenotype for 3–5 days or more than 8 days, respectively. Continuous treatment with TPA leads to down-regulation of PKC-α by proteolysis (data not shown) as has been described for many other systems (reviewed by Ohno *et al.*, 1991). Once PKC is down-regulated, as judged by Western blot analysis, the cells do not differentiate upon further TPA treatment. In contrast to PKC-α, we were unable to down-regulate PKC-δ even after continuous TPA treatment for 7 days, hence, the cells stay differentiated.

DISCUSSION

We have achieved overexpression of six of the eight known PKC isozymes in 32D cells, which enabled us to test whether overexpression of any of them could restore the ability to undergo TPA-induced macrophage differentiation seen in normal mouse myeloid progenitors. Although we found substantial differences in kinase activity among the cell lines that overexpressed the different PKC isozymes, we feel that these do not reflect comparable differences in the amount of overexpressed protein. Instead, they probably are due to the different activity that each PKC isozyme exhibits with respect to each substrate used as a target of phosphorylation (Kazanietz *et al.*, 1993). Furthermore, we observed that many isozymes, especially PKC-δ and -η, can be found in substantial quantities (sometimes more than 50%, as determined by Western blot, data not shown) in the Triton X-100-insoluble pellet of the cell lysate. This portion of the total PKC in the cell would not have been detected in our kinase assay. Thus, the signal intensity obtained from the Western blot analysis (Fig. 1) gives a better indication of the extent to which a particular member of the PKC gene family is overexpressed in any of the cell lines relative to the wild-type 32D cells, whereas the kinase assay principally indicates that the overexpressed protein is active.

Although data reported on *in vitro* phosphorylation of different substrates using purified PKC isozymes showed some variability in the effectiveness of phosphorylation, there have been no reports of any protein that was a substrate for phosphorylation by only certain PKC isozymes. These reports, nevertheless, indicate at least that purified isozymes show different affinities for particular substrates (Schaap *et al.*, 1989; Koide *et al.*, 1992; Kazanietz *et al.*, 1993). *In vivo*, both enzyme and substrate are usually present in much lower concentration and probably at specific subcellular locations to which only certain PKCs can translocate. Therefore, a much greater substrate specificity could be observed *in vivo* than *in vitro*.

Our results clearly indicate that the different members of the PKC gene family, although quite similar in structure, display dramatic biological differences *in vivo*. In an effort to summarize our observations on how overexpression and subsequent stimulation of the different PKC isozymes affected macrophage differentiation, we charted our observations over time after a single stimulation of each overexpresser with 10 ng/ml TPA (Fig. 6). This graph is not meant to be precise, but rather a summary description of the dynamic changes that we observed. We took increasing adherence, cell size, and vacuolation as well as the results of flow cytometry, lysozyme assays, and phagocytosis into consideration. The chart shows that only 32D clones that overexpress PKC-α and -δ attain the characteristics of mature macrophages, although most clones show a short-lived increase in adherence to the culture flask. It is not surprising that overexpression of PKC-ζ does not lead to TPA-induced differentiation, since this isozyme does not bind phorbol ester and, therefore, its kinase activity cannot be stimulated by TPA treatment (Ono *et al.*, 1989; Goodnight *et al.*, 1992). On the other hand, the clear involvement of only PKC-α and -δ in TPA-induced macrophage differentiation is quite remarkable. The inability of the other isozymes to induce differentiation is most likely due to their inability to phosphorylate an as yet unknown critical substrate and not due to faster down-regulation, since at least substantial amounts of all isozymes are still present in the

