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Introduction of the β isozyme of protein kinase C accelerates induced differentiation of murine erythroleukemia cells

(induction/commitment/hemoglobin/vincristine)

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ABSTRACT **Induction of differentiation in murine erythroleukemia cells (MELCs) involves a protein kinase C (PKC)-mediated step. Vincristine-resistant cells respond more rapidly to hybrid polar/apolar inducers than the parental cells. These vincristine-resistant MELCs contain elevated levels of the β isozyme of PKC (PKC-β). Exogenous homologous murine PKC-β, incorporated into permeabilized MELCs, accelerates induced differentiation. Neither rat PKC-β, nor mouse PKC-α, nor rat PKC-α, incorporated into permeabilized MELCs, is effective in altering the kinetics of induced differentiation. This provides direct evidence for a rate-limiting role for this PKC isozyme during *N,N'*-hexamethylenebisacetamide-mediated induced differentiation of a transformed cell.**

Terminal erythroid cell differentiation and loss of oncogenicity is induced in virus-transformed murine erythroleukemia cells (MELCs) by exposure to *N,N'*-hexamethylenebisacetamide (HMBA) or other hybrid polar/apolar compounds (1, 2). Although the molecular mechanism by which these agents initiate cell differentiation is not fully understood, it has been demonstrated that HMBA-induced commitment to MELC differentiation is a multistep process (3, 4). Commitment is defined as the irreversible capacity to express the differentiated phenotype despite removal of the chemical inducer (5, 6). Upon culture of the normally responsive MELC lines employed in our laboratories (DS19/Sc9 and N23; ref. 7) with HMBA, there is a latent period of 10–12 hr before commitment can be detected (6). This is followed by a period during which committed cells are progressively recruited to the differentiating population. Changes that occur during the latent period include alterations in membrane ion flux (8–10), cell volume (11), cyclic AMP concentration (12), and the expression of several genes including c-*myb*, c-*myc*, c-*fos*, and the gene encoding p53 (13–17).

Recently, we (11) and others (18) have provided evidence that a protein kinase C (PKC)-related signal is important in the early stages of induced MELC differentiation. The phorbol ester phorbol 12-myristate 13-acetate depletes cellular PKC activity and inhibits HMBA-mediated MELC differentiation. Upon removal from phorbol 12-myristate 13-acetate, MELCs progressively reaccumulate PKC activity and, in parallel, regain their inducibility by HMBA. Upon exposure to HMBA, MELCs exhibit a rapid and transient increase in membrane-associated PKC activity, generating in the cytosol a Ca^{2+}/phospholipid-independent kinase activity, followed by progressive decay of total PKC activity (13).

In the standard HMBA-responsive MELC strains employed in our laboratories (DS19/Sc9 and N23), PKC activity reflects principally the activities of two major isozymes, PKC-α and PKC-β, in a ratio of about 6:1 (19). Alteration in the relative activities of these PKC isozymes is characteristic of MELC variants selected for resistance to vincristine (VC). VC-resistant MELCs display acceleration of the rate of HMBA-induced differentiation, essentially eliminating the latent period prior to the initiation of commitment. VC-resistant MELCs also show enhanced sensitivity to low concentrations of HMBA (7, 19). In these cells PKC-β activity is significantly increased (PKC-α-to-PKC-β ratios approach 1:1), while the absolute level of PKC-α activity is essentially unchanged. In general, the level of PKC-β activity correlates with the rate of differentiation over a broad range of increases or decreases in that rate (19). These findings have suggested that PKC-β activity plays a critical role in the early stages of induction of terminal erythroid differentiation.

In the present studies, to test directly for a role for PKC-β in establishing the rate of induced differentiation in response to HMBA, we have examined the effect of incorporation of exogenous partially purified PKC-α or PKC-β on the rate of differentiation of a MELC line characterized by a relatively slow response to HMBA and two VC-resistant lines with increased inherent responsiveness to the inducer.

MATERIALS AND METHODS

Cell Culture. MELC cell lines N23, V3.17[7], and R1[VCR] were obtained as previously described (7, 19). They were maintained in α minimum essential medium (αMEM) supplemented with 10% (vol/vol) fetal calf serum and diluted when the cell density reached about 8×10^5 cells per ml. Cultures were initiated with an inoculum of 10^5 cells per ml, and HMBA was added at a final concentration of 5 mM.

