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Inositol lipids and phosphates in the regulation of the growth and differentiation of haemopoietic and other cells

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Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis is an important signalling reaction involved in the responses of cells to some, but not all, stimuli that promote cell proliferation. Active agents in this regard include antigens activating T and B lymphocytes, angiotensin (employing a receptor encoded by the *mas* oncogene), bombesin and platelet-derived growth factor PDGF). However, accumulating evidence suggests that inositol lipids and phosphates also have other roles in the regulation of cell growth and differentiation. Growth factor receptors that encode tyrosine kinases (such as that for PDGF) activate a kinase that synthesises phosphatidylinositol 3-phosphate, a novel lipid, and loss of this kinase-activating function abolishes growth-promoting activity. Human interleukin-4, a lymphokine that activates B lymphocytes, appears to employ phosphatidylinositol 4,5-bisphosphate hydrolysis as a brief initial signal that is followed by a sustained rise in cyclic adenosine monophosphate (cAMP): both signals are needed for the successful induction of the surface antigen CD23. Moreover, the same inositol lipid signalling pathway as is employed by antigen-stimulated mature T lymphocytes to provoke proliferation may be redeployed in immature T cells to trigger their elimination when they encounter self-antigens. Finally, studies of HL60 promyelocytic cells have shown that these cells contain high concentrations of inositol 3,4,5,6-tetrakisphosphate, 1,3,4,5,6-pentakisphosphate and hexakisphosphate, three inositol polyphosphates that are probably formed independently of inositol lipid metabolism. When these cells are induced to differentiate either towards neutrophils (in the presence of dimethylsulphoxide) or macrophages (in phorbol myristate acetate), cessation of growth and acquisition of differentiated characteristics are accompanied by large and different changes in the concentrations of these inositol phosphates that may be characteristic of these two pathways of differentiation.

Growth stimuli, oncogenes and inositol lipid signalling

The idea that inositol lipid hydrolysis is a key signal in the stimulation of quiescent lymphocytes into cell cycle was an inevitable development from the observation that inositol lipid turnover is accelerated in lymphocytes stimulated by the polyclonal mitogen phytohaemagglutinin (Fisher & Mueller 1968, 1971). This early experimental observation also helped to force the realization that stimulated inositol lipid hydrolysis is a receptor-coupled signalling reaction rather than an intracellular event implicated in exocytotic secretion (Michell 1975). Later work on a variety of cells and tissues then gradually led to recognition that rapid inositol lipid turnover, assumed to be a secondary response to a rapid generation of the cellular signals formed by inositol lipid hydrolysis, is a widespread characteristic of cells stimulated to

proliferate; for example by serum, platelet-derived growth factor (PDGF), low cell density or transformation by Rous sarcoma virus (see Michell (1982, 1985), for reviews of this early work).

In 1984, it was finally recognized that the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is a primary and widespread receptor-stimulated signalling reaction (Michell *et al.* 1981) whose products (inositol 1,4,5-trisphosphate and 1,2-diacylglycerol) are second messengers responsible for elevating the cytosolic Ca^{2+} concentration and activating protein kinase C in stimulated cells (Berridge 1984; Nishizuka, 1984; for recent reviews, see Berridge 1987; Berridge & Michell 1988; Michell *et al.* 1989; Kikkawa *et al.* 1989).

Initially, the most intense interest in the biological role of this signalling pathway was focussed on the regulation of short-term cell responses such as platelet activation and hepatic glycogenolysis. However, the publication in mid-1984 of two reports purporting to show that the known tyrosine kinases encoded by the *src* and *ros* oncogenes might also act as inositol lipid kinases, and therefore might supply substrate to the phosphatidylinositol 4,5-bisphosphate signalling pathway (Sugimoto *et al.* 1984; Macara *et al.* 1984), galvanized the research community into acceptance of a possibly pivotal role of inositol lipid hydrolysis in regulating cell proliferation. An immediate effect was to provoke speculation that some at least of the many oncogene-encoded proteins that had already been identified by that time might participate in an essential chain of signalling events that includes PtdIns(4,5)P_2 hydrolysis and leads to a temporal pattern of gene activation capable of driving cell proliferation (for example, Michell 1984, 1985; Berridge, 1984).

Subsequent work has confirmed some of the ideas generated at that time and has also added further, and still ill-understood, complexity to our ideas of how inositol lipids and phosphates participate in the control of cell proliferation and differentiation. We will first briefly review some of the recent work in this area and then outline some new experimental results from our own laboratories.

