

# An Activated Protein Kinase C $\alpha$ Gives a Differentiation Signal for Hematopoietic Progenitor Cells and Mimicks Macrophage Colony-stimulating Factor-stimulated Signaling Events

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**Abstract.** Highly enriched, bipotent, hematopoietic granulocyte macrophage colony-forming cells (GM-CFC) require cytokines for their survival, proliferation, and development. GM-CFC will form neutrophils in the presence of the cytokines stem cell factor and granulocyte colony-stimulating factor, whereas macrophage colony-stimulating factor leads to macrophage formation. Previously, we have shown that the commitment to the macrophage lineage is associated with lipid hydrolysis and translocation of protein kinase C  $\alpha$  (PKC $\alpha$ ) to the nucleus. Here we have transfected freshly prepared GM-CFC with a constitutively activated form of PKC $\alpha$ , namely PKAC, in which the regu-

latory domain has been truncated. Greater than 95% of the transfected cells showed over a twofold increase in PKC $\alpha$  expression with the protein being located primarily within the nucleus. The expression of PKAC caused macrophage development even in the presence of stimuli that normally promote only neutrophilic development. Thus, M-CSF-stimulated translocation of PKC $\alpha$  to the nucleus is a signal associated with macrophage development in primary mammalian hematopoietic progenitor cells, and this signal can be mimicked by ectopic PKAC, which is also expressed in the nucleus.

**H**EMATOPOIETIC growth factors (HGFs)<sup>1</sup> or cytokines can promote the survival, proliferation, and development of primitive cells (Ibelgafts, 1995). Although there is an increasing awareness of the molecular mechanisms that are activated and responsible for survival and proliferation, little is known of the signaling events leading to differentiation and development. Indeed, it is unclear if HGFs play an active role in the differentiation and development of multipotent hematopoietic progenitor cells, rather than simply acting as survival fac-

tors and mitogens (Cross et al., 1997). Erythropoietin, for example, is neither essential nor required for the formation of erythroid progenitor cells from more primitive multipotent cells (Wu et al., 1995). Furthermore, a multipotent cell has been shown in some instances to undergo differentiation to produce multiple cell lineages in the absence of HGFs, provided that they receive an appropriate survival stimulus (Fairbairn et al., 1993; Mayani et al., 1993). Although these and other data suggest that the differentiation of multipotent cells is a stochastic process, representing a consolidation of a randomly primed genetic program in the stem cells (Hu et al., 1997), data from the more developmentally restricted granulocyte macrophage colony-forming cells (GM-CFC) indicates that growth factors may indeed be able to influence lineage decisions (Williams et al., 1987; Cook et al., 1989; Heyworth et al., 1992, 1993; Metcalf, 1984, 1989; Metcalf and Nicola, 1991; Nicholls et al., 1994; Whetton et al., 1994).

GM-CFC proliferate and develop into either predominantly neutrophils in response to granulocyte colony-stim-

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1. *Abbreviations used in this paper:* G-CSF, granulocyte colony-stimulating factor; GM-CFC, granulocyte macrophage colony-forming cell; HGFs, hematopoietic growth factors; IL-3, interleukin-3; M-CSF, macrophage-CSF; MFI, mean fluorescence intensity; PKC, protein kinase C; SCF, stem cell factor.

ulating factor (G-CSF) and/or stem cell factor (SCF), or macrophages in response to macrophage-CSF (M-CSF; Heyworth et al., 1992; Whetton et al., 1994). We have previously reported that when activators of protein kinase C (PKC), such as TPA (phorbol-12-myristate-13-acetate) or bryostatin are added on their own to GM-CFC, they elicit a mild stimulation of proliferation and also induce the development of macrophages (Heyworth et al., 1993). When these PKC activators were added together with neutrophilic stimuli, such as SCF, there was a marked effect on lineage commitment, in that predominantly macrophages were formed. Furthermore, when calphostin C, a PKC inhibitor, was added in combination with M-CSF colonies containing both macrophages and neutrophils were formed. Thus, PKC inhibition partially abrogates the macrophage developmental lineage restriction imposed by M-CSF.

We have also found that the binding of M-CSF to its tyrosine kinase receptor, c-fms, (but not the binding of SCF to c-kit, also a tyrosine kinase receptor) stimulates a chronic increase in phosphatidylcholine breakdown and 1,2-diacylglycerol (the physiological activator of PKC) production in GM-CFC (Whetton et al., 1994), indicating the route whereby promacrophage developmental stimuli may induce PKC activation. Furthermore, we have found (using confocal microscopy) that stimulation with M-CSF and other cytokines promoting macrophage development from GM-CFC, resulted in a marked translocation of PKC $\alpha$  (but no other isoform) to the nucleus (Nicholls et al., 1994; Whetton et al., 1994). Treatment with calphostin C, however, inhibits the translocation to the nucleus and promotes the formation of neutrophils in cells treated with cytokines that normally promote macrophage development. The activation of PKC $\alpha$  and its translocation to the nucleus, therefore, appear to play a role in GM-CFC lineage commitment to macrophage development. To examine the potential role of PKC $\alpha$  in GM-CFC lineage restriction, we expressed a constitutively activated form of this enzyme which has had the regulatory domain deleted in a freshly prepared primary population of GM-CFC.

