The EMBO Journal vol.8 no.11 pp.3345-3350, 1989

Phospholipase C-γ, a substrate for PDGF receptor kinase, is not phosphorylated on tyrosine during the mitogenic response to CSF-1

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Communicated by J.Schlessinger

Quiescent mouse NIH3T3 cells expressing a transduced human c-*fms* gene encoding the receptor for colony stimulating factor-1 (CSF-1) were stimulated with mitogenic concentrations of platelet-derived growth factor (PDGF) or CSF-1. Immunoprecipitated phospholipase C-γ (PLC-γ) was phosphorylated on tyrosine and calcium was mobilized following treatment of intact cells with PDGF. In contrast, only trace amounts of phosphotyrosine were incorporated into PLC-γ and no intracellular calcium signal was detected after CSF-1 stimulation. Similarly, CSF-1 treatment did not stimulate phosphorylation of PLC-γ on tyrosine in a CSF-1-dependent, SV40-immortalized mouse macrophage cell line that expresses high levels of the CSF-1 receptor. In fibroblasts, antiserum to PLC-γ co-precipitated a fraction of the tyrosine phosphorylated form of the PDGF receptor (PDGF-R) after ligand stimulation, implying that phosphorylated PDGF-R and PLC-γ were associated in a stable complex. Pre-treatment of cells with orthovanadate also led to tyrosine phosphorylation of PLC-γ which was significantly enhanced by PDGF, but not by CSF-1. Thus, although the PDGF and CSF-1 receptors are structurally related and appear to be derived from a single ancestor gene, only PDGF-induced mitogenesis in fibroblasts correlated with tyrosine phosphorylation of PLC-γ.

Key words: CSF-1 receptor/PDGF receptor/tyrosine-specific protein kinase/phospholipase C-γ/phosphotyrosine

Introduction

The cell surface receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, insulin-like growth factor-1 (IGF-1) and colony stimulating factor-1 (CSF-1) are integral transmembrane glycoproteins with ligand-stimulated, tyrosine-specific protein kinase activities (Ushiro and Cohen, 1980; Ek *et al.*, 1982; Nishimura *et al.*, 1982; Kasuga *et al.*, 1982; Jacobs *et al.*, 1983; Rettenmier *et al.*, 1985; Sherr *et al.*, 1985). The PDGF and CSF-1 receptors (c-*fms* proto-oncogene product) show a relatively high degree of overall amino acid sequence similarity (Hampe *et al.*, 1984; Coussens *et al.*, 1986; Yarden *et al.*, 1986) and have been assigned to the same subfamily of receptor genes together with the c-*kit* proto-oncogene (Besmer *et al.*, 1986; Yarden *et al.*, 1987). In humans, the genes encoding the PDGF B-type receptor (here designated PDGF-R) and the CSF-1 receptor (CSF-1R) are arranged in tandem on the long arm of chromosome 5 where they appear to have arisen from a duplicated ancestral sequence (Roberts *et al.*, 1988). In contrast, the PDGF A-type receptor and the c-*kit* proto-oncogene have been assigned to human chromosome 4 (Yarden *et al.*, 1987; Matsui *et al.*, 1989). PDGF-R is normally expressed on mesenchymal cells and responds to PDGF elaborated by blood cells (Ross *et al.*, 1986), whereas CSF-1R is expressed on monocytes, macrophages, and their committed bone marrow progenitors and responds to CSF-1 produced by fibroblasts (Stanley *et al.*, 1983; Sherr and Stanley, 1989). When introduced into mouse NIH3T3 fibroblasts, the human CSF-1R gene enabled the cells to conditionally form colonies in semi-solid medium containing human recombinant CSF-1 (Roussel *et al.*, 1987). In chemically defined medium, physiologic concentration of CSF-1 can support the growth of these cells at the expense of PDGF and insulin. However, the cells undergo ligand-dependent morphologic transformation and are no longer contact-inhibited, implying that CSF-1 induces a genetic program different from that of PDGF (Roussel and Sherr, 1989).