A role for phosphatidylinositol 4,5-*bisphosphate or phosphatidylinositol* 4-*phosphate in cell proliferation?*

Probably the most direct attack on the question of whether these lipids play some essential role in cell proliferation has come from Takenawa *et al.* whose approach has been to determine whether the admission of polyphosphoinositide-directed monoclonal antibody into cells can interfere with their proliferation (Uno *et al.* 1988; Matuoka *et al.* 1988). Despite limited published evidence on the exact specificity of the antibody used, the results strongly support such a role. First, microinjection of anti-polyphosphoinositide antibody prevented the mitogenic responses of NIH 3T3 cells to bombesin and PDGF (Matuoka *et al.* 1988). Secondly, similar treatment of wild-type *Saccharomyces cerevisiae* temporarily blocked their growth: the reinitiation of growth after a lag might have been accomplished by the cells synthesizing new polyphosphoinositide to replace that bound by antibody. In support of the latter view, mutants defective in various aspects of inositol lipid metabolism lacked the ability to recover after antibody electroporation, but could in some cases be rescued by supplying either phosphatidylinositol 4,5-bisphosphate or a combination of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (Uno *et al.* 1988).

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The actions of receptor-tyrosine kinases and tyrosine kinase oncogenes

Contrary to the original suggestions of Macara *et al.* (1984) and Sugimoto *et al.* (1984), the proteins encoded by the *src* and *ros* oncogenes (and other tyrosine kinase-encoded oncogenes) do not catalyse the phosphorylation of inositol lipids. They do, however, coexist in plasma membranes with a phosphatidylinositol kinase (known as type I kinase) that is almost certainly a substrate for phosphorylation on tyrosine (Courtneidge & Heber 1987; Kaplan *et al.* 1987; Courtneidge *et al.* 1989; Roberts *et al.* 1989). Upon phosphorylation, this protein is activated and forms a tight association with the responsible tyrosine kinase. Surprisingly, type I kinase does not synthesize phosphatidylinositol 4-phosphate, the immediate precursor of receptor-sensitive phosphatidylinositol 4,5-bisphosphate, but phosphatidylinositol 3-phosphate, a novel phosphoinositide of unknown function (Whitman *et al.* 1988),

Despite its unknown function, one observation strongly suggests that this stimulation of the synthesis of phosphatidylinositol 3-phosphate has some essential role in one of the signalling pathways that can lead to cell proliferation. This is the fact that certain mutants of the platelet-derived growth factor receptor retain their ability to mediate PDGF-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate and phosphorylation of proteins on tyrosine, but fail to effectively activate the type I kinase or drive cells through the cell cycle. These mutants lack the 'kinase insert' sequence that normally divides the tyrosine kinase domain of this protein into two segments, and which therefore seems to play some key role in the regulation of phosphatidylinositol 3-kinase (Escobedo & Williams 1988; Coughlin *et al.* 1989).

One isoenzyme of phosphoinositidase C is a member of a family of proteins that have roles in growth regulation and share a (regulatory?) peptide domain

Activation of phosphoinositidase C, the enzyme responsible for receptor-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis, is usually mediated by an as yet undefined G protein (for example, Cockcroft & Stutchfield 1988) that can couple to one or more members of the ever-expanding family of seven-span receptors (Caron 1989): proven or likely members of this group that can be mitogenic in appropriate circumstances include receptors for vasopressin, bombesin (Rozengurt 1986) and angiotensin (the *mas* oncogene: Jackson *et al.* 1988).

However, phosphoinositidase C activity can also be switched on in appropriate cells by agents such as PDGF and epidermal growth factor, whose receptors are quite different in structure from the seven-span family, being single-span transmembrane proteins in which the cytoplasmic domain includes a growth factor-sensitive tyrosine kinase. Many possible mechanisms can be envisaged by which a tyrosine kinase might 'talk' to a G-protein-coupled enzyme, but a recent observation by Wahl *et al.* (1988, 1989) offers a possibility that could also account, in part at least, for the phosphoinositidase C heterogeneity that has recently been revealed by cloning studies. This is the EGF receptor-catalysed tyrosine phosphorylation of the phosphoinositidase C known as PLC-γ (or PLC-148), but not of PLC-β or PLC-δ, two of the other three widespread phosphoinositidase C isozymes that have been cloned to date (Stahl *et al.* 1988; Bristol *et al.* 1989; Katan *et al.* 1989; Suh *et al.* 1988; reviewed in Rhee *et al.* (1989)).

This possible regulatory mechanism may arise from one of the most intriguing themes to emerge in this field recently. It has been known for several years that three particular amino acid sequence motifs (termed A, B and C) are relatively highly conserved between $p60^{src}$ and

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many other oncogene-encoded tyrosine kinases but are unnecessary for enzyme activity. These also appear, in a rearranged order, in PLC-γ (Stahl *et al.* 1988; Bristol *et al.* 1989) and in the $p47^{gag\text{-}crk}$ product of the *crk* oncogene, which is not a tyrosine kinase (Mayer *et al.* 1988, 1989). Evidence from studies of the *fps* tyrosine kinase and from the *src* and *crk* proteins (summarized in Bristol *et al.* (1989)) is consistent with the notion that tyrosine phosphorylation within this putative modulatory domain might be an important regulator of the activity of these proteins. It will be intriguing to see whether phosphatidylinositol 3-kinase will turn out to be another member of this family