## Materials and Methods

### Preparation and Culture of Enriched GM-CFC

Cells from normal murine bone marrow enriched for GM-CFC were obtained by elutriation centrifugation as previously described (Williams et al., 1987; Cook et al., 1989). Soft agar colony-forming assays were performed as described previously (Sponcer et al., 1986). Colonies were picked out at random from agar plates for morphological analysis by cytospin preparation. Cytospin preparations of both liquid culture and colony samples were stained with May-Grunwald-Giemsa stain. The combined esterase stain was performed as previously described (Whetton et al., 1994).

### Retroviral Transfection

The constitutively active PKC $\alpha$  construct, PKAC, was kindly provided by M. Muramatsu (DNAX, Palo Alto, CA). The construct consists of a 253-amino acid truncation of the amino terminal regulatory domain of PKC $\alpha$  (Muramatsu et al., 1989). EcoRI linkers (Boehringer Mannheim, Mannheim, Germany) were added to the 1.8-kb PKAC construct, which was cloned into the EcoRI site of the retroviral vector pM5-neo to give pM5-PKAC. pM5 is a myeloproliferative sarcoma virus-based replication defective retroviral vector carrying the neomycin phosphotransferase gene as a selectable marker (Laker et al., 1987).

Stable transfected packaging cells were produced by G418 selection of GP + env86 cells (Markowitz et al., 1988b) infected with cell free superna-

tants from GP + envAM12 (Markowitz et al., 1988a) cultures, which had been lipofected (using Lipofectamine, GIBCO BRL, Paisley, Scotland) with either pM5-neo or pM5-PKAC plasmid DNA. Viral titres were determined by measuring the transfer of neomycin resistance to 3T3 cells in the presence of 8  $\mu$ g/ml of polybrene.

Infection of the GM-CFC was achieved by overnight culture in isoves medium with interleukin 3 (IL-3; 10 ng/ml), SCF (100 ng/ml) and FCS (10% vol/vol) containing cell free supernatants from the ecotropic producer cells GP + env86. Viral supernatants were added to give a final titre of  $\sim 10^6$  G418 resistant transfer units per ml. This method was also used to transfect the IL-3-dependent, haemopoietic cell line, IC2.9. After immunoprecipitation using anti-PKC $\alpha$  antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) the PKC activity in transfected IC2.9 cells was assayed by the phosphorylation of histone III $\alpha$ , as previously described (Evans et al., 1995).

### Flow Cytometry

Single cells were deposited in microwell plates using the automatic cell deposition unit of a FacsVantage flow cytometer (Becton Dickinson Co., Mountain View, CA). Single cell deposition into each well was checked by eye, using a microscope, 2 h after deposition. After 7 d, the morphology of the cells present in the wells was determined by spinning the plates and staining cells with May-Grunwald-Giemsa stain. The number of wells positive for cellular growth that initially contained a single cell was 83%.

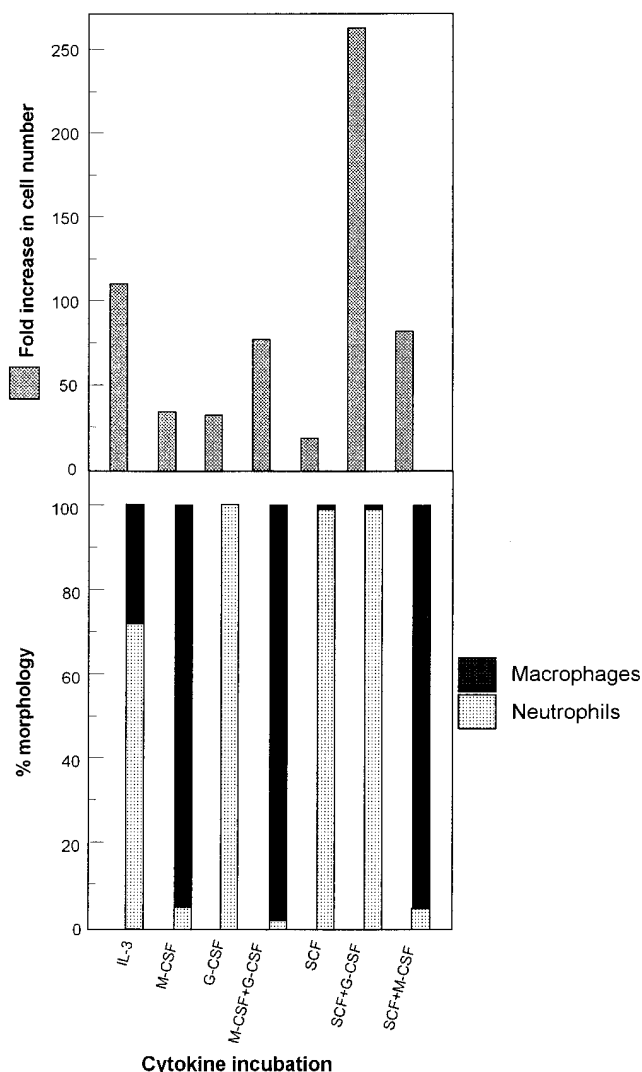
### Confocal Microscopy

The level of expression of PKC $\alpha$  and its localization within the cell was assessed using affinity-purified anti-PKC $\alpha$  specific antibodies and confocal microscopy. After an overnight transfection, with either pM5-PKAC, pM5-neo, or medium alone, slide preparations of transfected cells were made using a Shandon cytospin (500 rpm, 5 min). The cells were fixed for 10 min in ice cold methanol and incubated with primary antibody for 30 min. The PKC $\alpha$  antipeptide antibodies (GIBCO BRL, Paisley, Scotland) used recognized both the constitutively activated PKAC and native PKC $\alpha$ . The slides were then washed in PBS for 10 min. Samples were then blotted dry before incubating with FITC-conjugated sheep anti-rabbit IgG antibody (Binding Site, Birmingham, UK) for 30 min, and washed in PBS for 30 min. Nuclei were counterstained with propidium iodide for 1–2 min and fading of fluorescence was retarded with 2.4% DABCO (BDH Chemicals Ltd., Dagenham, UK) in 80% glycerol (Johnson et al., 1982). Stained cells were analyzed using an MRC 500 laser scanning confocal microscope (Biorad Laboratories, Hercules, CA).

