A Potential Role for Protein Kinase C-ε in Regulating Megakaryocytic Lineage Commitment*

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Multiple studies have shown that intracellular signal transduction by the protein kinase C (PKC) family participates in the initiation of megakaryocyte differentiation. In this study, multiple approaches addressed the functional contributions by specific PKC isozymes to megakaryocytic lineage commitment of two independent cell lines, K562 and human erythroleukemia (HEL). Pharmacologic profiles of induction and inhibition of megakaryocytic differentiation in both cell lines suggested a role for the calcium-independent novel PKCs, in particular PKC-ε. In transfection studies, the isolated variable domain of PKC-ε selectively blocked exogenous activation of the megakaryocyte-specific αIIb promoter. Constitutively active mutants of PKC-ε, but not of other PKC isozymes, cooperated with the transcription factor GATA-1 in the activation of the αIIb promoter. The functional cooperation between GATA-1 and PKC-ε displayed dependence on cellular milieu, as well as on the promoter context of GATA binding sites. In aggregate, the data suggest that PKC-ε specifically participates in megakaryocytic lineage commitment through functional cooperation with GATA-1 in the activation of megakaryocytic promoters.

A role for protein kinase C (PKC) signaling in megakaryocytic differentiation has been established by numerous experiments over the past two decades. In early studies, the PKC agonist phorbol diester selectively enhanced megakaryocyte colony formation by primary mouse bone marrow cells (1). More recent studies using primary human progenitors confirmed the promegakaryocytic effects of phorbol ester and showed such effects to be inhibitable by the PKC antagonists GF-109203X and Ro-31–8220 (2). In numerous cell line models of megakaryocytic differentiation, PKC activation induced an array of features including the following: cell cycle arrest, secretion of megakaryocytic cytokines, up-regulation of megakaryocytic surface antigens, cellular enlargement, polyploidization, development of proplatelet processes, and appearance of demarcation membranes (3, 4).

The PKC serine/threonine kinase family consists of at least 11 distinct isozymes organized into three subgroups, based on biochemical, pharmacologic, and structural properties (5). The classical or conventional PKCs (cPKCs) require diacylglycerol and Ca²⁺ for activation and consist of the α, β, βII, and γ isozymes. The novel PKCs (nPKCs) require only diacylglycerol for activation and consist of the δ, ε, η, and μ isozymes. The atypical PKCs lack responsiveness to diacylglycerol and Ca²⁺ and consist of the λ and ζ isozymes. Striking functional differences exist among PKC isozymes, with divergent functions documented even for factors with high structural homology (6–8).

PKC signaling may influence megakaryocytic differentiation through several isozymes. In K562 cells, PKC-mediated, sustained activation of the Raf-MEK-ERK signaling pathway is necessary for initiation of megakaryocytic differentiation (9, 10). Multiple PKC isozymes, in particular α, β, η, and δ, possess the capacity to activate the Raf-MEK-ERK pathway (11, 12). However, activation of the Raf-MEK-ERK pathway appears not to be required for megakaryocytic differentiation of primary progenitor cells, suggesting that PKC signaling plays an additional role in megakaryopoiesis independent of ERK activation (13).

To examine the contribution of specific PKC isozymes to megakaryocytic differentiation, we initially employed isozyme-selective pharmacologic agents in two independent cell line models of megakaryocytic differentiation, K562 and HEL. GF-109203X, an inhibitor of cPKCs and nPKCs but not of atypical PKCs, potently blocked megakaryocytic induction in both cell lines. By contrast, Go 6976, an inhibitor only of cPKCs (14), failed to block megakaryocytic differentiation, suggesting a specific requirement for nPKC signaling. For both cell lines, the PKC-ε-selective agonist, ingenol 3,20-dibenzoate (IDB) (15–17), induced megakaryocytic differentiation, as well as selective nuclear translocation of PKC-ε.