Physiologic substrates for receptor tyrosine kinases whose phosphorylation contributes to the mitogenic response remain poorly characterized. When A431 cells were treated with EGF, lysed, and tyrosine phosphorylated proteins were purified by adsorption and elution from an anti-phosphotyrosine matrix, the samples contained a phospholipase C activity capable of hydrolyzing phosphatidyl-inositol 4,5-diphosphate (PIP2) to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP3) (Wahl *et al.*, 1988). The latter compounds represent second messengers important in activating protein kinase C and in mobilizing calcium from intracellular stores (Berridge, 1987). Subsequent studies demonstrated that binding of EGF to cells overexpressing EGF receptors (EGF-R) resulted in rapid tyrosine phosphorylation of one of the PLC isoforms, PLC-γ (Wahl *et al.*, 1989a; Meisenheleder *et al.*, 1989; Margolis *et al.*, 1989; Rhee *et al.*, 1989). In addition, a kinase-inactive EGF-R mutant was unable to enhance PLC activity and Ca^{2+} release in living cells (Moolenaar *et al.*, 1988). These data implicated PLC-γ as a physiologic substrate of the EGF-R kinase and implied that phosphorylation of PLC-γ might thereby lead to increased phosphoinositide turnover in response to EGF stimulation.

PDGF also induces accelerated phosphoinositide turnover in fibroblasts (Habenicht *et al.*, 1981), and recent data has demonstrated tyrosine phosphorylation of PLC-γ in response to this growth factor (Meisenheleder *et al.*, 1989; Wahl *et al.*, 1989b). In contrast, CSF-1 has been reported to lack any effect on phosphoinositide turnover in mononuclear

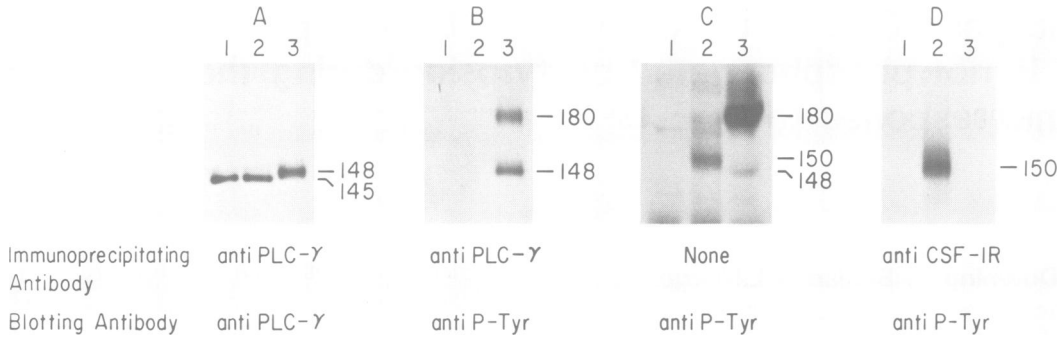


Fig. 1. Tyrosine phosphorylation of proteins from PDGF- and CSF-1-stimulated cells. Quiescent NIH3T3 cells were either unstimulated (**lanes 1**) or were stimulated for 5 min with either 0.4 μg/ml CSF-1 (**lanes 2**) or 0.1 μg/ml PDGF (**lanes 3**), lysed and precipitated with the antisera indicated beneath each panel. Following electrophoretic separation of the precipitates on denaturing polyacrylamide gels, the proteins were transferred to nitrocellulose and blotted with the antisera indicated below each panel. All immunoprecipitates (**panels A, B** and **D**) were prepared with 0.95 ml aliquots from 1 ml lysates obtained from replicate confluent cultures, each from 150 mm diameter dishes. The remaining aliquots of each lysate (0.05 ml, **panel C**) were applied to gels without immunoprecipitation. Molecular masses in kd calculated by calibration with known mol. wt markers are indicated at the right of each panel. Exposure times for autoradiography were 24 h.