Purification of PKC Isozymes. PKC isozymes were prepared from V3.17 MELCs and from rat and mouse brain sources. About 7×10^9 cells were collected when the cultures reached $7–8 \times 10^5$ cells per ml, washed three times with 100 ml of phosphate buffer (pH 7.5) containing 0.14 M sodium chloride, and suspended in 140 ml of 20 mM potassium phosphate buffer (pH 7.5) containing 10% (vol/vol) glycerol, 10 mM 2-mercaptoethanol, 1 mM EDTA, leupeptin (10 μg/ml), and 2 mM phenylmethylsulfonyl fluoride. The cells were lysed by sonication, and the particulate fraction was discarded after centrifugation at $100,000 \times g$ for 10 min at 5°C. The soluble fraction was chromatographed on a hydroxyapatite column (2×10 cm) as described (19). Fractions containing PKC-β (fractions 75–120) and PKC-α (fractions 160–220) were pooled and concentrated to about 5 ml by ultrafiltration (step I of purification). This PKC-β fraction was rechromatographed on hydroxyapatite for more com-

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Abbreviations: MELC, murine erythroleukemia cell; HMBA, *N,N'*-hexamethylenebisacetamide; PKC, protein kinase C; VC, vincristine.

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plete separation of the two isozymes. Fractions with PKC-β activity were pooled, concentrated, and dialyzed against complete Hepes buffer [10 mM Hepes (pH 7.5), containing 10% glycerol, 10 mM 2-mercaptoethanol, and 0.1 mM EDTA] (step II). This PKC-β fraction was chromatographed on a DEAE-52 column (1 × 7 cm) as described (19). Fractions with PKC activity (fractions 60–80) were pooled, concentrated, and dialyzed against complete Hepes buffer (step III). This PKC-β was chromatographed on a threonine-Sepharose (20) column (0.5 × 4 cm) previously equilibrated with dialysis buffer. The column was washed with buffer containing 50 mM sodium chloride, until the absorbance (280 nm) approached zero; the absorbed protein was eluted with a linear gradient (100 ml) of sodium chloride from 50 to 200 mM. Fractions containing PKC-β activity (fractions 41–54) were pooled, concentrated to 5 ml, and dialyzed against complete Hepes buffer. This enzyme (step IV) showed a single band on SDS/PAGE. Purified PKC-α was also prepared by this same protocol. The identity of each isozyme was confirmed by means of immunoblots employing isozyme-specific antipeptide antibodies (21) kindly provided by O. M. Rosen, as described (19). PKC activity was assayed as the pmol of ^{32}P incorporated into histone type III-S per min, as described elsewhere (13). The specific activities (units/mg of protein) for each of the step IV purified isozymes were as follows: PKC-β from V3.17 MELCs, 115,000; PKC-β from mouse brain, 122,000; PKC-β from rat brain, 108,000; PKC-α from V3.17 MELCs, 122,000; PKC-α from mouse brain, 130,000; PKC-α from rat brain, 98,000.

Incorporation of Exogenous PKC into MELC. Purified PKC isozymes, prepared as described above, were dialyzed for 6 hr against 10 mM Hepes (pH 7.5) containing 10% glycerol, 10 mM 2-mercaptoethanol, and 0.1 mM EDTA, then filtered through a Flow-pore filter (0.2-μm pore; Flow Laboratories). Approximately 1.5 × 10^6 MELCs were washed twice with 5 ml of 20 mM sodium phosphate buffer (pH 7.5) containing 0.14 M sodium chloride and permeabilized in glycerol and lysolecithin, as described by Nomura *et al.* (22). The permeabilized cells (in 2 ml) were incubated with the indicated amount of PKC isozyme (in 0.05 ml) for 18 hr at 37°C in a humidified incubator. Cells were washed twice with fresh complete αMEM, and viable cells (trypan blue exclusion test) were collected at the interface of a Ficoll gradient (two step; 12% upper and 18% lower phase) and centrifuged at 1200 × *g* for 20 min at 20°C. Cells were washed with fresh αMEM, and aliquots of 2 × 10^5 cells were suspended in 2 ml of culture medium with 5 mM HMBA. At the times indicated, the proportion of benzidine-reactive, hemoglobin-containing cells was determined in 0.1-ml aliquots, as previously described (6).

RESULTS AND DISCUSSION

Experiments were performed to determine the effect of exogenous PKC-α and PKC-β on the kinetics of HMBA-induced differentiation of three MELC lines: N23, a relatively slow-inducing variant, and V3.17[7] and R1[VCR], two VC-resistant, rapidly inducing lines, obtained as previously described (7, 19). Permeabilized cells were exposed, as described in *Materials and Methods*, to 60 units of PKC-α or PKC-β, purified from MELCs (step IV of purification, see *Materials and Methods*), and the kinetics of HMBA-induced differentiation (measured as the accumulation of hemoglobin-containing, benzidine-reactive cells) was followed over time (Fig. 1). In preliminary experiments we established that there was no depletion or translocation of endogenous PKC activity during permeabilization (data not shown).