## Results

### Response of Elutriated Cells to Stimulation with Various Cytokines

We have previously shown that the elutriated and highly enriched GM-CFC (produced by the method of Williams et al., 1987) proliferate in response to IL-3, GM-CSF, G-CSF, SCF, and M-CSF (Cook et al., 1989; Heyworth et al., 1992). [ $^3$ H]thymidine suicide, [ $^3$ H]thymidine incorporation, and anti-bromodeoxyuridine antibody staining assays all show a similar mitogenic response to SCF, SCF + G-CSF, or M-CSF, or SCF + M-CSF. Moreover, these are bipotent cells that can form macrophages or neutrophils (Whetton et al., 1994). SCF or SCF + G-CSF stimulates neutrophilic development and M-CSF (or SCF + M-CSF) stimulates macrophage development. Fig. 1 shows the effect of specific cytokines on the proliferation and development of the elutriated GM-CFC after 7 d in liquid culture. In the cultures stimulated with these cytokines, no apoptotic subpopulation was observed (viability remained >97% over 72 h) by staining the cells with acridine orange (Gregory et al., 1991), although in the absence of the appropriate cytokine, <1% of the cells were viable after 72 h and large numbers of apoptotic cells were present in the cul-



**Figure 1.** The effect of cytokines on the proliferation and development of elutriated GM-CFC in liquid culture. GM-CFC were cultured in liquid medium in the presence of the cytokines shown for 7 d. Cells were initially seeded at  $2 \times 10^4$  cells/ml and the fold increase in cell numbers over 7 d is shown. The percentage number of macrophage formed is displayed. The percentage number of blast cells was  $<4\%$  in all cultures, with the exception of M-CSF and SCF, which maintained 7 and 10%, respectively, blast cells. All other cells observed were neutrophils.

tures (results not shown). Thus, all the cells present can be stimulated to survive by either neutrophilic or macrophage developmental stimuli and, in the absence of these cytokine(s), they die.

### Transfection of Elutriated Cells with an Activated Protein Kinase C $\alpha$ , PKAC

These and other data show that specific cytokines stimulate the preferential development of either macrophages or neutrophils from GM-CFC. Because our previous work (Nicholls et al., 1994; Whetton et al., 1994) indicated that the translocation of PKC $\alpha$  to the nucleus was associated with commitment to macrophage development, we investigated whether PKC $\alpha$  translocation was a deterministic

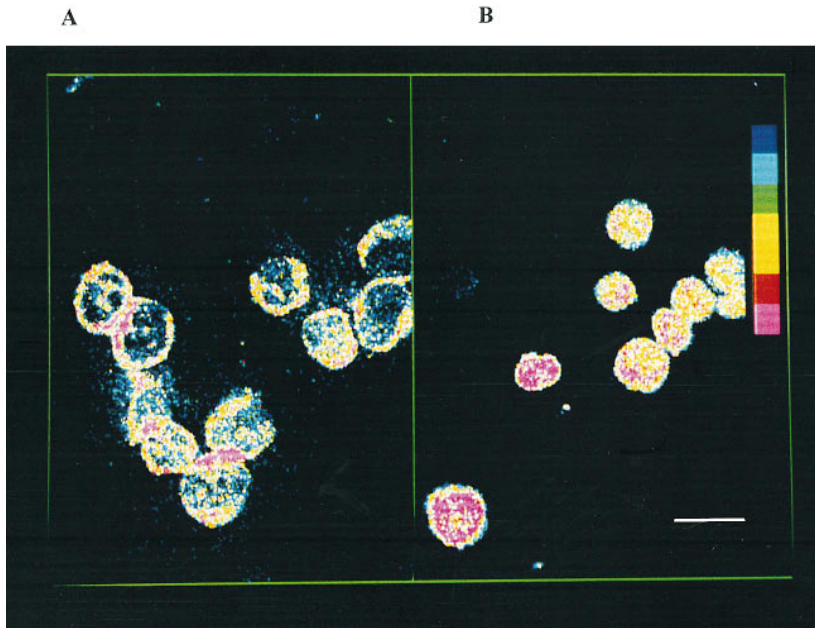
factor in the commitment of these cells. To achieve this we expressed a constitutively activated mutant of PKC $\alpha$ , PKAC, a deletion mutant that lacks the regulatory domain, in the GM-CFC. Because the GM-CFC are a transient cell population that, under our conditions, maintain their bipotentiality for only 48 h, it was not possible to select transfected cells using the neomycin drug resistance marker in the vector before investigating their response to macrophage or neutrophil stimuli. Furthermore, we observed in initial experiments that the addition of the neomycin analogue, G418 (1 mg/ml) to pM5-PKAC- and pM5-neo-transfected GM-CFC influenced cellular development. Subsequent experiments were therefore performed in the absence of this selection pressure.