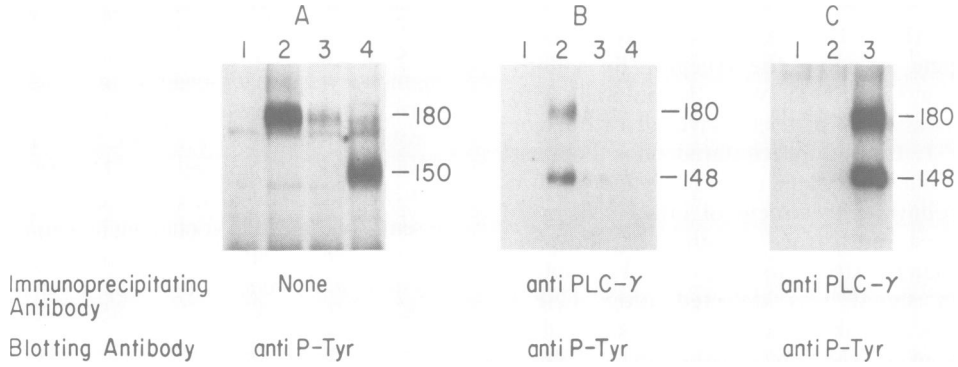


Fig. 2. Tyrosine phosphorylation of PLC-γ after stimulation with different concentrations of growth factors. (**Panels A** and **B**): lysates from unstimulated cells (**lanes 1**), from cells stimulated with 14 ng/ml PDGF (**lanes 2**), with 2.8 ng/ml PDGF (**lanes 3**) or with 0.4 μg/ml CSF-1 (**lanes 4**) were electrophoresed directly (**panel A**, 50 μl/lane) or were immunoprecipitated with antiserum to PLC-γ (**panel B**, 0.95 ml/lane). After transfer to nitrocellulose, the proteins were blotted with antiserum to phosphotyrosine (P-Tyr). (**Panel C**): lysates (total 3 ml) from triplicate cultures of unstimulated cells (**lane 1**) or from cells stimulated with 0.4 μg/ml CSF-1 (**lane 2**) or 0.1 μg/ml PDGF (**lane 3**) were precipitated with antiserum to PLC-γ, transferred to nitrocellulose and blotted with antiserum to P-Tyr. Molecular masses in kd calculated by calibration with known markers are indicated at the right of each panel. Exposure times for autoradiography were 24 h.

phagocytes (Whetton *et al.*, 1986). Since both PDGF and CSF-1 can induce a mitogenic response in NIH3T3 cells expressing a transduced human c-*fms* gene, we tested both growth factors for their ability to induce tyrosine phosphorylation of PLC-γ. We now show that PDGF, but not CSF-1, efficiently leads to PLC-γ phosphorylation on tyrosine, implying that CSF-1-induced mitogenesis in this system is not exerted through the agency of this regulatory enzyme.

Results

To determine if PLC was phosphorylated on tyrosine in response to PDGF or CSF-1 treatment, quiescent NIH3T3 cells expressing the c-*fms* proto-oncogene product were stimulated for 5 min with saturating concentrations of either PDGF (0.1 μg/ml) or CSF-1 (0.4 μg/ml), and cell lysates were immunoprecipitated with an antiserum to PLC-γ. The immunoprecipitated proteins were separated on denaturing polyacrylamide gels, transferred to nitrocellulose, and blotted with the same antiserum. Figure 1A shows that in the absence of growth factor treatment (lane 1), a single polypeptide of 145 kd was detected, corresponding to the expected molecular mass of PLC-γ (Suh *et al.*,1988; Rhee *et al.*, 1989). After CSF-1 stimulation (lane 2), the mobility of the 145 kd polypeptide was unchanged, whereas PDGF treatment resulted in a small increase in its mass to 148 kd (lane 3), suggesting that PLC-γ had undergone some modification. When parallel blots were incubated with antiserum to phosphotyrosine (Figure 1B), proteins precipitated with antiserum to PLC-γ were not detectably phosphorylated on tyrosine prior to growth factor treatment (lane 1) or in response to CSF-1 (lane 2). Following PDGF stimulation, however, the 148 kd PLC-γ polypeptide was found to be phosphorylated on tyrosine (lane 3). When cells were metabolically labeled with [^{32}P]orthophosphate and stimulated with PDGF, two-dimensional phosphoamino acid analysis confirmed that the immunoprecipitated PLC-γ band eluted from gels contained phosphotyrosine (data not shown).

A second tyrosine phosphorylated polypeptide of 180 kd was also immunoprecipitated with antiserum to PLC-γ (Figure 1B, lane 3). This band was assumed to represent PDGF-R since, after electrophoresis and transfer of proteins from total cell lysates, the 180 kd protein, corresponding in molecular mass to the receptor, was the major phosphotyrosine-containing species in PDGF-stimulated cells (Figure 1C, lane 3) (Yarden *et al.*, 1986). In agreement with this interpretation, the 180 kd protein was specifically recognized by an antiserum to a carboxy-terminal peptide of murine

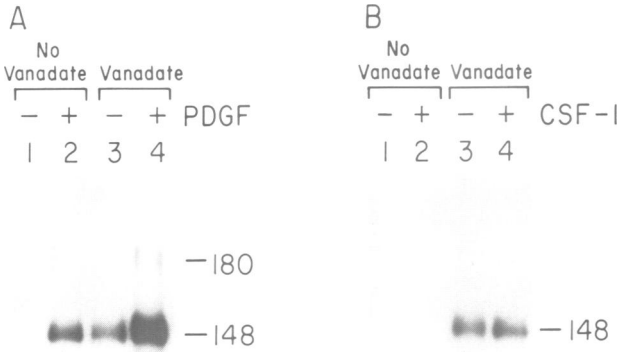


Fig. 3. Effect of phosphotyrosine kinase inhibitor on P-Tyr incorporation into PLC-γ. Cells pre-incubated for 16 h in complete medium containing 1% FCS without (**lanes 1** and **2**) or with (**lanes 3** and **4**) 50 μM sodium orthovanadate were stimulated for 5 min with 0.1 μg/ml PDGF (**panel A**) or 0.4 μg/ml CSF-1 (**panel B**) prior to lysis. Lysates (each 1 ml) were precipitated with antiserum to PLC-γ, transferred to nitrocellulose, and blotted with antiserum to P-Tyr. The calculated molecular masses of PLC-γ (148 kd) and PDGF-R (180 kd) were estimated from the positions of mol. wt markers run in adjacent lanes. Exposure times for autoradiography were 24 h.

