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Lineage commitment of transformed haematopoietic progenitors is determined by the level of PKC activity

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Our previous work showed that haematopoietic precursors transformed by the E26 avian leukaemia virus undergo multilineage differentiation in response to the phorbol ester phorbol 12-myristate 13-acetate (PMA). Treatment of the cells with high concentrations of PMA (100 nM) favours myelomonocytic differentiation, while lower concentrations (20 nM) induce predominantly eosinophil differentiation. Here we have investigated the role of protein kinase C (PKC) in this process and found that 100 nM, but not 20 nM, PMA dramatically down-regulates total cellular PKC activity, indicating that high PMA concentrations result in less efficient signalling than lower PMA concentrations. Consistent with these findings is the observation that very low PMA concentrations (1 nM), which presumably only moderately activate PKC, induce myeloid differentiation. This suggests the existence of two PKC thresholds which play a role in lineage commitment. To test the model, α- and ε-PKC isoforms were expressed in E26-transformed progenitors. These cells exhibited myelomonocytic differentiation even in the absence of PMA, while treatment with concentrations of PMA as high as 100 nM led to the differentiation of predominantly eosinophils and failed to down-regulate the exogenous PKC. Our results suggest that different levels of PKC activity result in three different phenotypes: (i) no PKC activity maintains the progenitor phenotype; (ii) low PKC activity favours myelomonocytic differentiation; (iii) high PKC activity favours eosinophil differentiation.

Keywords: haematopoietic precursors/multilineage differentiation/protein kinase C levels

Introduction

Haematopoietic differentiation is regulated by diffusible peptides which bind to specific transmembrane receptors, thus activating intracellular signal transduction pathways. These pathways are composed of a complex network of molecules whose expression pattern influences the cellular response to the extracellular signals. Phorbol esters have been shown to mimic such signals, and to influence maturation and commitment in several *in vitro* differentiation systems. This suggests that their intracellular receptor, protein kinase C (PKC; reviewed in Nishizuka, 1988), can function as a component of the signal transducing machinery. For example, the human promyelocytic cell line HL-60 can be induced to differentiate into macrophage-like cells by treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) (reviewed in Collins, 1987). Macrophage production is also observed upon PMA treatment of the U937 myelomonocytic cell line (Ways *et al.*, 1987), while differentiation along the megakaryocytic lineage has been described for the more immature progenitor line K562 (Koeffler *et al.*, 1981). Furthermore, recent evidence indicates that activation of PKC by cytokines plays a role in lineage determination during the differentiation of primary bipotent macrophage–granulocytic progenitors, with high activity leading to macrophage differentiation and no, or low, activity favouring granulocyte differentiation (Shearman *et al.*, 1993; Whetton *et al.*, 1994; Nicholls *et al.*, 1995).

The physiological activators of PKC are diacylglycerols (DAG) and unsaturated fatty acids, which are produced *in vivo* by membrane receptor-activated phospholipases (reviewed in Nishizuka, 1992). Binding of activators to the amino-terminal regulatory region of PKC induces profound conformational changes, resulting in activation of the kinase and unmasking of protein domains that mediate association with cellular membranes and increased sensitivity to proteolytic cleavage (Bell and Burns, 1991). Therefore, translocation of PKC is believed to be a reliable sign of activation. Because of its greater stability *in vivo*, PMA is more powerful than DAG in inducing translocation of the enzyme to the nuclear and plasma membranes (Aihara *et al.*, 1991). PMA-activated, membrane-associated PKC becomes a target for intracellular proteases and is quickly depleted from cells (Young *et al.*, 1987).

In some cases, treatment of cultured cells with different PMA concentrations results in different responses (reviewed in Clemens *et al.*, 1992). Dose dependence of the response to phorbol ester treatment has been observed in HL-60 cells, where high PMA concentrations inhibit proliferation and cause differentiation, while low concentrations are mitogenic and do not affect the phenotype (Trayner and Clemens, 1992). A similar mechanism has recently been proposed to control the choice between growth and differentiation in the neuronal precursor PC12 cell line. In this system, different levels of expression of the same growth factor receptor, activating the same intracellular signal transduction pathway to varying degrees, can elicit specific cellular responses (Dikic *et al.*, 1994; Traverse *et al.*, 1994; reviewed in Marshall, 1995).

Another PMA-inducible differentiation system is based on the ability of the E26 avian leukaemia virus to transform multipotent haematopoietic cells [Myb-Ets-transformed Progenitors (MEPs)] (Graf *et al.*, 1992). These cells,

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which are capable of differentiating along the erythroid and thrombocytic lineage, can be triggered by PMA treatment to differentiate along the myelomonocytic and eosinophilic pathways (Kraut *et al.*, 1994; Frampton *et al.*, 1995). Interestingly, treatment of MEP cells with lower concentrations of PMA (20 nM) yields mainly eosinophils, while high concentrations (100 nM) yield mainly myeloblasts (Graf *et al.*, 1992).

Here we have investigated the mechanism underlying the PMA dose dependence of MEP cell differentiation by measuring their intracellular PKC activity during PMA treatment and by overexpressing PKC. Our data suggest that eosinophilic differentiation results from sustained exposure to a high PKC signal, while myelomonocytic differentiation results from sustained exposure to a weaker PKC signal. This suggests a mechanism by which different degrees of activation of the same signalling pathway in haematopoietic progenitors favour their differentiation along different lineages.

