The EMBO Journal vol.9 no.8 pp.2423 – 2429, 1990

Colony-stimulating factor 1 activates protein kinase C in human monocytes

Kyoko Imamura, Ann Dianoux, Takashi Nakamura and Donald Kufe

Laboratory of Clinical Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA

Communicated by R.Kamen

Colony-stimulating factor 1 (CSF-1) is required for the survival, proliferation and differentiation of monocytes. We previously demonstrated that the CSF-1 receptor is linked to a pertussis toxin-sensitive G protein and that the induction of Na^+ influx by CSF-1 is a pertussis toxin-sensitive event. The present studies have examined activation of protein kinase C as a potential intracellular signaling event induced by the activated CSF-1 receptor. The results demonstrate that CSF-1 stimulates translocation of protein kinase C activity from the cytosol to membrane fractions. This activation of protein kinase C was sensitive to pretreatment of the monocytes with pertussis toxin. Lipid distribution studies demonstrated that phosphatidylcholine (PC) is the major phospholipid in human monocytes. Moreover, the results indicate that CSF-1 stimulation is associated with decreases in PC, but not in phosphatidylinositol (PI), levels. The absence of an effect of CSF-1 on PI turnover was confirmed by the lack of changes in inositol phosphate production. In contrast, CSF-1 stimulation was associated with increased hydrolysis of PC to phosphorylcholine and diacylglycerol (DAG) in both intact monocytes and cell-free assays. Furthermore, the increase in PC turnover induced by CSF-1 was sensitive to pertussis toxin. The results also demonstrate that the induction of Na^+ influx by CSF-1 is inhibited by the protein kinase C inhibitors staurosporine and the isoquinoline derivative H7, but not by HA1004. The present findings thus suggest that: (i) CSF-1 transduces signals via a pertussis toxin-sensitive G protein that result in the activation of protein kinase C; (ii) CSF-1-induced activation of protein kinase C is associated with increased PC turnover and DAG production; and (iii) this activation of protein kinase C plays a role in the stimulation of Na^+ influx.

Key words: colony stimulating factor/monocytes/pertussis toxin/protein kinase C

Introduction

Colony-stimulating factor 1 (CSF-1) regulates the survival, growth and differentiation of monocytes by binding to a single class of high affinity cell surface receptors (Stanley *et al.*, 1983). The CSF-1 receptor is identical to the product of the c-*fms* proto-oncogene (Sherr *et al.*, 1985) and is a member of a family of growth factor receptors with tyrosine kinase activity (Yarden and Ullrich, 1988). The detection of both CSF-1 and c-*fms* gene expression in cells differentiated along the monocytic lineage has suggested that CSF-1 may act by an autocrine mechanism in the regulation of monocytes (Nienhuis *et al.*, 1985; Sariban *et al.*, 1985; Wakamiya *et al.*, 1987; Horiguchi *et al.*, 1987, 1988).

There are presently few insights regarding the mechanisms whereby CSF-1 elicits its effects. CSF-1 enhances autophosphorylation of c-*fms* protein and down-regulates this receptor on macrophages (Guilbert and Stanley, 1986). Furthermore, receptor down-regulation is associated with CSF-1 internalization and degradation (Guilbert and Stanley, 1986). Other studies have demonstrated that CSF-1 stimulates protein synthesis (Tushinski and Stanley, 1983, 1985), glucose uptake (Hamilton *et al.*, 1988) and Na^+, K^+-ATPase activity (Vairo and Hamilton, 1988) during induction of macrophage DNA synthesis. Moreover, recent work has suggested that the CSF-1 receptor is linked to a pertussis toxin-sensitive G protein, and that a similar or identical protein is involved in CSF-1-induced Na^+ influx (Imamura and Kufe, 1988). Other studies have demonstrated that CSF-1-induced proliferation is a pertussis toxin-sensitive event (Imamura and Kufe, 1988; He *et al.*, 1988). While these findings indicated that a pertussis toxin-sensitive G protein is involved in transducing signals from the stimulated CSF-1 receptor, it was not clear whether other events are induced by CSF-1 in this signaling pathway.

Certain polypeptide growth factors stimulate mitogenesis by activating a phospholipase C that hydrolyzes phosphatidylinositol 4,5 diphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Macara, 1985; Berridge, 1987). However, other mitogens such as insulin appear to stimulate proliferation in the absence of PIP_2 turnover (L'Allemain and Pouyssegur, 1986). While v-*fms* transformation has been associated with activation of a PIP_2-specific phospholipase C (Jackowski *et al.*, 1986), studies with CSF-1 have demonstrated that proliferation of mouse bone marrow-derived macrophages is not associated with hydrolysis of inositol lipids (Whetton *et al.*, 1986). These findings have suggested that alternative CSF-1 receptor-mediated events may result in mitogenesis. For example, other work has demonstrated that platelet-derived growth factor or serum stimulation is associated with activation of a phosphatidylcholine (PC)-specific phospholipase C that catalyzes the formation of DAG and phosphorylcholine (Besterman *et al.*, 1986). Moreover, recent studies have demonstrated that interleukin 1 (IL-1) stimulates PC, but not PI, hydrolysis in T lymphocytes (Rosoff *et al.*, 1988). This effect is responsible for increased DAG production in IL-1-treated cells and the subsequent activation of protein kinase C.

The present studies demonstrate that CSF-1 activates protein kinase C in human monocytes. This activation of protein kinase C is associated with increased PC hydrolysis and DAG formation. We also demonstrate that these events are sensitive to pertussis toxin.

Results

Effects of CSF-1 on activation of protein kinase C

Previous studies have demonstrated that several growth factors stimulate proliferation via protein kinase C activation (Rozengurt, 1986; Whetton *et al.*, 1988). To examine if CSF-1 activates protein kinase C in human monocytes, we stimulated cells with CSF-1 and measured this enzyme activity in both the membrane and cytosol fractions. CSF-1 treatment was associated with an increase in membrane-bound protein kinase C activity (Figure 1A). Furthermore, this transient increase in membrane-bound protein kinase C was accompanied by a decline in cytosolic enzyme activity (Figure 1A). While this finding indicated that CSF-1 activates protein kinase C, the relationship of this event to signaling events transduced by G proteins was unclear. However, the translocation of protein kinase C activity by CSF-1 was inhibited by pretreatment of the monocytes with pertussis toxin (Figure 1B). Taken together, these findings indicated that CSF-1 also activates protein kinase C by a pertussis toxin-sensitive mechanism, possibly through enhanced DAG formation.