The role of $p21^{ras}$ proteins in regulation of phosphoinositidase C

The $p21^{ras}$ proteins encoded by *ras* oncogenes and proto-oncogenes are GTP-binding proteins with GTPase activity, smaller than the G-proteins that couple receptors to effector systems, but homologous in sequence. Mutant RAS proteins in which the low GTPase activity characteristic of normal cellular $p21^{ras}$ is essentially abolished are frequently found in human tumours, and these mutant proteins also transform cultured cells. For biological activity, these proteins must localize to the plasma membrane as a result of fatty acylation. Given their plasma membrane localization and partial resemblance to the G-proteins that couple receptors to effector systems such as adenylate cyclase and phosphoinositidase C, an obvious hypothesis has been that $p21^{ras}$ can couple cell-surface receptors to some signal pathway capable of driving cell proliferation: phosphoinositidase C activation is an obvious candidate (Michell 1984). However, the evidence that has accumulated on this point is very mixed (Hall *et al.* 1989; Downward & Weinberg, 1989). On the one hand, at least some of the evidence that initially pointed to a role for $p21^{ras}$ in the coupling of certain receptors to phosphoinositidase C (Wakelam *et al.* 1986; Chiarugi *et al.* 1986; Parries *et al.* 1987) seems to have been dependent on an unexpected *ras*-induced increase in receptor numbers in the transfected cells (Downward & Weinberg 1989). On the other hand, there still seems to be a real tendency for cells that express a transforming mutant $p21^{ras}$ to show an enhanced basal turnover of inositol lipids in the absence of any overt receptor-directed stimulus of phosphoinositidase C (Fleischman *et al.* 1986; Wolfman & Macara 1987; Hall *et al.* 1989). In particular, Wakelam (1989) has shown that *ras* transfection leads to an enhanced yield of inositol phosphates when fluoroaluminate is used to directly activate the G-protein that controls phosphoinositidase C: this seems likely to involve some action of *ras* at an early post-receptor step in the signalling sequence.

An intriguing new twist to this story has been provided by the discovery of GTPase-activating protein (GAP), a cytosolic protein that shows the expected characteristics of an intracellular target of *ras* action. This 120 (kilodalton) kDa protein specifically interacts with both normal and transforming variants of $p21^{ras}$ but it only activates a GTPase in the normal version of $p21^{ras}$ (and its yeast homologue RAS1): transforming variants show no GTPase activity even when associated with GAP, and certain biologically disabled $p21^{ras}$ variants fail to interact with GAP (McCormick *et al.* 1989; Hall *et al.* 1989; Sigal *et al.* 1989). The cloning of GAP has revealed that it possesses the B and C elements of the *src*-like putative regulatory domain that is a potential site of tyrosine phosphorylation: it is yet another member of the growing family of proteins that are both intimately involved in cellular growth control and share this structural motif (Vogel *et al.* 1988; Rhee *et al.* 1989).

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Signalling by human interleukin-4

A novel sequence of second messenger changes is seen in human B cells stimulated by recombinant human interleukin-4 (rhIL-4). In these cells, rhIL-4 provokes growth (O'Garra *et al.* 1988), and a relatively early event in this growth stimulation is the expression of the glycoprotein CD23. This is a cell-surface component that serves both as a low-affinity cell-surface receptor for immunoglobulin E and, after release by proteolytic cleavage at the external surface of the plasma membrane, as a low molecular mass B-cell growth and differentiation factor (Gordon *et al.* 1989; Delespesse *et al.* 1989). Expression of CD23 is a response that can serve as a convenient test of the efficacy of added agents to mimic the actions of IL-4.

In a collaborative study involving three laboratories in Birmingham, Paris and London, it has been observed that rhIL-4 evokes the following train of responses in quiescent human tonsil B cells (M. Finney, G. R. Guy, R. H. Michell, J. Gordon, B. Dugas, K. P. Rigley & R. E. Callard, unpublished data). Within seconds of the addition of rhIL-4, there is a rapid accumulation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) that is accompanied by a simultaneous rise in cytoplasmic Ca^{2+} concentration. After a peak at about 15 s, these responses decline to basal values by about 60 s. There is then a substantial rise in the concentration of cyclic AMP (to 1.8–2.5 times the basal value), which starts 5–10 min after the addition of rhIL-4 and is maintained for at least 20 min. Induction of the expression of CD23 occurs during the following few hours. Remarkably, the stimulatory actions of murine IL-4 on mouse B cells appear not to involve this signalling pathway.

