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Haemopoietic stem cell development to neutrophils is associated with subcellular redistribution and differential expression of protein kinase C subspecies

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SUMMARY

Multipotential FDCP-Mix A4 (A4) cells can be induced either to self-renew or to differentiate and develop into mature neutrophils in liquid culture, depending on the haemopoietic growth factors with which they are cultured. When cultured in low concentrations of interleukin 3 (IL-3, 1 unit/ml)) plus Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) and Granulocyte-CSF (G-CSF), A4 cells proliferate with accompanying development to form cells which resemble mature, postmitotic neutrophils. The presence of high concentrations of IL-3 (100 units/ml) blocks the development of A4 cells even in the presence of GM-CSF plus G-CSF. A4 cell development to neutrophils is accompanied by major changes in the expression of protein kinase C (PKC) subspecies in these cells. The predominant subspecies present in multipotent A4 cells, as judged by direct chromatographic analysis, was the type III enzyme (α) subspecies, whereas in mature A4 cell neutrophils, the type II (βI + βII) enzymes were predominant. Phorbol esters added to immature A4 cells resulted in a proliferative response, but when added to postmitotic A4 cells resembling neutrophils they elicited a large increase in reactive oxygen intermediate production. This suggests that the type III (α) subspecies may mediate proliferative responses in stem cells, whilst the type II (βI + βII) enzymes are more important for the mature cell functions of postmitotic neutrophils. In cultures containing IL-3 (100 units/ml) both the type III, and also the type II subspecies were predominantly membrane-associated for prolonged periods (>24 hours). The addition of IL-3 (100 units/ml) to FDCP-Mix A4 cells starved of haemopoietic growth factors led to the rapid translocation of protein kinase C from the cytosol to the membrane; no such effect was observed with GM-CSF or 1 unit/ml IL-3. Under conditions where differentiation and development were induced (1 unit/ml of IL-3 plus GM-CSF and G-CSF), there was a redistribution of all PKC subspecies to the cytosol from the membrane. Thus, IL-3 preserves the multipotential nature of A4 cells and translocates PKC to the membrane. As GM-CSF cannot stimulate the translocation of protein kinase C, the differential biochemical and developmental effects of these growth factors on multipotent cells may in part be mediated by the activation of protein kinase C.

Key words: neutrophils, protein kinase C, haemopoietic stem cells

INTRODUCTION

Multipotent haemopoietic cells can undergo a process of differentiation, proliferation and development to form postmitotic mature myeloid cells (Dexter, 1991; Metcalf, 1990, 1991; Migliaccio et al., 1991; Whetton and Dexter, 1989). The commitment of a multipotent cell to a specific program of development can be regulated by exogenous stimuli such

as haemopoietic growth factors (see Dexter, 1991; Metcalf, 1990, 1991; Whetton and Dexter, 1989). Progenitor cells committed to a specific developmental pathway also require growth factors for survival, proliferation and development (Burgess and Nicola, 1983; Cook et al., 1989). Evidence from both *in vivo* and *in vitro* studies has shown that growth factors such as Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Granulocyte Colony Stimulating Factor (G-CSF) and interleukin 3 (IL-3) can promote the proliferation and development of committed neutrophil/macrophage precursor cells (Cook et al., 1989; Metcalf, 1990, 1991; Whetton and Dexter, 1989). There are, however, some differences in the response of common target cells to these cytokines, in that the mitogenic response elicited by IL-3 or GM-CSF is markedly greater than that observed with G-CSF (Cook et al., 1989) and the proportion of neutrophils or macrophages formed from GM-CFC (Granulocyte Macrophage Colony Forming Cells) can be controlled by the type of growth factor added (Cook et al., 1989; Dexter, 1991; Metcalf, 1990, 1991).

This differential response of haemopoietic progenitor cells to specific growth factors may be associated with the activation of different signalling events within the same cells upon occupation of their respective receptors at the cell surface. Recent evidence suggests that there is a family of cytokine receptors that share some structural similarities, this family includes the receptors for IL-3 and GM-CSF (Nicola and Metcalf, 1991). Human IL-3, IL-5 and GM-CSF receptors share a common β_c subunit, which associates with the ligand-specific α subunit (Takaki et al., 1991; Tavernier et al., 1991; Van et al., 1991) to form a high-affinity receptor capable of eliciting a mitogenic response (Kitamura et al., 1991a). Competition between the three distinct ligand binding α subunits for the β_c subunit has been reported, suggesting that a single pool of the β_c subunit can elicit *common* signalling events for all three of these cytokines in human cells (Kitamura et al., 1991b; Tavernier et al., 1991). However, there are *distinct* forms of the *murine* IL-3 receptor that do not share a common β subunit with the GM-CSF receptor (Hara and Miyajima, 1992; Park et al., 1992). Furthermore, distinct forms of the human GM-CSF α subunit have now been cloned and sequenced (Crosier et al., 1991). This receptor heterogeneity may explain why some of the biological responses to growth factors such as GM-CSF and IL-3 are different in the *same* target cell population. For example, the response of highly enriched, multipotent murine progenitor cells to these two growth factors is markedly different, in that IL-3 is by far the more potent mitogenic and developmental stimulus (Heyworth et al., 1988; Moore, 1988; Whetton and Dexter, 1989).

We have investigated the relative abilities of haemopoietic cell growth factors to modulate self-renewal, differentiation and development using a multipotent haemopoietic cell line, FDCP-Mix A4 (A4) (Heyworth et al., 1990, 1991). In the presence of high concentrations of IL-3 (high [IL-3]), A4 cells will proliferate whilst undergoing self-renewal, i.e. the multipotent nature of the cells is preserved. When GM-CSF is added to the cells there is a relatively modest rate of proliferation but the cells develop into mature myeloid cells. When high concentrations of IL-3 are present together *with* GM-CSF, A4 cells undergo self-renewal, i.e. IL-3 can suppress the developmental response elicited by the GM-CSF (Heyworth et al., 1990).