Although all permeabilized cell lines displayed a longer latent period before onset of differentiation than the unmanipulated parental MELC lines, the introduction of PKC-β resulted in acceleration of the initial rate of HMBA-induced differentiation in all three MELC lines tested (Fig. 1), as compared with permeabilized control cells, which had not received exogenous PKC. By comparison, the introduction of PKC-α did not alter the kinetics of inducer-mediated differentiation. For each cell line, both control and PKC-

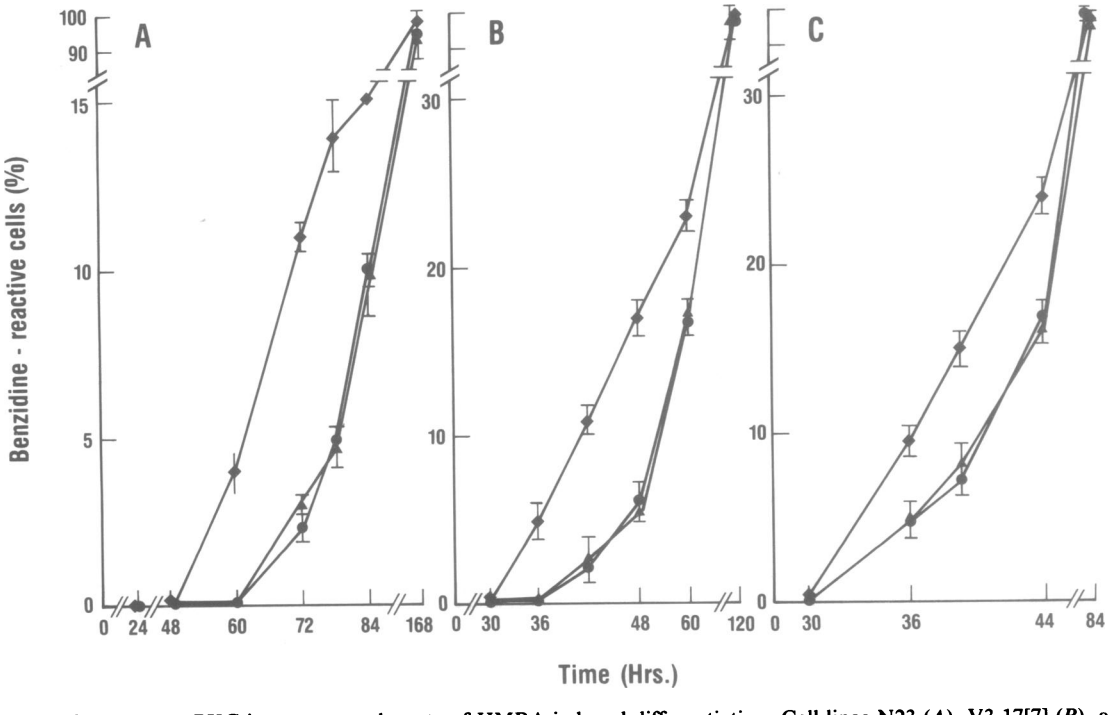

FIG. 1. Effect of exogenous PKC isozymes on the rate of HMBA-induced differentiation. Cell lines N23 (*A*), V3.17[7] (*B*), and R1[VCR] (*C*) were permeabilized and exposed to 60 units of partially purified PKC-β or PKC-α. The figure illustrates the progressive accumulation of benzidine-reactive cells in response to 5 mM HMBA in populations of MELCs that have incorporated exogenous PKC-β (◆), PKC-α (▲), or no PKC (●). Each point represents the mean ± SD of three separate experiments.

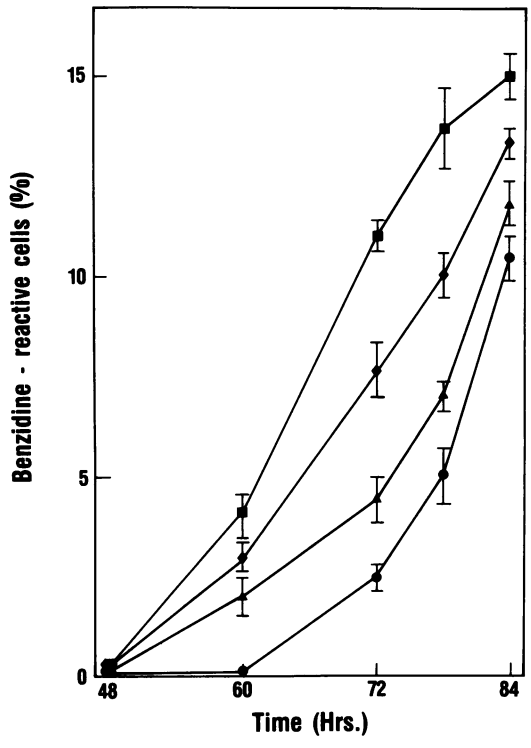

FIG. 2. Rate of HMBA-induced differentiation of N23 MELCs in response to increasing amounts of incorporated exogenous PKC-β. MELCs (1.5×10^6) were permeabilized, incubated with different amounts of purified PKC-β, then cultured with 5 mM HMBA, as described in Fig. 1. At the times indicated, the proportion of benzidine-reactive cells was determined. The amounts of exogenous PKC to which cells were exposed were 60 units (■), 45 units (◆), 30 units (▲), and no PKC-β (●). Each point is the mean ± SD of three independent experiments.