Results

PMA dose-dependent regulation of PKC levels in MEPs

Since chronic exposure to phorbol esters causes an increased rate of PKC proteolysis in many cell types, leading to a decrease in expression levels (Young *et al.*, 1987), we tested whether the specific effect of different PMA concentrations on the differentiation of MEP cells might be the result of differences in the down-regulation of PKC. For this purpose, two pools of three MEP clones were each treated with 20 or 100 nM PMA and analysed for the level of PKC present over a period of 54 h. As most mouse isozyme-specific anti-PKC antibodies tested failed to cross-react with chicken PKC (expressed in brain extracts), we used an alternative strategy to measure endogenous PKC protein levels. Total cell extracts were prepared at different time points after PMA treatment, and the lipid- and Ca^{2+}-dependent kinase activity was determined as a measure of the total amount of PKC. As shown in Figure 1a, a dramatic difference was observed between the two PMA concentrations used: while after 54 h of treatment ~70% of the original level was still detectable with 20 nM PMA, only 20% of the enzyme was retained after the same time period with 100 nM PMA.

The observed differences in total PKC levels are also reflected in the amount of membrane-bound, active PKC detected after PMA treatment of MEP cells. After 28 h of exposure to 20 nM PMA, ~12% of the enzyme present at the beginning of the treatment was found in the membrane-associated fraction and ~70% in the cytoplasm. However, with 100 nM PMA, after an initial activation peak, only background levels of activity were found in the membrane fraction and ~6% in the cytoplasm. This indicates that treatment of MEP cells with lower concentrations of PMA, which cause a moderate PKC down-regulation, results in more efficient signalling than treatment with higher concentrations, which dramatically reduces PKC levels. We do not think that the observed early transient activation of PKC with 100 nM is biologically relevant in the system studied since periods of PMA treatment shorter than 3–4 days did not cause MEP cells to differentiate [Graf *et al.* (1992) and our unpublished data].

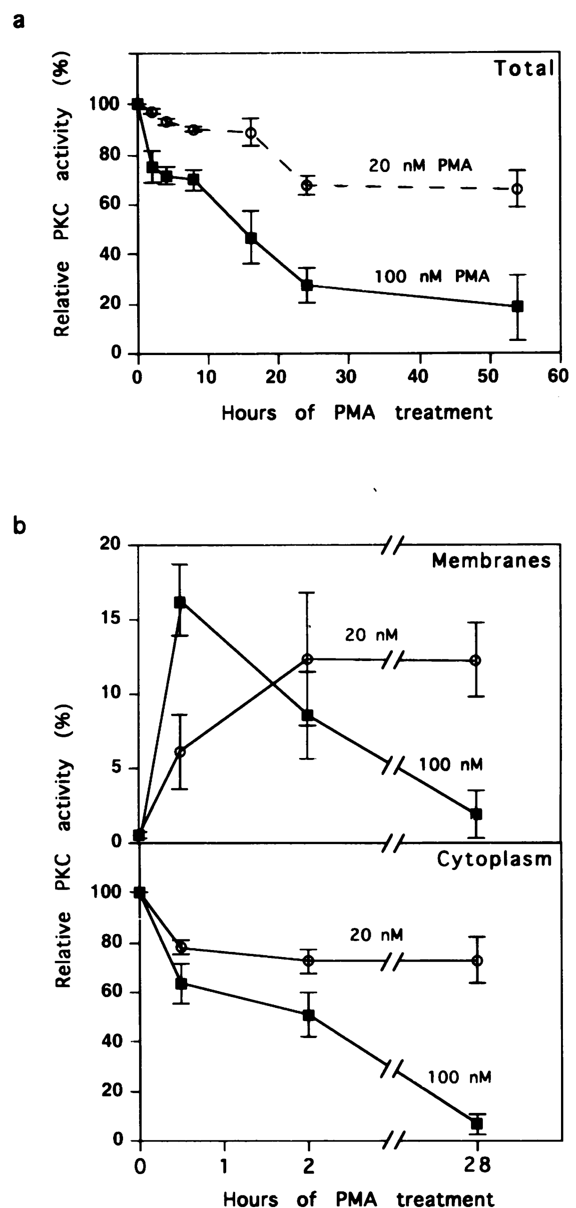

Fig. 1. Down-regulation of PKC in MEPs upon treatment with different PMA concentrations. (**a**) Two pools of three clones each were treated with either 20 or 100 nM PMA and lysed at different time points. The Ca^{2+}- and phospholipid-dependent kinase activity in the crude extracts was determined, and values obtained are expressed as a percentage of the activity present in untreated cells. All values were normalized to the proportion of viable cells (as judged by trypan blue staining). (**b**) The subcellular distribution of PKC in a pool of 15 MEP clones treated with either 20 or 100 nM PMA was determined at different time points. The values are expressed as a percentage of the activity present in the cytoplasmic fraction of untreated cells, normalized to total protein concentration.

Forced expression of PKC in Myb-Ets-transformed cells

Next we tested whether forced expression of PKC would influence the differentiation of MEPs. PKC is a family of at least 12 related polypeptides with a variable tissue distribution and differences in their biochemical regulation. The α- and ε-PKC genes were selected as prototypes for this study as they encode Ca^{2+}-dependent and Ca^{2+}-independent isozymes, respectively (Nishizuka, 1988).