Effects of CSF-1 on intracellular DAG formation

The finding that CSF-1 stimulates protein kinase C activation prompted us to study DAG production in monocytes. The cells were labeled with [^{3}H]glycerol for 48 h and then stimulated with 1000 U/ml CSF-1 for up to 30 min. An increase in [^{3}H]DAG production was detectable at 5 min of CSF-1 treatment (Figure 2A). Moreover, DAG levels remained elevated in the CSF-1 treated cells for the 30 min incubation period. In contrast, treatment of monocytes with boiled CSF-1 or lipopolysaccharide (2.5 fg/ml) had little if any effect on DAG production (data not shown). Similar experiments were performed using monocytes pretreated with pertussis toxin. While pertussis toxin had no detectable effect on [^{3}H]DAG levels in unstimulated monocytes, this toxin inhibited the increases in [^{3}H]DAG production induced by CSF-1 (Figure 2B). These findings were thus in concert with the demonstration that CSF-1 activates protein kinase C by a pertussis toxin-sensitive mechanism.

Effects of CSF-1 on membrane phospholipids

Previous studies have demonstrated that certain growth factors stimulate proliferation by activation of signaling pathways that involve hydrolysis of membrane phospholipids (Macara, 1985; Berridge, 1987). In this regard, we first analyzed the distribution of lipids in resting human monocytes. Monocytes were labeled with [^{3}H]oleic acid or [^{14}C]stearic acid and the lipids extracted for analysis by thin layer chromatography. Incorporation of these two fatty acids into lipids revealed similar distribution patterns with PC representing the major component (data not shown). The monocytes were also incubated with [^{3}H]glycerol to study further phospholipid distribution, as well as the effects of CSF-1 stimulation. In these experiments, PC was similarly identified as the major phospholipid (Figure 3). Furthermore, treatment of these monocytes with 1000 U/ml CSF-1 for 30 min was associated with an ~40% decrease in PC level (Figure 3). In contrast, there was little if any effect of CSF-1 on other membrane phospholipids. These findings suggested that CSF-1 treatment of monocytes is associated with alterations in PC, but not PI, metabolism.

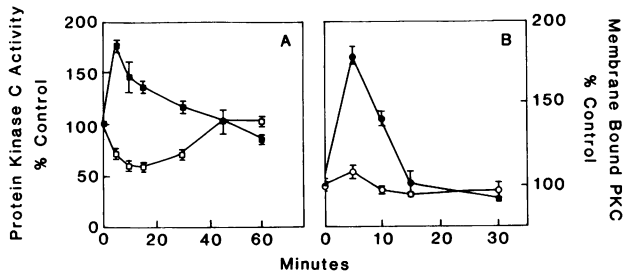

Fig. 1. Effects of CSF-1 on protein kinase C activity. (**A**) Monocytes were treated with 1000 U/ml CSF-1 for the indicated times and assayed for protein kinase C activity in the cytosol (□) and membrane (■) fractions. Protein kinase C activity in the cytosol and membrane fractions of unstimulated or control cells was 3570 and 2216 pmol/mg protein/min, respectively. (**B**) Monocytes were pre-incubated in the absence (●) and presence (○) of 100 ng/ml pertussis toxin for 4 h at 37°C before adding 1000 U/ml CSF-1 for the indicated times. Protein kinase C (PKC) activity was monitored in the membrane fraction. The results represent the mean ± SD of three determinations.

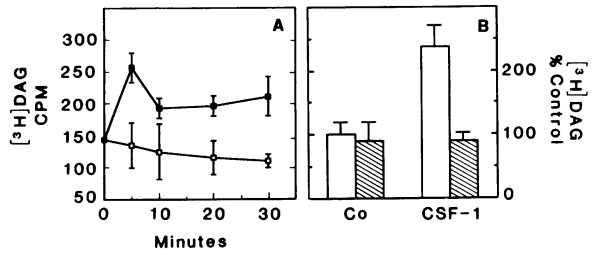

Fig. 2. Effects of CSF-1 on DAG production. (**A**) Monocytes were labeled with [^{3}H]glycerol for 48 h and then incubated in the absence (□) and presence (■) of 1000 U/ml CSF-1 for the indicated times. The lipids were extracted for analysis of [^{3}H]DAG. (**B**) Monocytes were labeled with [^{3}H]glycerol for 48 h and then incubated in the presence (open bars) and absence (striped bars) of 100 ng/ml pertussis toxin for 4 h. After stimulation with CSF-1 for 30 min, the cells were analyzed for [^{3}H]DAG production. These results (mean ± SD) are from five separate experiments each performed in triplicate.

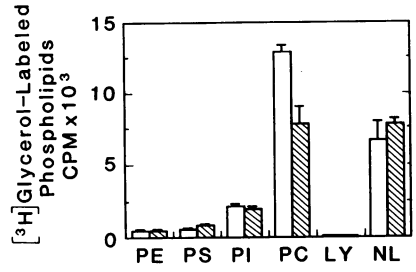

Fig. 3. Effects of CSF-1 on phospholipid distribution. Monocytes were labeled with [^{3}H]glycerol for 48 h and then incubated in the absence (open bars) or presence (striped bars) of 1000 U/ml CSF-1 for 30 min. Lipids were extracted and analyzed by TLC. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; LY, lysophospholipids; and NL, neutral lipids. The results are expressed as the mean ± SD of three determinations. Similar findings were obtained in four separate experiments.

