The EMBO Journal vol.12 no.7 pp.2681 – 2690, 1993

Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and *src*-related tyrosine kinase(s) in human myeloid derived cells

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Communicated by R.H.Michell

The signalling pathways used by the GM-CSF receptor are currently unknown. Here we show that in human myeloid derived cells GM-CSF can stimulate; (i) the accumulation of PtdIns(3,4,5)P_3; (ii) increases in p53/p56^{lyn} and p62$^{c\text{-}yes}$ directed protein tyrosine kinase activities in anti-*lyn* and anti-c-*yes* antibody directed immunoprecipitates, respectively and; (iii) increases in phosphoinositide 3OH-kinase activity in anti-phosphotyrosine, anti-p53/p56^{lyn} and anti-p62$^{c\text{-}yes}$ antibody directed immunoprecipitates. These results suggest that GM-CSF can stimulate formation of protein tyrosine kinase co-ordinated signalling complexes, that contain p53/p56^{lyn}, p62$^{c\text{-}yes}$ and an activated PtdInsP_2 directed phosphoinositide 3OH-kinase, which can drive the accumulation of the putative second-messenger PtdIns(3,4,5)P_3.

Key words: GM-CSF/neutrophils/PtdIns 3-kinase/*lyn*, *yes*/PtdIns(3,4,5)P_3

Introduction

The cytokine GM-CSF (granulocyte macrophage-colony stimulating factor) has a variety of actions on myeloid derived cells; it has been shown to stimulate mitogenesis, differentiation, increased cell viability, increased synthesis/release of other extracellular mediators and priming/activation of mature cell function (reviewed in Gasson, 1991; Rapoport, 1992). The basic lack of understanding of how many of these responses are controlled and, more subtly, the fact that some of them involve effects on the signalling pathways of other ligands (e.g. directly, in the process of priming, or indirectly, by release of other ligands) has made it difficult to define clearly those signals that are primary responses to GM-CSF (Weisbart *et al.*, 1985, 1987; Corey and Rosoff, 1989; Wirthmueller *et al.*, 1989). Consequently, despite its biological and clinical significance, GM-CSF's signalling pathways are unknown.

The genes encoding the human GM-CSF receptor have recently been cloned so that the heterodimeric complex is now known to consist of an 85 kDa ligand binding subunit (α, showing low affinity for GM-CSF; Di Persio, 1988) and a 120 kDa presumptive signal transducing subunit (β or KH97, the presence of which increases the affinity of the receptor for GM-CSF, Gorman *et al.*, 1990; Hayashida *et al.*, 1990). These polypeptides show sequence homology with other cytokine receptors including those for G-CSF (granulocyte-colony stimulating factor), interleukin 2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, growth hormone, prolactin, erythropoietin and CNTF (ciliary neurotrophic factor; Davis *et al.*, 1991; Hunter, 1991; Rapoport *et al.*, 1992; Renauld *et al.*, 1992). An unusual feature of this superfamily appears to be the sharing of subunits by receptors to different ligands; thus the β subunit of the GM-CSF receptor is also used in the IL-3 and IL-5 receptor complexes (see Kitamura *et al.*, 1991; Nicola and Metcalf, 1991). The primary structures of this family of receptors do not contain domains with known signalling functions (e.g. seven-transmembrane-segment domains characteristic of activators of heterotrimeric G-proteins or domains known to encode intrinsic protein tyrosine kinases) but, several lines of evidence suggest that they may use receptor-associated protein tyrosine kinases (PTKs) to transduce their signals. Thus, all of these ligands, including GM-CSF (e.g. Gomez-Cambronero *et al.*, 1989; Kanakura *et al.*, 1990; Berkow, 1992) have been shown to induce rapid tyrosine phosphorylation in their target cells and one member of this family, the IL-2 receptor, has been shown to associate physically with and activate *src*-type PTKs (e.g. p56^{lck} in a T-cell line; Hatakeyama *et al.*, 1991). Further, a burgeoning and diversely structured group of receptors (e.g. mIgM, mIgE and CD3/TCR receptors) are now thought to transduce their signals via the activation of receptor-associated, often *src*-type, PTKs (e.g. Samelson *et al.*, 1990; Burkhardt *et al.*, 1991; Cooke *et al.*, 1991; Yamanashi *et al.*, 1991; Eiseman and Bolen, 1992; Thompson *et al.*, 1992; Tsygankov *et al.*, 1992). Despite the growing list of receptors with which the GM-CSF receptor can be compared, the diversity of effects they produce means that neither the identity of the particular receptor-associated PTKs, nor the set of second and third level messenger systems that might be regulated by GM-CSF, can currently be predicted on the basis of sequence homologies alone.

A large variety of receptors which utilize PTKs to transduce their signals can stimulate accumulation of PtdIns(3,4,5)P_3 (Cantley *et al.*, 1991). This event is not elicited by other known signalling pathways and has been postulated to be a critical intracellular signal (Cantley *et al.*, 1991; Downes and Carter, 1991; Stephens *et al.*, 1991). PtdIns(3,4,5)P_3 can be synthesized by an agonist-sensitive phosphoinositide 3OH-kinase (PI3K), which readily 3OH-phosphorylates PtdIns, PtdIns4P and PtdIns(4,5)P_2 *in vitro*, but appears to be PtdIns(4,5)P_2 directed *in vivo* (Auger *et al.*, 1989; Carpenter *et al.*, 1990; Stephens *et al.*, 1991; Hawkins *et al.*, 1992). This paper describes experiments which were designed to establish whether the GM-CSF

receptor can elicit a 'PtdIns(3,4,5)P_3 response' and the transduction reactions which might be implicated in activation of the PI3K responsible.

Results

GM-CSF stimulated PtdIns(3,4,5)P_3 accumulation in human neutrophils and U937 cells

Freshly isolated human neutrophils were labelled to steady-state with [^{32}P]P_i. The prelabelled cells were then challenged with various agonists and the levels of ^{32}P-labelled phospholipids determined. GM-CSF caused significant accumulation of [^{32}P]PtdIns(3,4,5)P_3 and [^{32}P]PtdIns(3,4)P_2 without a significant effect on [^{32}P]PtdIns3P, [^{32}P]PtdIns4P, [^{32}P]PtdIns(4,5)P_2 or [^{32}P]PtdOH levels (see Figure 1A). In contrast, tumour necrosis factor-α (TNF-α; 1000 U/ml), CSF-1 (colony stimulating factor-1/macrophage colony stimulating factor; 4000 U/ml), IGF-1 (50 ng/ml) and insulin (10 μg/ml) did not cause a significant accumulation of [^{32}P]PtdIns(3,4)P_2 or [^{32}P]PtdIns(3,4,5)P_3 (incubations with agonists were for 3 min; data not shown). The levels of PtdIns(3,4,5)P_3 attained after 3 min stimulation with GM-CSF were typically 20–40% of the amount of PtdIns(3,4,5)P_3 produced after 8 s challenge with a maximal dose of FMLP (e.g. see Figures 3 and 4).

GM-CSF produced a very similar 'PtdIns(3,4,5)P_3 response' (to that described in neutrophils above) in U937 cells (see Figure 1B), whereas TNF-α had no significant effect (data not shown). The accumulation of PtdIns(3,4,5)P_3 occurring in U937 cells in response to 2 min exposure