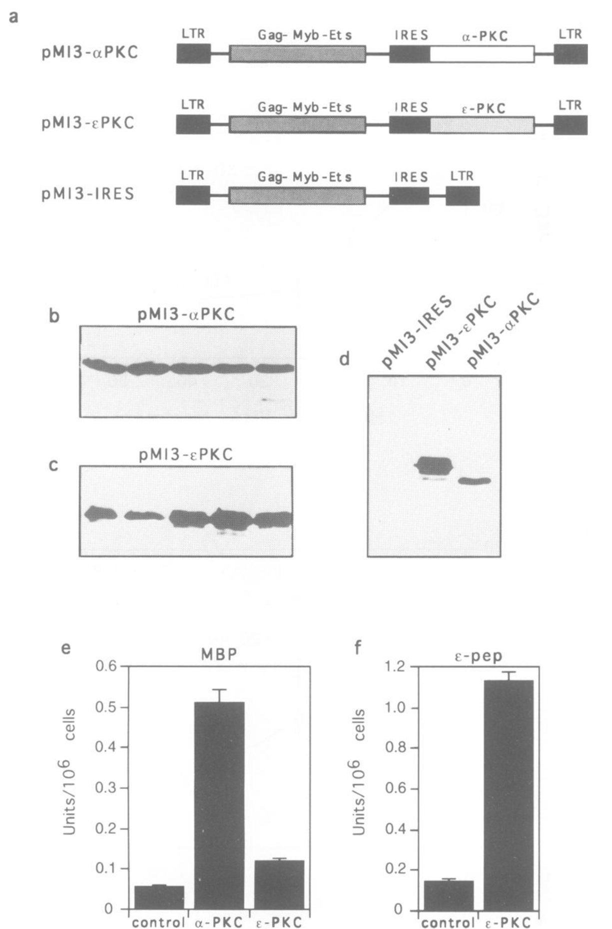

Fig. 2. (**a**) Schematic diagram of the structure of E26-PKC viruses and levels of PKC expression in transformed MEPs. Viral constructs. pMI3 is a derivative of wild-type E26 containing AEV LTRs (long terminal repeats) IRES = internal ribosomal entry site. (**b** and **c**) Western blot analysis of five individual clones transformed by α- and ε-PKC-containing viruses probed with the corresponding isozyme-specific antibodies. (**d**) Western blot analysis of pools of 10 clones each transformed by different constructs, probed with a mixture of anti α- and ε-PKC antibodies. (**e** and **f**) Comparison of Ca^{2+}- and phospholipid-dependent kinase activity in extracts from PKC expressors and from control cells. Partially purified PKC was assayed on a synthetic myelin basic protein-derived peptide (MBP) or on a substrate peptide derived from the ε-PKC pseudosubstrate site (ε-pep). One unit of activity is defined as the amount of enzyme required to transfer 1 nmol [^{32}P]phosphate into substrate per minute. Each bar represents the average of triplicate samples.

Furthermore, these two isozymes have been reported to have different substrate specificities *in vitro* (Schaap *et al.*, 1989) and *in vivo* (Chin *et al.*, 1993; Hata *et al.*, 1993), raising the possibility that they have different functions in haematopoietic lineage commitment. Therefore retroviruses were constructed which, as illustrated in Figure 2a, produce a bicistronic RNA that contains coding sequences for both the Gag-Myb-Ets oncoprotein and for either α- or ε-PKC. The translation of the 3′ coding sequence is initiated by an encephalomyocarditis virus internal ribosomal entry site (IRES) (Ghattas *et al.*, 1991). Owing to the presence of both coding sequences on the same transcript, every transformed cell should also express the second protein. A pMI3-based vector containing Gag-Myb-Ets and the IRES element, but lacking PKC sequences, was constructed as control virus.

Of 50 clones transformed by each construct and analysed by Western blotting with isozyme-specific antibodies, all were found to express the expected PKC form (Figure 2b–d). In order to test whether the expressed proteins were active, three clones for each construct were pooled and the activatable PKC in the corresponding extracts was assayed *in vitro* using a myelin basic protein (MBP)-derived peptide as substrate. Clones expressing α-PKC were found to contain ~10 times more kinase activity than control and ε-PKC clones (Figure 2e). Consistent with previously published results (Schaap *et al.*, 1989), a similar increase in PKC activity relative to control cells could be detected in the ε-PKC expressors with a synthetic ε-pseudosubstrate peptide, but not with the MBP peptide (Figure 2f). These results show that the exogenously expressed PKCs are functional and retain their substrate specificities.

PKC-expressing MEPs differentiate along the myelomonocytic lineage

In order to determine whether PKC expression influences the phenotype of Myb-Ets-transformed MEP cells, blastoderm cultures were infected with the different constructs and incubated for 10 days. A total of 130–160 transformed colonies for each construct were picked and plated individually in liquid culture medium containing chicken myelomonocytic growth factor (cMGF), a factor required for the proliferation of Myb-Ets-transformed myeloblasts (Leutz *et al.*, 1984; Metz *et al.*, 1991; Sterneck *et al.*, 1992). Cells were then expanded for an additional 4–6 days in this medium and their phenotype was assessed by flow cytometric analysis (FACS) with monoclonal antibodies against the myelomonocytic specific surface marker MYL51/2 (Kornfeld *et al.*, 1983), the MEP marker MEP21 and the eosinophilic marker EOS47 (McNagny *et al.*, 1992). Both PKC-containing viruses were found to yield 2- to 3-fold higher proportions of transformed myeloblast versus MEP colonies compared with the control virus lacking PKC (Figure 3a). Overexpression of PKC α with a point mutation in the ATP binding site, which destroys the kinase activity of the enzyme (Pears and Parker, 1991), gives a myeloblast to MEP ratio that is indistinguishable from control virus (our unpublished results). This suggests that overexpression of PKC favours commitment along the myeloid lineage, requiring intact PKC kinase activity.