PDGF-R (data not shown). Even when total cytoplasmic proteins from PDGF-stimulated cells were immunoblotted with antiserum to phosphotyrosine, a band corresponding in mobility to PLC-γ was detected (Figure 1C, lane 3). Since the data shown in panel C were obtained with 5% of the cell lysate, whereas those in panel B were obtained with quantitative immunoprecipitates of the remainder, only a relatively small fraction of the total tyrosine-phosphorylated PDGF-R was present in immune complexes with PLC-γ. In agreement with the data shown in Figure 1B (lane 2), a tyrosine phosphorylated form of PLC-γ was not observed in cell lysates from CSF-1-stimulated cells, whereas a band corresponding to CSF-1R at 150 kd was detected (Figure 1C, lane 2). Immunoprecipitation with an antiserum to CSF-1R (Figure 1D) confirmed that the latter band represented the receptor.

Previous Scatchard analyses indicated that the NIH3T3 cells used in these studies expressed ~5-fold more PDGF than CSF-1 binding sites at 4°C (Bowen-Pope and Ross, 1982; Roussel *et al.*, 1988). The data of Figure 1C similarly indicated that more tyrosine phosphate was incorporated into PDGF-R than into CSF-1R after stimulation with their respective ligands. In addition, relatively more tyrosine phosphate was incorporated into other cellular proteins in response to PDGF treatment (data not shown). Stimulation of the cells with higher concentrations of PDGF (0.5 μg/ml) or of CSF-1 (4 μg/ml) did not further increase tyrosine phosphorylation of the receptors or of heterologous proteins, confirming that the concentrations of growth factors used for the experiments shown in Figure 1 were sufficient to saturate both classes of binding sites.

To verify that phosphorylation of PLC-γ on tyrosine would still be observed if the 'signal strength' of PDGF-R were reduced to that of CSF-1R, cells were stimulated with decreasing concentrations of PDGF, and lysates were blotted with antiserum to phosphotyrosine. Figure 2A shows that decreasing levels of phosphotyrosine were detected in PDGF-R after treatment of the cells with 14 ng/ml (lane 2) and 2.8 ng/ml of PDGF (lane 3), respectively. Concentrations of PDGF between 3 and 5 ng/ml represent a full mitogenic dose for cultures of NIH3T3 cells in serum-free medium (Zhan and Goldfarb, 1986). CSF-1 at a concentration of 100 ng/ml can replace the PDGF requirement for growth of NIH3T3 cells that express human CSF-1R (Roussel and Sherr, 1989). Equivalent levels of tyrosine phosphorylation were obtained for PDGF-R with 14 ng/ml PDGF (lane 2) and for CSF-1R with 0.4 μg/ml CSF-1 (lane 4), respectively. When the identical cell lysates were immunoprecipitated with antiserum to PLC-γ, tyrosine phosphate was still detected in 148 kd PLC-γ only after PDGF, but not after CSF-1, stimulation (Figure 2B, lanes 2−4). Again, the tyrosine phosphorylated form of PDGF-R was detected in anti-PLC-γ immunoprecipitates, and at each concentration of PDGF used to stimulate the cells, only a fraction of the total tyrosine phosphorylated form of the receptor was present in anti-PLC-γ immune complexes.

To increase the sensitivity of the immunoblot procedure, triplicate 150-mm diameter cultures rather than single plates were stimulated with saturating concentrations of CSF-1 or PDGF, and pooled lysates (3 ml) were precipitated with an excess of antiserum to PLC-γ and applied to single lanes of a polyacrylamide gel. After electrophoresis and transfer to nitrocellulose, blotting with anti-phosphotyrosine antibody readily demonstrated phosphorylation of both PLC-γ and PDGF-R in lysates of PDGF-stimulated cells (Figure 2C, lane 3). Under these conditions, trace quantities of phosphorylated PLC-γ were now detected after CSF-1 stimulation (lane 2), but were not seen in immunoprecipitates from unstimulated cultures (lane 1). Thus, while we cannot exclude that PLC-γ is a substrate for the CSF-1R kinase, these data underscore the differential effects of PDGF and CSF-1 on PLC-γ tyrosine phosphorylation.