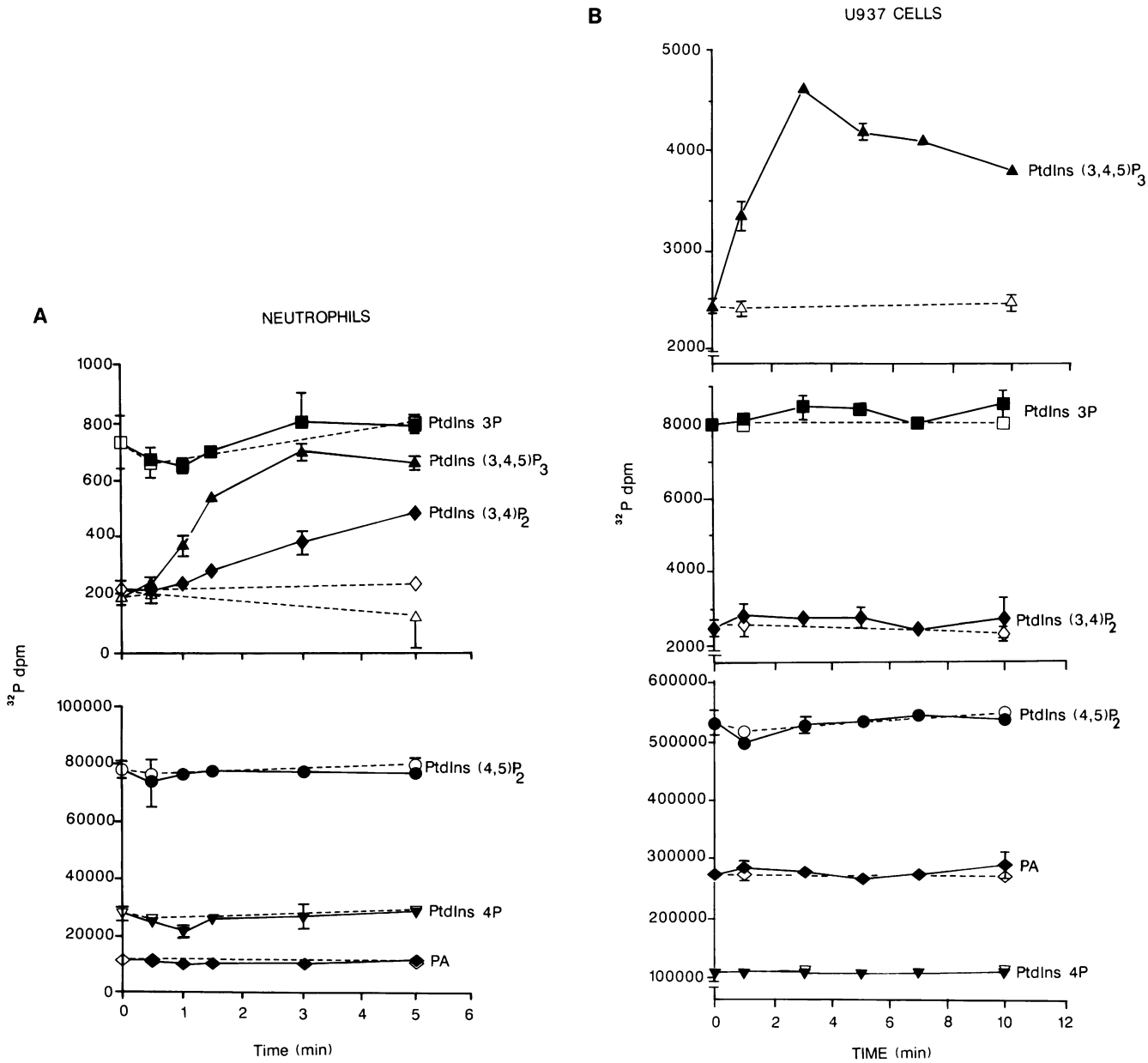

Fig. 1. The effects of GM-CSF on the levels of [^{32}P]-polyphosphoinositides and [^{32}P]PtdOH in human neutrophils and U937 cells. Human neutrophils (**A**) and U937 cells (**B**) were labelled to steady state with [^{32}P]Pi, washed and stimulated with a maximal effective dose of GM-CSF (final concentration of 100 ng/ml; solid lines) or vehicle alone (dashed lines). After various times incubations were quenched, [^{32}P]polyphosphoinositides and [^{32}P]PtdOH were quantified as described in Materials and methods. Data shown are means ± SE (n = 3). Similar results were obtained in two further experiments.

to GM-CSF was dose dependent with a maximal response apparent at 60 ng/ml (which was unaltered at 500 ng/ml) and a 50% maximal effect at ~5–10 ng/ml (data not shown).

GM-CSF does not stimulate a phosphoinositidase C

The lack of effect of GM-CSF on PtdIns4P, PtdIns(4,5)P_2 and PtdOH levels indicates that GM-CSF has failed to activate phosphoinositidase C (PIC) in either neutrophils or U937 cells. In view of the potential significance of this observation (see Discussion) and the fact that the above data are not sensitive or unambiguous indicators of PIC activation, we have measured the rate of [^{3}H]inositol phosphate accumulation (as the most sensitive and reliable index of PIC activation available) in human neutrophils and U937 cells in response to GM-CSF (see Figure 2). These data confirm that GM-CSF, in contrast to FMLP or ATP, does not significantly stimulate PIC in neutrophils or U937 cells (Sullivan *et al.*, 1987; Corey and Rosoff, 1989).

Pharmacological sensitivity of GM-CSF stimulated PtdIns(3,4,5)P_3 accumulation

Pertussis toxin, an agent that can ADP-ribosylate and inhibit the actions of a specific subset of G-proteins in human neutrophils, blocked the ability of FMLP to stimulate PtdIns(3,4,5)P_3 accumulation (Traynor-Kaplan *et al.*, 1989; see Figure 3) but had no effect on the response elicited by GM-CSF in the same preparations of cells (Figure 3; similar results were obtained with U937 cells; data not shown). These results clearly suggest that the GM-CSF receptor in either U937 cells or human neutrophils is not coupled to PtdIns(3,4,5)P_3 accumulation via a pertussis toxin-sensitive G-protein.

Staurosporine, a potent, although unselective inhibitor of protein kinases and genistein, a reputedly more selective PTK inhibitor (Akiyama *et al.*, 1987), have been shown to block GM-CSF stimulated accumulation of tyrosine phosphorylated proteins in neutrophils (Berkow, 1992) and also IL-2 stimulated PTK activity and association of PI3K with anti-phosphotyrosine antibody directed immunoprecipitates in a T cell line (Merida *et al.*, 1992). Both reagents completely abolished GM-CSF stimulated PtdIns(3,4,5)P_3 accumulation in human neutrophils and staurosporine also abolished GM-CSF stimulated PtdIns(3,4,5)P_3 accumulation in U937 cells (see Figure 4). In contrast, neither reagent substantially affected the ability of FMLP to stimulate PtdIns(3,4,5)P_3 accumulation in neutrophils. At the concentrations at which genistein was used, it (i) reduced the levels of PtdIns(4,5)P_2 in intact neutrophils (Figure 4), and (ii) inhibited a neutrophil derived, PtdIns(4,5)P_2 directed PI3K activity by 28% (assayed *in vitro* with added phospholipids). These factors probably account for the fact that genistein reduced the absolute levels of PtdIns(3,4,5)P_3 in both control and stimulated neutrophils; it did not, however, significantly alter their responsiveness to FMLP (in terms of fold response above control). The positive internal control afforded by the FMLP response in these experiments shows that, under the conditions we have employed, the protein kinase inhibitors can effectively distinguish between the pathways connecting the FMLP and GM-CSF receptors to PI3K activity and suggest that a protein kinase(s) may be implicated in the mechanism by which GM-CSF activates PtdIns(3,4,5)P_3 production.

TPA has been reported to rapidly and specifically inhibit GM-CSF and IL-3 enhanced tyrosine phosphorylation of proteins (Kanakura *et al.*, 1991). The precedent set by similar effects of TPA on EGF and insulin receptors suggests there may be an inhibitory PKC target site in the GM-CSF receptor regulating its coupling to signalling systems (Kasuga *et al.*, 1982; Downward *et al.*, 1985; Sibley *et al.*, 1988). We find brief pretreatment of neutrophils with TPA (50 ng/ml for 3 min) can completely abolish GM-CSF but not FMLP stimulated PtdIns(3,4,5)P_3 accumulation [inhibition of PtdIns(3,4,5)P_3 accumulation in the presence of TPA was (mean ± range, n = 2): 101 ± 11% for a 3 min stimulation with 50 ng/ml GM-CSF and 29 ± 3% for 8 s stimulation with 1 μM FMLP]. Hence this result serves to consolidate both the notion that the GM-CSF receptor receives a functionally significant input from PKC and the relationship between GM-CSF stimulated tyrosine phosphorylation of proteins and activation of a PtdInsP_2 directed PI3K activity in myeloid derived cells.