To examine the differentiation potential of MEPs further, 10 MEP clones transformed by each construct were cultured for an additional 2 weeks in the presence of cMGF. Only one of the clones transformed by the control virus contained a myelomonocytic subpopulation (17% of the cells), while nine out of 10 of both the α- and ε-PKC clones contained myeloid cells (12–63%). Furthermore, the two single α- and ε-PKC-containing clones that retained the MEP phenotype were found to be completely refractory to PMA treatment (data not shown), raising the possibility that they contained a defect in the signal transduction pathway downstream of PKC.

PKC is known to be activated in response to myelomonocytic growth factors (Whetton *et al.*, 1994). It is therefore possible that exogenous PKC expression influences the cellular response to cMGF in our system. For example, increased production of myelomonocytic cells

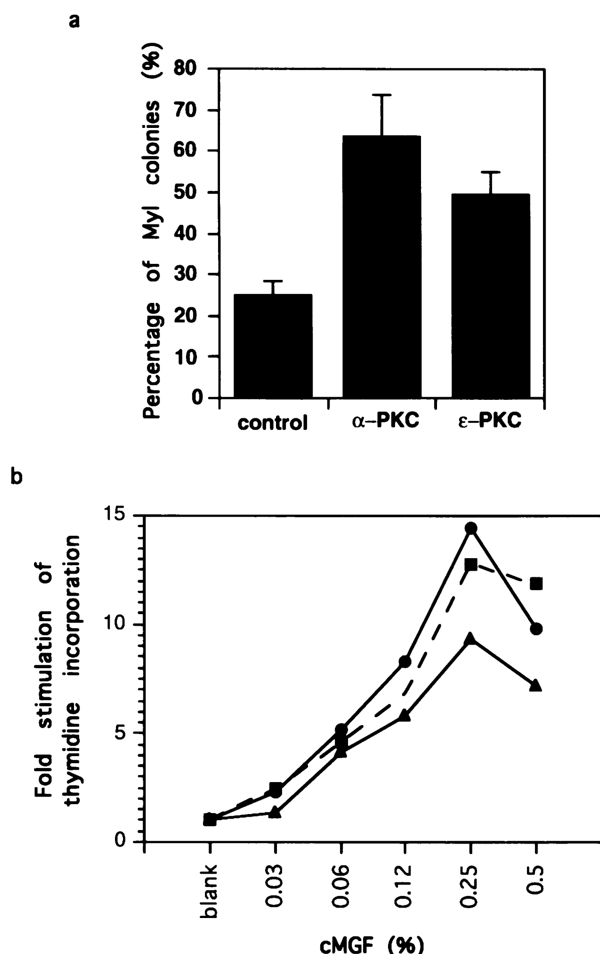

Fig. 3. Transforming ability of PKC-encoding and control E26 viruses to and growth response of transformed myeloid clones. (**a**) Percentage of transformed myeloblast (Myl) colonies obtained with the different constructs. Each bar represents the average of numbers obtained in three experiments. A total of 156 individual colonies transformed by the PMI3 control virus, 160 colonies transformed by pMI3-α PKC and 137 colonies transformed by pMI3-ε PKC virus were analysed. (**b**) cMGF dependence of myeloid clones transformed by PKC-expressing viruses and control E26 virus. Pools of five independent clones were grown for 2 days in the presence of various cMGF concentrations and the cells pulse labelled with [^{3}H]thymidine. The results shown represent thymidine incorporation relative to cells maintained in the absence of cMGF. pMI3-IRES (-■-), pMI3-αPKC (-▲-), pMI3-εPKC (-●-).

in our assays might have been caused by an increased ability of PKC-expressing myeloblasts to proliferate in response to cMGF or to become factor independent. This has been shown to be the case in E26 myeloblasts superinfected with kinase-type oncogenes, where the cells become growth factor independent due to the production of their own cMGF (Adkins *et al.*, 1984). We therefore assessed the responsiveness of myeloblasts to cMGF by a [^{3}H]thymidine incorporation assay. As shown in Figure 3b, no significant difference between control myeloblasts and PKC overexpressors was detected, showing that forced PKC expression does not induce factor independence. This observation supports the notion that PKC overexpression in MEPs induces their commitment along the myelomonocytic lineage without inducing autocrine growth and without significantly altering their growth properties.