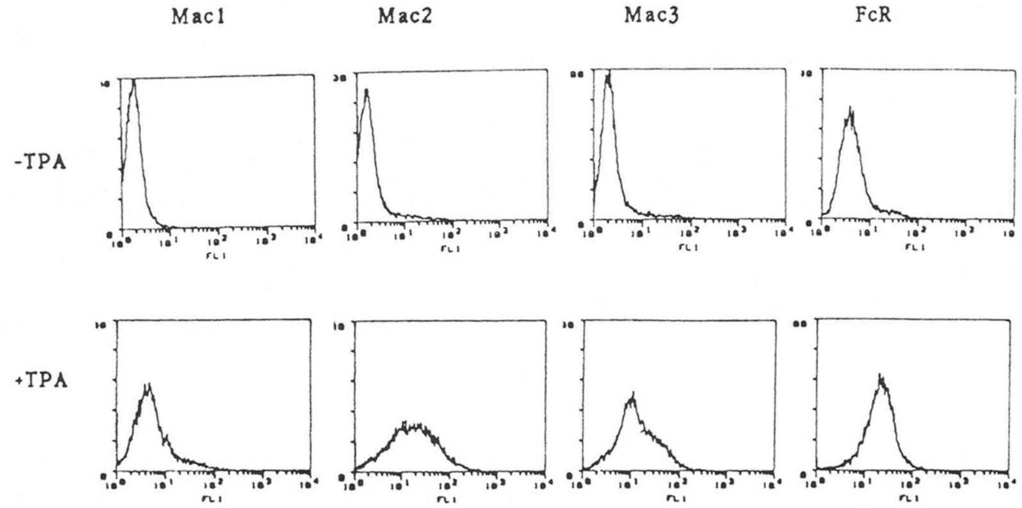

FIG. 4. **Flow cytometric analysis.** 32D-LTR-α cells were stained with fluorescein isothiocyanate-labeled antibodies as indicated above each pair of patterns. The patterns of unstimulated cells (*−TPA*) are shown above those obtained after overnight stimulation with 10 ng/ml TPA (*+TPA*).

TABLE II

Macrophage markers observed 6 h after TPA activation of 32D clones that overexpress PKC isoforms

	wt	α	βII	δ	ϵ	ζ	η
Increased adherence	+[a]	++++	++[a]	++++	++	+[a]	++
Increased cell volume[b]	−	++++	−	++++	+	−	+
Macrophage morphology[b]	−	++++	−	++++	−	−	−
Phagocytosis[c]	−	++++	−	++++	−	−	−
Lysozyme production[d]	+	+++	+	+++	+	+	+

[a] Transiently seen between 0.5 and 2 h after TPA addition.
[b] See Fig. 2.
[c] See Fig. 5.
[d] Observations made 18 h after TPA addition.

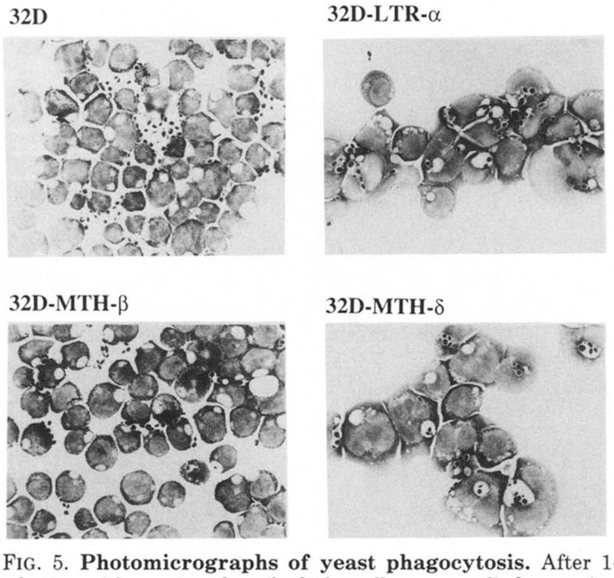

FIG. 5. **Photomicrographs of yeast phagocytosis.** After 1-h incubation with yeast as described, the cells were applied to a microscopic slide by cytospin and stained with Wright-Giemsa. TPA-activated 32D-LTR-α and 32D-MTH-δ have taken up almost all yeast cells in their vacuoles, whereas TPA-activated wild-type 32D and 32D-MTH-β have not removed the yeast cells from the surrounding medium.

cell lysates 6 h after TPA stimulation (Fig. 3).

It has been suggested that PKC-β mediates macrophage differentiation, because variants of HL-60 that do not differentiate upon TPA treatment lack PKC-β expression (Nishikawa *et al.*, 1990; Tonetti *et al.*, 1992). These results were not confirmed in another published report, in which it was suggested, instead, that the defect in the resistant HL-60 cell lines lies downstream of PKC (McSwine-Kennick *et al.*, 1991). We were also unable to find any differences in PKC-β expression between TPA-sensitive and TPA-resistant HL-60 cell lines.[2] Closer examination of the results reported by Tonetti *et al.* (1992) reveals that the TPA-resistant HL-60 lines have substantially lower levels of PKC-α and PKC-δ expression than the TPA-sensitive lines. Thus our findings that PKC-α and -δ, but not PKC-β, are involved in TPA-induced myeloid differentiation might also apply to HL-60 differentiation, and the previously reported data on this subject have to be reevaluated.