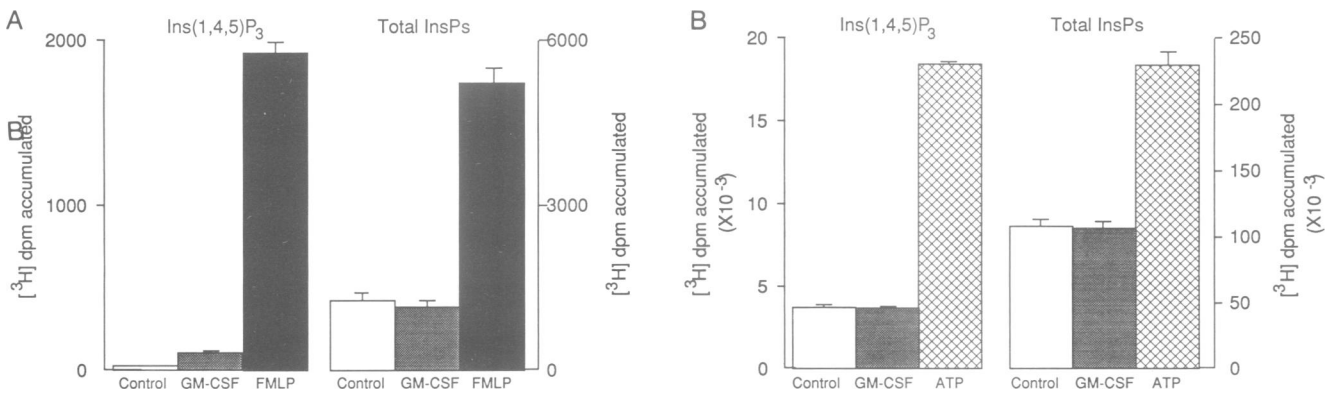

Fig. 2. GM-CSF does not activate phosphoinositidase C in either human neutrophils or U937 cells. (**A**) PIC activation in GM-CSF and FMLP stimulated human neutrophils. Human neutrophils were labelled with [^{3}H]Ins, then washed and challenged with GM-CSF (for 3 min with 100 ng/ml, final concentration), FMLP (for 8 s, 1 μM final concentration) or vehicle alone (3 min). (**B**) PIC activation in GM-CSF and ATP stimulated U937 cells. U937 cells were labelled with [^{3}H]Ins then washed and challenged with GM-CSF (for 3 min, 100 ng/ml final concentration), MgATP (for 15 s, 250 μM final concentration) or vehicle alone (3 min). Reactions were quenched and [^{3}H]inositol phosphates were quantified as described in Materials and methods. Data shown are mean d.p.m. accumulated (n = 2) (ranges are indicated by error bars) in Ins(1,4,5)P_3 and in a total inositol phosphate pool.

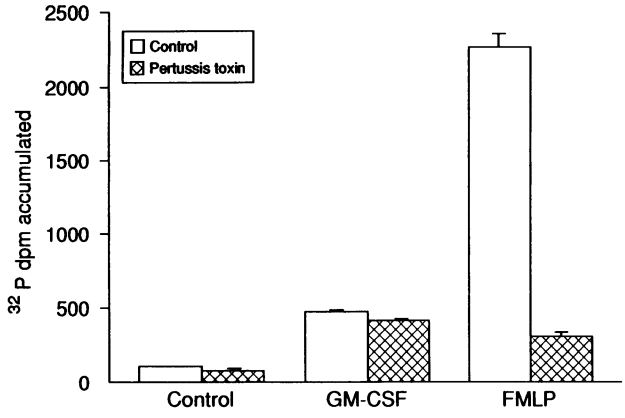

Fig. 3. Effect of pertussis toxin on GM-CSF stimulated [^{32}P]PtdIns(3,4,5)P_3 accumulation in human neutrophils. Human neutrophils were incubated with [^{32}P]Pi, in the presence or absence of pertussis toxin, washed and challenged with GM-CSF (50 ng/ml final concentration for 2.5 min) or FMLP (1 μM final concentration for 8s). The reactions were quenched and [^{32}P]lipids were quantified as described. Data presented are mean d.p.m. in PtdIns(3,4,5)P_3 (n = 2; the ranges of the individual samples are shown by error bars). The effects of pertussis toxin pretreatment on FMLP stimulated PtdIns(3,4,5)P_3 production were examined in four additional experiments, which yielded similar results. Pertussis toxin pretreatment had no significant effect on the amount of [^{32}P]PtdIns(4,5)P_2 in unstimulated neutrophils (data not shown).

The mechanism(s) by which GM-CSF activates PtdIns(3,4,5)P_3 accumulation

GM-CSF stimulates increases in PI3K activity in anti-phosphotyrosine antibody directed immunoprecipitates. The vast majority of agonists that have been demonstrated to stimulate 3-phosphorylated inositol lipid metabolism also stimulate increases in PI3K activity in anti-phosphotyrosine antibody directed immunoprecipitates (e.g. Carpenter and Cantley, 1990). Hence, although data from these assays is ambiguous (as indirect immunoprecipitation can occur; see Discussion) they represent an indicator of the involvement of tyrosine phosphorylation in receptor regulation of a PI3K activity.

GM-CSF stimulated an increase in PI3K activity in anti-phosphotyrosine antibody directed immunoprecipitates from lysates of both human neutrophils and U937 cells (see Figure 5). Furthermore, staurosporine completely abolished these effects of GM-CSF in neutrophils.

The identity of the PTK(s) activated by GM-CSF and IL-3. Although the primary structure of the GM-CSF receptor contains no evidence for an intrinsic PTK activity, the data we show above clearly suggests it can regulate a PI3K activity by a mechanism involving tyrosine phosphorylation. The signal transducing β subunit of the IL-2 receptor, which possesses strong homologies with the shared β chain of the IL-3 and GM-CSF receptors (Hunter, 1991; Rapoport *et al.*, 1992), has been shown to activate non-receptor, *src*-related PTKs (Hatakeyama *et al.*, 1991; Torigoe *et al.*, 1992), hence it is likely that the IL-3 and GM-CSF receptors will use non-receptor PTKs to transduce their signals. We have screened GM-CSF stimulated human neutrophils, TF-1 cells, AML-93 and M-O7e cells, and also IL-3 stimulated M-O7e and TF-1 cells, with a panel of antibodies developed against a number of *src*-type PTKs for evidence of ligand dependent activation of the PTKs present in immunoprecipitates prepared with each of the members of the panel of antisera.

Stimulation with GM-CSF resulted in increased recovery of a p53/p56^{lyn} targeted PTK activity that specifically associated with anti-p53/p56^{lyn} antibody directed immunoprecipitates from lysates of neutrophils, TF-1 cells, AML-193 cells and M-O7e cells; see Figure 6 and data not shown (similar data were obtained with IL-3 stimulated TF-1 and M-07e cells; data not shown). However, the quantity of p53/p56^{lyn} that could be detected in Western blots of anti-p53/p56^{lyn} antibody directed immunoprecipitates did not change after 1, 5 or 15 min stimulation with GM-CSF (data pooled from all times was a mean of 95 ± 15% of the unstimulated samples). Additionally, GM-CSF caused a similar increase in a p62$^{c\text{-}yes}$-targeted protein kinase activity associated with anti-p62$^{c\text{-}yes}$ antibody directed immunoprecipitates from human neutrophils (Figure 6). Antisera against the proteins encoded by *blk*, *fgr*, *fyn*, *lck*, *fes* and *hck* failed to immunoprecipitate analogous cytokine dependent protein kinase activity from lysates derived from any of the cells (data not shown). The characteristics of the phosphoproteins detected in these assays (Figure 6A), in the context of much previous work (e.g. Burkhardt *et al.*, 1991; Hatakeyama *et al.*, 1991; Yamanashi *et al.*, 1991; Eiseman and Bolen, 1992; Thompson *et al.*, 1992; Torigoe *et al.*, 1992), suggests that the GM-CSF stimulated recovery of PTK activity in these immunoprecipitates is a manifestation of an increased capacity of the relevant *src*-type PTK (i.e. p53/p56^{lyn} or p62$^{c\text{-}yes}$ in anti-p53/p56^{lyn} or anti-p62$^{c\text{-}yes}$ antibody directed immunoprecipitates, respectively) to autophosphorylate.

Association of a PI3K with anti-p53/p56^{lyn} and anti-p62$^{c\text{-}yes}$ antibody directed immunoprecipitates. PI3K activity can tightly associate with both cellular and transforming *src*-type PTKs *in vivo* and be tyrosine phosphorylated by them *in vitro* (e.g. Cantley *et al.*, 1991; Gout *et al.*, 1992). GM-CSF receptor stimulation caused increased association of an 85 kDa protein, likely to be the regulatory subunit of PI3K, with anti-p53/p56^{lyn} antibody directed immunoprecipitates from neutrophils (Figure 7A). We could also detect GM-CSF stimulated increases in PI3K activity in anti-p53/p56^{lyn} antibody directed immunoprecipitates from both neutrophils and TF-1 cells (see Figure 7B; IL-3 was also able to stimulate this response in TF-1 cells, data not shown). Additionally, GM-CSF was able to stimulate an increase in PI3K activity in anti-p62$^{c\text{-}yes}$ immunoprecipitates prepared from neutrophils (Figure 7B). This suggests that GM-CSF can cause translocation of PI3K into complexes containing p53/p56^{lyn} and p62$^{c\text{-}yes}$.