Effects of CSF-1 on phosphatidylinositol turnover

Previous studies have demonstrated that CSF-1-induced proliferation of mouse bone marrow-derived macrophages is not associated with PI hydrolysis (Whetton *et al.*, 1986). However, the finding that v-*fms* transformation activates a

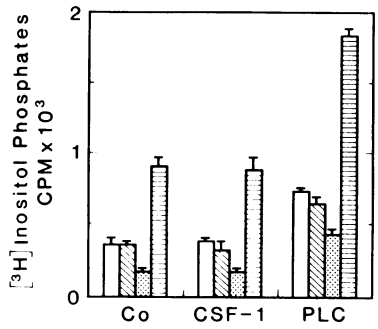

Fig. 4. Effects of CSF-1 and exogenous phospholipase C on inositol phosphates production. Monocytes were labeled with myo[2-^{3}H]inositol for 48 h and then 10 mM lithium chloride for 10 min before adding 1000 U/ml CSF-1 or 1 U/ml phospholipase C (PLC). After 10 min the lipids were extracted and analyzed for IP_1 (□), IP_2 (▨), IP_3 (▦) or total inositol phosphate (□) production on Dowex-1 formate columns. These results expressed as the mean ± SD of three determinations are representative of three separate experiments.

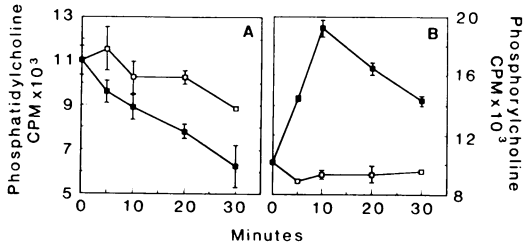

Fig. 5. Effects of CSF-1 on PC hydrolysis and phosphorylcholine production. Monocytes were labeled with [^{3}H]choline chloride for 48 h and then incubated in the absence (□) and presence (■) of 1000 U/ml CSF-1 for the indicated times. Following extraction, [^{3}H]PC was identified in the organic fraction (**A**) and [^{3}H]phosphorylcholine in the aqueous fraction (**B**) by TLC. These results (mean ± SD) are representative of four experiments. The decrease in PC was 30.9 ± 10.9% and the increase in phosphorylcholine was 142.8 ± 9.0% at 30 min of CSF-1 treatment for the four experiments.

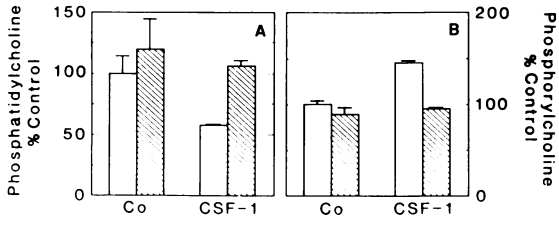

Fig. 6. Effects of pertussis toxin on CSF-1-stimulated phosphatidylcholine hydrolysis. Monocytes were labeled with [^{3}H]choline for 48 h and then incubated in the absence (open bars) and presence (striped bars) of 100 ng/ml pertussis toxin for 4 h. After stimulation with 1000 U/ml CSF-1 for 30 min, the cells were extracted and analyzed for [^{3}H]PC (**A**) and [^{3}H]phosphorylcholine (**B**). These results of three determinations (mean ± SD) are representative of three separate experiments.

PI-specific phospholipase C (Jackowski *et al.*, 1986) prompted examination of PI turnover in CSF-1-stimulated human monocytes. Monocytes were labeled with [^{3}H]inositol and following CSF-1 treatment the lipids were extracted for analysis of inositol 1-monophosphate (IP_1), inositol 1,4-bisphosphate (IP_2) and IP_3 by anion-exchange column chromatography. Stimulation of monocytes with 1000 U/ml CSF-1 for 10 min had no detectable effect on production of IP_1, IP_2 or IP_3 (Figure 4). In contrast, exposure of the labeled monocytes to exogenous phospholipase C (Stone *et al.*, 1988) resulted in significant increases in each of these inositol phosphates (Figure 4). Similar findings were obtained at 1, 3 and 5 min of CSF-1 exposure (data not shown). Taken together with the absence of an effect on PI levels, these findings suggested that stimulation of both human monocytes and murine macrophages (Whetton *et al.*, 1986) with CSF-1 is not associated with detectable hydrolysis of this phospholipid.

Effects of CSF-1 on phosphatidylcholine hydrolysis

In view of the finding that CSF-1 treatment was associated with enhanced DAG production (Figure 2) and decreases in the level of PC (Figure 3), we examined in further detail the possibility that hydrolysis of this phospholipid is induced by CSF-1. In these experiments, monocytes were labeled with [^{3}H]choline for 48 h and then stimulated with CSF-1 for up to 30 min. CSF-1 treatment was associated with decreases in PC levels to ~70% that of control cells at 30 min (Figure 5A). This effect of CSF-1 was also associated with a stimulation of phosphorylcholine production (Figure 5B). In contrast, these effects on PC turnover and phosphorylcholine production were not detected when monocytes were exposed to: (i) CSF-1 that had been boiled for 60 min; or (ii) lipopolysaccharide at a 10-fold higher concentration (2.5 fg/ml) than that in 1000 U/ml CSF-1 (data not shown).

Our previous studies indicated that the CSF-1 receptor is linked to a pertussis toxin-sensitive GTP-binding protein (Imamura and Kufe, 1988). Consequently, it was of interest to determine whether CSF-1-induced PC hydrolysis was also sensitive to this toxin. Monocytes were therefore pretreated with pertussis toxin and then monitored for PC turnover after stimulation with CSF-1 for 30 min. Pertussis toxin had little if any effect on PC levels in unstimulated monocytes (Figure 6A). In contrast, the decrease in PC level associated with CSF-1 treatment was inhibited by the toxin (Figure 6A). Furthermore, the increase in phosphorylcholine production was also inhibited by pertussis toxin (Figure 6B). These findings suggested that CSF-1-induced stimulation of PC hydrolysis is regulated by a pertussis toxin-sensitive G protein.