Although both these cytokines are capable of eliciting a proliferative response the *developmental response* observed is plainly markedly different. The A4 cell line therefore offers an opportunity to study the possible differences between the signalling events elicited by GM-CSF and IL-3, which may have important developmental consequences for the multipotent cell.

One candidate intracellular regulatory molecule which may be involved in the development of myeloid cells (Aihara et al., 1991; Balazovich et al., 1987; Beckman et al., 1990; Girard et al., 1987; Hashimoto et al., 1990; Hocevar and Fields, 1991; Katayama et al., 1989; Melloni et al., 1989) and be differentially regulated by GM-CSF and IL-3, respectively, in some cell types is the serine- and threonine-specific protein kinase, protein kinase C (PKC) (Brizzi et al., 1991; Coffey et al., 1988; Fields et al., 1989; Sullivan et al., 1987; Whetton et al., 1988). A role for this enzyme has already been implied for the IL-3-stimulated proliferation of multipotent haemopoietic stem cells (Spivak et al., 1989; Whetton et al., 1986), and myeloid precursor cell lines (Boswell et al., 1989; Evans et al., 1986; Farrar et al., 1985; Linnekin and Farrar, 1990; Robinson et al., 1991).

PKC is now known to comprise a family of several subspecies, of which the α, βI, βII and τ-subspecies form the major subgroup (Kikkawa et al., 1989). Whilst PKC activation can stimulate proliferation, mature postmitotic neutrophils also express protein kinase C subspecies that are capable of coupling receptors (e.g. Platelet Activating Factor and chemotactic peptide receptors) to functional activation (e.g. the oxidative burst and reactive oxygen intermediate production) (Lew, 1989; Tauber et al., 1989).

Here we have attempted to identify the various subspecies of PKC that may be associated with the proliferation and functional activation of myeloid cells and the relative effects of GM-CSF and IL-3 on this protein kinase family.

MATERIALS AND METHODS

Conditions for FDCP-Mix A4 cell development

Cells from cultures of FDCP-Mix A4 cells maintained in Fischer's medium plus 20%(v/v) horse serum plus 100 units/ml IL-3 were taken during the logarithmic growth phase and washed twice. A4 cells were then resuspended in Iscove's medium plus 20% (v/v) fetal calf serum and 1 unit/ml IL-3, 50 units/ml GM-CSF and 1000 units/ml G-CSF (Heyworth et al., 1990). Cells were then incubated at 37°C in a gassed incubator containing 5% CO_2 in air. Recombinant GM-CSF and IL-3 were obtained from Biogen SA (Geneva, Switzerland) at 2.1×10^7 units/mg and 1.25×10^6 units/mg, respectively, whilst recombinant G-CSF was a kind gift from Amgen (Thousand Oaks, CA) at 1×10^8 units/mg. The suppliers of other tissue culture reagents were as described previously (Heyworth et al., 1990).

Morphological analysis of A4 cells

Morphological analysis of A4 cells was performed as described by Spooncer et al. (1986), using a cytospin centrifuge and *O*-dianisidine stain followed by May-Grunwald-Giemsa stain.

Determination of DNA synthesis

DNA synthesis was assessed using the [^{3}H]thymidine incorporation assay as described previously (Whetton et al., 1986). When A4 cells which were undergoing development to neutrophils were employed, they were first washed free of the medium in which they were cultured, then replated in Fischer's medium plus horse serum (20%), plus any other additive, for 18 hours prior to the addition of [^{3}H]thymidine for a further 4 hours before the radioactivity associated with acid-insoluble material was determined.

A4 cell fractionation and the determination of protein kinase C activity

The preparation of cytosolic and membrane fractions, and the partial purification and resolution of PKC subspecies from A4 cells, was performed essentially as described previously (Shearman et al., 1989). Enzyme activity was assayed by measuring the incorporation of $^{32}P_i$ into either H1 histone or myelin basic protein, from [^{32}P]ATP. The final composition of the reaction mixture was varied to compensate for the relative amount of enzyme activity in the samples studied. The range of conditions utilized was as follows: enzyme fraction, 20-50 μl; calcium chloride, 0.1-0.3 mM; phosphatidylserine, 2-20 μg/ml; diolein, 0.8-8 μg/ml; incubation time, 10-30 minutes; specific radioactivity 200-600 cts/min per pmol. PKC activity is defined as that measured in the presence of phosphatidylserine, diolein and calcium chloride minus that measured in the presence of 0.5 mM EGTA

Assay of reactive oxygen intermediate production by developing FDCP-Mix A4 cells

Measurement of reactive oxygen intermediate (ROI) production was performed as described previously (Heyworth et al., 1990). Briefly, the A4 cells were washed by centrifugation at 1800 ***g*** for 5 minutes and then resuspended in Fischer's medium plus 1% (w/v) bovine serum albumin (final cellular concentration 1×10^5/ml). A4 cells (1 ml) were then incubated for 5 minutes at 37°C prior to the addition of luminol (final concentration, 250 μM), incubated for 1 minute and then agonists (i.e. either the chemotactic peptide, FMLP, or TPA) were added and the increase in chemiluminescence associated with oxygen radical production determined. In experiments where PKC was down-modulated, TPA (100 ng/ml) was added for the final 6 hours of the incubation in the medium employed to induce development of the A4 cells. This resulted in the depletion of all detectable PKC activity in the cells.

RESULTS

A4 cells differentiate and develop into mature postmitotic neutrophils

In the presence of high concentrations of IL-3 (>100 units/ml) A4 cells undergo continuous self-renewal (Heyworth et al., 1990); the further addition of GM-CSF plus G-CSF did not induce differentiation. When IL-3 concentrations are progressively lowered, then its inhibitory effects on differentiation and development in liquid culture can be overcome by GM-CSF plus G-CSF, leading to the formation of mature postmitotic neutrophils. This effect is dependent on the concentration of IL-3 and is independent of the medium or type of serum (horse or fetal calf) used (Heyworth et al., 1990). When G-CSF is omitted from such cultures there is some development of the cells to mature neutrophils over 7 days, but a significant percentage of the cells remain as blast cells. (For this reason we included G-CSF in all our experiments in order to allow the development of *all* the FDCP-Mix A4 cells to mature cells within 8 days.) Furthermore, the low dose of IL-3 in cultures which were induced to differentiate was necessary to prevent the death of a discrete subset of A4 cells (<20%).