Discussion

The GM-CSF stimulated accumulation of PtdIns(3,4,5)P_3 in both U937 cells and neutrophils provides the best evidence so far for the identity of a second-messenger mediating some of GM-CSF's actions. Several features of this response suggest that, unlike many reported effects of GM-CSF on signalling reactions (e.g. Corey and Rossoff, 1989; McColl *et al.*, 1989), it is not generated by a release of secondary extracellular mediators, such as LTB_4 or PAF. Both LTB_4 and PAF activate neutrophils via a pertussis toxin-sensitive G-protein (Traynor-Kaplan *et al.*, 1989; data not shown) and stimulate accumulation of inositol phosphates in parallel with

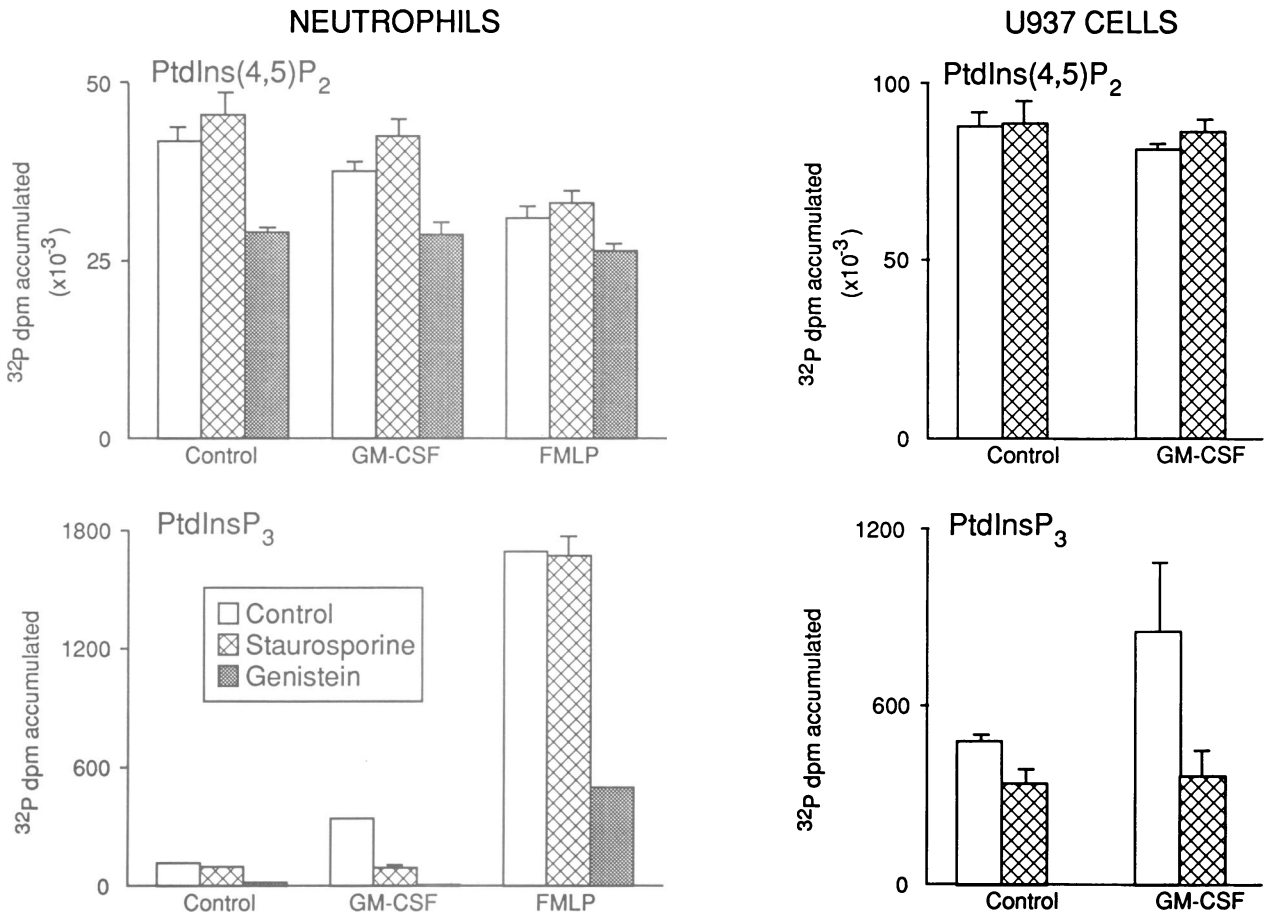

Fig. 4. Effects of staurosporine and genistein on FMLP and GM-CSF stimulated [^{32}P]PtdIns(3,4,5)P_3 and [^{32}P]PtdIns(4,5)P_2 accumulation in human neutrophils and U937 cells. Freshly isolated human neutrophils were labelled with [^{32}P]Pi, washed and incubated (37°C) with staurosporine (5 μM final concentration for 6 min in total) or genistein (400 μM final concentration for 6 min in total) or vehicle alone (20% v/v, DMSO in *N*-HBBSS, for 6 min in total). After 3 min with staurosporine, genistein or vehicle the cells were challenged with GM-CSF (100 ng/ml final concentration for 3 min) or, after 5 min 52 s, with FMLP (1 μM final concentration, for 8 s). U937 cells were labelled with [^{32}P]Pi, washed and preincubated (37°C) with staurosporine (5 μM final concentration, for 3 min) or vehicle (3 min) then stimulated with GM-CSF (100 ng/ml final concentration for 2 min) or vehicle alone (2 min). Incubations were quenched, and [^{32}P]phospholipids were quantified as described. Data presented are mean d.p.m. (n = 2) in PtdIns(3,4,5)P_3 (lower panels) or PtdIns(4,5)P_2 (upper panels), the ranges of the individual data points are indicated by error bars. The data for neutrophils incubated with or without staurosporine were reproduced in one further experiment for GM-CSF and three further experiments for FMLP.

PtdIns(3,4,5)P_3. Hence, the evidence that GM-CSF stimulates a pertussis toxin-insensitive accumulation of PtdIns(3,4,5)P_3, without a co-incident accumulation of inositol phosphates, suggests that the rise in PtdIns(3,4,5)P_3 is unlikely to be a secondary response. Moreover, this is supported by the relatively rapid kinetics of this rise in PtdIns(3,4,5)P_3, the only comparably rapid responses to GM-CSF are in tyrosine phosphorylation of proteins (e.g. Berkow, 1992).

In both GM-CSF stimulated neutrophils and U937 cells, PtdIns(3,4)P_2 accumulates significantly more slowly and to a lower extent than PtdIns(3,4,5)P_3 [indeed, in U937 cells the accumulation of PtdIns(3,4)P_2 is barely detectable]. Further, GM-CSF does not significantly affect PtdIns3P levels in either cell type. This pattern of changes in 3-phosphorylated inositol lipids is consistent with previous work that has shown that agonists can stimulate accumulation of PtdIns(3,4,5)P_3 by activating a PtdIns(4,5)P_2 directed PI3K activity and that PtdIns(3,4)P_2 accumulation is probably fueled by PtdIns(3,4,5)P_3 dephosphorylation (Auger *et al.*, 1989; Stephens *et al.*, 1991; Hawkins *et al.*, 1992). This PtdIns(4,5)P_2 directed PI3K activity is likely to be the 'phenotypic manifestation' in intact cells of the PI3K activity which has been extensively characterized *in vitro* (e.g. Carpenter *et al.*, 1990; Stephens *et al.*, 1991; Hiles *et al.*, 1992).