Our strategy was to obtain verifiable expression of PKAC in the majority of cells during the period before which lineage commitment occurs. This was achieved by culturing the cells during retroviral transfection in SCF (100 ng/ml) and IL-3 (10 ng/ml), a growth factor combination, which we have previously shown to maintain not only the viability but also the bipotentiality of these cells (Kan et al., 1991), and using confocal microscopy to quantitate expression.

### Characterization of Transfected GM-CFC by Confocal Microscopy

To quantify the efficiency of retroviral infection, that is, the number of cells expressing increased levels of PKC $\alpha$ , GM-CFC from 16-h cultures with either pM5-PKAC, pM5, or medium alone were subjected to immunostaining. After washing, cells were cytospun, stained with anti-PKC $\alpha$  antibody and then subjected to analysis by an MRC 500 laser scanning confocal microscope. Fig. 2 illustrates the staining achieved in GM-CFC incubated for 16 h with medium alone or the pM5-PKAC retrovirus. The photographs demonstrate, as previously reported, the endogenous expression of PKC $\alpha$  in GM-CFC (Whetton et al., 1994). After a 16-h incubation with viral supernatant,  $>95\%$  of PKAC-transfected GM-CFC contain  $>200\%$  of the levels of PKC $\alpha$ , compared to pM5-neo and medium alone-treated controls (Table I). The mean increase observed in pM5-PKAC-treated cells was 3.3-fold. Furthermore, a nuclear localization of PKC $\alpha$  was notable in PKAC-transfected GM-CFC, the mean fluorescence intensity (MFI) of nuclear PKC $\alpha$  was 5.0-fold higher in pM5-PKAC-treated cells compared to control cells. Careful analysis of PKC $\alpha$  localization in the enriched GM-CFC confirmed that there was no membrane association. Furthermore, in other experiments with enriched GM-CFC, we have observed PKC $\beta$  translocation to the plasma membrane after a 10-min incubation with SCF (150 ng/ml; results not shown). Thus, although we can positively identify plasma membrane association of particular PKC isoforms in GM-CFC with antibody staining, it was not seen with PKC $\alpha$  in PKAC-transfected cells. The pM5-neo-transfected cells showed a slightly higher proportion of PKC $\alpha$  in the nucleus than observed in the control cells.

The number of GM-CFC that can be purified precluded a direct assessment of PKC activity in the transfected cells. To correlate the changes in the MFI with an increase in PKC activity we transfected an IL-3-dependent haemopoietic cell line, IC2.9, with the pM5-PKAC construct.



**Figure 2.** PKC $\alpha$  expression and localization in transfected highly enriched GM-CFC. The figure illustrates the levels of PKC $\alpha$  expression and localization following incubation with either (A) medium alone or (B) medium containing pM5-PKAC. The plates show representative fields of samples from cells that were used for the experiments described. The images are shown in pseudocolour to display the level and distribution of PKC $\alpha$  within the cell. Low levels are represented in blue and the highest levels in yellow. Quantitated figures for the level of expression of PKC $\alpha$  are shown in Table I.

This cell line was chosen as it resembles the GM-CFC morphologically. We observed a twofold increase in histone III $\alpha$  phosphorylation in lysates from PKAC-transfected cells (compared to controls), which corresponded to a doubling in the MFI. This infers that we achieved around a threefold increase in PKC $\alpha$  activity in the transfected GM-CFC.

With the transfection procedure used we have therefore achieved our objectives: to express PKAC in the majority of the GM-CFC population within the nucleus during the period when these bipotential cells undergo lineage commitment.

#### **Effect of PKAC Transfection on GM-CFC in Liquid Culture**

To first determine if the supernatant obtained from cultures of the retroviral packaging cell line produced any factors that affected GM-CFC development, we incubated the GM-CFC in the presence of the cytokines listed in Fig. 1 plus the supernatant obtained from the packaging cell line GP + env86. No effect was seen on GM-CFC development, in terms of colony-forming ability and morphology

in comparison to medium alone-treated control cultures (data not shown).

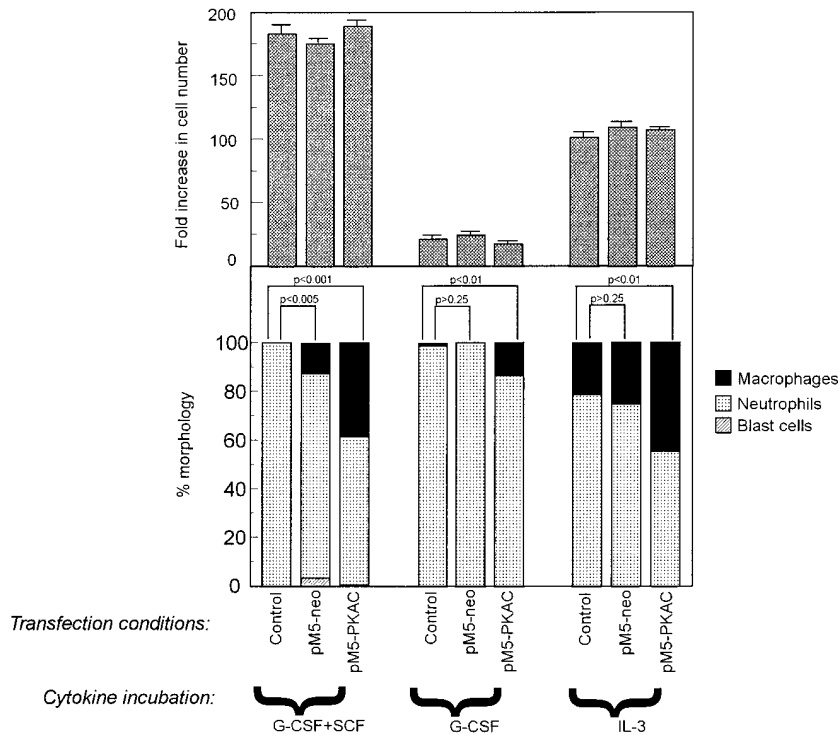
The effect of pM5-PKAC-transfection on the elutriated GM-CFC was then analyzed in liquid culture (Fig. 3). Incorporation and expression of PKAC did not affect the proliferative ability of GM-CFC in response to the cytokines, IL-3, G-CSF, and G-CSF + SCF. The fold increase in cell number observed in control, pM5-neo- and pM5-PKAC-transfected cells was similar in all cases. In all three cytokine combinations tested, there was also little evidence of apoptosis (<1% of total cell number were apoptotic). However, in the absence of growth factors, GM-CFC treated with medium alone, pM5-neo, or pM5-PKAC died within 24 h. Staining these cells with acridine orange (Gregory et al., 1991) showed the morphology typical of cells that had undergone apoptosis (data not shown). Therefore, overexpression of activated PKC $\alpha$  in enriched GM-CFC did not elicit increased survival in the elutriated cells, nor did it result in enhanced proliferation.