enriched cells reached full differentiation (90–95% benzidine-reactive cells) at about the same time. This suggests that not all cells contain the same level of extra, exogenous PKC-β, because the exogenous PKC-β is randomly distributed among the permeabilized MELCs. Transient acceleration of differentiation may also be explained if, as is likely, the exogenous PKC-β is only transiently effective, due to dilution during subsequent cell divisions and to instability of the exogenous PKC-β activity. Induction of differentiation in MELCs is a stochastic process; cells are progressively recruited to become irreversibly committed to differentiate during their exposure to inducer (6, 23). It is proposed that those cells destined to differentiate early, at a time when incorporated exogenous PKC-β activity is relatively high, will do so at an accelerated rate, while those destined for later differentiation, by which time incorporated exogenous PKC-β activity has fallen, will do so at a rate similar to that of control cells, which have not received exogenous PKC-β.

Table 1. Effect on HMBA-induced differentiation of incorporated exogenous PKC-β from preparations of enzyme of increasing specific activity

Purification step	PKC-β recovery,* units	Specific activity, units/mg	% benzidine-reactive cells†
I	35,000	1,129	11 ± 2
II	25,000	2,450	13 ± 3
III	15,000	13,640	12 ± 2
IV	8,000	114,300	12 ± 3
Control‡	—	—	3 ± 1

This isozyme was purified, as described in *Materials and Methods*, from the VC-resistant MELC variant V3.17[44]; this variant was selected because of its relatively high content of PKC-β (19). Permeabilized N23 cells were exposed to 60 units of PKC-β, from each step of increasing enzyme purification.

*Units of PKC activity are defined as pmol of ^{32}P incorporated into histone type III-S per min, as previously described (13).

†The percentage of benzidine-reactive cells was determined after 72 hr of exposure to 5 mM HMBA.

‡N23 cells were permeabilized and then exposed to HMBA, but exogenous PKC-β was not added.

Table 2. Effect of exogenous PKC isozymes purified from different sources on HMBA-induced differentiation of N23 cells

Addition	Source	Units of PKC added	% benzidine-reactive cells
No PKC			3 ± 1
PKC-β	MELCs	60	11 ± 2
PKC-β	Mouse brain	60	3 ± 1
		120	6 ± 2
		240	8 ± 2
		60	3 ± 1
		120	3 ± 1
PKC-β	Rat brain	240	3 ± 2
		60	3 ± 1
		120	3 ± 1
PKC-α	MELCs	240	3 ± 1
PKC-α	Mouse brain	60	3 ± 1
		120	3 ± 1
PKC-α	Rat brain	120	3 ± 1

PKC-α and PKC-β were partially purified from several sources, as described in the text, and exposed, in the amounts indicated, to permeabilized N23 cells. The proportion of benzidine-reactive cells was determined after 72 hr of incubation with 5 mM HMBA, and the values represent the mean ± SD for three independent experiments.

Acceleration in the rate of induced differentiation by incorporation of exogenous PKC-β is dose dependent (Fig. 2). The magnitude of the effect on the rate of differentiation is related to the amount of PKC-β presented for incorporation and is not related to the relative purity of the PKC-β preparation (Table 1). This strongly supports the conclusion that it is the PKC-β itself that affects induced differentiation and that there is a critical relationship between the level of cellular PKC-β activity and the kinetics of induced differentiation.

The effectiveness of incorporated exogenous PKC-β is also determined by the source of the isozyme (Table 2). MELC-derived PKC-β has a more pronounced effect than mouse brain PKC-β, even when the latter is incorporated at higher concentration, whereas rat brain β isozyme has no detectable effect. There is no effect detected from PKC-α whether derived from rat brain, mouse brain, or MELCs. These results suggest that there is a degree of species specificity to the PKC-β effect on inducer-mediated MELC differentiation. The differences between mouse brain and mouse erythroleukemia-derived PKC-β may reflect differences in the proportion of the known PKC-β isoforms, PKC-βI and PKC-βII (24), present in preparations of kinase from the two murine sources and reported to be differentially expressed in different tissues (25).

This study demonstrates that the introduction of exogenous PKC-β purified from MELC, but not PKC-α, into permeabilized MELCs results in an increase in the rate of HMBA-induced differentiation. These data provide direct evidence to support the conclusion that PKC-β plays an important role in the signaling mechanisms by which HMBA induces cell differentiation.

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