A number of pieces of evidence point to the involvement of PTKs, or more specifically to *src* family PTKs, in the mechanism by which the GM-CSF receptor stimulates PtdIns(3,4,5)P_3 accumulation. Hence, not only is this response blocked by PTK inhibitors but GM-CSF can stimulate staurosporine-sensitive increases in a PI3K activity in anti-phosphotyrosine antibody directed immunoprecipitates. Furthermore, the 'PtdIns(3,4,5)P_3 response' elicited by activation of the GM-CSF receptor develops significantly more slowly than the responses to stimulation of other receptors (e.g. Jackson *et al.*, 1992; Ruderman *et al.*, 1990; Traynor-Kaplan *et al.*, 1989), with the exceptions of the IL-2, CD3/TCR and CD2 receptors, which, although structurally diverse, are all thought to utilize *src* family PTKs to transduce their signals (Remillard *et al.*, 1991; Ward *et al.*, 1992). This circumstantial evidence is hardened by the findings that GM-CSF or IL-3 stimulation of a number of myeloid derived cells leads to an apparent increase in the

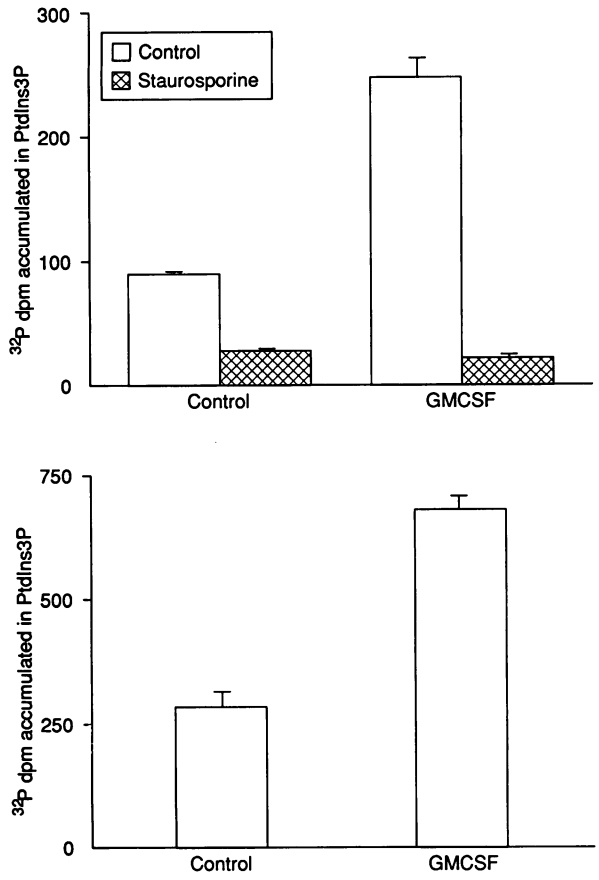

Fig. 5. GM-CSF stimulated appearance of PI3K activity in anti-phosphotyrosine antibody directed immunoprecipitates from human neutrophils and U937 cells. Upper panel: human neutrophils were treated with either vehicle or staurosporine (5 μM final concentration) for 7 min, the last 3 min of which was in the presence of GM-CSF (100 ng/ml). Lower panel: suspensions of U937 cells were stimulated with either vehicle (3 min) or GM-CSF (100 ng/ml final concentration for 3 min). Incubations were conducted and anti-phosphotyrosine antibody directed immunoprecipitates prepared and assayed for PI3K activity as described in Materials and methods. The data represent the amount of [^{32}P]PtdIns3P formed during the assays (upper panel, mean n = 2, ± range; lower panel, mean n = 4, ± SE). 'Non-immune' controls (GM-CSF stimulated samples containing an appropriate mixture of PBS/azide in place of the anti-phosphotyrosine antibody during the immunoprecipitation) contained virtually undetectable PI3K activity (data not shown).

capacity of the *src*-type PTK p53/p56^{lyn} (and additionally for p62$^{c\text{-}yes}$ in GM-CSF stimulated neutrophils) to autophosphorylate when assayed *in vitro*. Analogous increases in autophosphorylating activity are not seen in a variety of other *src*-type PTKs, suggesting that p53/p56^{lyn} and p62$^{c\text{-}yes}$ are specifically implicated in the signal transducing machinery used by the GM-CSF receptor. Further, GM-CSF stimulation can induce formation of a complex containing p53/p56^{lyn}, an 85 kDa protein (immunologically related to the 85 kDa subunits of PI3Ks which have been purified from several sources) and PI3K activity (and also complexes containing p62$^{c\text{-}yes}$ and PI3K activity in neutrophils).

These observations are consistent with recent data suggesting that thrombin and IL-2 receptors and CD3/TCR and mIgM may also stimulate formation of complexes containing PI3K activities and *src* family PTKs (Gutkind *et al.*, 1990; Augustine *et al.*, 1991; Thompson *et al.*, 1992; Yamanashi *et al.*, 1992). However, in none of these latter cases have all of the relevant data connecting receptor stimulation with (i) 'activation' of an *src*-type PTK; (ii) the recruitment of a PI3K activity into tight complexes with the activated PTK and (iii) enhanced accumulation of a 3-phosphorylated inositol lipid in cells, been documented. A precisely analogous, but receptor independent, series of connections between increased accumulation of PtdIns(3,4,5)P_3 and activation of *src*-type PTKs is also evident in cells transformed by v-*src*, v-*yes* or middle T antigen-activated c-*src*, or cells overexpressing c-*fyn* (Sugimoto *et al.*, 1984; Whitman *et al.*, 1985; Kaplan *et al.*, 1986; Cantley *et al.*, 1991; Fukui *et al.*, 1991; Gout *et al.*, 1991). The simplest explanation for these data is that in myeloid derived cells GM-CSF stimulation can lead to 'activation' of p53/p56^{lyn} and p62$^{c\text{-}yes}$ and that these *src* family PTKs then form phosphotyrosine co-ordinated signalling complexes into which a PI3K is recruited, activated and hence drives the accumulation of PtdIns(3,4,5)P_3 (see Figure 8). The pattern of events presented in Figure 8 draws strength from evidence suggesting the interactions which are depicted can occur in other situations and furthermore might supply an 'activating' signal to a PI3K activity. However, the physical connections that are made in these signalling complexes are not clear. This is because complexes of proteins associating via SH2 domain− tyrosine phosphate interactions can be co-immunoprecipitated by antibodies directed aginst any one of their members (when the immunoprecipitates are washed according to conventional protocols). Hence, as PI3K and *src*-type PTKs can possess SH2 domains and/or tyrosine phosphates, evidence that they can be co-localized in this manner does not establish either the direct contacts, or the 'polarity' of the SH2−tyrosine phosphate interactions, that are implicated. Morever, this problem means additional, currently unrecognized proteins could mediate these associations (for example like protein '?' in Figure 8) or be responsible for the activation of the *src*-type PTKs and/or PI3K. However, these potential elaborations, although plausible, are currently unnecessarily complicated explanations for our data.

The demonstration that GM-CSF stimulates the synthesis of PtdIns(3,4,5)P_3 is a step forward in elucidating its signalling mechanisms. Unfortunately however, whilst a great deal of work suggests that PtdIns(3,4,5)P_3 will turn out to be a 'second-messenger' of some description (e.g. see Cantley *et al.*, 1991; Downes and Carter, 1991), we still have little idea of what its intracellular targets may be. Recent reports of a high degree of homology between the gene encoding the catalytic subunit of a mammalian PI3K and a gene defined by a vacuolar protein sorting mutant in yeast (Herman and Emr, 1990; Hiles *et al.*, 1992) have suggested that 3-phosphorylated inositol lipids may have a role in 'vesicle management' or 'protein sorting'.

Materials and methods

Materials

Standard reagents were from sources described in Stephens *et al.* (1990) and Jackson *et al.* (1992). Radiochemicals and enhanced chemiluminescence detection reagents were from Amersham International Plc. Phosphatidylinositol was from Sigma or Avanti Lipids. PEI cellulose TLC plates were from CamLab. Cytokines and growth factors were very generously supplied by the following sources: GM-CSF (human, recombinant), Robert Markus (Dept Haematology, Addenbrookes, Cambridge, UK) and Steve Clark (Genetics Institute, Cambridge, MA); IL-3, Douglas Williams (Immunex, Seattle, WA); IGF-1