That the above sequence of changes in intracellular messenger levels is essential to the actions of rhIL-4 has been confirmed by two types of observation. First, the induction of CD23 triggered by rhIL-4 can be prevented if the transient Ca^{2+} signal triggered by IL-4 is suppressed by the use of an intracellular Ca^{2+} buffer such as indo-1. Secondly, when the observed messenger changes are mimicked by added pharmacological agents, CD23 is again induced. The effective treatment involved briefly treating cells with phorbol dibutyrate (to activate protein kinase C) and ionomycin (to mimic the Ins(1,4,5)P_3-triggered Ca^{2+} transient), then washing these agents away and incubating the cells with dibutyryl cyclic AMP: application of the same agents in the reverse order was ineffective. However, despite the successful induction of CD23 by this sequence of treatments, the same exposure of B cells to phorbol dibutyrate and ionomycin failed to provoke a delayed rise in cyclic AMP similar to that triggered by rhIL-4. It therefore seems that there must be at least one more element, still to be identified, in the hIL-4 signalling cascade: this might be either another output signal from the phosphatidylinositol 4,5-bisphosphate hydrolysis system or an independent and parallel signal pathway that links the hIL-4 receptor to cyclic AMP accumulation.

Signalling through the antigen receptors of mature and immature lymphocytes

The antigen receptors of B cells and of T cells are clonally distributed variants of membrane immunoglobulins and of the alpha (α) and beta (β) T-cell receptor (TcR) chains of the CD3–TcRαβ complex, respectively. The first indication that these might transmit their signals into cells through the widespread inositol lipid signal-transducing pathway came from Fisher & Mueller (1968, 1971) who showed that phytohaemagglutinin, a plant lectin that is a

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polyclonal mitogen for lymphocytes, provokes a very rapid phosphatidylinositol turnover in human blood lymphocytes. The speed of the response suggested that it was a direct consequence of phytohaemagglutinin's interaction with the cells. Later studies showed that this response probably occurs both in B cells and in T cells (Maino *et al.* 1975); that it is accompanied by an elevation of cytosolic Ca^{2+} concentration in the stimulated cells (Crumpton *et al.* 1975; Greene *et al.* 1976), but that elevation of the cytosolic Ca^{2+} concentration is not an intermediate step in the triggering of phytohaemagglutinin-stimulated phosphatidylinositol turnover (Allan & Michell 1977). These shared characteristics revealed an unexpected similarity between the responses of quiescent lymphocytes to agents that brought them into the cell cycle and the responses of diverse other cells to various hormones and neurotransmitters, so suggesting that receptor-stimulated inositol lipid hydrolysis might be an essential step in lymphocyte activation (Michell 1975; Allan & Michell 1977). In recent years, this view has been confirmed: reviews of the central role of antigen receptor-triggered phosphatidylinositol 4,5-bisphosphate hydrolysis in the rousing of quiescent B cells and T cells can be found in Moller (1987), Klaus *et al.* (1987), Harnett & Klaus (1988) and Guy *et al.* (1989).

Work on T-cell development in the thymus has recently revealed a second function of the CD3–TcRαβ antigen receptor complex. Soon after T-cell progenitors carrying the original germ line genes for the receptor α and β chains populate the foetal thymus, the cells multiply and the genes are rearranged to produce the polyclonal pattern of receptors characteristic of functional mature T cells. At this stage it is inevitable that a proportion of the randomly rearranged genes will encode αβ combinations that recognize self antigens, and the cells bearing these must be eliminated before expression of mature immune responses (von Boehmer *et al.* 1989). This elimination of anti-self cells appears to require the normal recognition of antigens by the CD3–TcRαβ receptor complexes of the immature T cells in the thymic medulla. However, in this unusual prenatal situation in which the only antigens available to be recognized are components of self, activation of the receptors triggers the apoptotic destruction of the reactive immature cells (Smith *et al.* 1989; Owen *et al.* 1989), a process that involves hydrolysis of genomic DNA into nucleosomal fragments in the manner first described by Wyllie (1980), rather than the clonal expansion characteristic of adult cells.

We have therefore initiated experiments, in collaboration with Owen *et al.* (this symposium) to determine whether the antigen-induced 'suicide' of immature T cells occurs: (i) because the CD3–TcRαβ antigen receptor complex of these cells substitutes a different transmembrane signalling system for the phosphatidylinositol 4,5-bisphosphate hydrolysis that is employed by the CD3–TcRαβ receptors of mature T cells, or (ii) because the same output signals from phosphatidylinositol 4,5-bisphosphate hydrolysis can somehow trigger cell proliferation in mature T cells but initiate apoptosis in immature T cells. Our initial experiments have employed cultured foetal mouse thymi in which the addition of anti-CD3 antibodies provokes the elimination by apoptosis of approximately half of the immature T cell population and proliferation of very few cells (Smith *et al.* 1989; Owen *et al.* this symposium). In these circumstances, two different anti-CD3 monoclonal antibodies that are capable of triggering apoptosis also stimulate an obvious accumulation of lipid-derived inositol phosphates (assayed as the inositol mono- and bisphosphates that accumulate at a Li^+ block: see figure 1). This response is easily detected within 1 h, which is much earlier than the hydrolysis of DNA into nucleosomal fragments becomes apparent. Preliminary studies suggest that a much smaller, but similar, response is probably provoked by staphylococcal enterotoxin B, which serves as an oligoclonal antigen that is recognized only by a much smaller subset of cells whose