In transfection assays, the isolated variable domain of PKC-ε, but not that of PKC-α, completely blocked exogenous activation of the megakaryocyte-specific αIIb promoter. Constitutively active mutants of PKC-ε activated the αIIb promoter 3–6-fold. We also addressed whether PKC-ε signaling influenced the function of GATA-1, a transcription factor known to play a
critical role in megakaryopoiesis and in activation of the αIIb promoter (18–20). Indeed, GATA-1 and constitutively active PKC-ε showed synergistic activation of the αIIb promoter. Notably, the ability to synergize with GATA-1 distinguished PKC-ε from other PKC isozymes, dependent on cellular milieu, and depended on the context of GATA binding sites within the promoter.

MATERIALS AND METHODS

Cell Culture—K562 and HEL, obtained from the ATCC, were grown in RPMI 1640 with 10% fetal bovine serum at 37 °C, 5% CO2. C3H10T1/2, obtained from ATCC, was grown in Dulbecco’s modified Eagle’s medium with 10% neonatal calf serum at 37 °C, 5% CO2, 9293T, provided by Dr. Kevin Lynch (Department of Pharmacology, University of Virginia School of Medicine), was grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C, 5% CO2. All experiments using K562 and HEL employed mid-log phase cells at a density of 0.5–1 x 106 cells/ml. Conditioned media was obtained as described previously by 72 h of treatment of either K562 or HEL cells with 25 nM 12-O-tetradecanoylphorbol-13-ester (Sigma) followed by harvesting and dialysis of supernatant (9).

For megakaryocytic induction, cells were resuspended in conditioned media and incubated at 37 °C, 5% CO2 for 1–3 days, as indicated. The compounds GF-109203X, Go6976, and IDB were purchased from LC Laboratories. The PKC inhibitors GF-109203X and Go6976 were added, at indicated concentrations, to conditioned media at the initiation of megakaryocytic induction.

Flow Cytometry—Staining of cells for surface CD41 employed the fluorescein isothiocyanate-conjugated antibody PT1/PTC (Coulter) at 25 μg/ml. Staining of cells for surface glycophrin A employed the phycoerythrin-conjugated antibody GA-R2-PE (Pharmingen) at 10 μg/ml. Appropriate fluorochrome-conjugated, isotype-matched antibody controls were used at concentrations identical to the corresponding experimental antibodies. Flow cytometric analysis was performed on a FACScan system utilizing Lysys II software (Becton Dickinson).

Immunofluorescent Staining for PKC—Treated cells were cytospun onto glass slides and fixed for 2 min in ice-cold methanol followed by 2 min in ice-cold acetone. After blocking for 30 min at room temperature with 1% normal goat serum in phosphate-buffered saline, primary antibodies in 0.1% normal goat serum/phosphate-buffered saline were applied for 1 h at room temperature. Murine monoclonal antibodies to PKC-ε (Santa Cruz Biotechnology, Santa Cruz, CA) and to PKCα (Transduction Laboratories, Lexington, KY) were used at 200 ng/ml and at 1.25 μg/ml, respectively. Control murine antibody (NOR 3.2; BIO SOURCE International) was used at 1 μg/ml. Secondary antibody, consisting of phycoerythrin-conjugated goat anti-mouse (Tago, Inc.) diluted 1:100 in 0.1% normal goat serum/phosphate-buffered saline, was applied for 30 min at room temperature. For nuclear visualization, 4,6-diamidino-2-phenylindole was included in the coverslip mounting medium. Cells were visualized by confocal laser scanning fluorescence microscopy on a Zeiss LSM 410 (Jena, Germany) using Zeiss LSM analysis software.

Plasmid Constructs—The αIIb-luciferase reporter constructs were made by polymerase chain reaction amplification of bases 598 to +32, bases 98 to +32, or bases 98 to +32 from human αIIb promoter fragment kindly provided by Dr. Samuel Santoro (21). The polymerase chain reaction products were co-digested with XhoI plus HindIII and ligated into the corresponding sites of pGL3-Basic (Promega). The β-galactosidase expression vector consisted of pCMVβ (CLONTECH). The GATA-1 expression vector employed the EF-1-α-neo expression plasmid and has been previously described (22).