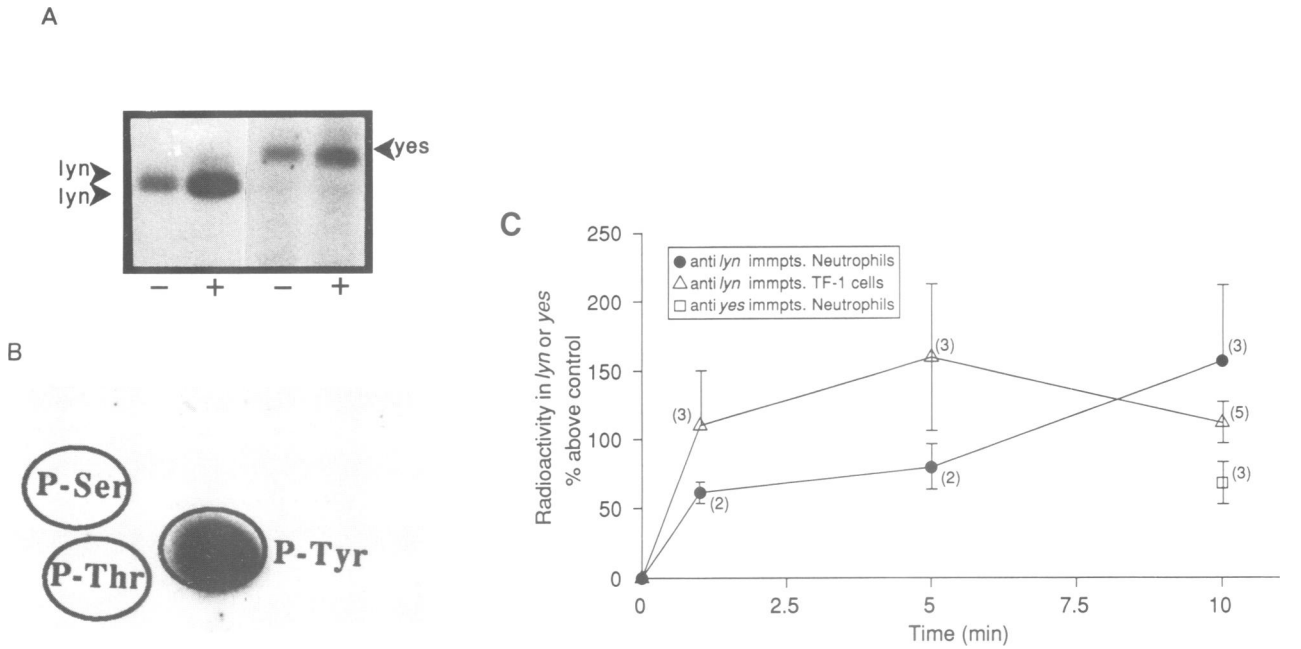

Fig. 6. GM-CSF stimulated protein phosphorylation in anti-p53/56^{lyn} and anti-p62$^{c\text{-}yes}$ antibody directed immunoprecipitates from myeloid derived cells. (**A**) Human neutrophils were incubated with GM-CSF (+, 10 ng/ml) or vehicle (−) for 10 min, then lysed and anti-p62$^{c\text{-}yes}$ or anti-p53/p56^{lyn} antibody directed immunoprecipitates prepared and assayed for PTK activity (see Materials and methods). An autoradiograph of the gel in which the products of the PTK assays were resolved was prepared and the section corresponding to the position to which p53/p56^{lyn} or p62$^{c\text{-}yes}$ had migrated is shown. (**B**) Shows the results of an analysis of the [^{32}P]phosphoamino acids present in p53/p56^{lyn} derived from a PTK assay performed with an anti-p53/p56^{lyn} antibody directed immunoprecipitate prepared from GM-CSF stimulated (10 ng/ml for 10 min) TF-1 cells (see Materials and methods). (**C**) Human neutrophils (●, □) and TF-1 cells (△) were incubated with GM-CSF (10 ng/ml or vehicle) for various times. The cells were then lysed and either anti-p53/p56^{lyn} or anti-p62$^{c\text{-}yes}$ antibody directed immunoprecipitates were prepared and assayed for p53/p56^{lyn} (●, △) or p62$^{c\text{-}yes}$ (□) phosphorylation, respectively (see Materials and methods). The results shown are for the ^{32}P contents of p53/p56^{lyn} or p62$^{c\text{-}yes}$ in GM-CSF stimulated samples, expressed as percentage increases above their relevant controls: the data are means ± SEM for (*N*) observations. Taken collectively (all time points pooled and subjected to a non-orthogonal analysis of variance), the ^{32}P contents of the relevant *src*-type PTKs were significantly higher in GM-CSF stimulated samples than in unstimulated samples; for both p53/p56^{lyn} in TF-1 samples ($F_{1,10} = 42.21$, $P < 0.001$), and p53/p56^{lyn} ($F_{1,6} = 14.9$, $P < 0.01$) and p62$^{c\text{-}yes}$ ($F_{1,2} = 34.99$, $P < 0.05$) in neutrophil samples.

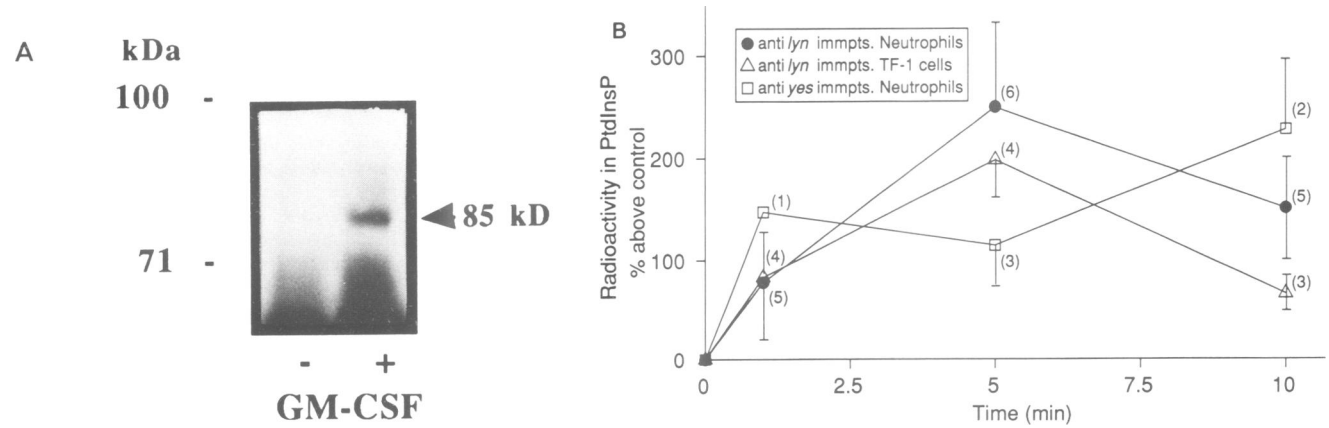

Fig. 7. PI3K associates with anti-p53/p56^{lyn} and anti-p62$^{c\text{-}yes}$ antibody directed immunoprecipitates from GM-CSF stimulated cells. (**A**) Human neutrophils were incubated with GM-CSF (10 ng/ml or vehicle for 10 min, then lysed and anti-p53/p56^{lyn} immunoprecipitates prepared (see Materials and methods). The immunoprecipitates were extracted with SDS sample buffer, resolved by SDS−PAGE and electrophoretically transferred to a nitrocellulose filter. The filter was then probed with a p85 antiserum (against the p85 subunit of rat liver PI3K; see Materials and methods). The secondary antibody was detected by enhanced chemiluminescence assay, a photograph of which is shown. The position of p85 kDa is marked. (**B**) Human neutrophils (●, □) and TF-1 cells (△) were incubated with GM-CSF (10 ng/ml) or vehicle for various times. The cells were then lysed and either anti-p53/p56^{lyn} (●, △) or anti-p62$^{c\text{-}yes}$ (□) antibody directed immunoprecipitates were prepared and assayed for PI3K activity (see Materials and methods). The results shown are for the amount of PtdInsP formed in assays of GM-CSF stimulated samples, expressed as percentage increases above their relevant controls: the data are means ± SEM for (*N*) observations. Non-immune controls (GM-CSF stimulated samples containing no anti-*src*-type PTK antibody during the immunoprecipitation) contained virtually undetectable PI3K activity (data not shown). A similar statistical analysis of this data to that described in Figure 6C (i.e. combining all time points), indicates that the PI3K activity in GM-CSF stimulated samples is significantly higher than in unstimulated samples, in both anti-p53/p56^{lyn} immunoprecipitates from neutrophils ($F_{1,15} = 12.75$, $P < 0.01$) and TF-1 cells ($F_{1,10} = 12.35$, $P < 0.01$), and anti-p62$^{c\text{-}yes}$ immunoprecipitates from neutrophils ($F_{1,5} = 15.1$, $P < 0.05$).