PMA induces preferential eosinophilic differentiation in MEPs overexpressing PKC

To determine the effect of PMA on PKC overexpressors, MEP colonies were isolated and expanded in the absence of cMGF, and then treated with various concentrations of PMA in the presence of cMGF for 8 days. The resulting populations were analysed for the expression of lineage-specific cell surface antigens and Figure 4 shows a comparison of three representative clones. Compared with control clones, both α- and ε-PKC overexpressors showed a lower level of MEP21 expression in the absence of PMA, probably reflecting leaky PKC activity in a subset of the cells. When the PKC overexpressors were treated with a low concentration of PMA (5 nM), which was innocuous in control clones, a complete down-regulation of MEP21 antigen was observed and most cells then expressed the eosinophilic marker EOS47 (Figure 4a and b). In addition, in the ε-PKC overexpressors, even the highest concentration of PMA tested (100 nM) induced predominantly eosinophilic differentiation, in contrast to pMI3-IRES control clones, which became predominantly myeloid at this concentration. The α-PKC overexpressors showed a similar, although less pronounced, effect. Significantly, in both types of PKC overexpressors the exogenous enzyme could still be detected by Western blot even after 8 days of treatment with 100 nM PMA. Only in the α-PKC overexpressors was a slight down-regulation observed, correlating with a decrease in eosinophilic differentiation (Figure 4c). This suggests that overexpressed PKC can escape PMA-induced proteolysis, the mechanism of which remains unknown.

Treatment of MEPs with 'subthreshold' PMA concentrations results in myelomonocytic differentiation

The observations presented show that high levels of PKC expression correlate with the commitment of MEP cells to differentiate along the eosinophilic lineage, while low levels correlate with the formation of predominantly myelomonocytic cells. Although in previous experiments no induction of differentiation had been observed in MEPs treated with <20 nM PMA (Graf *et al.*, 1992), the model developed in this study predicts that prolonged treatment of MEPs with 'subthreshold' PMA concentrations should lead to the production of myeloblasts, but not eosinophils. To test this prediction, PMA concentrations ranging from 0.3 to 100 nM were tested on control virus-transformed MEP clones and on α-PKC overexpressors. As shown in Figure 5, we indeed observed the formation of significant proportions of myeloblasts in control virus-transformed MEPs at concentrations between 0.3 and 3 nM. In contrast, the α-PKC overexpressors differentiated into predominantly myeloblasts in the total absence of PMA and into eosinophils at concentrations exceeding 3 nM. As expected, the population of myelomonocytic cells predominated again upon treatment with 100 nM PMA, a concentration which is able to partially down-regulate the exogenous α-PKC (Figure 4c and data not shown).

Discussion

Our results indicate a correlation between the net PKC activity in MEPs and their commitment to either the

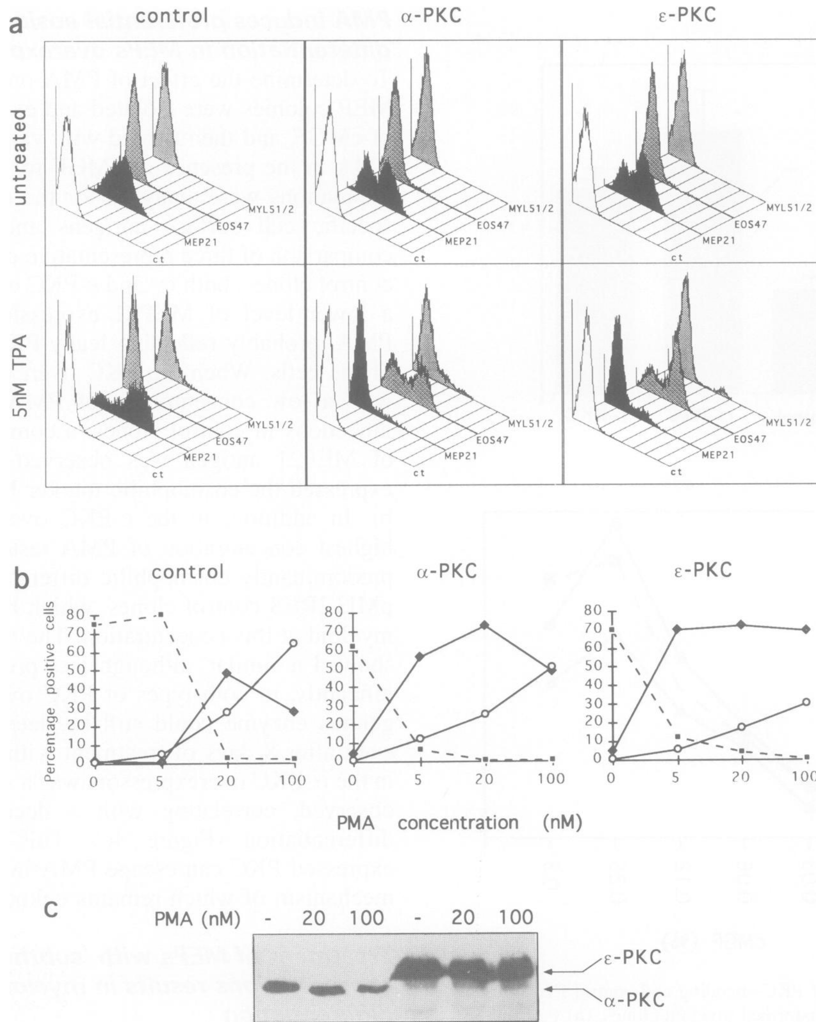

Fig. 4. Effects of PMA on the differentiation of PKC-expressing or control MEP clones. (**a**) Flow cytometric analysis of lineage-specific cell surface markers upon treatment of PKC-expressing and control MEPs grown in the absence or presence of 5 nM PMA for 8 days. MEP21 antibody identifies progenitor cells, EOS47 eosinophils and MYL51/2 myeloid cells. The vertical axis represents cell number, the horizontal axis fluorescence (logarithmic scale). (**b**) Distribution of differentiated cells after treatment with different concentrations of PMA for 8 days. The percentage of cells stained by MEP21 (-■-), EOS 47 (-◆-) and MYL51/2 (-O-) is shown. (**c**) Western blot analysis of PKC levels at day 8 of PMA treatment.