Effects of CSF-1 on cell-free phospholipase activity

The demonstration that CSF-1 stimulates PC hydrolysis was also examined by monitoring phospholipase activity in cell lysates. Monocytes were treated with CSF-1 and sonicates were assayed for phospholipase activity by monitoring hydrolysis of [^{14}C]PC to [^{14}C]DAG. CSF-1-treated monocytes had an increase in phospholipase activity that was detectable by 5 min and reached maximal levels by 20 min of stimulation (Figure 7A). The pretreatment of these monocytes with pertussis toxin had no detectable effect on levels of constitutive phospholipase-mediated PC hydrolysis (Figure 7B). In contrast, pertussis toxin pretreatment inhibited the increase in [^{14}C]DAG formation associated with CSF-1 stimulation (Figure 7B). These findings further supported the involvement of a pertussis toxin-sensitive G protein in transducing signals from the ligand-activated CSF-1 receptor to a phospholipase that hydrolyzes PC.

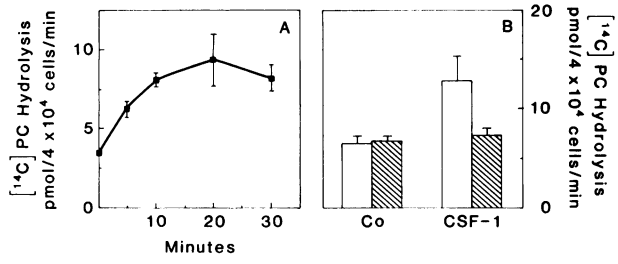

Fig. 7. Effects of CSF-1 on [^{14}C]PC hydrolysis. (**A**) Monocytes (14×10^4) were treated with 1000 U/ml CSF-1 for the indicated times. The cells were then sonicated and incubated with [^{14}C]PC. The release of [^{14}C]DAG was monitored by TLC and used to determine phospholipase activity. (**B**) Monocytes were pre-incubated in the absence (open bars) and presence (striped bars) of 100 ng/ml pertussis toxin for 4 h before adding 1000 U/ml CSF-1 for 30 min. The cells were then assayed for [^{14}C]PC hydrolysis. The results are expressed as the mean ± SD of three determinations. These results are representative of four separate experiments.

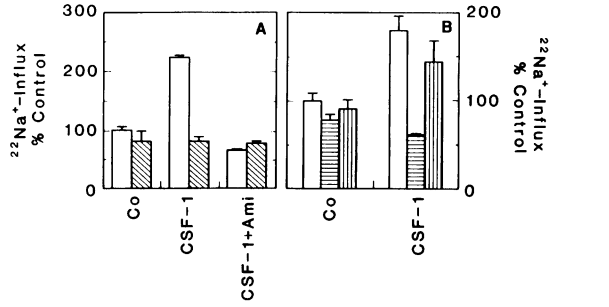

Fig. 8. Effects of protein kinase C inhibitors on CSF-1-induced Na^+ influx. (**A**) Monocytes were incubated in the absence (open bars) and presence (striped bars) of 1 μM staurosporine at 37°C for 5 min. The monocytes were then stimulated with CSF-1 for 20 min and then monitored for $^{22}Na^+$ influx. The effect of 2 mM amiloride was determined by pretreating cells with this agent for 5 min before washing and adding the CSF-1. The $^{22}Na^+$ influx in untreated or control cells was 11 686 c.p.m./mg protein/20 min. The results are expressed as the mean ± SD of three determinations. (**B**) Monocytes were incubated in medium (□), 10 μM H7 (▤) or 10 μM HA1004 (▥) for 5 min. The cells were then stimulated with CSF-1 for 20 min and monitored for $^{22}Na^+$ influx. The $^{22}Na^+$ influx in control cells was 12 440 c.p.m./mg protein/20 min. These results (mean ± SD of three determinations) are representative of three separate experiments.

Relationship between activation of protein kinase C and Na^+ influx

Previous studies have demonstrated that CSF-1 stimulates Na^+ influx into monocytes (Imamura and Kufe, 1988; Vaira and Hamilton, 1988) and that signaling of this event involves transduction by a pertussis toxin-sensitive G protein (Imamura and Kufe, 1988). Other work has demonstrated that activation of protein kinase C can be an intermediate event between ligand-induced receptor stimulation and enhanced Na^+/H^+ exchange (Besterman and Cuatrecasas, 1984; Grinstein and Rothstein, 1986; Rozengurt, 1986). Consequently, we studied the relationship of CSF-1-induced protein kinase C activation and Na^+ influx. This issue was first approached by treating monocytes with staurosporine, an inhibitor of protein kinase C (Tamaoki *et al.*, 1987; Vegesna *et al.*, 1988). Staurosporine inhibited membrane-bound protein kinase C activity in unstimulated and CSF-1-stimulated monocytes (data not shown). This agent had little if any effect on Na^+ influx into unstimulated monocytes (Figure 8A). Moreover, staurosporine had no detectable effect on Na^+ influx into monocytes treated with amiloride, an inhibitor of Na^+/H^+ exchange (data not shown). In contrast, while CSF-1 stimulated Na^+ influx into these cells, staurosporine completely inhibited this effect (Figure 8A). The CSF-1-stimulated Na^+ influx was also inhibited by amiloride and similar results were obtained when these cells were treated with both amiloride and staurosporine (Figure 8A).

Since staurosporine is a nonspecific protein kinase inhibitor, we further addressed this issue with H7, a structurally distinct isoquinolinesulfonamide derivative that also inhibits protein kinase C (Hidaka *et al.*, 1984). H7 had little effect on constitutive Na^+ influx into unstimulated monocytes, while this agent inhibited the increases in Na^+ uptake induced by CSF-1 (Figure 8B). Since H7 can inhibit other protein kinases, we also used similar concentrations of HA1004, a more selective inhibitor of cGMP- and cAMP-dependent protein kinases (Asano and Hidaka, 1984). HA1004 had minimal effects on Na^+ influx into unstimulated or CSF-1-stimulated monocytes (Figure 8B). Taken together, these findings indicated that staurosporine and H7 inhibit Na^+ influx induced by CSF-1 and that CSF-1-induced Na^+ influx is dependent upon activation of protein kinase C. These findings were also in concert with the demonstration that both CSF-1-induced activation of protein kinase C and enhanced Na^+ influx are pertussis toxin-sensitive events.