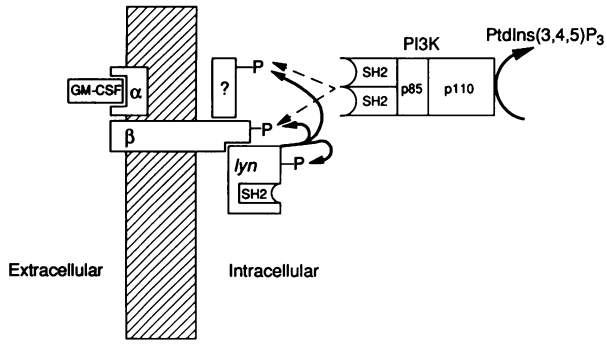

Fig. 8. Activation of PI3K by GM-CSF. This is a schematic diagram designed to portray one currently plausible mechanism for how the GM-CSF receptor activates PtdIns(3,4,5)P_3 accumulation. There are two major considerations for portraying the connections as described. The first of these is a desire to build into the model a mechanism for receptor-specific use of the *src*-type PTKs (this is achieved by allowing the kinase to phosphorylate the receptor or a receptor directed protein); this is apparently required by observations of the way p56^{lck} and p59^{fyn} are used to 'activate' PI3K in T-cells by CD3/TCR, the IL-2 receptor and CD4 (Augustine *et al.*, 1991; Hatakeyama *et al.*, 1991; Thompson *et al.*, 1992; Tsygankov *et al.*, 1992). The second consideration is to provide a possible mechanism for the activation of PI3K: recent studies have shown that PI3K can be activated by the docking of its SH2 domains on to specific tyrosine phosphates (Backer *et al.*, 1992; C.L.Carpenter, K.R.Auger, Chaudhuri, B.Schaffhausen, S.Schoelson and L.C.Cantley, submitted; although the GM-CSF receptor does not contain a currently accepted tyrosine phosphate consensus sequence for PI3K binding i.e. a YXXM motif; Cantley *et al.*, 1991); this would not be possible if it was a tyrosine phosphate on PI3K that was bound by the SH2 domain of a *src*-type PTK. It is not clear how receptor-activation may stimulate the activity of the receptor-associated *src*-type PTKs; analogy with other systems suggests that it may involve an allosteric interaction leading to the release of an internal, inhibitory, SH2−tyrosine phosphate bond within the *src*-type PTK (e.g. Matsuda *et al.*, 1990; Samelson *et al.*, 1990; Yamanashi *et al.*, 1991; Beyers *et al.*, 1992; Veillette *et al.*, 1992). It has been shown that PI3K can be extracted from cells phosphorylated on tyrosine residues and that it can be phosphorylated *in vitro* by association with appropriately activated PTKs, including those of the *src* family (e.g. Cantley *et al.*, 1991; Gout *et al.*, 1992). Recent studies however, suggest that stimulation of PI3K by growth factors utilizing intrinsic receptor-PTKs does not lead to a measurable increase in its overall tyrosine phosphate content (Backer *et al.*, 1992; Hu *et al.*, 1992).

(human,recombinant) and insulin, Tony Corps (AFRC Institute, Cambridge, UK); CSF-1 (purified from conditioned media), David Linch (University College, London); TNF-α (human, recombinant), Genzyme Plc.

Preparation and/or culture of cells

TF-1 cells (a GM-CSF and IL-3 dependent human leukaemic cell line kindly provided by Toshio Kitamura, DNAX, Palo Alto, CA), M-07e cells (a GM-CSF and IL-3 dependent human leukaemic cell line kindly provided by Luigi Pegoraro, University of Turino, Italy) and AML-193 cells (a GM-CSF dependent cell line obtained from the ATCC, Bethesda, MD) were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM glutamine, and 1% penicillin/streptomycin and 1−5 ng/ml of recombinant human GM-CSF. U937 cells (kindly provided by Lyndsey Needham, British Biotechnology Ltd, England) were grown in RPMI 1640 with 5% heat inactivated fetal calf serum (Imperial Laboratories Ltd) at a density of between 1 and 4 × 10^5 cells/ml. All cell lines were cultured in an atmosphere of 95% air/5% CO_2. U937 cells were routinely prepared for experiments by serum-starving them for 16 h (at 3 × 10^5 cells/ml in RPMI 1640, 0.1% w/v fatty acid-free BSA) and then washing them twice in a HEPES-buffered balanced salts solution (HBBSS) of the following composition: 140 mM NaCl, 5 mM KCl, 2.8 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.06 mM $MgSO_4$, 15 mM HEPES, 5.6 mM glucose, 0.1% w/v fatty acid-free BSA, pH 7.2 at 37°C with NaOH. The HBBSS was filtered (0.2 μm Sartorius, Minisart) immediately prior to use. Freshly prepared human neutrophils were prepared for anti-PTK antibody immunoprecipitations as described previously (Corey and Rosoff, 1989). For all other experiments, neutrophils were freshly prepared as described (Stephens *et al.*, 1991) and finally resuspended in 0.2 μm-filtered *N*-HBBSS (final composition: 110 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1.5 mM $CaCl_2$, 30 mM HEPES, 0.1% w/v fatty acid-free BSA, pH 7.4 at 37°C with NaOH). Neutrophil suspensions were routinely >93% pure.

[^{3}H]Ins and [^{32}P]Pi labelling of U937 cells and human neutrophils

To label U937 cells with [^{3}H]Ins they were grown as described above (but in the presence of inositol-free medium and dialysed fetal calf serum) for 48 h, and then their medium was replaced with the same but containing [^{3}H]Ins (2 μCi/ml). After 48 h labelling, the cells were serum-starved (in inositol-free medium), washed twice with HBBSS and resuspended (5 × 10^6 cells/ml, see above). Aliquots (1 ml) were challenged with agonists or their vehicle, quenched and processed for anion-exchange HPLC as described previously (Stephens *et al.*, 1990).

U937 cells were labelled with [^{32}P]Pi (Amersham Plc; PBS-13) by incubating them in HBBSS (containing 0.3 mCi/ml [^{32}P]Pi and 0.5% fatty acid-free BSA, at 2.5 × 10^7 cells/ml, at 37°C). After 70 min the cells were washed three times, resuspended in HBBSS (containing 0.5% fatty acid-free BSA at 2.5 × 10^7 cells/ml) and equilibrated at room temperature for 5 min before aliquoting the cells into glass tubes prewarmed to 37°C (150 μl aliquots in 5 ml microcap vials, CamLab). After 2 min at 37°C, agonists were added (contained in 10−20 μl of incubation medium), the cells mixed and incubations finally quenched with 3.75 vols of chloroform/methanol (1/2; v/v; establishing a homogeneous primary extraction phase; Bligh and Dyer, 1959).

Human neutrophils were labelled with: (i) [^{32}P]Pi (typically at a final concentration of 2.0 mCi/ml) for 70 min or (ii) [^{3}H]Ins (typically at a final concentration of 1 mCi/ml for 3 h) as described (Stephens *et al.*, 1991). If the neutrophils were being simultaneously labelled with [^{32}P]Pi and treated with pertussis toxin, the incubation conditions were precisely as described for the toxin pretreatment (below) except that the balanced salts solution contained 2.5 mCi/ml [^{32}P]Pi. After the labelling period the cells were suspended (at 4 × 10^7/ml) in filtered *N*-HBBSS containing 0.5% fatty acid-free BSA and stimulated with agonists as described above. Neutrophils (2 × 10^7/ml) were pretreated with pertussis toxin (List Biologicals; 1.5 μg/ml, in the presence of 6.3 mg/ml cytochrome C) for 2.5 h; cells treated with heat-inactivated pertussis toxin were used as controls.

Extraction and resolution of PtdIns(3,4,5)P_3

The quenched samples were mixed with (in volumes relative to the original assay volumes), 3.75 vols of chloroform (containing 5000−8000 d.p.m. [^{3}H]PtdIns(4,5)P_2 to act as an internal marker for recovery of lipids through the extraction, and carrier phospholipids containing 2−5 μg of phosphorus; Stephens *et al.*, 1991) and 0.875 vols of 2.4 M HCl, 5 mM tetrabutylammonium sulfate (to establish a stable two phase solvent system; Folch *et al.*, 1957). The samples were vortexed for 2 min, centrifuged and the lower phases were removed to fresh vials where they were washed with fresh upper phase (3.66 vols) before being moved to a third vial. The original upper phases were washed with fresh lower phase (5.72 vols) which was then transferred to the secondary vials to wash the upper phases they contained before being pooled with the original lower phase and dried down under vacuum. The dry lipids were deacylated with 200 μl of monomethylamine reagent for 30 min as described previously (Clarke and Dawson, 1981; Stephens *et al.*, 1991) and the resulting [^{32}P]glycerophosphoinositol phosphates were resolved by HPLC as described (Stephens *et al.*, 1991). Data were routinely corrected for losses during extraction by means of the internal [^{3}H]PtdIns(4,5)P_2 spikes. When quantitative comparisons between different preparations of [^{32}P]Pi-labelled neutrophils were made, data were normalized by reference to the levels of [^{32}P]PtdIns(4,5)P_2 in control samples.