myelomonocytic or eosinophilic lineage. As summarized in the model of Figure 6, this suggests the existence of distinct PKC activity thresholds that determine differentiation decisions along distinct lineages. Depending on the amount of PMA present in the culture (horizontal axis), the level of PKC activity (vertical axis, closed circles) falls into three partially overlapping regions, separated by two thresholds (T1 and T2). PKC activity within each of these regions correlates with a different probability of differentiation. In the absence of PMA (no PKC activity), the MEP phenotype is maintained. In contrast, either low or high PMA concentrations (which result in a low net PKC activity) favour myelomonocytic differentiation, while intermediate concentrations of PMA (high net PKC activity) favour eosinophilic differentiation. The effects of PKC overexpression in MEPs are also schematized in Figure 6. Increased expression levels of PKC in the absence of PMA favour myeloid differentiation, probably because of a moderate spontaneous activation of over-expressed PKC. This is consistent with results observed in other systems (Mischak *et al.*, 1993a; Akita *et al.*, 1994; Goode *et al.*, 1994; Borner *et al.*, 1995). Finally, middle to high PMA concentrations favour eosinophil differentiation, most likely because PKC remains activated due to the observed inefficient down-regulation of the exogenously expressed enzyme.

The observation that PKC-overexpressing myeloblasts are still factor dependent shows that the PMA-induced differentiation of MEPs is not a consequence of activating an autocrine growth mechanism in myeloid cells. It is also consistent with the finding that myeloblasts obtained by treatment of MEPs with high PMA concentrations are still cMGF dependent (Graf *et al.*, 1992). This result therefore differs from the observed induction of myelo-monocytic/eosinophilic differentiation of MEPs obtained by overexpression of constitutively active kinase-type oncogenes, where the resulting myeloblasts (and possibly eosinophils) grow in an autocrine fashion due to the production of cMGF (Adkins *et al.*, 1984; Metz *et al.*, 1991; Sterneck *et al.*, 1992).

The finding that α- and ε-PKC isozymes have indistinguishable effects on the differentiation of MEP cells

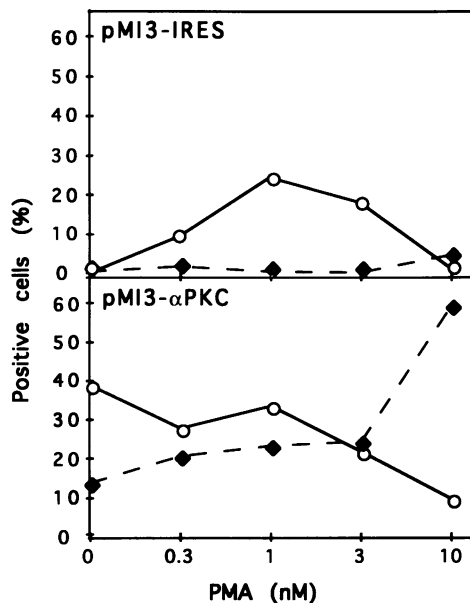

Fig. 5. Effects of 'subthreshold' PMA concentrations. MEP cells were incubated with PMA at the concentrations indicated for 7 days (pMI3-αPKC cells) or 14 days (pMI3-IRES cells). The percentage of cells stained by EOS47 (-◆-) and MYL51/2 (-○-) is indicated.