Discussion

CSF-1 is necessary for the growth and differentiation of monocytes (Stanley *et al.*, 1983; Becker *et al.*, 1987). However, few details are available regarding the mechanisms by which this growth factor elicits these effects. The CSF-1 receptor is identical to the product of the c-*fms* proto-oncogene (Sherr *et al.*, 1985) and undergoes autophosphorylation on tyrosine following ligand stimulation (Guilbert and Stanley, 1986). Recent studies have examined phosphorylation events following binding of CSF-1 to receptors on the BAC1.2FS murine macrophage cell line (Downing *et al.*, 1988; Sengupta *et al.*, 1988). Multiple substrates have been identified, including autophosphorylation of the CSF-1 receptor (Downing *et al.*, 1988; Sengupta *et al.*, 1988). Other studies in v-*fms* transformed NIH3T3 cells have similarly demonstrated the presence of multiple proteins with increased phosphotyrosine content (Morrison *et al.*, 1988). However, the identity of these substrates, as well as the function of the c-*fms* tyrosine kinase activity in intracellular signaling, remains unclear.

The present studies have examined the role of phospholipid hydrolysis in CSF-1-induced signaling events. Certain polypeptide growth factors stimulate mitogenesis by phospholipase C-mediated hydrolysis of phosphoinositides to DAG and inositol phosphates (Macara, 1985; Berridge, 1987). However, previous studies have demonstrated that CSF-1-stimulated proliferation of mouse macrophages is unrelated to turnover of inositol lipids (Whetton *et al.*, 1986). Similar findings have been obtained in the present work. In this regard, there was no detectable decline in PI levels in CSF-1-treated human monocytes. Furthermore, there were no detectable increases in IP_1, IP_2 or IP_3 levels following CSF-1 stimulation. Taken together, these findings would thus

suggest that ligand-induced activation of the CSF-1 receptor is not associated with stimulation of PI-specific phospholipase C activity in monocytes. Nonetheless, other work has indicated that constitutive activation of this receptor in v-*fms* transformed mink lung cells is associated with increased activity of a PIP_2-specific phospholipase C (Jackowski *et al.*, 1986). Thus, stimulation of PI-specific phospholipase C activity may play a role in v-*fms*-induced transformation, but not in CSF-1-stimulated signaling events.

Lipid distribution studies in the present experiments demonstrated that PC is a major component in [^{3}H]oleic acid and [^{14}C]stearic acid labeled resting human monocytes. PC was also identified as the major phospholipid in monocytes labeled with [^{3}H]glycerol. Interestingly, CSF-1 stimulation was associated with decreases in PC level, while there was little if any effect on levels of PI, phosphatidylserine and phosphatidylethanolamine. This increase in PC turnover was also associated with enhanced production of phosphorylcholine. Phosphorylcholine arises from the hydrolysis of PC by phospholipase C, as well as from choline kinase activity (Warden and Friedkin, 1985). The increase in phosphorylcholine production was greater than that expected from the decreases in PC level. This finding may be related to differences in sensitivity of the assays used to measure these metabolites. Alternatively, phosphorylcholine may be produced as a result of increases in both PC hydrolysis and choline kinase activity. In any event, the demonstration that CSF-1 stimulates phospholipase-mediated hydrolysis of [^{14}C]PC in cell-free extracts supports the production of phosphorylcholine, at least in part, by CSF-1-induced PC turnover in intact monocytes.

Studies monitoring DAG production were also performed in CSF-1-treated monocytes to further examine the induction of phospholipase-mediated PC hydrolysis. In these experiments with [^{3}H]glycerol labeled monocytes, an increase in [^{3}H]DAG production was detectable at 5 min of CSF-1 treatment. This effect was associated with a translocation of protein kinase C activity from the cytosol to the membrane fraction. The increase in membrane protein kinase C activity was also maximal at 5 min of CSF-1 stimulation. Taken together, these findings suggested that CSF-1 stimulates phospholipase-mediated PC turnover in human monocytes. Furthermore, CSF-1-induced PC hydrolysis and DAG formation appear to be responsible for the activation of protein kinase C. Serum and platelet-derived growth factor have similarly been reported to stimulate PC hydrolysis and thereby activate protein kinase C as a result of increased DAG formation (Besterman *et al.*, 1986). Moreover, the present findings are similar to IL-1-induced protein kinase C activation in T lymphocytes by stimulation of PC hydrolysis and DAG production (Rosoff *et al.*, 1988).

The activation of protein kinase C in CSF-1-stimulated monocytes further suggested that the induction of other intracellular events might be related to this finding. For example, a variety of growth factors stimulate mitogenesis by increasing Na^+ influx coupled to H^+ efflux via an amiloride-sensitive antiport (Grinstein and Rothstein, 1986). The stimulation of this Na^+/H^+ exchange has been associated with the activation of protein kinase C in certain systems (Besterman and Cuatrecasas, 1984; Grinstein and Rothstein, 1986; Rozengurt, 1986). In contrast, certain mitogens have been shown to stimulate the Na^+/H^+ antiport in the absence of protein kinase C activation or after down-regulation of this enzyme (Vara *et al.*, 1984; Hesketh *et al.*, 1985; Vara and Rozengurt, 1985; Daniel and Ives, 1986). Previous work has demonstrated that CSF-1 stimulates Na^+ influx and Na^+, K^+-ATPase activity during induction of DNA synthesis (Imamura and Kufe, 1988; Vairo and Hamilton, 1988). However, the relationship of this event to activation of protein kinase C has been unknown. In the present studies, staurosporine inhibited CSF-1-induced activation of both protein kinase C and Na^+ influx. While this agent is a nonspecific protein kinase inhibitor (Tamaoki *et al.*, 1987; Vegesna *et al.*, 1988) other studies with H7, a structurally distinct inhibitor of protein kinase C (Hidaka *et al.*, 1984), also blocked the stimulation of Na^+ influx by CSF-1. These findings and the absence of an effect with HA1004, a more selective inhibitor of cyclic nucleotide-dependent kinases (Asano and Hidaka, 1984), would suggest that CSF-1-induced Na^+ influx is associated with the activation of protein kinase C by this factor. In this regard, treatment of human monocytes with TPA also stimulates amiloride-sensitive Na^+ influx (data not shown).