Anti-phosphotyrosine antibody directed immunoprecipitations from U937 cells and human neutrophils and assay of PI3K activity

Aliquots (0.8 ml) of U937 cells (1.4 × 10^7 cell/ml) and human neutrophils (1.25 × 10^7 cells/ml; pretreated with 1 mM di-isopropylfluorophosphate for 5 min at 20°C) were transferred into 2.2 ml microfuge tubes (containing 50 μl of 17 mM orthovanadate in *N*-HBBSS or HBBSS) in a 37°C water bath. After 2 or 3 min agonist additions (10 μl) commenced and at the appropriate time reactions were quenched by pelleting the cells (4 s in a microcentrifuge), removing the supernatant, adding 1 ml of ice-cold lysis buffer [137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (w/v) NP40, 10% w/v glycerol, 1 mg/ml BSA, 20 mM Tris, 0.5 mM orthovanadate, 0.2 mM PMSF, 10 μg/ml leupeptin, antipain, pepstatin A and aprotinin, pH 8.0 4°C], vortexing fiercely and finally placing them on ice (total time from water bath to ice bath was ~35 s). After 10 min samples were centrifuged (30 min, 4500 *g* at 0°C) and 0.8 ml aliquots of the supernatants (typically containing 0.55−0.65 mg/ml neutrophil, or

1 mg/ml U937 cell derived protein) were mixed with 8 μl of anti-phosphotyrosine monoclonal antibody (PY20, ICN) and 40 μl of a 1/1 (v/v; packed beads/lysis buffer) suspension of protein A−sepharose CL4B beads (Pharmacia; pre-equilibrated for 2 h in lysis buffer) then end on end mixed. After 2 h (at 0°C) the beads were washed (at 4°C), as follows: three times with lysis buffer; twice with 0.5 M LiCl, 0.1 M Tris (pH 8.0, 4°C); once with 0.15 M NaCl, 10 mM Tris, 1 mM EDTA (pH 7.6, 4°C); once with 20 mM HEPES, 1 mM DTT, 5 mM $MgCl_2$ (pH 7.6, 4°C). Finally, all of the supernatant was removed, 40 μl of ice-cold assay buffer (20 mM β-glycerophosphate, 5 mM pyrophosphate, 30 mM NaCl, 1 mM DTT, pH 7.2, 4°C) and 20 μl of PtdIns/cholate [containing 3 mg/ml PtdIns; a dry film of PtdIns was bath-sonicated into assay buffer, containing 1% (w/v) cholate] were added and the assay tubes then mixed thoroughly and transferred to a 37°C water bath. After 5 min 40 μl of a solution containing 3 μM ATP, 7.5 mM $MgCl_2$ and 0.25 mCi/ml of [γ-^{32}P]ATP (PB 10168 Amersham Plc) was added to initiate the assay. After 15 min the reactions were quenched with 500 μl of chloroform/methanol/H_2O [made up from 97.88 ml of chloroform/methanol, 1/2 (v/v) and 2.12 ml of H_2O] to yield a homogeneous primary extraction phase (Bligh and Dyer, 1959; we assumed 20 μl of aqueous buffer was carried into the final assay with the beads). The samples were mixed thoroughly, then resolved into two phases by the addition of 489 μl of chloroform (in some experiments this included 5000 d.p.m. of [^{3}H]PtdIns4P) and 114 μl of 2.4 M HCl, 5 mM tetrabutylammonium sulfate. Extractions were completed according to the protocol defined above. The final dry lipid film was either dissolved in 25 μl of chloroform/methanol (2/1, v/v), applied to a TLC plate (silica gel 60; Merck) and resolved and quantified by scraping as described (Jackson *et al.*, 1992), or deacylated and resolved by anion-exchange HPLC (together with internal [^{3}H]GroPIns3P and [^{3}H]GroPIns4P standards) as described (Stephens *et al.*, 1991). 85−90% of the [^{32}P]GroPInsP products eluted with the [^{3}H]GroPIns3P standard in both control and stimulated samples, confirming that a PI3K activity was being assayed.

Assay of PTK and PI3K activities in anti-src-type PTK antibody directed immunoprecipitates

Both TF-1 and AML-193 cells were washed three times in sterile PBS and incubated in PBS for 3 h at 37°C in a 5% CO_2/95% air incubator. M-O7e cells were washed three times in sterile PBS and incubated overnight in RPMI 1640 and 10% FCS. The growth factor starved cells or freshly purified neutrophils were stimulated with 10 ng/ml of GM-CSF or IL-3 at 37°C. The cells were lysed at 4°C in a buffer containing 20 mM Tris−HCl (pH 7.4), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mM sodium vanadate, 1 mM PMSF and 10 μg/ml aprotinin, pepstatin and leupeptin. The lysates were incubated with antisera directed against the *src*-related PTKs (Bolen *et al.*, 1991) for 90 min at 4°C (4 μg/ml of lysate) and then for 60 min at 4°C with protein A−sepharose. The immunoprecipitates were washed three times with PBS, 1% NP-40; twice with 0.5 M LiCl, 100 mM Tris−HCl (pH 7.5) and twice with 100 mM NaCl, 1 mM EDTA, 10 mM Tris−HCl (pH 7.4). Protein kinase reactions were performed in the presence of 20 mM Tris−HCl (pH 7.5), 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 μM ATP and 10−30 μCi of [γ-^{32}P]ATP. Reactions were carried out for 20 min at room temperature, terminated by the addition of 2 × Laemmli buffer, boiled for 5 min and resolved by SDS−PAGE (10% resolving gel). Dried gels were autoradiographed (Kodak X-OMAT AR film) for 1−2 days. The ^{32}P contents of bands corresponding to p53/p56^{lyn} or p62$^{c\text{-}yes}$ were determined either by excising the appropriate bands and counting for radioactivity, or by densitometric scanning of the autoradiograms. To establish the identity of the amino acids phosphorylated in receptor-activated assays of p53/p56^{lyn}, segments (corresponding to autoradiographically localized p53/p56^{lyn}) of the polyacrylamide gels were excised, rehydrated with 30% methanol, dried, digested with 50 μg/ml trypsin in 50 mM NH_4HCO_3, and finally hydrolysed with 6N HCl for 90 min at 100°C. The hydrolysis products were resolved by 2D chromatography (Draetta *et al.*, 1988; electrophoresis at pH 1.9 followed by TLC with isopropanol/HCl/H_2O, 70/15/15). The dried chromatogram was autoradiographed and the positions of authentic internal phosphoamino acid standards were marked.

Anti-*src*-related PTK antibody directed immunoprecipitates were assayed for PI3K activity as described (Auger *et al.*, 1989). The [^{32}P]lipid formed in these assays was established to be [^{32}P]PtdIns3P (Auger *et al.*, 1989; data not shown).

To estimate the quantity of the regulatory subunit of PI3K (p85 protein) in the anti-p53/p56^{lyn} antibody directed immunoprecipitates, the washed immunoprecipitates were dissolved in SDS sample buffer, electrophoretically resolved in a 10% polyacrylamide gel in the presence of SDS and transferred to a nitrocellulose membrane which was then blocked with 5% (w/v) dried skimmed milk and 0.2% (w/v) Tween-20 in Tris-buffered saline. The membrane was incubated for 90 min with a rabbit antiserum prepared against the purified 85 kDa subunit of PI3K (Carpenter *et al.*, 1990). A secondary incubation with a goat anti-rabbit immunoglobulin coupled to horseradish peroxidase (Bio-Rad) was followed by detection via enhanced chemiluminescence (ECL, Amersham). To estimate the quantity of *src*-related PTKs in anti-*src*-related PTK antibody directed immunoprecipitates, the immunoprecipitates were conducted with antibodies crosslinked (by dimethylpimelimidate; as described, Harlow and Lane, 1988) to protein A−sepharose. The immunoprecipitates formed by the covalently attached antibodies still demonstrated GM-CSF stimulation of *src*-related PTK activity (data not shown). Western blots were prepared from the immunoprecipitates formed by the covalently crosslinked antibodies as described for transfer of the p85 subunit of PI3K. The resulting membranes were washed, blocked (see above) and then probed with anti-*src*-related PTK antibodies followed, after further washing, by [^{125}I]Protein A. After 90 min with [^{125}I]Protein A the Western blots were washed again and ^{125}I was localized autoradiographically and quantitated by densitometric scanning.

Acknowledgements

We would like to thank Christopher Carpenter, Stephan Soltoff, Rosana Kapeller, Brian Duckworth, Candy Corey, Kurt Auger and Lyuba Varticovski for their advice and helpful comments and David Brown for statistical analyses. L.R.S. would like to thank Amersham International Plc for the award of a collaborative grant. A.E. was supported by an EMBO short-term fellowship. P.T.H. is a Lister Research Fellow; T.R.J. is a Royal Society Mr and Mrs J.Jaffé Research Fellow. Some of this work was supported by grants NIH Kll-HL02303 (SJC), NIH GM41890 (LCC), Whitaker Health Sciences Fund (SJC) and Comunidad de Madrid C181/91 (FM).

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Received on August 25, 1992; revised on March 23, 1993