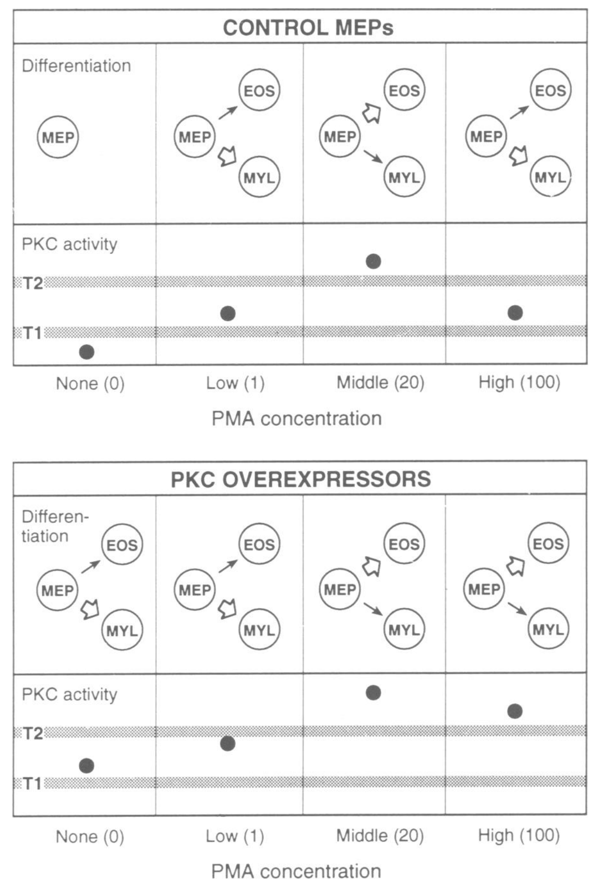

Fig. 6. Model diagram representing the results described. Different concentrations of PMA used to treat MEP cells (horizontal axis, nM) result in different levels of PKC activation (filled circles). In the case of the PKC overexpressors, it has been assumed that PKC activities are already elevated in the absence of PMA and that PKC activities remain high even in the presence of 100 nM PMA, although this has not actually been measured. Depending on whether PKC activity levels exceed a first threshold (T1) or a second threshold (T2, indicated as grey lines) myeloid or eosinophil differentiation is favoured. This is represented in the upper part of the figures. Small arrows, low probability of differentiation; large arrows, high probability of differentiation. For further explanations, see the text.

suggests that they act on a common substrate. Although α-PKC, but not ε-PKC, is able to trigger macrophage differentiation of the mouse myeloid cell line 32D (Mischak *et al.*, 1993b), and differences have been described in their substrate specificity (Schaap *et al.*, 1989; Razin *et al.*, 1994; Hirano *et al.*, 1995), common substrates also exist (Hata *et al.*, 1993; Wotton *et al.*, 1993; Decock *et al.*, 1994). In particular, the TCRβ enhancer is stimulated by both PKC isozymes in a Raf-1/MAPKKK-dependent manner (Wotton *et al.*, 1993), and PKC directly activates Raf-1 *in vivo* (Carroll and May, 1994). These observations, combined with our earlier findings that v-Raf and its upstream activator v-Ras are also capable of inducing MEP cell differentiation (Graf *et al.*, 1992), suggest that Raf-1 acts as a common substrate for α- and ε-PKC.

Most ligand-activated membrane receptors are able to activate Raf-1 through Ras, and it is possible that the effects of PMA treatment observed in tissue culture cells reflect the ability of PKC to mimic signalling from one or more cytokine receptors. Interestingly, the receptors for GM-CSF, a granulocyte/monocyte differentiation factor, and for IL-5, an eosinophil differentiation factor, share the same signalling subunits and activate the Ras/MAP kinase pathway (Satoh *et al.*, 1991), indicating that they activate the same intracellular pathway(s). Consistent with this idea, it has been shown that triggering of epidermal growth factor and nerve growth factor receptors in PC12 cells elicits different responses, and that this is due to quantitative differences in activation of the MAP kinase pathway (reviewed in Marshall, 1995). These observations raise the possibility that quantitative differences in the signalling strength of different receptors, resulting in different levels of PKC activation, play a role in mediating specific effects.

A possible nuclear target for PKC signalling is the Gag-Myb-Ets fusion protein itself. Proteins of the Ets family are among the known nuclear targets of the MAP kinase pathway (Lai and Rubin, 1992; Brunner *et al.*, 1994) and the Ets portion of the E26 oncoprotein is required for maintaining MEP cells in an immature state (Kraut *et al.*, 1994; F.Rossi, K.M.McNagny, C.Logie, A.F.Stewart and T.Graf, submitted). Alternatively, the transcription factors GATA-1 and C/EBPβ might be modulated by PKC during MEP cell differentiation. Thus, GATA-1 is highly expressed in MEPs, moderately in transformed eosinophils and not at all in myeloblasts, while C/EBPβ is expressed in myeloblasts and eosinophils, but not in MEPs. Treatment of MEPs with PMA leads to the down-regulation of GATA-1 and to the rapid appearance of C/EBPβ. Furthermore, forced expression of GATA-1 in myeloblasts leads to the production of eosinophils and MEPs (Kulessa *et al.*, 1995), and forced expression of C/EBPβ in MEPs leads to the appearance of eosinophils (Müller *et al.*, 1995). Finally, C/EBPβ is dependent on MAP kinase signalling for transcriptional activation and nuclear translocation (Katz *et al.*, 1993; Kowenz-Leutz *et al.*, 1994). Thus, PKC-mediated inactivation of GATA-1 and/or activation of C/EBPβ might be responsible for at least some of the PKC effects described.

Regardless of what the critical PKC target(s) are, the observation that different levels of PKC determine the cell's differentiation phenotype suggests that they must ultimately result in a change in the balance between specific transcription factors. The challenge is now to understand how the strength of the signal delivered by a single signalling component is translated into different patterns of gene expression during haematopoietic differentiation.

Materials and methods

Virus construction and DNA protocols

The pMI3-IRES virus was obtained by inserting the IRES-containing *PvuII* fragment from the pCITE-2a plasmid (Novagen) in a blunted Asp718 site at position 11025 of pMI3 (Introna *et al.*, 1990). Bovine α-PKC cDNA in pSP64 plasmid, mouse ε-PKC in pMT2 plasmid and α-PKC kin⁻ cDNA in pKS1 (Pears and Parker, 1991) were generously provided by Dr Peter Parker. They were cut out as *NcoI–XbaI* fragments using the *NcoI* site at the ATG and cloned in pMI3-IRES immediately 3′ of the IRES sequence to generate pMI3-αPKC, pMI3-αPKC kin⁻ and pMI3-εPKC. Cloning was performed according to standard techniques (Sambrook *et al.*, 1989).