We considered the possibility that the failure to readily observe CSF-1R-induced phosphorylation of PLC-γ might be due to the activity of tyrosine phosphatases. Cells were therefore pre-incubated for 16 h in medium containing sodium orthovanadate, a tyrosine phosphatase inhibitor, prior to stimulation with PDGF or CSF-1. Cell lysates were then immunoprecipitated with antiserum to PLC-γ and immunoblotted with antiserum to phosphotyrosine. As shown above, in the absence of vanadate, the tyrosine phosphorylated form of PLC-γ was detected only after PDGF stimulation (Figure 3A, lanes 1 and 2). In contrast, vanadate treatment resulted in stable tyrosine phosphorylation of PLC-γ in the absence of PDGF (lane 3) with further phosphate incorporation after exposure to the growth factor (lane 4). A tyrosine phosphorylated form of PLC-γ was not observed after CSF-1 stimulation in the absence of vanadate (Figure 3B, lanes 1 and 2), and no CSF-1-induced increase in tyrosine phosphorylation was seen in the presence of vanadate (lanes 3 and 4). As shown previously (Downing *et al.*, 1988), significant increases in tyrosine phosphorylation of other cellular proteins were induced by CSF-1 in the presence of vanadate (data not shown). Therefore, the differences in PLC-γ phosphorylation in response to PDGF and CSF-1 were maintained in the presence of a tyrosine phosphatase inhibitor.

PLC-γ cleaves PIP2 to yield diacylglycerol and IP3. Since the latter mobilizes calcium from intracellular stores, we determined whether PDGF-induced PLC-γ phosphorylation correlated with a detectable calcium signal. Cells were

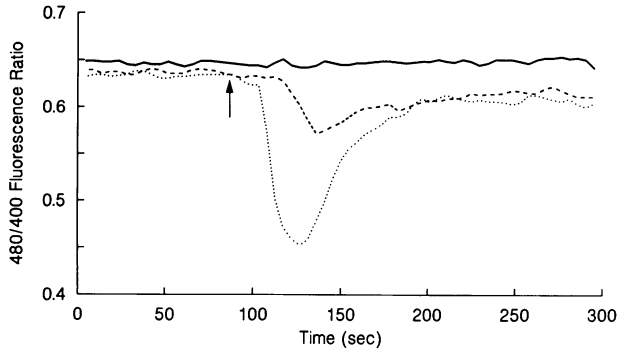
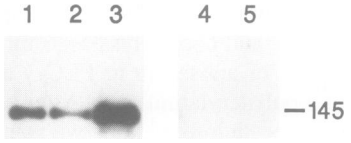


Fig. 4. Mobilization of intracellular calcium by PDGF but not CSF-1. Cells pre-incubated for 30 min in complete medium containing 10 μg/ml INDO-1 were treated at the time point indicated by the arrow with either CSF-1 (—, 0.4 μg/ml), porcine PDGF (-----, 0.1 μg/ml) or ionomycin (·····, 8.3 μg/ml). Induction of intracellular calcium flux, demonstrated by reduction in the INDO-1 fluorescence ratio (480 nm/400 nm), was detected after addition of PDGF or calcium ionophore, but not after CSF-1 addition.



Immunoprecipitating Antibody	**Anti PLC-γ**	**Anti PLC-γ**
Blotting Antibody	**Anti PLC-γ**	**Anti P-Tyr**

Fig. 5. Lack of CSF-1-stimulated tyrosine phosphorylation of PLC-γ in BAC1.2F5 murine macrophages. Quiescent BAC1.2F5 (**lanes 1, 2, 4** and **5**) or NIH3T3 (**lane 3**) cells were either lysed directly (**lanes 1, 3, 4**) or were stimulated for 5 min with 0.45 μg/ml human recombinant CSF-1 (**lanes 2** and **5**) prior to lysis. Lysates (1 ml) from replicate cultures were precipitated with antiserum to PLC-γ, and the precipitated proteins were separated on denaturing gels, transferred to nitrocellulose, and blotted either with antiserum to PLC-γ (**lanes 1–3**) or to phosphotyrosine (**lanes 4** and **5**). Parallel blots of immunoprecipitated CSF-1R confirmed that it contained phosphotyrosine only after CSF-1 stimulation (Downing *et al.*, 1988; data not shown). Exposure times for autoradiography were 24 h.

incubated for 30 min with a membrane-permeable calcium binding dye, INDO-1, and after uptake, were stimulated with either PDGF, CSF-1, or a calcium ionophore. Mobilization of calcium into the cytoplasm results in a shift in the fluorescence emission spectrum of the dye which can be accurately measured by flow cytometry. Within 45 s of addition, a calcium signal was detected using ionomycin or PDGF, but not CSF-1 (Figure 4). Thus PDGF, which increases phosphoinositide turnover in fibroblasts (Habenicht *et al.*, 1981), was capable of mobilizing calcium, whereas CSF-1, which lacks this activity in macrophages (Whetton *et al.*, 1986), was not.