We had predicted that PKC-α or -β might be able to imbue 32D with TPA-inducible differentiation ability, because these two isozymes were present only in vanishingly small quantities in wild-type 32D cells. Since PKC-α, but not -β, can fulfill this function, it is likely that PKC-α normally is the principal isoform involved in myeloid differentiation. It was unexpected that, in addition to PKC-α, PKC-δ can also perform this function, inasmuch as endogenous PKC-δ is abundant in wild-type 32D, as it is in all myeloid cell lines examined as well as normal mouse myeloid cells (Mischak *et al.*, 1991b). Probably, PKC-δ, if overabundant, can phosphorylate substrates that are usually phosphorylated by PKC-α. It is also interesting to note that the overexpression and subsequent stimulation of PKC-δ leads to cell cycle arrest in CHO (Chinese hamster ovary) cells (Watanabe *et al.*, 1992) and complete growth inhibition of NIH 3T3 cells (Mischak *et al.*,

[2] H. Mischak and J. Goodnight, unpublished observations.

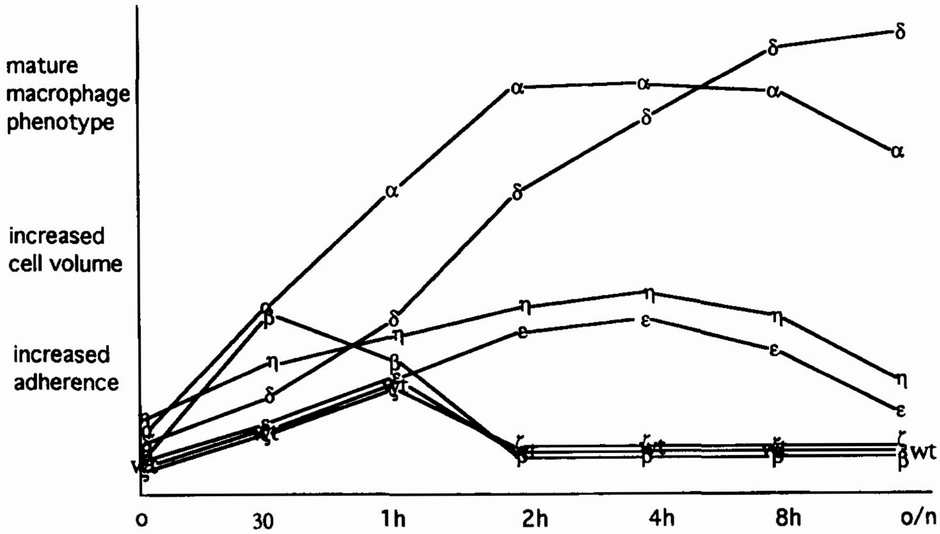

FIG. 6. **Kinetics of TPA-induced differentiation.** A compilation of observations of the effects observed after treatment with 10 ng/ml TPA of wild-type 32D cells (*wt*) or cell lines that overexpress the different PKC isozymes (α, 32D-LTR-α; β, 32D-MTH-β; δ, 32D-MTH-δ; ϵ, 32D-LTRe; ζ, 32D-MTH-ζ; η, 32D-MTH-η).

1993), phenomena that closely correlate with TPA-induced terminal differentiation.

Our data is best explained by a mechanism in which phosphorylation of critical substrates can be performed only by certain PKC isozymes. These data also suggest that the lack of expression of certain PKC isozymes, in particular of PKC-α, in most mouse early myeloid cell lines contributes to the inability of these cell lines to differentiate in response to TPA, and, possibly also to normal differentiation signals. An analogous inability to differentiate, which might involve changes in PKC expression or mutations in PKC, may be one of the steps that lead to the development of myeloid leukemias.

Our results also indicate that it is important to identify which members of the PKC family are involved in experiments that show that certain biological functions are mediated by PKC activation. Most likely, other PKC isozymes will be shown to have similarly specialized functions in other systems. This is the first report in which a TPA-induced biological function that had been lost in a cell line could be restored by substituting the missing members of the PKC gene family. Further examination of the different PKC overexpressing 32D cells may enable us to identify the critical substrates of PKC-α and -δ that are the downstream signal mediators of TPA-induced myeloid differentiation.

Acknowledgments—We thank Dr. John Knopf for kindly providing the PKC-βII cDNA, Dr. Nachman Mazurek for the pLTR, pLTR-α, and pLTR-B25 expression vectors, Dr. Grace Shen-Ong for the pMTH expression vector, and Dr. Denise Cooper for the anti-PKC-β antiserum.

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