Mammalian expression of a full-length constitutively active (CA) mutant of PKC-ε employed Srd-rfK55A/R156A/A159E (AE3), kindly provided by Dr. S. Ohno (23). Expression of a full-length CA mutant of PKC-ε employed Srd-8R144/144A (DRA), also kindly provided by Dr. S. Ohno (23). Expression of a full-length CA mutant of PKC-ε employed rpe-CMV-PKC-ε A26E, kindly provided by Dr. Gottfried-Baier (24). Expression of a full-length CA mutant of PKC-ε employed rpe-CMV-PKC-ε R145A/R146W, kindly provided by Dr. J. Anthony-Watson (25). Mammalian expression constructs for HA-epitope-tagged, isolated PKC catalytic and regulatory domains have been previously described (26). In brief, fragments encoding the catalytic domains of α (amino acids 326–762) and ε (amino acids 395–737) were ligated into the pHANE vector. The fragment encoding the catalytic domain of δ (amino acids 334–674) was ligated into the pHACE vector. Fragments encoding the variable domains of α (amino acids 2–325) and ε (amino acids 2–394) were ligated into the pHANE vector.

RESULTS

Pharmacologic Implication of nPKC in Megakaryocytic Differentiation—Previous work in our laboratory indicated that sustained activation of the Raf-MEK-ERK pathway in the K562 hematopoietic cell line resulted in production of autocrine factors promoting megakaryocytic maturation (9). To identify
signaling pathways triggered by such autocrine factors, we analyzed the effects of pharmacologic inhibitors on megakaryocytic induction in two independent cell lines, K562 and HEL. The induction stimulus consisted of conditioned media from 12-O-tetradecanoylphorbol-13-ester-treated HEL cells, which show identical activity to that previously reported for K562 cells (9).

The only compound in our screening that potently and specifically blocked megakaryocytic induction by conditioned media was GF-109203X. GF-109203X inhibits both cPKC and nPKC isozymes, as well as pp90rsk2 (14, 28). Therefore, parallel experiments were carried out using the compound Go6976, known to inhibit cPKCs and pp90rsk2 but not nPKCs (14, 28). As shown in the flow cytometric profiles in Fig. 1, conditioned media alone induced up-regulation of the megakaryocyte surface antigen CD41 and down-regulation of the erythroid surface antigen glycophorin A. Whereas GF-109203X completely blocked both responses, Go6976 at similar doses showed no inhibition of either response.

Analysis of cellular morphology supported the flow cytometric results in Fig. 1. In particular, HEL cells exposed to conditioned media undergo spreading and enlargement as part of their megakaryocytic differentiation. As shown in Fig. 2, the morphologic changes induced in HEL cells by conditioned media were abrogated by GF-109203X. By contrast, Go6976 strikingly enhanced the cellular spreading and enlargement induced by conditioned media. These data confirm that biologically active doses of Go6976 acted to enhance rather than inhibit features of megakaryocytic differentiation.

In an alternative approach, the isozyme-selective PKC agonist IDB was applied directly to K562 and HEL cells in standard growth media. Multiple previous studies have indicated that IDB is a selective activator of nPKCs, particularly PKC-e (15–17). As shown in Fig. 3, IDB caused CD41 up-regulation and glycophorin A down-regulation in K562 and HEL cells. In addition, HEL cells treated with IDB manifested the standard morphologic changes seen with megakaryocytic induction (not shown). Immunofluorescent staining (Fig. 4) showed that treatment of K562 cells with IDB induces rapid nuclear translocation of PKC-e but no change in the subcellular localization of PKC-a. Rapid nuclear translocation of PKC-e was also observed in HEL cells treated with IDB (data not shown). Thus, both agonists and antagonists implicate nPKC, in particular PKC-e, in the induction of megakaryocytic differentiation.