The inability of human CSF-1R to phosphorylate PLC-γ in mouse fibroblasts could reflect mobilization of the receptor across cell- and species-specific barriers. We therefore performed parallel experiments using an SV40-immortalized mouse macrophage cell line, BAC1.2F5 (Morgan *et al.*, 1987), that expresses high levels of murine CSF-1R and strictly depends on exogenous CSF-1 for its survival and proliferation in culture. The cells were starved for CSF-1 for 18 h to up-regulate their cell surface receptors and were then stimulated with saturating concentrations of the growth factor for 5 min. Under these conditions, mouse CSF-1R becomes phosphorylated on tyrosine and induces tyrosine phosphorylation of a series of heterologous cellular proteins (Downing *et al.*, 1988). Detergent lysates were immunoprecipitated with antiserum to PLC-γ, and the precipitated proteins were separated on denaturing polyacrylamide gels, transferred to nitrocellulose, and reacted with antisera to PLC-γ or to phosphotyrosine.

Figure 5 shows that BAC1.2F5 macrophages express immunoprecipitable PLC-γ (lane 1) with an electrophoretic mobility indistinguishable from that expressed in mouse NIH3T3 cells (lane 3). Following stimulation of BAC1.2F5 cells with CSF-1, the apparent mol. wt of macrophage PLC-γ was unchanged (lane 2). Immunoblotting with antiserum to phosphotyrosine failed to detect immunoprecipitated PLC-γ either before (lane 4) or after (lane 5) CSF-1 stimulation of the cells. Thus, although PLC-γ is expressed in a mouse macrophage cell line, it was not detectably phosphorylated on tyrosine by murine CSF-1R.

Discussion

Certain polypeptide growth factors, such as EGF (Pike and Eakes, 1987; Hepler *et al.*, 1987; Johnson and Garrison, 1987; Olashaw and Pledger, 1988; Habenicht *et al.*, 1981) and PDGF (Habenicht *et al.*, 1981), can enhance the rate of formation of IP3, leading to mobilization of calcium from intracellular stores. The hydrolysis of PIP2 by PLC also produces 1,2-diacylglycerol, another second messenger that activates protein kinase C (Berridge, 1987). In intact A431 cells, stimulation of PLC activity by EGF correlates with the rapid EGF-dependent phosphorylation of both EGF-R and PLC-γ on tyrosine and with the formation of immunoprecipitable complexes containing these two tyrosine phosphorylated proteins (Wahl *et al.*, 1988, 1989a; Meisenheleder *et al.*, 1989; Margolis *et al.*, 1989). Although it has not been demonstrated that phosphorylation of PLC-γ on tyrosine by EGF-R either enhances its enzymatic activity, changes its substrate specificity or alters its subcellular topology, both the IP3 response and the activation of protein kinase C by diacylglycerol might be coupled to the activity of receptor tyrosine kinases through such a mechanism.

A related issue is whether other receptors of the tyrosine kinase family, or the non-receptor tyrosine kinases encoded by certain other proto-oncogenes, are able to exert any of their mitogenic effects through the agency of phospholipase C isoforms. PLC-γ also appears to be a physiologic substrate of the PDGF-R tyrosine kinase. Recent studies (Meisenheleder *et al.*, 1989; Wahl *et al.*, 1989b), in agreement with results reported here, indicated that PDGF, like EGF, induced tyrosine phosphorylation of PLC-γ in fibroblasts and that a fraction of the total tyrosine phosphorylated PDGF receptor could be co-precipitated with PLC-γ. Although other PLC isozymes were not detectably phosphorylated in response to EGF in living cells (Margolis *et al.*, 1989), PDGF also induced incorporation of trace amounts of phosphotyrosine into PLC-β1 (Meisenheleder *et al.*, 1989). It is possible that some PDGF-R molecules associate with PLC-γ prior to PDGF stimulation but cannot be detected in our assay until they become phosphorylated on tyrosine. Alternatively, PLC-γ may associate with PDGF-R only after the receptor undergoes a ligand-induced conformational that

correlates with up-regulation of its kinase activity (Keating *et al.*, 1988; Williams, 1989). Clearly, only a small fraction of the tyrosine phosphorylated form of the receptor was co-precipitated with antiserum to PLC-γ, suggesting that variables other than tyrosine phosphorylation of PDGF-R itself must determine the ability of the receptor to associate with PLC-γ.