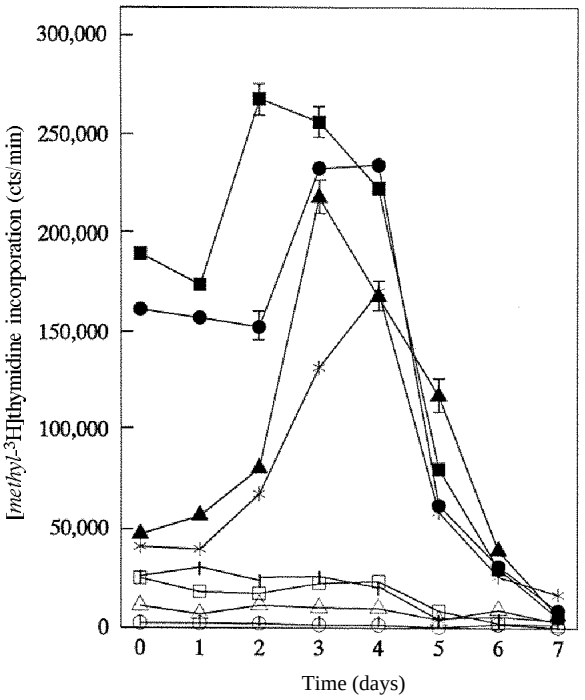

Fig. 1. Proliferative potential of A4 cells in response to haemopoietic growth factors or TPA. A4 cells were cultured in conditions which stimulated their development to neutrophils over a 7-day time course (see Materials and methods). The proliferative response of these cells in response to haemopoietic growth factors or the phorbol ester TPA (100 ng/ml) was assessed, each day, by the level of [^{3}H]thymidine incorporation (see Materials and methods). The concentrations employed gave maximal responses in all cases, except IL-3 (1 unit/ml). The effect of the combination of growth factors known to be required for the optimal development of A4 cells to neutrophils is also shown. Results are the mean±standard deviation of at least three experiments.(∗-∗) GM-CSF (50 units/ml); (○-○) no addition; (□-□) G-CSF (5000 units/ml); (△-△) IL-3 (1 unit/ml); (●-●) IL-3 (100 units/ml); (■-■) IL-3 (100 units/ml) + GM-CSF (50 units/ml); (▲-▲) IL-3 (1 unit/ml) + GM-CSF (50 units/ml) + G-CSF (5000 units/ml); (+-+) TPA.

Development of the cells is accompanied by proliferation: cell numbers increased by approximately 46-fold during the 7 days in which the cells formed mature neutrophils. Initially, the FDCP-Mix A4 cells exhibit a mitogenic response to IL-3, GM-CSF, G-CSF and also TPA (12-*O*-tetradecanoylphorbol 13-acetate). The dose of these agents required to elicit a maximal mitogenic response did not vary during the development of the cells to neutrophils. There is, however, a loss of proliferative potential during the seven-day period of development such that none of these agents is capable of increasing the rate of [^{3}H]thymidine incorporation significantly beyond the seventh day (Fig.1). TPA-stimulated ROI production, however, steadily increased from an immeasurably small level on days 0, 1 and 2 to a substantial rate on day 7 (Table 1). Thus, as

Table 1. Chemotactic peptide-stimulated reactive oxygen intermediate production is sensitive to the down modulation of protein kinase C

	ROI production (cts/min×10^3)		
Time (days)	FMLP-stimulated	FMLP-stimulated in PKC down-modulated cells	TPA-stimulated
1	1.2	0.6	1.2
2	8.7	0.6	17.5
3	12.5	0.0	30.0
4	52.5	1.8	182.5
5	68.7	2.5	315.1
6	67.5	3.4	302.5
7	90.0	3.2	795.0

A4 cells were cultured under conditions which stimulated their development to neutrophils over a 7-day period. ROI production was determined as described in Materials and methods. Results shown are representative of three independent experiments. The chemotactic peptide, FMLP, and TPA were employed at final concentrations of 10 μM and 100 ng/ml, respectively.

would be anticipated in haemopoietic cell development, proliferative capacity is diminished as the functions and morphology of mature, postmitotic cells are acquired (see Table 1; and Heyworth et al., 1990, 1991).

Expression of protein kinase C subspecies during development of A4 cells into neutrophils

By direct chromatographic analysis of PKC activity, using sequential anion exchange and hydroxyapatite chromatography, we have assessed the relative levels of PKC subspecies in the combined cytosolic and membrane fractions during A4 cell development. In comparison to a preparation of rat whole brain enzyme, which expresses three major peaks of activity, corresponding to the type I (τ), type II (βI + βII) and type III (α) enzymes (Fig.2A), the A4 cells were found to express two major peaks of enzyme activity. By a number of criteria, namely their elution position from the hydroxyapatite column, enzymological characteristics and immunopositive reaction to subspecies-selective antibodies (data not shown), these enzymes were shown to correspond to the brain type II (β) and type III (α), respectively. The type II (β) enzyme was a mixture of the βI and βII subspecies; however, we did not attempt to distinguish these subspecies on a routine basis. As the A4 cells were stimulated to develop into neutrophils, the total recoverable PKC activity in the cells remained constant, but the relative abundance of the two enzymes was found to change dramatically. At day 0 (Fig.2B), the ratio of activities of the type II (β):type III (α) enzymes was 0.4:1, at day 4 (Fig.2D) 1:1 and at day 7 (Fig.2F) 1.5:1, representing a significant shift in the relative activity of the distinct subspecies during development.