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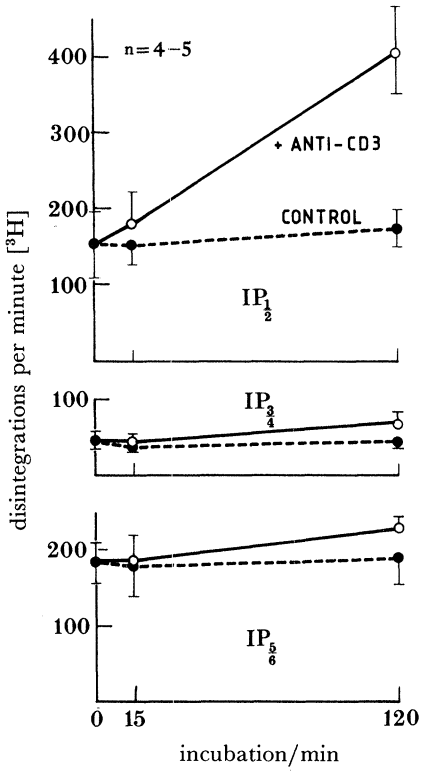

FIGURE 1. Anti-CD3 antibody stimulates inositol mono- or bisphosphate accumulation in immature mouse thymic lobes. 14-day foetal thymi from CBA mice were cultured for seven days with [^{3}H]inositol to label all inositol-containing components of the proliferating immature T cells to isotopic equilibrium. The apoptosis of CD3–TcRαβ-bearing cells was initiated by the addition of anti-CD3 monoclonal antibodies to thymi incubated in the presence of 10 mM LiCl: the response shown could be produced by either a hamster anti-mouse CD3 antibody or a rat anti-mouse CD3 IgG2b monoclonal, but not by a rat anti-mouse CD4 IgG2b monoclonal. Inositol phosphates were extracted in acid medium and separated on small Dowex 1 anion-exchange columns. Given the relatively long periods of stimulation, it is to be expected that the inositol phosphates generated in a sustained manner during stimulation would be trapped in the presence of 10 mM LiCl at the level of inositol monophosphates and bisphosphates (Ins$P_{\frac{1}{2}}$), whereas inositol pentakis- and hexakisphosphates (Ins$P_{\frac{5}{6}}$) should provide a relatively stable reference signal consisting mostly of slowly metabolized inositol polyphosphates.

CD3–TcRαβ receptor complexes have β chains of the Vβ8 group (Fleischer & Schrezenmeier 1988; White *et al.* 1989).

So far, we have not directly demonstrated the rapid phosphatidylinositol 4,5-bisphosphate hydrolysis which we presume is the initiating reaction that leads to this harvest of inositol phosphates, mainly because the rapidly growing cell population in the cultured thymic lobes only tolerates a low level of labelling with [^{3}H]inositol. However, if it is assumed that this is indeed the initiating reaction, then it must transmit the usual inositol 1,4,5-trisphosphate/Ca^{2+} and 1,2-diacylglycerol/protein kinase C signals to the cell interior. Smith *et al.* (1989) have shown that elevation of cytoplasmic [Ca^{2+}] in the immature thymic T cells with an ionophore is alone capable of mimicking the anti-CD3-induced apoptotic response, and that activation of protein kinase C by phorbol esters does not produce this response. As the Ca^{2+} signal is downstream of phosphatidylinositol 4,5-bisphosphate in the normal signalling cascade, it was to be expected that exposure to Ca^{2+} and ionomycin should fail to mimic the inositol lipid hydrolysis provoked by anti-CD3, and this was the result observed.

These results provide an initial indication that the same phosphatidylinositol 4,5-

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bisphosphate-derived intracellular signals are probably generated by activation of the CD3–TcRαβ receptor complex in both immature and mature T cells, with the subsequent choice between apoptosis of immature T cells and proliferation of mature T cells dictated either by the immediate downstream targets of a rise in cytosolic [Ca^{2+}] or a divergence in the signalling cascade at an even later stage. The latter possibility is favoured by the ability of cyclosporin A to block the TcR-mediated responses of both immature and mature T cells, probably at a site downstream of the initial receptor-generated second messengers (Takeuchi *et al.* 1988; Gao *et al.* 1988).