**Blockade of Megakaryocytic Promoter Activation by the Regulatory Domain of PKC-e**—The amino-terminal regulatory sequences of PKCs, when expressed as isolated fragments, function as dominant-negative PKC inhibitors (29, 30). Accordingly, we examined whether transfection of isolated regulatory domains from either PKC-a or PKC-e could interfere with exogenous activation of the megakaryocyte-specific αIb-598 promoter (31). In cells transfected with control vector, 24 h of conditioned media caused an ~14-fold up-regulation of the αIb-598-luciferase reporter activity (Fig. 5A). Expression in cells of the isolated α regulatory fragment (α-Reg) almost completely eliminated responsiveness to the conditioned media stimulus. Immunoblotting showed similar expression levels of the HA-epitope-tagged α and e regulatory domains in HEK-293T transfectants (Fig. 5B).

**Specific Cooperation of PKC-e with GATA-1 in Activation of a Megakaryocytic Promoter**—We next tested whether constitutively active PKC mutants could activate the αIb megakaryocytic promoter. Fig. 6A demonstrates the similar results obtained with two different types of constitutively active mutants, inhibitory domain point mutants (CA mutants in left graph), and isolated PKC catalytic domains (CAT mutants in...
The inhibitory domain point mutants consist of full-length PKCs with point mutations in the autoinhibitory regulatory domains (23–25). The isolated PKC CAT completely lack regulatory domains, which have also been implicated in Ca\(^{2+}\) and lipid binding, interaction with RACKs, and kinase-independent signaling (26). Using the megakaryocyte-specific \(\alpha\)IIb-598 reporter, we tested the effects of PKC isozymes alone and in conjunction with GATA-1, a known positive regulator of the \(\alpha\)IIb promoter (18, 19). As shown in Fig. 6A, constitutively active PKC-\(\epsilon\) mutants alone modestly activated the \(\alpha\)IIb promoter (3–6-fold) but demonstrated clear functional cooperation with GATA-1. In fact, coexpression of GATA-1 with either CA or CAT mutants of \(\epsilon\) led to levels of reporter activation analogous to those obtained with conditioned media induction. Functional cooperation with GATA-1 was clearly isozyme-restricted in that constitutively active mutants of \(\alpha\), \(\delta\), and \(\theta\) all failed to augment GATA-1-mediated \(\alpha\)IIb activation. In fact, \(\delta\) caused a 2–3-fold inhibition of GATA-1 activation. Immunoblot analysis demonstrated equivalent levels of GATA-1 expression in all transfectants, indicating that the differential effects of the PKC isozymes were not because of differences in GATA-1 levels (Fig. 6B). In addition, immunoblot demonstrated analogous expression levels of the HA-epitope-tagged \(\epsilon\), \(\alpha\), and \(\delta\) catalytic domains in HEK-293T cells (Fig. 6C).
Cellular Context and Promoter Context Influence PKC-ε/GATA-1 Cooperativity—To determine whether functional interaction between PKC-ε and GATA-1 occurred also in non-hematopoietic cells, cotransfections were carried out in C3H10T1/2 fibroblasts rather than in K562 hematopoietic cells. The full-length CA ε mutant, as well as the isolated ε CAT, failed to augment GATA-1-mediated αIIb activation in C3H10T1/2 fibroblasts (Fig. 7A). Immunoblotting demonstrated equivalent expression of GATA-1 in all of the transfectants (Fig. 7B). Thus, the functional interaction of PKC-ε with GATA-1 clearly depends on the cell type employed for transfection.

The αIIb promoter contains multiple GATA binding sites, including a functional site within the promoter-proximal −98 fragment (18, 19). To determine whether specific promoter regions were required for PKC-ε/GATA-1 cooperativity, 5′ truncated reporter constructs, αIIb-98 and αIIb-348, were compared with αIIb-598 for responsiveness to PKC-ε ± GATA-1 in K562 cells. Surprisingly, the αIIb-98 and αIIb-348 reporters showed full activation by GATA-1 alone but no evidence of augmentation by PKC-ε (Fig. 7C). Thus, the functional GATA binding sites in the αIIb −348 to +32 fragment were insufficient to mediate PKC-ε/GATA-1 cooperativity.