In addition to the enzymes described above, a third peak of enzyme activity, eluting between the type II (β) and type III (α) subspecies, appeared to be expressed in increasing abundance during development of the A4 cells (see Fig.2C,D and F). The resolution of this novel enzyme from the type II (β) PKC subspecies was not achieved in a consistent fashion using the standard gradient elution programme. The identity of the enzyme has not been confirmed, but it has been shown to be activated by phosphatidylserine and diacylglycerol in a calcium-independent manner, and to respond to arachidonic acid (data not shown).

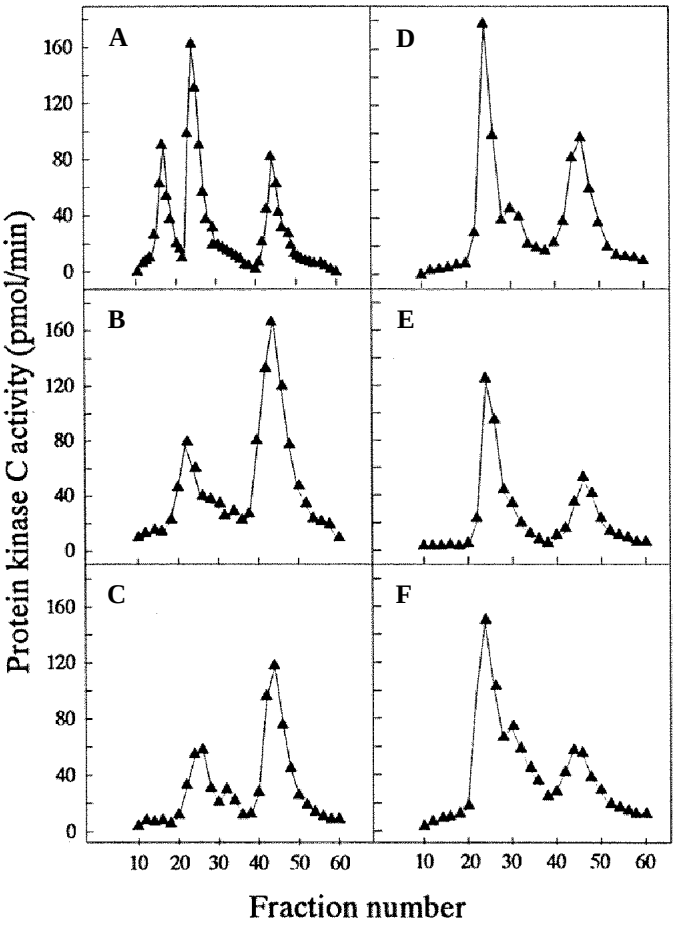

Fig. 2. Expression of protein kinase C activity during A4 cell development. PKC activity from the combined cytosolic and membrane fractions of rat whole brain tissue (A) and A4 cells at day 0 (B), day 1 (C), day 4 (D), day 6 (E) and day 7 (F) of development into neutrophils was partially purified, then applied to an hydroxyapatite column and eluted with a gradient of increasing concentrations of potassium phosphate (Shearman *et al.*, 1989). The data shown are representative of three independent experiments.

Cytosol/membrane distribution of protein kinase C is modulated by interleukin 3, but not GM-CSF

High concentrations of IL-3 (100 units/ml) have been shown to stimulate the self-renewal of A4 cells and to abrogate their ability to undergo differentiation in response to growth factors such as GM-CSF and G-CSF (Heyworth et al., 1990). We assessed, therefore, the effect of high and low concentrations of IL-3 on the cytosol/membrane distribution of PKC activity in these cells. In A4 cells cultured under conditions to induce constant self-renewal (100 units/ml IL-3 in the absence of any other haemopoietic growth factor) (Fig.3A) the majority of the activity (>90% of total activity) was recovered in the membrane fraction.

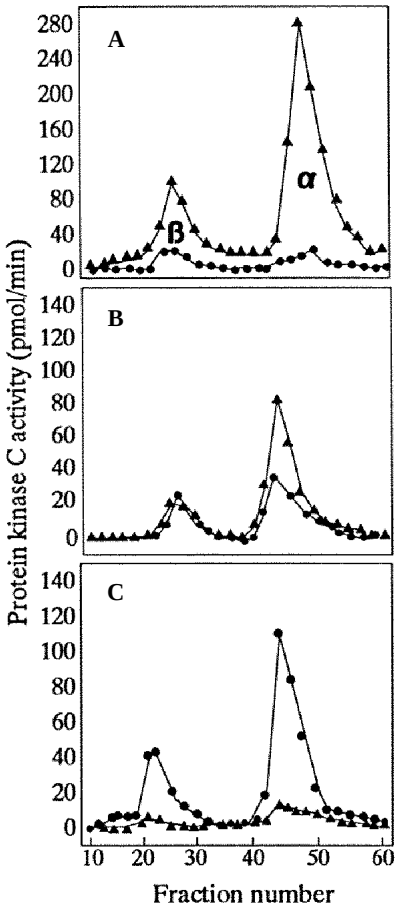

Fig. 3. The effect of culture conditions on the subcellular distribution of protein kinase C in A4 cells. The distribution of PKC between the cytosolic and membrane fractions was assessed following the culture of A4 cells for 24 hours in IL-3 (100 units/ml) (A); IL-3 (100 units/ml) plus GM-CSF (50 units/ml) plus G-CSF (5000 units/ml) (B); and IL-3 (1 unit/ml) plus GM-CSF (50 units/ml) plus G-CSF (1000 units/ml) (C). These culture conditions gave the following cellular morphologies after 7 days culture: A, 98% blast cells, 2% early granulocytes, 0% late granulocytes; B, 87% blast cells, 7% early granulocytes, 6% late granulocytes; C, 2% blast cells, 10% early granulocytes, 88% late granulocytes. Results shown are representitive of three individual experiments. (●-●) Cytosol; (▲-▲) membrane.