However, when aliquots were removed for cytological analysis to determine whether constitutively active PKC $\alpha$  had any effect on cellular morphology, profound differences in the types of cells formed from the elutriated cells were observed. In the presence of stimuli, which have previously been shown to promote neutrophilic development (Fig. 1), the infection protocol had little effect on the number and type of mature cells formed from the elutriated GM-CFC (Figs. 1 and 3). However, when transfected with the pM5-PKAC, a significant number of macrophages could be identified in the cultures in the presence of either SCF + G-CSF or G-CSF alone. When the cells were cultured in IL-3, which promotes the formation of neutrophils and macrophages from the elutriated cells, there was a significantly increased proportion of macrophages in the cultures after a 7-d incubation. In pM5-neo-treated cultures in G-CSF + SCF, there was an increase in macrophages seen compared to control cultures that may be associated with the modest nuclear translocation of PKC $\alpha$

**Table I.** The Expression of Native and Ectopically Expressed PKAC in the Nucleus of Elutriated GM-CFC

Pretreatment	Nuclear PKC $\alpha$ (MFI)	MFI Per Cell (Percent of Control)
Control	8,480 $\pm$ 480	100
pM5-neo treated	10,200 $\pm$ 340	106
pM5-PKAC treated	42,400 $\pm$ 1,590	332

Elutriated GM-CFC were incubated overnight, as described in the Materials and Methods section, with either medium alone and no retrovirus (*control*), pM5-neo, or pM5-PKAC. Samples were washed and then cytospin preparations taken and stained with anti-PKC $\alpha$  antibody, which recognizes both the mutant and native forms of PKC $\alpha$ . Cells were counterstained with propidium iodide, to define the nucleus, and the mean fluorescence intensity (MFI;  $\pm$  SEM) in the nucleus determined for >50 cells for each incubation from three different experiments. Greater than 95% of the pM5-PKAC-transfected cells showed at least a doubling in the MFI per cell.



**Figure 3.** The effect of constitutively activated PKAC expression on GM-CFC development. After culturing with the appropriate retrovirus, the elutriated GM-CFC were washed and cultured in liquid culture conditions as described (Heyworth et al., 1992). The top panel shows the fold increase in cell numbers after 7 d, from cells initially seeded at  $2 \times 10^4$  cells/ml. The bottom panel shows the mean percentage morphology of the cells formed, as analyzed using cytospin preparations. Results show the mean  $\pm$  SEM from three experiments. The *P* values shown were calculated using the Student's *t*-test.

observed in these cells (Table I). When cultured in the absence of any growth factors there were no macrophages present after 24 h, only apoptotic, nonviable cells. The elutriated cells, therefore, respond to the expression of activated PKC $\alpha$  by the preferential production of macrophage, but with no effect on suppression of apoptosis or changes in the rate of proliferation.

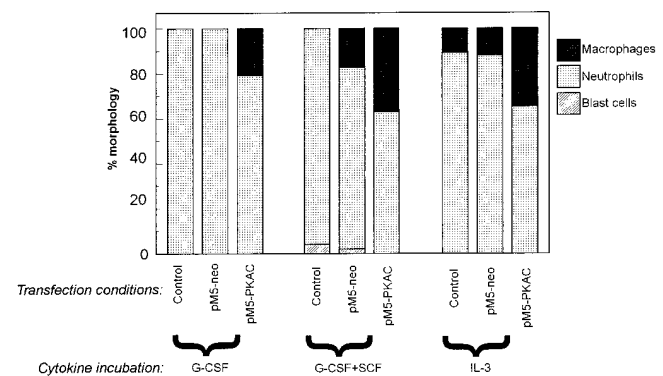
### Effect of PKAC Transfection on GM-CFC Development in Colony-forming Assays

To determine whether or not there was an effect of PKAC expression on the proliferative potential of individual clonogenic elutriated cells, the transfected cells were plated out in soft gel assays. In these assays the number of cells per colony was not significantly different in control, pM5-neo- and pM5-PKAC-transfected cells. Using the enriched GM-CFC population, the plating efficiency was 30–40% in the presence of IL-3 in soft agar (Williams et al., 1987; Cook et al., 1989). When cells were preincubated with pM5-PKAC or the pM5-neo, the plating efficiency was unchanged (when plated in IL-3, pM5-PKAC and pM5-neo pretreatment gave  $111 \pm 4\%$  and  $107 \pm 7\%$  of control colony formation,  $n = 5$  mean  $\pm$  SEM). In the absence of growth factors, medium alone-treated, pM5-neo- and pM5-PKAC-transfected GM-CFC were unable to form clusters or colonies in vitro over a 2–7-d period. When cells from colonies grown in the presence of IL-3 for 7 d were collected and replated in soft agar with IL-3, no colonies were formed. These data show that the transduced activated PKC $\alpha$  did not influence the proliferative potential of the colony-forming cells.