The present findings thus suggest that ligand-induced stimulation of the CSF-1 receptor is associated with a cascade of intracellular signaling events. Previous studies suggested that the CSF-1 receptor is linked to a pertussis toxin-sensitive G protein and that CSF-1-induced Na^+ influx is a pertussis toxin-sensitive event (Imamura and Kufe, 1988). The present results would indicate that a G protein transduces signals from the activated CSF-1 receptor to a phospholipase that hydrolyzes PC. In this context, CSF-1-induced PC turnover, DAG formation and activation of protein kinase C were all sensitive to pertussis toxin. Heterotrimeric G proteins transduce signals from cell surface receptors to a variety of enzyme effector systems, such as adenylate cyclase, phospholipase C and phospholipase A_2 (Gilman, 1984; Casey and Gilman, 1988). These proteins have also been implicated in the regulation of K^+ and Ca^{2+} channels (Neer and Clapham, 1988). Previous studies have demonstrated that PI-specific phospholipase C activity is regulated in certain systems by a pertussis toxin-sensitive G protein (Johnson *et al.*, 1986; Kikuchi *et al.*, 1986). PC hydrolysis in rat liver membranes is also regulated by a G protein, although this activity is insensitive to pertussis toxin (Irving and Exton, 1987). In contrast, the present findings suggest that phospholipase-mediated PC turnover in CSF-1-treated monocytes is coupled to a pertussis toxin-sensitive G protein. Two pertussis toxin substrates of M_r 41 000 and 40 000 have been identified in human monocytes (Imamura and Kufe, 1988) that are probably similar to G proteins designated $alpha_i$-2 and $alpha_i$-3 (Didsbury and Snyderman, 1987; Didsbury *et al.*, 1987; Backlund *et al.*, 1988). However, the identity of the G protein linked to the CSF-1 receptor remains unclear.

Other tyrosine kinase receptors have been linked to regulatory G proteins. The epidermal growth factor receptor activates both PI turnover and phospholipase A_2 activity via pertussis toxin-sensitive G proteins (Johnson *et al.*, 1986; Teitelbaum, 1990). EGF-dependent proliferation of human breast cancer cells is also sensitive to pertussis toxin (Church and Buick, 1988). Furthermore, the insulin receptor may interact with a G protein by mechanisms not directly involving its tyrosine kinase (Rothenberg and Kahn, 1988). However, there is as yet no direct evidence that tyrosine kinase receptors are coupled to G proteins. Indeed, the

available evidence suggests that these receptors may activate PI turnover by tyrosine phosphorylation of phospholipase C (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989). Pertussis toxin can elevate cAMP in certain cells, including the RAW264.7 murine macrophage cell line (Burch *et al.*, 1988). Moreover, recent studies have demonstrated that this cyclic nucleotide inhibits CSF-1-induced macrophage proliferation (Vairo *et al.*, 1990; Jackowski *et al.*, 1990). However, there is no detectable change in cAMP levels following treatment of human monocytes with pertussis toxin alone or in combination with CSF-1 (unpublished data). Thus, the present findings would suggest that the ligand-stimulated CSF-1 receptor activates phospholipase-mediated PC turnover via a pertussis toxin-sensitive G protein. Finally, the hydrolysis of PC to DAG and phosphorylcholine can occur through the activation of a PC-specific phospholipase C and/or through the combined activities of phospholipase D and phosphatidate phosphohydrolase. Further studies are needed to distinguish the involvement of these two pathways in CSF-1-stimulated human monocytes.

Materials and methods

Monocyte purification and culture

Human peripheral blood monocytes were separated by Ficoll-Hypaque gradient centrifugation and purified by adherence for 1 h. The adherent cell population was collected with a plastic policeman, repurified by adherence for 1 h, and then washed with three changes of medium. The final purified cell population consisted of >96% monocytes by morphologic examination of Wright–Giemsa stains. Cell purification and culturing were performed in RPMI 1640 medium with 10% fetal bovine serum (FBS). The monocytes were treated with 1000 U/ml human recombinant CSF-1 (Lot # FAP-805, 1.7 × 10^8 U/ml CSF-1, endotoxin <0.04 ng/ml; Dept. of Process and Product Development, Cetus Corp., Emeryville, CA), 1 μM staurosporine (Kyowa Hakko, Tokyo), 10 μM H7 (Seikagaku America, Inc.), 10 μM HA1004 (Seikagaku) and 2.5 fg/ml lipopolysaccharide (Sigma).

Measurement of protein kinase C activity

Monocytes were suspended in serum-free RPMI 1640 medium and incubated in the presence of CSF-1 or other agents at 37°C. The cells (10^7) were then collected immediately in a microcentrifuge. The cell pellets were suspended in 100 μl of distilled water, homogenized by passage through a 25-gauge needle and immediately reconstituted in buffer A (20 mM Tris–HCl, pH 7.5, 100 μg/ml aprotinin, 0.25 mM leupeptin and 1 mM phenylmethylsulfonylfluoride). The cytosolic fraction was obtained after centrifugation and the particulate fraction was solubilized in buffer A containing 1% Triton X-100 for 15 min on ice. Protein kinase C was separated by DEAE–cellulose column chromatography and elution with 80 mM NaCl. Aliquots of DEAE-purified protein kinase C were assayed for cytosolic or membrane activity in the presence of 10 mM $MgCl_2$, 1 mM $CaCl_2$, ~10^6 c.p.m. [γ-^{32}P]ATP (335 c.p.m./mmol; Amersham Corp., Arlington Heights, IL) and 20 μg of histone H1, with or without 8 μg/ml phosphatidylserine and 11.7 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA). After incubation at 30°C for 10 min, protein kinase C activity was determined by subtracting the amount of ^{32}P incorporation into histone H1 in the absence of added phospholipids and calcium from that in the presence of these agents.