Both the type II (β) and type III (α) enzymes showed a similar distribution, with the latter enzyme being more enriched in the membrane fraction. If these cells were cultured for a prolonged period under the same 'self-renewal' conditions (Fig.3B) the enzyme activity remained predominantly associated with the membrane fraction (65% of total activity), although the type II (β) subspecies showed an approximately equal distribution between the cytosolic and membrane fractions. If, on the other hand, A4 cells were cultured under conditions to induce differentiation (1 unit/ml IL-3, plus GM-CSF and G-CSF) (Fig.3C) the enzyme activity shifted to a predominantly cytosolic location (>90% of total activity), with both subspecies behaving in a similar manner.

The observation of membrane-associated PKC during A4 cell self-renewal, followed by a shift to a predominantly

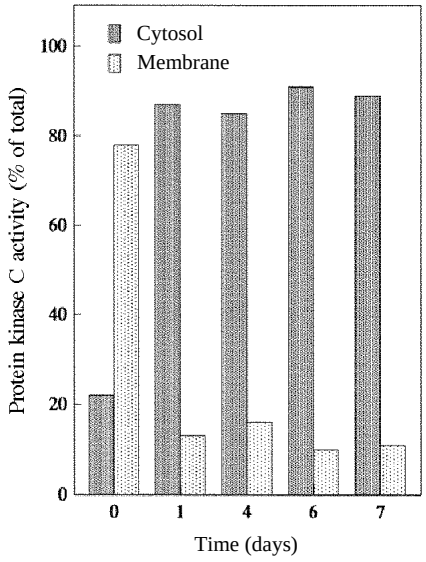

Fig. 4. The cytosol:membrane distribution of protein kinase C in FDCP-Mix A4 cells as they develop from multipotent cells to neutrophils. A4 cells cultured in conditions which stimulated their development to neutrophils were assayed for the subcellular localization of the PKC at the time points shown. Results shown are representative of three separate experiments.

cytosolic location of the enzyme during the first day of differentiation, was found to hold throughout the maturation of the cells to neutrophils. When assayed at days 1, 4, 6 and 7 the enzyme activity was found to be more than 80% cytosolic (Fig.4). This altered distribution of PKC activity was not associated with a differential proliferative status of the cells, as [^{3}H]thymidine incorporation assays indicated that cells from day 0 to day 4 exhibited a strong response to the growth stimuli (1 unit/ml IL-3, 50 units/ml GM-CSF and 5000 units/ml G-CSF) in which they were cultured (Fig.1).

In order to determine if the long-term effect of lowering the IL-3 concentration on PKC localization correlated with an *acute* effect on redistribution of this enzyme from the cytosol to the membrane, we assessed the ability of GM-CSF and IL-3, respectively, to translocate PKC to the membrane. In order to do this, cells were pretreated without haemopoietic growth factors for 6 hours so that PKC was found predominantly in the cytosol. After this preincubation the cells were treated with GM-CSF or IL-3 for 15 minutes. Only the cells treated with IL-3 or TPA showed any redistribution of PKC activity to the membrane fraction (see Table 2). Thus IL-3, but not GM-CSF, is capable of modulating the subcellular localization of PKC in both an acute (15 minute) and a chronic (24 hour) fashion (Table 2 and Fig.3).

Down-modulation of protein kinase C decreases IL-3-, but not GM-CSF-stimulated [^{3}H]thymidine incorporation

PKC activity, however, can be down-modulated by treating the parent A4 cells with TPA. We found that 6 hours of incubation with TPA removed all *measurable* PKC activity from the cells (results not shown). Furthermore,

Table 2. The effect of IL-3 and GM-CSF, respectively, on the acute redistribution of protein kinase C activity from the cytosol to the membrane fraction

Additive	Membrane-associated PKC (% of total)
No addition	8±6
IL-3	29±2
GM-CSF	8±6
TPA	54±6

The effects of IL-3 (100 units/ml), TPA (100 ng/ml) and GM-CSF (50 units/ml) on the redistribution of PKC activity were assessed after a 15-minute incubation. The results shown are the mean ± standard deviation of at least three experiments.

western blot analysis confirmed that there was no detectable PKC present in the cells. The down-modulation of PKC with phorbol ester had a differential effect on GM-CSF- and IL-3-stimulated [^{3}H]thymidine incorporation. Compared to control cultures PKC-down-modulated cells exhibited a 35±5% decrease in [^{3}H]thymidine incorporation in the presence of 100 units/ml IL-3, whilst down-modulated cells incubated with GM-CSF (50 units/ml) showed only a 6±3% decrease (mean±s.e.m., n=4). Thus, whilst GM-CSF-mediated mitogenic signalling is not grossly affected by the removal of PKC, there is certainly an inhibitory effect observed when IL-3 is employed as the mitogenic stimulus.

Down-modulation of protein kinase C abrogates chemotactic peptide-stimulated reactive oxygen intermediate production in developing FDCP-Mix A4 cells

Whilst the results given above suggest that there is an effect of IL-3 on PKC activity it is also clear that there is a role for PKC in the functional activation of neutrophils. The developing neutrophilic FDCP-Mix A4 cell acquires an ability to respond to agonists that potentiate the functional activation of mature blood cells (Heyworth et al., 1990). Some of these agents, such as the chemotactic peptide, FMLP, can activate PKC. We compared the ability of FMLP to functionally activate normal and PKC-down-modulated cells. It was clear that as the A4 cells acquired the ability to respond to FMLP, the down-modulation of PKC reduced the ability of the cells to produce activated oxygen species in response to this agonist (Table 1). This indicates that the PKC in the developing A4 cells can couple to receptors associated with functional activation of the cells. IL-3 was incapable of activating ROI production at any stage of A4 cell development (data not shown), and must therefore have a different effect on the PKC isoforms to that observed with FMLP.