Although there was no effect of PKAC on colony formation or proliferative ability, there was a marked effect on the colony morphology. Whereas in neutrophilic stimuli, SCF + G-CSF or G-CSF alone, the colonies formed

from the control or pM5-neo-transfected cells contained predominantly neutrophils. Those transfected with pM5-PKAC contained a large number of neutrophil/macrophage colonies. Fig. 4 shows the constituent cells of colonies grown from transfected and control-elutriated cells. The most notable feature, with all three cytokine combinations, was the fact that the proportion of macrophages increased in every case in colonies grown from pM5-PKAC-transfected elutriated GM-CFC. Thus, clonogenic cells appear to alter their response to neutrophilic stimuli, such as SCF + G-CSF, when the cells are transfected with an activated PKC $\alpha$ .

Culture of transfected elutriated cells in the presence of 100 U/ml M-CSF did not result in any change in colony-



**Figure 4.** Morphology analysis of colonies formed from highly enriched GM-CFC: the effect of constitutively activated PKAC expression. Soft agar colony-forming assays were performed as described previously (Sponcer et al., 1986). Colonies were picked out at random from agar plates for morphological analysis by cytospin preparation. The percentage cell type present in each colony was determined for 40 colonies. Results shown are from a typical experiment of four.

forming ability ( $110 \pm 6\%$  of IL-3 response, mean  $\pm$  SEM,  $n = 3$ ) and the morphology of colonies formed after all three transfection protocols (medium alone or pM5-neo or pM5-PKAC) was  $>98\%$  macrophage.

### Single Cell Analysis of the Effect of PKAC Transfection on GM-CFC

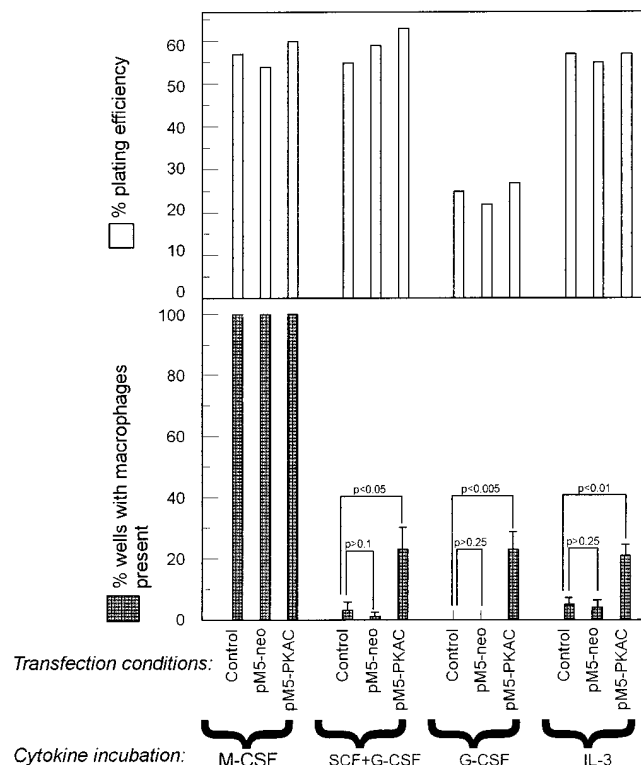
Although the elutriated GM-CFC population were  $>98\%$  blast cells, this does not exclude the possibility that accessory cell populations may contribute to the effects reported above, via paracrine production of factors that influence lineage commitment, in both liquid and soft gel cultures. The cell densities used in these assays are so low, that this seems unlikely. However, to address this issue, the direct effect of activated PKC $\alpha$  on GM-CFC at the single cell level was examined. This was achieved using the automated cell deposition unit of a flow cytometer to deposit single GM-CFC into the wells of a 96-wellplate, after the retroviral transfection procedure. The results are displayed in Fig. 5.

Single cell cultures of GM-CFC in IL-3 produced plating efficiencies between 50–60% that were not statistically different (assessed by Student's *t* test) between controls, pM5-neo-, and pM5-PKAC-transfected GM-CFC (Fig. 5). A similar plating efficiency was seen with G-CSF + SCF cultures or M-CSF single cell cultures, in which there was also no effect of transfection on plating efficiency. In the presence of G-CSF alone, the plating efficiency was  $\sim 25\%$  with again no effect of the transfection procedure on this figure. Furthermore, there was no evidence of apoptosis in control, pM5-neo, or pM5-PKAC cultures over a 72-h period, arguing against survival of macrophage precursors stimulated by PKAC being an important element in the types of cells formed after 7 d.