Although the genes encoding the receptors for the PDGF B chain and CSF-1 are closely related in their structure, chromosomal location and organization (Yarden *et al.*, 1986; Roberts *et al.*, 1988), their patterns of tissue-specific expression are divergent and possibly mutually exclusive. In adult animals, CSF-1R is restricted in its expression to monocytes, macrophages and their committed bone marrow progenitors where it supports cell proliferation, differentiation and survival (Stanley *et al.*, 1983; Sherr and Stanley, 1989). In bone marrow-derived macrophages, CSF-1 stimulates proliferation without inducing inositol lipid breakdown (Whetton *et al.*, 1986), suggesting that CSF-1R is not directly coupled to phospholipase C. In agreement, SV40-immortalized murine macrophages were found to express an immunoprecipitable 145 kd PLC isoform which was indistinguishable in molecular mass from PLC-γ detected in NIH3T3 cells and was not detectably phosphorylated on tyrosine following CSF-1 stimulation.

When human CSF-1R was transduced and expressed in mouse NIH3T3 fibroblasts, we were similarly unable to demonstrate more than trace levels of CSF-1-dependent phosphorylation of PLC-γ or calcium mobilization under conditions in which PDGF stimulation led to phosphorylation of PLC-γ on tyrosine and a detectable calcium signal. In normalized reactions, where the levels of tyrosine phosphorylation of CSF-1R and PDGF-R were equivalent, no PLC-γ tyrosine phosphorylation was induced by saturating doses of CSF-1. In contrast, limiting doses of PDGF induced readily detectable PLC-γ tyrosine phosphorylation. Even when PLC-γ was stably phosphorylated on tyrosine after vanadate treatment of intact cells, addition of high doses of CSF-1 prior to cell lysis led to no detectable increase in PLC-γ phosphorylation, in spite of the fact that, under these conditions, other cellular proteins undergo rapid phosphorylation on tyrosine (Downing *et al.*, 1988). PLC-γ phosphorylation on tyrosine was similarly not detected in NIH3T3 cells expressing 4-fold higher levels of an oncogenic human CSF-1R mutant allele (Roussel *et al.*, 1988) (data not shown). Thus, when co-expressed in NIH3T3 cells, CSF-1R and PDGF-R show differential behavior with respect to PLC-γ phosphorylation, even though both receptors readily mediate ligand-induced mitogenic responses.

We cannot exclude the possibility that CSF-1R or its oncogenic derivatives can interact with other PLC isoforms or indirectly affect PLC activity. For example, mink lung fibroblasts stably transformed by the feline v-*fms* gene were found to express higher levels of membrane-bound, guanine nucleotide-dependent PLC activity and exhibited faster rates of phosphoinositide turnover than their parental non-transformed counterparts (Jackowski *et al.*, 1986). In contrast, after introduction of human CSF-1R into CSF-1-unresponsive Chinese hamster lung fibroblasts, CSF-1 induced DNA synthesis in serum-starved cells without directly triggering inositol phosphate formation. However, CSF-1 slightly enhanced the activity of PLC agonists, such as thrombin, whereas pertussis toxin partially inhibited both CSF-1-induced DNA synthesis and activation of Na^+/H^+ exchange, pointing to the possibility of 'cross-talk' between different signaling pathways (Hartmann *et al.*, 1989). The simplest interpretation is that CSF-1-induced mitogenesis does not require accelerated phosphatidylinositol turnover (Whetton *et al.*, 1986), but rather depends upon another second messenger system.