Inositol lipids and phosphates in differentiation

Inositol lipids and phosphates not only play a key role in triggering short-term cell responses and cell proliferation, but also in some way help to regulate processes involved in the commitment of cells to particular pathways of differentiation. The best illustration of this is the ability of injected Li^+ to redirect the developmental fate of certain cells in the *Xenopus* embryo. This effect appears to be caused by an intracellular sequestration of cellular inositol supplies as inositol phosphates: this is a result of Li^+ inhibition of inositol monophosphate phosphatase and 1,4-bisphosphate/1,3,4-trisphosphate 1-phosphatase. This teratogenic effect of Li^+ can be overcome either by the co-injection of inositol or by administration of phorbol myristate acetate (PMA), which is assumed to substitute as a protein kinase C stimulus in the place of a 1,2-diacylglycerol signal that fails to be generated in a normal manner in cells that have been made functionally inositol-depleted (Busa & Gimlich 1989).

Inositol phosphates in myeloid cell differentiation

Multipotent haemopoietic stem cells give rise to several different cell lineages by a relatively ill-understood process of lineage commitment that is only partially reproducible in isolated cell lines. One interpretation of this process is that the lineage commitment of these cells is a random process, with any individual multipotent cell by chance becoming restricted to differentiation along only one of several initially available pathways (Ogawa 1983). An alternative view, which was recently developed, partly to account for the restricted range of lineages open to variant 'haemopoietic' cell lines such as HL60 human promyelocytic leukaemia cells (Birnie 1988), is that individual stem cells pass along some sort of developmental progression, and that at each stage in this sequence they possess the potential to differentiate only towards a limited subset of lineages (Brown *et al.* 1985, 1987). Evidence in favour of the latter view is of two types. First, a linear series of relations can be inferred between different haemopoietic progenitor cells, both from the particular pairings of developmental fates that are available to different bipotent progenitor cells and from the cell behaviours seen in myelodysplastic and myeloproliferative disorders (Brown *et al.* 1985). Secondly, it can be argued, both from studies of lymphocyte behaviour and from other developmental studies (for example, of the nervous system, intestinal cells, leech embryos and slime moulds), that choices during cell diversification arise in a preprogrammed manner (Brown *et al.* 1988).

One of our aims has been to discover what roles, if any, are played by inositol phosphates in these developmental decisions. In particular, we have little understanding of the biological functions of many of the more highly phosphorylated inositol derivatives that are found in mammalian and other eukaryote cells, and we have focussed much of our attention on these.

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Among these are several compounds, notably inositol 3,4,5,6-tetrakisphosphate, inositol 1,3,4,5,6-pentakisphosphate and inositol hexakisphosphate, that are present in relatively high concentrations, turn over relatively slowly and are probably formed by routes that do not involve receptor-stimulated inositol lipid hydrolysis (Heslop *et al.* 1985; Szwergold *et al.* 1987; Vallejo *et al.* 1987; Stephens *et al.* 1988; Irvine *et al.* 1988). The function(s) of these materials remain unknown, but one obvious possibility is that they might play some role in longer term regulation of cell behaviours such as those involved in either the commitment of cells to differentiation or the expression of differentiated characteristics. Two earlier studies have suggested that induction of HL60 cell differentiation towards either neutrophils or macrophages involves a decrease in throughput of signals along the inositol lipid signalling pathway. In the study by Porfiri *et al.* (1988), each cell tended to contain progressively smaller concentrations of inositol mono-, bis-, tris- and tetrakisphosphates as the cells ceased division during the first two days after addition of dimethylsulphoxide to induce neutrophil differentiation: the level of diacylglycerol also decreased during this period. The less detailed study by Geny *et al.* (1988) suggested a much more rapid inhibition of the steady-state rate of phosphoinositidase C-mediated inositol lipid hydrolysis when HL60 cells cease division in response to PMA, an inducer of macrophage differentiation. Although partly similar results had been obtained in an earlier study on Friend erythroleukaemia cells (Faletto *et al.* 1985), the different behaviour of these cells (which are induced to differentiate by dimethylsulphoxide and prevented from differentiating by PMA), leaves open interesting questions about their interpretation. The changes that were seen in a previous study from this laboratory of the neutrophil differentiation of HL60 cells were much more complex, and included large changes in the levels of some of the most abundant and highly phosphorylated inositol compounds, including inositol 3,4,5,6-tetrakisphosphate (the predominant isomer in these cells), pentakisphosphate and hexakisphosphate (Michell *et al.* 1988).

It has long been known that neutrophils display enhanced inositol phospholipid turnover both during phagocytosis (Karnovsky & Wallach 1961; Sastry & Hokin 1966) and in response to stimuli that induce chemotaxis, secretion and the generation of oxygen radicals (Cockcroft *et al.* 1981; Cockcroft & Stutchfield 1988). These receptor-mediated responses are also induced effectively in HL60 cells: undifferentiated HL60 cells display a typical generation of inositol phosphates and resulting Ca^{2+} elevation in response to ATP, and cells that have been differentiated toward neutrophils in the presence of DMSO both retain this response and acquire responsiveness to the chemoattractant peptide *f*Met-Leu-Phe. Pittet *et al.* (1989) recently presented a very detailed analysis of the relationships, in *f*Met-Leu-Phe-stimulated HL60 'neutrophils', between the changing cellular levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and the regulation of cytosolic Ca^{2+} homeostasis.