Morphological analysis of cells formed after a 7-d incubation in IL-3 after exposure to medium alone, compared to pM5-neo virus, did not produce any significant changes in cell morphology. However, transfection with pM5-PKAC caused a significant increase in the number of colonies containing macrophages to  $21 \pm 3\%$  of the total in the presence of IL-3. In the presence of the neutrophilic stimulus, SCF + G-CSF, the proportion of colonies containing macrophages increased from  $3 \pm 2\%$  in control cultures to  $23 \pm 7\%$  in pM5-PKAC-treated cells. To further characterize the cells formed in these clonal assays, we used the combined esterase stain which allows a distinction to be made between cells of the monocyte/macrophage lineage (which stain a dark color) and the neutrophilic lineage (which stain a reddish color). The adherent cells, which stained as macrophages (Figs. 4–6), also stained positive for nonspecific esterase (grey black stain), the marker for macrophages (Fig. 6).

When cultured in M-CSF, all the colonies formed contained macrophages regardless of the preincubation treatment, whereas in G-CSF-stimulated cultures, transfection with pM5-PKAC again resulted in an increase in the number of colonies containing macrophages. These results corroborated those seen in liquid and soft gel assays; typical colonies are shown in Fig. 6.

The effects of pM5-PKAC transfection on colony development are, therefore, not due to release of cytokines from

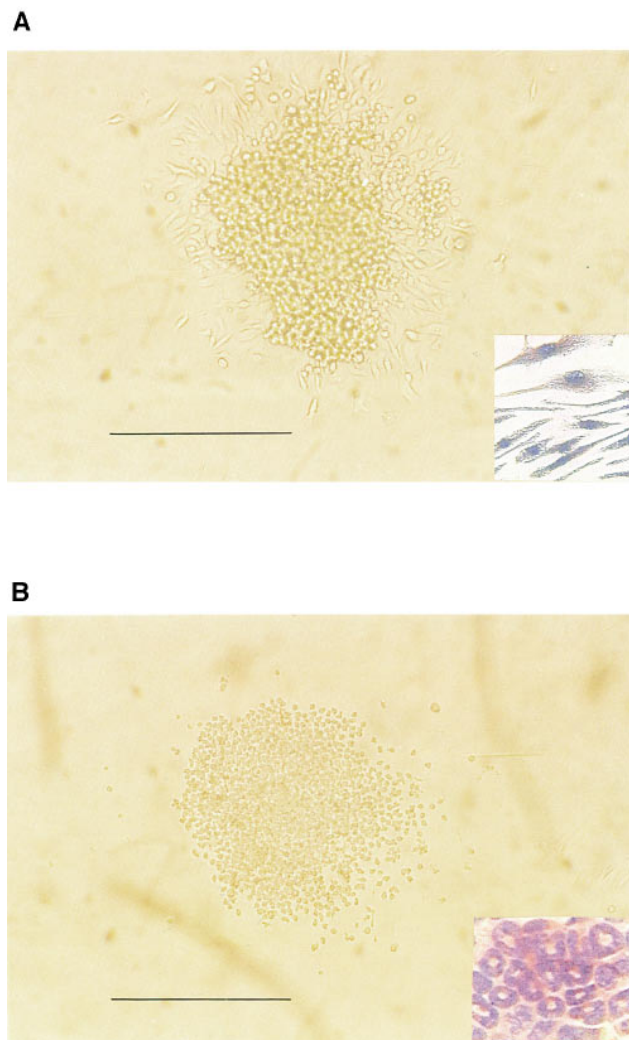


**Figure 5.** The effect of constitutively activated PKAC expression on single GM-CFC in culture. Single GM-CFC were deposited into wells using the automatic cell deposition unit of a Becton Dickinson FacsVantage flow cytometer. GM-CFC were plated in microtiter wells of a 96-well plate in a total volume of 250  $\mu$ l of isocoves medium containing 20% (vol/vol) FCS plus cytokines. One 96-well plate was used for each condition that is pM5-PKAC, pM5-neo, and medium alone control. The top panel shows the percentage-plating efficiency from single cells deposited in wells, and the bottom panel shows the percentage of the colonies formed, which contained macrophages. The *P* values shown were calculated using the Student's *t*-test from three experiments.

accessory cell populations. Given the plating efficiencies observed, it is also extremely unlikely that pM5-PKAC selects specific clonogenic cells with macrophage potential to survive, proliferate, and develop while inhibiting the development of monopotent neutrophilic precursor cells.

### Discussion

Several PKC isoforms have been reported to be involved with developmental pathways. The general inhibition of classical PKCs ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) in transgenic *Drosophila melanogaster* inhibits neuronal process development (Broughton et al., 1996). PKC $\alpha$  has also been associated with neural induction in *Xenopus* (Otte and Moon, 1992), and in keratinocyte maturation (Rutberg et al., 1996). PKC $\beta$  has been implicated in death/survival signaling (Evans et al., 1995; Pongracz et al., 1995, 1996), which may be linked to its apparent role in the G2/M transition of the cell cycle (Thompson and Fields, 1996) in addition to the TPA-induced differentiation of HL60 cells (Macfarlane and Manzel, 1994). PKC  $\theta$  has been implicated in the TGF $\beta$ -induced inhibition of differentiation in myoblasts (Zappelli et al., 1996). Furthermore, PKC $\alpha$  and  $-\epsilon$  induce myelomonocytic



**Figure 6.** Micrographs of colonies formed from single enriched GM-CFC. Single GM-CFC were deposited into wells using the automatic cell deposition unit of a Becton Dickinson FacsVantage flow cytometer. GM-CFC were plated in microtiter wells of a 96-well plate in a total volume of 250  $\mu$ l of iscoves medium containing 20% (vol/vol) FCS + G-CSF and SCF. The micrographs are of representative colonies ( $\times 100$ ) from PKAC-transfected GM-CFC (A) and nontransfected GM-CFC (B). Cells were stained using the combined esterase stain, inset micrographs are of cells from representative colonies of PKAC-transfected GM-CFC (A) and nontransfected GM-CFC (B). Neutrophilic stains are shown stained reddish, whereas the monocytic cells in A are stained brown/black.  $\times 400$ . Bars, 100  $\mu$ m.

differentiation in E26 avian leukemia virus-transformed hematopoietic precursor cells (Rossi et al., 1996). Different PKC isoforms thus appear to be associated with developmental pathways in numerous systems, including hematopoietic cell development.