Materials and methods

Cell culture and lysis conditions

NIH3T3 cells transfected with the human c-*fms* gene (Coussens *et al.*, 1986; Roussel *et al.*, 1987) and expressing $\sim 2 \times 10^4$ CSF-1 binding sites per cell (Roussel *et al.*, 1988) were maintained in 150 mm diameter culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), glutamine and antibiotics. Sixteen hours prior to addition of growth factors, subconfluent cultures were transferred to complete medium containing 1% FCS to render them quiescent. In some experiments, 50 μM Na orthovanadate was also added to metabolically inhibit phosphotyrosine phosphatase activity. Cells were stimulated at 37°C for 5 min by addition of the indicated concentrations of purified porcine PDGF (B chain homodimer) (ICN Biochemicals, Costa Mesa, CA) or with a saturating concentration (4 nM) of human recombinant CSF-1 produced in mammalian CHO cells (generously provided by Dr Steven Clark, Genetics Institute, Cambridge, MA). Although NIH3T3 cells synthesize CSF-1, the mouse growth factor does not bind to the human receptor with high enough affinity to elicit a biological response (Roussel *et al.*, 1987, 1988). Cells were rinsed at 4°C with phosphate-buffered saline (PBS) and scraped from the plates in lysis buffer (1 ml per culture dish) containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride (PMSF) (protease inhibitors from Sigma Chemicals, St Louis, MO), 100 mM NaF, 6 mM tetrasodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate and 0.1 mM Na orthovanadate. The lysates were incubated for 5 min on ice and centrifuged at 12 000 *g* for 5 min to remove nuclei and debris.

The SV40-immortalized mouse macrophage cell line, BAC1.2F5, absolutely requires CSF-1 for its proliferation and survival in culture and expresses $0.5-1.0 \times 10^5$ CSF-1 binding sites per cell (Morgan *et al.*, 1987). BAC1.2F5 cultures were maintained in DMEM containing 10% FCS, glutamine, antibiotics and 2000 units/ml (0.9 nM) recombinant CSF-1. Subconfluent cultures were transferred to complete medium lacking CSF-1 for 18 h and then stimulated for 5 min with 5 nM of the growth factor prior to lysis as above.

Immunoprecipitation and immunoblotting

Immunoadsorbants were prepared by incubating 3 μg of protein A–Sepharose (Sigma Chemicals) in 20 mM HEPES, pH 7.5, with empirically determined quantities of the indicated rabbit antisera (15–100 μl depending on titer) for 20 min at 22°C. The adsorbed Sepharose beads were washed three times with 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100 [HNTG Buffer] and added to cell lysates (3 μg immunoadsorbant per ml lysate) for 90 min at 4°C. Under these conditions, immunoprecipitations performed with rabbit antisera to a carboxy-terminal peptide of rat brain PLC-γ (Sung *et al.*, 1987) or to a v-*fms*-coded polypeptide produced in bacteria (Furman *et al.*, 1986) were quantitative, with no residual antigen remaining in the fluid phase. Immunoprecipitates were washed three times with HNTG buffer, resuspended in Laemmli's buffer (Laemmli, 1970), boiled and separated under reducing conditions on denaturing polyacrylamide gels containing sodium dodecyl sulfate (SDS) (Anderson *et al.* 1984). Total cell lysates (50 μl) were diluted with an equal volume of 2 × Laemmli buffer, boiled and separated on denaturing polyacrylamide gels as above. The separated proteins were transferred to nitrocellulose, and blotted (Roussel *et al.*, 1984) either with rabbit antiserum to porcine brain PLC-γ or with antiserum prepared to phosphotyrosine coupled to keyhole limpet hemocyanin (Margolis *et al.*, 1989). Bound antibodies were detected with [^{125}I]protein A (Amersham, Arlington Heights, IL).

Measurements of calcium flux

Cells were incubated at 37°C for 30 min in complete medium supplemented with 10 mM HEPES and 10 μg/ml INDO-1 (acetoxymethyl ester, Molecular Probes, Junction City, OR) and then centrifuged, resuspended at 10^6 cells/ml in the same medium and placed on ice. Fluorescence measurements

were performed on an EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL), using UV laser excitation (351.1–363.8 nm, 70 mW) and simultaneous detection of fluorescence emission at 400 nm ± 20 nm and 480 ± 20 nm. The ratio of 480/400 fluorescence was recorded and displayed as a function of time. After establishing a stable baseline reading for each sample, either human recombinant CSF-1 (final concentration = 0.4 μg/ml), porcine PDGF (final concentration = 0.1 μg/ml) or the calcium ionophore, ionomycin (final concentration = 8.3 μg/ml; Behring Diagnostics, San Diego) was injected and sampling was continued. A shift in the INDO-1 fluorescence emission spectrum accompanies binding to calcium ions, resulting in a reduction of the recorded fluorescence ratio corresponding to the increase in intracellular calcium. The mean fluorescence ratio was computed as a function of time after factor addition.

Acknowledgements

We thank John Kayoma for excellent technical assistance, Dr Steven Clark of Genetics Institute, Cambridge, MA, for providing purified human recombinant CSF-1 and Dr E.Richard Stanley for providing BAC1.2F5 cells. This work was supported in part by NIH grants CA-01429 (J.R.D.) and CA-47064 (C.J.S.), by Cancer Center Core Grant CA-21765 and by the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital.

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Received on May 29, 1989; revised on July 10, 1989