To examine in detail the changes in inositol phosphate levels that accompany differentiation and stimulation, we labelled HL60 cells to equilibrium with 1 mg l^{-1} ^{3}H-labelled inositol, the lowest concentration compatible with sustained growth at the normal rate, in a serum-free medium containing insulin, transferrin and selenium: HL60 cells make little or no inositol from glucose, so they must use exogenous labelled inositol to synthesize all inositol-containing constituents. The labelled cells were then induced to differentiate towards neutrophils by 0.9% (by volume) dimethylsulphoxide (DMSO) or towards macrophages by 10 nM phorbol myristate acetate (PMA), in the continued presence of the growth medium containing labelled inositol. To confirm that normal differentiation patterns were maintained in this serum-free

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environment, we monitored cell numbers, phagocytic index, development of the ability to elevate cytosolic Ca^{2+} in response to *f*met-leu-phe, and unspecific acid esterase, with satisfactory results (not shown). To maximize the resolution of the complex inositol phosphate complement of the cells, we used an Hplc method capable of resolving an extended spectrum of isomeric inositol phosphates, including inositol tetrakis-, pentakis- and hexakisphosphates. The typical chromatogram shown in figure 2 provokes two immediate conclusions. First, acid extracts of growing HL60 cells contain a very large number of inositol phosphates, to which we are now attempting to assign definitive identities: suggested identities mentioned herein are tentative, and based purely on chromatographic comparisons with known compounds. Secondly, inositol tetrakisphosphate (intracellular concentration approximately 4 μM), pentakisphosphate (approximately 20 μM) and hexakisphosphate (approximately 50 μM) were the most abundant inositol polyphosphates in these cells, being present at far higher concentrations than the inositol 1,4,5-trisphosphate (approximately 0.2 μM) that is assumed to be responsible for regulating cytosolic Ca^{2+} level (French *et al.* (1988)).

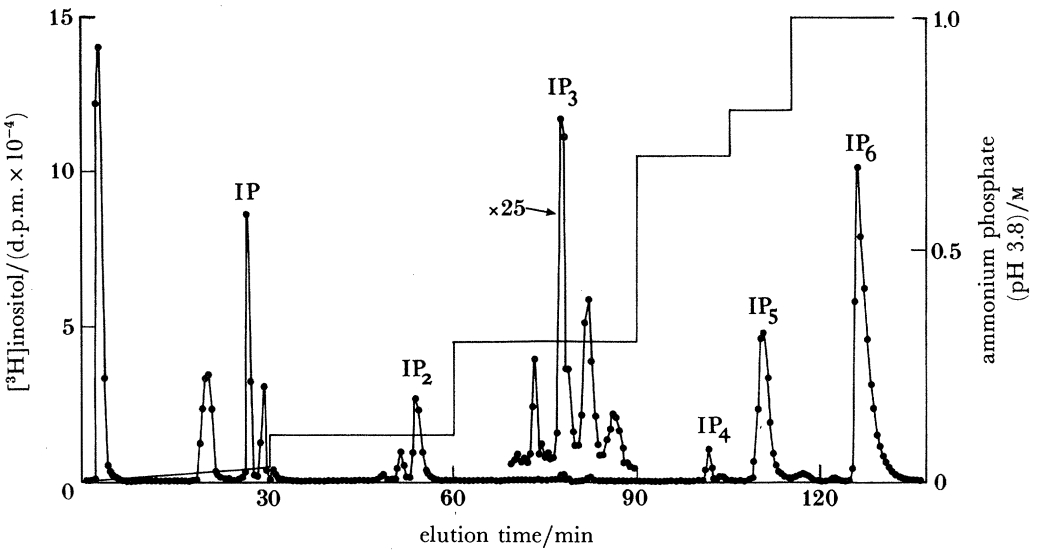

FIGURE 2. High performance liquid chromatographic separation of the inositol phosphates of equilibrium-labelled HL60 promyelocytic cells on a Partisil 10SAX column eluted with the illustrated ammonium phosphate initial gradient and subsequent steps at pH 3.8. Major groups of peaks are identified as, for example, inositol monophosphates (InsP) and inositol trisphosphates (IP_3, also shown expanded 25-fold for clarity): although we have tentatively assigned isomeric identities to many of the peaks (Michell *et al.* 1988; French *et al.* 1988), these have not yet been rigorously checked by chemical degradation.