**DISCUSSION**

Involvement of PKC signaling in hematopoietic lineage commitment decisions has been well documented. In progenitors transformed by the E26 avian leukemia virus, thresholds of PKC activity correlated with cell fate determinations as follows: (a) no kinase activity was associated with undifferentiated cells; (b) low activity was associated with myelomonocytic differentiation; and (c) high activity was associated with eosinophil differentiation (32). In primary, bipotential granulocyte macrophage colony-forming cells, activation of PKC-α induced commitment to the macrophage lineage (33). Similarly, our data suggest that signaling via PKC-ε may promote megakaryocytic lineage commitment of the bipotential BFU-E/MK progenitor, a cell with capability for either erythroid or megakaryocytic differentiation (34). Previous studies have notably shown that PKC-ε undergoes down-regulation during erythroid differentiation and that inhibition of PKC-ε specifically enhances erythroid differentiation (35, 36).

Mechanisms by which PKC-ε signaling might contribute to the activation of the megakaryocytic αIIb promoter remain unclear. Earlier studies with isolated catalytic domains have shown PKC-ε to activate multiple pathways that converge on the serum-response element of the c-fos promoter, c-Raf, MEK1-ERK, MEK kinase 1-stress-activated protein kinase kinase-c-Jun NH₂-terminal kinase, and rhoA (26). However, those studies showed equivalent activation of the various pathways by the PKC-α catalytic domain. Our results, by contrast, show no activation of the αIIb promoter by the PKC-α catalytic domain. The rapid nuclear translocation of PKC-ε observed with megakaryocytic induction by IDB (Fig. 4) raises the possibility that PKC-ε itself might act directly upon critical nuclear substrates.

The functional cooperation of PKC-ε with GATA-1 raises a number of mechanistic possibilities. One scenario is that PKC-ε signaling targets a transcriptional complex containing GATA-1 and enhances GATA-1 function by phosphorylation of one of the members of this complex, such as GATA-1 itself or the cofactor friend of GATA-1. The absence of functional interaction in C3H10T1/2 cells argues against direct phosphorylation of GATA-1 by PKC-ε as a sufficient mechanism. This scenario also fails to account for the dependence of PKC-ε signaling on promoter context, as illustrated in Fig. 7C. Accordingly another possibility is that PKC-ε signaling targets GATA-1 complexes binding to specific regions of the αIIb promoter. A recent study employing embryonic stem cell hematopoiesis has defined within the human αIIb promoter a 200-base pair critical enhancer region, −398 to −598, that is necessary and sufficient
for megakaryocyte-specific transgene expression (31). Interestingly, our data indicate that a similar region (from −348 to −598 of the human αIIb promoter) is required for responsiveness to PKC-ε signaling. Future studies will attempt to correlate PKC-ε response elements within the αIIb promoter with megakaryocyte-specific enhancer function.

A major question in the molecular characterization of hematopoietic lineage commitment is how two lineages with highly similar arrays of transcription factors can show non-overlapping, indeed mutually exclusive, patterns of gene expression. Erythroid and megakaryocytic cells share expression of the highly restricted factors GATA-1, GATA-2, Lmo2, NF-E2, friend of GATA-1, and SCL/tal. Most striking among these factors is GATA-1, which dominantly activates erythroid genes only in erythroblasts and dominantly activates megakaryocytic genes only in megakaryocytes. Our current data raise the possibility that isozyme-specific signaling by PKC may modify GATA function in accordance with promoter context. In particular, PKC-ε signaling might specifically augment GATA-1 function in the context of megakaryocytic promoters, thereby redirecting the entire transcriptional program of a cell from erythroid to megakaryocytic.

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PKC-ε in Megakaryocytic Differentiation

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