DISCUSSION

The multipotent A4 cell line can be induced to differentiate and develop along several haemopoietic lineages. Differentiation is accompanied by proliferation until a post-mitotic, functionally active cell is formed. We have characterized the conditions required to preferentially induce neutrophil development, and have shown that both types of growth factor, as well as their concentrations, are critical in this respect (Heyworth et al., 1990, 1991). This focuses considerable interest on the cellular signalling pathways activated by these growth factors, and on how the relative influence of the functionally active components alter during development of the myeloid cell. We have chosen to study the role of PKC, as this protein kinase is known to modulate the proliferative responses of many distinct cell types to growth factors including IL-3 (Boswell et al., 1989; Evans et al., 1986; Farrar et al., 1985; Linnekin and Farrar, 1990; Robinson et al., 1991), yet it is also activated by agonists (such as chemotactic peptides or Platelet Activating Factor; Lew, 1989; Tauber et al., 1989) that evoke functional responses in mature cells without increased DNA synthesis.

The *response* of A4 cells to IL-3 and GM-CSF is markedly different: IL-3 promotes self-renewal whilst GM-CSF stimulates development. The molecular basis for these profound differences is extremely relevant to the regulation of haemopoiesis. The data from experiments where PKC activity was down-modulated indicate that there is a greater requirement for this kinase in IL-3-stimulated proliferation than is the case when GM-CSF is employed as a mitogen. It is possible therefore that the differential effects of IL-3 and GM-CSF are due to their activated occupied receptors coupling to signal transduction mechanisms that have some important differences, such as PKC activation.

It has been reported that the human and murine, IL-3 and GM-CSF receptors may share a common subunit, which has been suggested to elicit all the cellular signalling events within the target cell (Hara and Miyajima, 1992; Nicola and Metcalf, 1991; Park et al., 1992). This observation could not account for the differential effects of GM-CSF and IL-3 on the multipotent murine A4 cells. Data recently obtained by Hara and Miyajima (1992) indicate that there is more than one type of the *murine* high-affinity IL-3 receptor and suggest a possible mechanism whereby some but not all of the signalling events elicited by these two cytokines can be held in common. High-affinity forms of the GM-CSF and IL-3 receptors that share a common β subunit have also been characterised, and it is possible that these receptors activate the same transmembrane signalling events upon ligand binding (Hara and Miyajima, 1992; Park et al., 1992). Thus, the specific effects of IL-3 that we observe on A4 cells may be elicited by the AIC2A and SUT-1 proteins, which individually bind IL-3 (but not GM-CSF) with low affinity, but, when combined, form high-affinity IL-3-specific receptors that share no subunit in common with GM-CSF receptors (Hara and Miyajima, 1992).

It is also possible to infer from our data that only one specific isoform of the IL-3 receptor may activate PKC in A4 cells. It will now be of interest to establish if the isoforms of the high-affinity IL-3 receptor alter during development of the cells to neutrophils and whether this correlates with a change in the cellular signalling events elicited by IL-3.

The self-renewal/differentiation balance of the A4 cells is controlled by the presence of IL-3: high concentrations of this cytokine abrogate the ability of A4 cells to develop into mature neutrophils (i.e. IL-3 promotes self-renewal). In culture conditions designed to promote A4 self-renewal

and block development, the PKC activity was found to be associated mainly with the membrane fraction of the cell, presumably in an activated state (Fig.3). When the culture conditions were changed to allow the differentiation and maturation of these cells to neutrophils, the PKC activity was recovered mainly in the cytosolic fraction. This suggests that, whilst undergoing IL-3-stimulated self-renewal, PKC is activated in the A4 cells, but when cultured in low concentrations of IL-3, which permit commitment to development, the enzyme returns to its inactive, cytosolic form.

Although our data suggest that there is a role for PKC in the mode of action of IL-3, other reports using other cell lines suggest that this is not the case (Kraft et al., 1990). One possibility of explaining these discrepancies is that there may be differential abilities of IL-3 receptor isoforms (expressed on different cell types) to couple to PKC activation. Another possibility is that different isoforms of PKC are expressed in specific IL-3-dependent cell lines, only some of which are *both* activated *and* can elicit a mitogenic response to this growth factor. We have shown that the PKC enzymes expressed in A4 cells change markedly during development to neutrophils. It is quite possible that the subspecies expressed in the late committed neutrophilic progenitor A4 cells (the β isoforms) have a different function to that of the α isoforms, and whilst both isoforms are associated with the membrane in cells treated with high concentrations of IL-3, this does not necessarily mean that both of these PKC subtypes contribute to a mitogenic response. Thus the decrease in PKC α isotype may contribute to the cells becoming postmitotic; alternatively, the onset of the postmitotic phenotype could diminish the requirement for this enzyme.

The suggestion that PKC β has no role in IL-3-mediated mitogenic signalling is substantiated by the observation that transfection of PKC β into an IL-3-dependent cell line had no effect on survival or proliferation (Kraft et al., 1990). It is interesting to note that expression of a *specific* PKC subspecies (PKC α) is associated with neural induction and competence in *Xenopus* (Otte and Moon, 1992), suggesting that this family of enzymes may indeed have an important role in developmental decisions in a number of different tissues. Another intriguing aspect of the role of PKC subspecies in developing myeloid cells is the observation that PKC activation by phorbol esters can promote not only [^{3}H]thymidine incorporation but also the production of ROI in developing A4 cells. It is a possibility, although the evidence is only circumstantial at present, that the increased expression of the β subspecies during development is required to couple efficiently the receptors for agonists, such as FMLP, to the NADPH oxidase system (Lew, 1989; Tauber et al., 1989). On the other hand, the greater expression of the α subspecies in the precursor cells may be important for coupling growth factor receptors to cellular proliferation. Another possibility is that the unidentified PKC subspecies which elutes slightly later than the β subspecies on hydroxyapatite column chromatography may have the potential to be activated by phosphatidylinositol 4,5-bisphosphate phospholipase C-linked receptors, such as the FMLP receptor (Lew, 1989; Tauber et al., 1989) and *also* to couple to the NADPH oxidase system, leading to its activation and the production of oxygen radicals. The observation that IL-3 cannot promote the production of ROI in A4 cells suggests either that PKC subspecies are differentially activated by agonists such as IL-3 and FMLP, or that the cells at this stage in their development exhibit a modified signal transduction response to IL-3. The A4 cell line now offers us the opportunity to dissect out the various roles for PKC subspecies by modulating the expression of these enzymes in the developing myeloid cell.