Cell labeling and lipid extraction

Monocytes were labeled for 48 h in RPMI 1640 medium with 10% FBS containing 1 μCi/ml [2-^{3}H]glycerol (1 Ci/mmol), 2 μCi/ml [9,10(n)-^{3}H]oleic acid (5 Ci/mmol) or 0.1 μCi/ml [1-^{14}C]stearic acid (56 mCi/mmol) (Amersham Corp.). After washing three times in RPMI 1640, the monocytes were treated with various reagents at 37°C in RPMI 1640 without serum. At the end of each incubation, the cells were homogenized in 20 vol of chloroform/methanol (2:1, v/v) (Bligh and Dyer, 1959). The chloroform fraction was washed once with 0.2 vol of salt solution (0.02% $CaCl_2$, 0.017% $MgCl_2$, 0.29% NaCl, 0.37% KCl) and three times with solvent (chloroform:methanol:water 3:48:47, v/v/v) (Folch *et al.*, 1956). After centrifugation, the organic phase was removed and evaporated. Samples were solubilized in chloroform/methanol (2:1, v/v) and separated by thin layer chromatography (TLC).

Lipid separation by thin layer chromatography

Neutral lipids were separated on a Silica Gel G plate (Whatman, Clifton, NJ) with a solvent system of petroleum ether/diethyl ether/methanol/acetic acid (180:40:6:4, v/v/v/v) (Habenicht *et al.*, 1981). Separation of phospholipids by TLC was accomplished by modification of a procedure (Skipski *et al.*, 1964) using chloroform/ethanol/acetic acid/water (25:15:8:2, v/v/v/v). Authentic markers were co-chromatographed with the lipid fraction and visualized by iodine vapor. The appropriate regions were then scraped from the plate and monitored for radioactivity.

Phosphatidylinositol hydrolysis

Monocytes were labeled with 1 μCi/ml myo-[2-^{3}H]inositol (Amersham Corp.) for 48 h. The medium was replaced with RPMI 1640 containing 10 mM lithium chloride and the cells incubated for 1, 3, 5 and 10 min before adding 1000 U/ml CSF-1 or 1 U/ml phospholipase C (Sigma). The reactions were terminated after 10 min by centrifugation. The lipids were extracted as already described and the aqueous layer applied to 0.5 × 2 cm Dowex-1 formate columns (Bio-Rad, Richmond, CA). The columns were washed with 8 ml of water to remove free inositols. Deacylated inositol lipids were then eluted with 16 ml of 60 mM ammonium formate/5 mM disodium tetraborate, inositol monophosphates eluted with 8 ml of 200 mM ammonium formate/100 mM formic acid, inositol diphosphates eluted with 8 ml of 400 mM ammonium formate/100 mM formic acid and inositol trisphosphates eluted with 8 ml of 800 mM ammonium formate/100 mM formic acid. Two ml fractions were collected for determination of radioactivity.

Phosphatidylcholine hydrolysis

Monocytes were labeled for 48 h in RPMI 1640 medium with 10% FBS containing 1 μCi/ml [methyl-^{3}H]choline chloride (81.8 Ci/mmol, Amersham Corp.). The lipids were extracted following CSF-1 treatment and monitored for PC and phosphorylcholine production (Yavin, 1976). The organic phase was monitored for changes in PC as already described in this section. The aqueous fraction was evaporated and dissolved in 50% ethanol. Aliquots were applied to silica gel plates and developed in methanol/0.5% NaCl/ammonia (100:100:2, v/v/v). Phosphorylcholine was identified by iodine vapor using an authentic marker and the appropriate fraction collected for determination of radioactivity.

Measurement of cell free phospholipase activity

Phospholipase-mediated PC hydrolysis in cell-free extracts was determined as described (Clark *et al.*, 1986). Monocytes were suspended in Puck's saline F which contained 10 μM phenylmethylsulfonylfluoride, 100 μg/ml bacitracin, 1 mM benzamidine and 5 μg/ml soybean trypsin inhibitor. The cells were sonicated and 20 μl of the preparation (equivalent to 4 × 10^4 cells) was added to 24 μl of the reaction mixture which contained 20 mM HEPES buffer, pH 7.0, 1 mM $CaCl_2$, 100 mM NaCl, 1 mM deoxycholate, and 10 μM 1-palmitoyl-2-[1-^{14}C]palmitoyl 1-3-phosphatidylcholine (55 mCi/mmol, Amersham Corp.). The reaction was incubated at 37°C for 30 min and then stopped by the addition of 50 μl of chloroform/methanol (2:1, v/v) followed by adding 50 μl of chloroform and 50 μl of 4 M KCl. After centrifugation, the organic phase was applied to a Silica Gel TLC plate and chromatographed in petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v). Authentic standards were co-chromatographed with the lipid extract. The appropriate fraction was located by iodine vapor, scraped from the plate and analyzed by scintillation counting. Phospholipase activity was quantitated by the release of [^{14}C]DAG from the parent phospholipid.

Measurement of Na^+ uptake

Monocytes were incubated in 200 μl buffer containing 20 mM HEPES (pH 7.4), 2 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 5.5 mM D-glucose, 135 mM choline chloride and 1 mM ouabain at 37°C. The monocytes were preincubated in the presence and absence of 2 mM amiloride for 5 min at 37°C before adding 0.5 μCi $^{22}Na^+$ (364 μCi/μg; Amersham Corp.). The reaction was terminated after 20 min by washing three times in ice-cold 0.1 M $MgCl_2$. The monocytes were solubilized in 0.2 N NaOH and monitored for $^{22}Na^+$ uptake.

Acknowledgements

This investigation was supported by United States Public Health Service Grants CA-34183 and CA-42082 awarded by the National Cancer Institute, and by a Burroughs Wellcome Clinical Pharmacology Award (to D.K.)

Materials and methods

Strains

E.coli HJM114 (Δlacpro)F′ (lacpro), JM103(Δ(lacpro)thi, strA, supE, endA, sbcB, hsdR, traD36, proB, laclqZM15), and LC137(htpR, lon, lac_{am}, trp_{am}, pho_{am}, mal_{am}, rpsL, $supC^{ts}$, tsc:Tn*10*) were from our collection. CJ105 ($secA^{ts51}$) and CJ107 ($secY^{ts24}$) have been described (Wolfe *et al.*, 1985).