We have previously shown that PKC $\alpha$  is associated with hematopoietic progenitor cell development in that it is translocated to the nucleus in response to cytokines that induce macrophage development in GM-CFC (Whetton et al., 1994). PKC $\alpha$  is also translocated in response to phorbol esters, which biases the development of GM-CFC to the macrophage lineage. Conversely, inhibitors of PKC

promote GM-CFC development along the neutrophilic pathway (Heyworth et al., 1993). These observations support the hypothesis that PKC $\alpha$  is involved in signal transduction events leading to lineage commitment in primary hematopoietic cells.

To test this hypothesis further, we have now expressed an activated PKC $\alpha$  in the GM-CFC, a normal, primary, bipotent hematopoietic progenitor cell population. To achieve this we used a 253-amino acid NH<sub>2</sub>-terminal truncated form of PKC $\alpha$ , namely PKAC (Muramatsu et al., 1989). This lacks most of the regulatory domain, which has been replaced with 17-NH<sub>2</sub>-terminal residues of the PKA catalytic domain. Potential problems due to the myristoylation of the PKA leader sequence, thus giving altered localization and hence substrate availability, were not evident in this system, as both endogenous activated PKC $\alpha$  and the PKAC localized to the nucleus and had the same biological effect, i.e., macrophage development. These results reflect the observations of James and Olson (1992) who reported that the myristoylation of such PKC $\alpha$  constructs did not affect nuclear localization.

Having established an efficient transfection procedure for the GM-CFC, giving us >95% transfection, the technique was used to demonstrate that the expression of an activated PKC $\alpha$  leads to a significant increase in the proportion of the cells forming macrophages in the presence of stimuli, which normally promote only neutrophilic development. We have shown previously that the elutriated GM-CFC undergo lineage commitment after undergoing one or several cell divisions (Heyworth et al., 1994) supporting the earlier elegant work of Metcalf and Burgess (1982). Our data now indicate that some of the first, second, or third generation cells commit to macrophage development as a consequence of the expression of PKC $\alpha$ , which lacks the regulatory domain. Thus the probability of elutriated GM-CFC forming neutrophils is reduced and macrophages are formed even in the presence of neutrophil development stimuli, such as G-CSF  $\pm$  SCF. The interesting data are that, whereas G-CSF + SCF gave predominantly neutrophil colonies from elutriated GM-CFC, a significant proportion of the colonies formed under these conditions after transfection with PKAC contained neutrophils and macrophage. The constitutively activated PKAC construct was insufficient to induce all of the bipotent cells formed in the early stages of development to commit to macrophage development. This remains to be explained, but it has some resonance with the work of Rossi et al. (1996), who found that the degree of PKC activation in avian hematopoietic precursor cells influenced lineage commitment. It is also possible that the PKC $\alpha$  signal transduction pathway is not the only means whereby macrophage developmental stimuli influence lineage commitment. Nonetheless all of our data to date (Heyworth et al., 1993; Nicholls et al., 1994; Whetton et al., 1994) strongly indicates a major role for this enzyme in lineage determination. Perhaps a second M-CSF-stimulated signaling pathway is required in concert with PKC $\alpha$  activation to more markedly affect the probability of lineage commitment.

We found, in initial experiments with G418, that only 50% of the colonies formed from elutriated transfected cells were neomycin resistant (although >95% expressed high levels of PKC $\alpha$  after transfection with pM5-PKAC),

indicating that transient expression of PKC $\alpha$  occurred in some cells. This may explain why some colonies contained no macrophages; i.e., there was insufficient PKC $\alpha$  expression to influence lineage commitment in the first and second generation cells. The relationship between expression levels and lineage commitment cannot be described from our data, as we cannot subject the same cells to both quantitative immunocytochemical analysis and culture them thereafter. Transient expression, however, may reflect the situation in normal cells, as the levels of PKC $\alpha$  have been shown to diminish as multipotent cells undergo maturation to phagocytic cells (Shearman et al., 1993). This is compatible with a role for this kinase in developmental decisions, as opposed to maturation. The fact that some leukaemic myeloid cell lines do not express PKC $\alpha$  (Mischak et al., 1991) may, therefore, be a reason for their lack of ability to differentiate and develop to mature cells.

Our data implicate PKC $\alpha$  in a deterministic signaling pathway for lineage commitment in GM-CFC. Current studies are aimed at identifying the substrates for PKC $\alpha$  in the nucleus and determining how their phosphorylation could modify their activity, thereby increasing the probability of the cell committing to macrophage development.

We thank Gerald Johnson and Stella Pearson for their technical assistance, and Jaleel Miyan for helpful discussion.

This work was supported by the Leukaemia Research Fund and the Cancer Research Campaign. J.M. Lord is a Royal Society University Research Fellow. T.M. Dexter is a Gibb Fellow of the Cancer Research Campaign.

Received for publication 4 June 1997 and in revised form 23 December 1997.

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