Cells

All cells were derived from White Leghorn Spafas chickens maintained in Heidelberg. The Q2bn packaging cell line (Stoker and Bissel, 1988) was grown in Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal calf serum, 2% chicken serum and 10 mM HEPES. Transformed primary cells were grown in 'blastoderm medium' (Graf *et al.*, 1992) with or without ~10 U/ml crude cMGF (Leutz *et al.*, 1984).

Production of infectious virus and blastoderm transformation assay

Blastoderm cells were obtained from 2-day-old chick embryos (stage 10–12) as previously described (Graf *et al.*, 1992). Viruses were produced by transiently transfecting plasmids containing proviral sequences in the Q2bn packaging cell line (Stoker and Bissel, 1988) by $Ca_3(PO_4)_2$ precipitation (Graham and van der Eb, 1973). Thirty-six hours after transfection, mitomycin C was added at a concentration of 2 μg/ml in order to block cell proliferation. The next morning, the cells were washed four times in medium before adding the freshly prepared blastoderm cells. After 24 h of co-cultivation in the presence of 2 μg/ml polybrene (Sigma, No. H 9268), suspension cells were collected and plated in blastoderm medium containing 0.8% methocel as described earlier (Graf *et al.*, 1992). Transformed colonies were isolated after ~10 days using a drawn-out Pasteur pipette and were seeded in microtitre wells for expansion. Since we noticed that co-cultivation of transfected fibroblasts with blastoderms yielded relative small proportions of MEP colonies versus myeloblast colonies, probably because of the production of cytokines that facilitate the MEP to myeloblast transition (F.Rossi and J.Frampton, unpublished data), we used pools of transformed, mitomycin-treated myeloblasts as an alternative source of virus in all experiments where the transformed cell phenotype was investigated.

PKC activity assay

PKC activity was determined using a PKC assay system kit (Gibco, No. 3161SA). Synthetic peptides corresponding to amino acids 4–14 of myelin basic protein (provided with the kit) or the ε-peptide (Schaap *et al.*, 1989) were used as PKC substrates. Briefly, cells were lysed by homogenization in 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 10 mM β-mercaptoethanol, 25 mg/ml aprotinin and leupeptin, and cellular debris was eliminated by centrifugation. Where indicated, PKC was partially purified by loading the supernatant on a DEAE (Whatman DE52) column equilibrated in 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA and 10 mM β-mercaptethanol. Columns were washed with 10 vol of the same buffer and PKC was eluted in a single step with 5 column vol of the same buffer containing 0.3 M NaCl. Appropriate dilutions of the column eluates or the crude extracts were incubated at 25°C in the presence of the substrates, 20 mM ATP, 1 mM CaCl, 20 mM $MgCl_2$ and 5 μCi/ml [γ-^{32}P]ATP with or without activators (10 mM PMA, 280 mg/ml phosphatidyl serine, Triton X-100 mixed micelles). Lipid-dependent activity was determined by subtracting the values obtained without activators from the values obtained with the activators. All experiments were performed in triplicate.

Subcellular fractionation

The cells were lysed by homogenization in 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 25 mg/ml aprotinin and leupeptin, and cellular debris was eliminated by low-speed centrifugation. The extracts were then centrifuged at 100 000 *g* in a Beckman table-top ultracentrifuge and 0.5% Triton X-100 was added to the supernatant (cytoplasmic fraction). The membrane-containing pellets were resuspended in lysis buffer containing 0.5% Triton X-100, vortexed for 30 min at 4°C and centrifuged again. The resulting supernatant represented the membrane-associated fraction. Appropriate dilutions of the two fractions were assayed for PKC activity as described above.

Thymidine incorporation assay

Samples of 10^4 cells for each pool of clones were plated in DMEM containing 2% FCS plus an appropriate dilution of crude cMGF (Leutz *et al.*, 1984). After 2 days, [^{3}H]thymidine (29 Ci/mmol, Amersham) was added at a concentration of 12 μCi/ml. Two hours later, cells were harvested and processed with a SKATRON cell harvester. Thymidine incorporation was quantified in the presence of scintillation liquid. Values represent the average of triplicate determinations.

Antibodies and immunofluorescence

Anti α- and ε-PKC rabbit polyclonal antibodies were a kind gift of Dr Peter Parker. MEP21, EOS47 and MYL51/2 mouse monoclonals were produced in this laboratory and have been described previously (Kornfeld *et al.*, 1983; Graf *et al.*, 1992; McNagny *et al.*, 1992). Indirect immunofluorescence was performed on living cells. Cells were incubated for 10–30 min in an appropriate dilution of the first antibody, washed in medium, centrifuged as described above and resuspended in an appropriate dilution of fluorescein-labelled goat anti-mouse second antibody. After an additional 10–30 min incubation, cells were washed twice, resuspended in medium and propidium iodine was added to a concentration of 50 mg/ml. Samples were analysed on a Beckman FACScan fluorocytometer using live gating to exclude dead cells.

Western blotting

Cells were lysed in 0.1% Triton X-100 [0.1% Triton X-100, 25 mM Tris–HCl (pH 7.6), 1 mM EDTA (pH 8), 200 mM NaCl, 50 mM NaF, 0.1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Trasylol (Bayer)], the extracts fractionated by SDS–PAGE and blotted onto Immobilon-P membrane (Millipore). Immunodetection was performed by enhanced chemiluminescence (ECL, Amersham) as recommended by the manufacturer.

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