When considering the changes occurring during differentiation (figure 3), it should be noted that dimethylsulphoxide-induced differentiation of HL60 cells towards neutrophils involves a progressive decrease in cell volume, particularly after 1–2 days as the cells cease dividing (figure 3*b*, open symbols): to maintain a stable intracellular concentration of any inositol phosphate during this process, the total quantity present would therefore have to approximately halve. A number of inositol phosphates tended to show this pattern: these include inositol 1,4-bisphosphate, inositol 3,4-bisphosphate and inositol 1,3,4-trisphosphate (data not shown), and inositol 3,4,5,6-tetrakisphosphate and hexakisphosphate (figure 3*a*, *c*). In marked contrast, the total inositol pentakisphosphate content of cells was maintained over the first two days during which the cells ceased dividing and decreased in size (that is, the intracellular concentration must have arisen substantially), and there was then net synthesis to further increase its level

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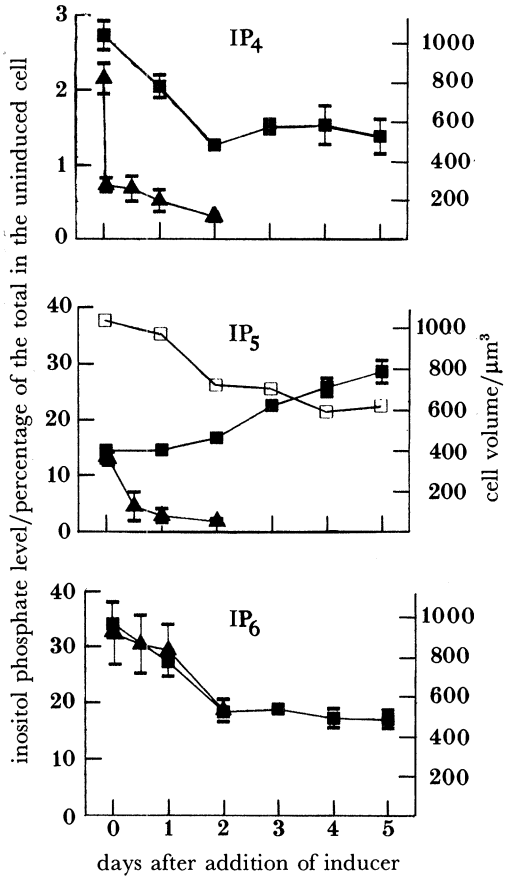

FIGURE 3. Changes in the inositol tetrakisphosphate (IP_4), inositol pentakisphosphate (IP_5) and inositol hexakisphosphate (IP_6) levels (■) and the cell volumes (□) of HL60 cells differentiating towards neutrophils in 0.9% (v/v) dimethylsulphoxide and in the inositol polyphosphate levels of cells differentiating towards macrophages in the presence of 10 nM TPA (▲). Results for each inositol phosphate are expressed per cell, as a ratio related to the total radioactivity in all inositol phosphates in growing cells at the time of addition of the inducer of differentiation: most data are mean values derived from analysis of triplicate cell samples in each of four independent experiments. Note that cessation of growth is immediate in the presence of TPA and the cells are fully differentiated within two days, whereas cells continue to grow for 1–3 days after dimethylsulphoxide addition (increasing in number about fivefold) and acquire neutrophil characteristics progressively from about two days onwards.

over the following days during which the cells acquired differentiated characteristics (figure 3*b*).

Upon addition of PMA to induce differentiation towards macrophages, HL60 cells stop dividing almost immediately, and their cell volume probably does not change markedly. Accompanying this, there is a rapid decline, within 1 h, in the levels of several inositol phosphates, including the 1,3-, 1,4- and 3,4- isomers of inositol bisphosphate (not shown) and inositol 3,4,5,6-tetrakisphosphate (figure 3*a*). Other compounds, such as the 1,4,5- and 1,3,4- isomers of inositol trisphosphate, showed no obvious change (not shown). Like inositol 3,4,5,6-tetrakisphosphate, inositol pentakisphosphate was substantially depleted, though more slowly (figure 3*b*): this was in sharp contrast with its accumulation in dimethysulphoxide-differentiated cells. Inositol hexakisphosphate initially showed little change, but declined substantially during the second day of exposure to PMA (figure 3*c*).

What are we to make of these large changes in the levels of the most abundant inositol

phosphates? At present, we do not know whether these patterns of change in inositol phosphate levels represent metabolic behaviours essential to differentiation along the neutrophil and macrophage pathways or simply responses to the perturbation of cells by exogenous chemical agents. This question should be resolved by two types of experiment: (i) employing other agents to induce the two pathways of differentiation; (ii) comparing the inositol phosphate responses, to differentiation inducers, of normal cells and of variant cell lines that fail to differentiate in response to these agents. At best, we may have identified functional changes in inositol phosphate levels that are essential in driving cells towards their differentiated fates. If this is the case, these studies will open a path to identification of the cellular functions of these unexpectedly abundant intracellular polyphosphates. At worst, we may simply have identified foreign compounds that perturb the synthesis and degradation of inositol polyphosphates. This could be of considerable value, given the current dearth of information both on the pathways of synthesis and degradation of these compounds, into which cells put an unexpected metabolic investment, and on the regulation of these reactions.

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