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REFERENCES

Aihara, H., Asaoka, Y., Yoshida, K. and Nishizuka, Y. (1991). Sustained activation of protein kinase C is essential to HL-60 cell differentiation to macrophage. *Proc. Nat. Acad. Sci. USA* **88**, 11062-11066.

Balazovich, K. J., Portnow, D., Boxer, L. A. and Prochownik, E. V. (1987). Changes in protein kinase C activity are associated with the differentiation of Friend erythroleukemia cells. *Biochim. Biophys. Acta* **927**, 247-55.

Beckman, B. S., Mason, G. M., Martinez, M. I. and Fermin, C. (1990). Enhanced expression of the beta II subspecies of protein kinase C in differentiating erythroleukemia cells. *Exp. Cell Res.* **191**, 319-22.

Boswell, H. S., Harrington, M. A., Burgess, G. S., Nahreini, T. L., Derigs, H. G., Hodges, T. D., English, D., Crean, C. D. and Gabig, T. G. (1989). A mutant RAS gene acts through protein kinase C to augment interleukin-3 dependent proliferation in a fastidious immortal myeloid cell line. *Leukemia* **3**, 662-8.

Brizzi, M. F., Arduino, C., Avanzi, G. C., Bussolino, F. and Pegoraro, L. (1991). GM-CSF and phorbol esters modulate GM-CSF receptor expression by independent mechanisms. *J. Cell. Physiol.* **148**, 24-34.

Burgess, A. W. and Nicola, N. A. (1983). Effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on the proliferation of granulocyte macrophage colony forming cells. *Blood* **61**, 575-579.

Coffey, R. G., Davis, J. S. and Djeu, J. Y. (1988). Stimulation of guanylate cyclase activity and reduction of adenylate cyclase activity by granulocyte-macrophage colony-stimulating factor in human blood neutrophils. *J. Immunol.* **140**, 2695-701.

Cook, N., Dexter, T. M., Lord, B. I., Cragoe, E. J. J. and Whetton, A. D. (1989). Identification of a common signal associated with cellular proliferation stimulated by four haemopoietic growth factors in a highly enriched population of granulocyte/macrophage colony-forming cells. *EMBO J.* **8**, 2967-74.

Crosier, K. E., Wong, G. G., Mathey, P. B., Nathan, D. G. and Sieff, C. A. (1991). A functional isoform of the human granulocyte/macrophage colony-stimulating factor receptor has an unusual cytoplasmic domain. *Proc. Nat. Acad. Sci. USA* **88**, 7744-8.

Dexter, T. M. (1991). Growth and differentiation in the haemopoietic system. *Biochem. Soc. Trans.* **19**, 303-6.

Evans, S. W., Rennick, D. and Farrar, W. L. (1986). Multilineage hematopoietic growth factor interleukin 3 and direct activators of protein kinase C stimulate phosphorylation of common substrates. *Blood* **68**, 906-13.

Farrar, W. L., Thomas, T. P. and Anderson, W. B. (1985). Altered

cytosol/membrane enzyme redistribution on interleukin-3 activation of protein kinase C. *Nature* **315**, 235-7.

Fields, A. P., Pincus, S. M., Kraft, A. S. and May, W. S. (1989). Interleukin-3 and bryostatin 1 mediate rapid nuclear envelope protein phosphorylation in growth factor-dependent FDC-P1 hematopoietic cells. A possible role for nuclear protein kinase C. *J. Biol. Chem.* **264**, 21896-901.

Girard, P. R., Stevens, V. L., Blackshear, P. J., Merrill, A. J., Wood, J. G. and Kuo, J. F. (1987). Immunocytochemical evidence for phorbol ester-induced directional translocations of protein kinase C in HL60, K562, CHO, and E7SKS cells: possible role in differentiation. *Cancer Res.* **47**, 2892-8.

Hara, T. and Miyajima, A. (1992). Two distinct functional high affinity receptors for mouse Interleukin 3 (IL-3). *EMBO J.* **11**, 1875-84.

Hashimoto, K., Kishimoto, A., Aihara, H., Yasuda, Y., Mikawa, K. and Nishizuka, Y. (1990). Protein kinase C during differentiation of human promyelocytic leukemia cell line, HL-60. *FEBS Lett.* **263**, 31-34.

Heyworth, C. M., Dexter, T. M., On, K. and Whetton, A. D. (1990). The role of haemopoietic growth factors in self-renewal and differentiation of IL-3 dependent multipotential stem cells. *Growth Factors* **2**, 197-211.

Heyworth, C. M., Hampson, J., Dexter, T. M., Walker, F., Burgess, A. W., Kan, O., Cook, N., Vallance, S. J. and Whetton, A. D. (1991). Development of multipotential haemopoietic stem cells to neutrophils is associated with increased expression of receptors for granulocyte macrophage colony-stimulating factor: altered biological responses to GM- CSF during development. *Growth Factors* **5**, 87-98.

Heyworth, C. M., Ponting, I. L. and Dexter, T. M. (1988). The response of haemopoietic cells to growth factors: developmental implications of synergistic interactions. *J. Cell Sci.* **91**, 239-247.

Hocevar, B. A. and Fields, A. P. (1991). Selective translocation of beta II-protein kinase C to the nucleus of human promyelocytic (HL60) leukemia cells. *J. Biol. Chem.* **266**, 28-33.

Katayama, N., Nishikawa, M., Minami, N. and Shirakawa, S. (1989). Putative involvement of protein kinase C in proliferation of human myeloid progenitor cells. *Blood* **73**, 123-30.

Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989). The protein kinase C family: heterogeneity and its implications. *Annu. Rev. Biochem.* **58**, 31-44.

Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K. and Miyajima, A. (1991a). Reconstitution of functional receptors for human granulocyte/macrophage colony-stimulating factor (GM-CSF): evidence that the protein encoded by the AIC2B cDNA is a subunit of the murine GM-CSF receptor. *Proc. Nat. Acad. Sci. USA* **88**, 5082-6.

Kitamura, T., Sato, N., Arai, K. and Miyajima, A. (1991b). Expression cloning of the human IL-3 receptor cDNA reveals a shared beta subunit for the human IL-3 and GM-CSF receptors. *Cell* **66**, 1165-74.

Kraft, A. S., Wagner, F. and Housey, G. M. (1990). Overexpression of protein kinase C beta 1 is not sufficient to induce factor independence in the interleukin-3-dependent myeloid cell line FDC- P1. *Oncogene* **5**, 1243-6.

Lew, D. P. (1989). Receptor signalling and intracellular calcium in neutrophil activation. *Eur. J. Clin. Invest.* **19**, 338-346.

Linnekin, D. and Farrar, W. L. (1990). Signal transduction of human interleukin 3 and granulocyte-macrophage colony-stimulating factor through serine and tyrosine phosphorylation. *Biochem. J.* **271**, 317-24.

Melloni, E., Pontremoli, S., Vioti, P. L., Patrone, M., Marks, P. A. and Rifkind, R. A. (1989). Differential expression of protein kinase C isozymes and erythroleukemia differentiation. *J. Biol. Chem.* **264**, 18414-18418.

Metcalf, D. (1990). The induction and inhibition of differentiation in normal and leukaemic cells. *Phil. Trans. R. Soc. Lond.* **327**, 99-109.

Metcalf, D. (1991). Control of granulocytes and macrophages: molecular, cellular and clinical aspects. *Science* **254**, 529-533.

Migliaccio, G., Migliaccio, A. R., Druzin, M. L., Giardina, P. J. V., Zsebo, K. M. and Adamson, J. W. (1991). Effects of recombinant human stem cell factor (SCF) on the growth of human progenitor cells in vitro. *J. Cell. Physiol.* **148**, 503-509.

Moore, M. A. S. (1988). Interleukin 3: an overview. *Lymphokines* **15**, 219-283.

Nicola, N. A. and Metcalf, D. (1991). Subunit promiscuity among hemopoietic growth factor receptors. *Cell* **67**, 1-4.

Otte, A. P. and Moon, R. T. (1992). Protein kinase C isozymes have distinct roles in neural induction and competence in Xenopus. *Cell* **68**, 1021-29.

Park, L. S., Sorensen, M. U., Luhrs, R., Morrissey, P. J., Cosman, D. and Larsen, A. (1992). Cloning of the low affinity murine Granulocyte Macrophage Colony Stimulating Factor receptor and reconstitution of a high affinity complex. *Proc. Nat. Acad. Sci. USA* **89**, 4295-4299.

Robinson, M., Chen, T. and Warne, T. R. (1991). IL-3-induced generation of alkylacylglycerol and diacylglycerol in an IL-3- dependent cell line. *J. Immunol.* **147**, 2624-9.

Shearman, M. S., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1989). A rapid method for the resolution of protein kinase C subspecies from rat brain tissue. *Methods Enzymol.* **168**, 347-351.

Spivak, J. L., Hogans, B. B. and Stuart, R. K. (1989). Tumor-promoting phorbol esters support the in vitro proliferation of murine pluripotent hematopoietic stem cells. *J. Clin. Invest.* **83**, 100-7.

Spooncer, E., Heyworth, C. M., Dunn, A. and Dexter, T. M. (1986). Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors. *Differentiation* **31**, 111-8.

Sullivan, R., Griffin, J. D., Simons, E. R., Schafer, A. I., Meshulam, T., Fredette, J. P., Maas, A. K., Gadenne, A. S., Leavitt, J. L. and Melnick, D. A. (1987). Effects of recombinant human granulocyte and macrophage colony-stimulating factors on signal transduction pathways in human granulocytes. *J. Immunol.* **139**, 3422-30.

Takaki, S., Mita, S., Kitamura, T., Yonehara, S., Yamaguchi, N., Tominaga, A., Miyajima, A. and Takatsu, K. (1991). Identification of the second subunit of the murine interleukin-5 receptor: interleukin-3 receptor-like protein, AIC2B is a component of the high affinity interleukin-5 receptor. *EMBO. J.* **10**, 2833-8.

Tauber, A. I., Cox, J. A., Curnutte, J. T., Carrol, P. M., Nakakuma, H., Warren, B., Gilbert, H. and Blumberg, P. M. (1989). Activation of human neutrophil NADPH-oxidase in vitro by the catalytic fragment of protein kinase-C. *Biochem. Biophys. Res. Commun.* **158**, 884-90.

Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van, d. H. J., Fiers, W. and Plaetinck, G. (1991). A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5- specific alpha chain and a beta chain shared with the receptor for GM-CSF. *Cell* **66**, 1175-84.

Van, d. H. J., Devos, R., Plaetinck, G., Fache, I., Fiers, W. and Tavernier, J. (1991). Characterization of the murine IL-5 receptor complex with the use of a panel of monoclonal antibodies. Relationship to the murine IL-3 receptor. *J. Immunol.* **147**, 3413-8.

Whetton, A. D. and Dexter, T. M. (1989). Myeloid haemopoietic growth factors. *Biochim. Biophys. Acta* **989**, 111-32.

Whetton, A. D., Heyworth, C. M. and Dexter, T. M. (1986). Phorbol esters activate protein kinase C and glucose transport and can replace the requirement for growth factor in interleukin-3-dependent multipotent stem cells. *J. Cell Sci.* **84**, 93-104.

Whetton, A. D., Monk, P. N., Consalvey, S. D., Huang, S. J., Dexter, T. M. and Downes, C. P. (1988). Interleukin 3 stimulates proliferation via protein kinase C activation without increasing inositol lipid turnover. *Proc. Nat. Acad. Sci. USA* **85**, 3284-8.

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