Materials

Proteinase K was from Boehringer Mannheim. Phenylmethylsulfonyl fluoride (PMSF) was from Sigma. [^{35}S]methionine (1000 Ci/mmol) and [^{35}S]dATP were from Amersham. DNA polymerase I (Klenow fragment), T4 kinase and T4 ligase were from New England Nuclear and Boehringer Mannheim, respectively. Oligonucleotides were synthesized at the Biocenter, Basel and at the Chemical Instrument Center at Ohio State University.

Plasmids

The pQN plasmids containing the procoat gene or the procoat−lep hybrid gene were described in Kuhn *et al.* (1986a). Gene VIII of M13 coding for procoat was subcloned into the pJF119 HE plasmid (Fürste *et al.*, 1986) containing the *tac* promoter.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed as described in Dalbey and Wickner (1987). Following mutagenesis, the template was isolated from the phage of single plaques and the mutants identified by DNA sequencing (Sanger *et al.*, 1977). Restriction enzymes were purchased from BRL, New England Biolabs, Pharmacia and Boehringer Mannheim. For all DNA manipulations, we followed the procedure described by Maniatis *et al.* (1982). The method of Cohen *et al.* (1973) was used for DNA transformations. After subcloning all mutations were verified by plasmid sequencing as described by Chen and Seeburg (1985).

Media, labeling and protease accessibility experiments

M9 minimal media (unless indicated) was prepared as described in Miller (1972) and was supplemented with 0.5% fructose and 50 μg/ml of each amino acid (without methionine). For radioactive labeling studies, the cells bearing a plasmid encoding procoat were grown in M9 media to the early log phase at 37°C. At an A_{600} of approximately 0.2, the plasmid encoded proteins were expressed by either 0.2% arabinose (procoat−lep) or 1 mM IPTG (procoat). The cells were then labeled with [^{35}S]methionine for the indicated times. For protease accessibility studies, cells were labeled for 1 min and subsequently incubated with 500 μg/ml methionine. An aliquot (0.5 ml) of cells was transferred to an equal volume of 40% sucrose, 60 mM Tris, and 20 mM EDTA pH 8.0 (ice cold) to permeabilize the outer membrane of the cells. Samples were then analyzed by immunoprecipitation (Wolfe *et al.*, 1982), SDS−PAGE and fluorography (Ito *et al.*, 1980).

Acknowledgements

This work was supported by a National Science Foundation Grant (DCB-8718578), an American Cancer Society Junior Faculty award, a seed grant from the Office of Research and Graduate Studies, a Basil O'Connor starter grant from the March of Dimes, a pilot research grant from the American Cancer Society (Ohio State University) and a grant from the American Cancer Society (Ohio Division). AK would like to acknowledge technical help of Helga Jütte and typing of Elvira Amstutz. This work was also supported by a grant (3.533-0.86) from the Swiss National Science Foundation.

References

Bakker,E. and Randall,L.L. (1984) *EMBO J.*, **3**, 895−900.
Chen,E. and Seeburg,P.H. (1985) *DNA (NY)*, **4**, 165−170.
Cobet,W.W.E., Mollay,C., Müller,G. and Zimmermann,R. (1989) *J. Biol. Chem.*, **264**, 10169−10176.
Cohen,S.N., Chang,A.C.Y. and Han,L. (1973) *Proc. Natl. Acad. Sci. USA*, **69**, 2110−2114.
Dalbey,R.E. and Wickner,W. (1987) *Science*, **235**, 784−787.
Daniels,C.J., Bole,D.G., Quay,S.L. and Oxender,D.L. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 5396−5400.
Date,T., Goodman,J.M. and Wickner,W. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4669−4673.
Fürste,J.P., Pansegrau,W., Frank,R., Blocker,H., Scholz,P., Bagdasarian, M. and Lanka,E. (1986) *Gene*, **48**, 119−131.
Gallusser,A. and Kuhn,A. (1990) *EMBO J.*, **9**, in press.
Ito,K., Date,T. and Wickner,W. (1980) *J. Biol. Chem.*, **262**, 2123−2130.
Kuhn,A. (1987) *Science*, **228**, 1413−1415.
Kuhn,A. (1988) *Eur. J. Biochem.*, **177**, 267−271.
Kuhn,A. and Wickner,W. (1985) *J. Biol. Chem.*, **260**, 15914−15918.
Kuhn,A., Wickner,W. and Kreil,G. (1986a) *Nature*, **322**, 335−339.
Kuhn,A., Kreil,G. and Wickner,W. (1986b) *EMBO J.*, **5**, 3681−3685.
Kuhn,A., Kreil,G. and Wickner,W. (1987) *EMBO J.*, **6**, 501−505.
Li,P., Beckwith,J. and Inouye,H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7685−7689.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miller,J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor. NY.
Randall,L.L. and Hardy,S.J.S. (1986) *Cell*, **46**, 921−928.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.
Von Heijne,G. (1986a) *J. Mol. Biol.*, **192**, 287−290.
Von Heijne,G. (1986b) *EMBO J.*, **5**, 3021−3027.
Wickner,W. (1989) *Trends Biochem. Sci.*, **14**, 280−283.
Wolfe,P.B., Silver,P. and Wickner,W. (1982) *J. Biol. Chem.*, **257**, 7898−7902.
Wolfe,P.B., Rice,M. and Wickner,W. (1985) *J. Biol. Chem.*, **260**, 1836−1841.
Yamada,H., Tokuda,H. and Mizushima,S. (1989a) *J. Biol. Chem.*, **264**, 1723−1728.
Yamada,H., Matsuyama,S., Tokuda,H. and Mizushima,S. (1989b) *J. Biol. Chem.*, **264**, 18577−18581.
Yamane,K. and Mizushima,S. (1988) *J. Biol. Chem.*, **263**, 19690−19696.
Zimmermann,R., Watts,C. and Wickner,W. (1982) *J. Biol. Chem.*, **257**, 6529−6536.

Received on April 5, 1990; revised on May 10, 1990