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The kinase insert domain of colony stimulating factor-1 receptor is dispensable for CSF-1 induced phosphatidylcholine hydrolysis

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Mouse NIH 3T3 fibroblasts transfected with human colony stimulating factor-1 receptor produced diacylglycerol in response to CSF1 and this correlated with elevated phosphatidylcholine hydrolyzing activity measured in an in vitro assay. Treatment of cells with the isoflavone derivative genistein attenuated PC hydrolysis in vitro suggesting a role for CSF1R tyrosine kinase activity. A CSF1R mutant lacking 67 amino acids of the kinase insert domain, which may affect the association of receptor with certain substrates, stimulated PC hydrolysis in response to CSF1. Coupling to PC hydrolysis is likely a general property of CSF1R and the kinase insert domain is dispensable for this activity.

Colony stimulating factor-1 receptor; Tyrosine kinase; Phosphatidylcholine hydrolysis; NIH 3T3 cell

1. INTRODUCTION

Colony stimulating factor-1 is a lineage-specific hemopoietin required for the maturation of cells of the mononuclear phagocyte lineage [1]. CSF1 binds with high affinity to a 972 amino acid transmembrane receptor expressed on macrophages and their precursors [2]. CSF1 binding stimulates receptor tyrosine kinase activity and a host of cellular responses believed to be important in transducing receptor signals [1]. As with other members of the class III receptor tyrosine kinases (PDGFR and KIT), the CSF1R cytoplasmic kinase domain is split into two segments by a hydrophilic sequence of approximately 70 amino acids termed the kinase insert domain [2]. The KI domain of CSF1R may have an important role in substrate recognition. For example, KI domain deletion mutants of CSF1R associated poorly with phosphatidylinositol 3-kinase [3,4], a novel enzyme with a possible role in growth regulation and transformation by viral oncogenes.

Many polypeptide growth factors stimulate a phospholipase C that hydrolyzes phosphatidylinositol 4,5-bisphosphate in the plasma membrane to yield inositol 1,4,5-trisphosphate and diacylglycerol [5]. IP_3 stimulates release of Ca^{2+} from intracellular stores while DAG activates protein kinase C [6]. A phosphatidylinositol-specific PLC is a direct substrate for PDGFR and EGFR [7], and tyrosine phosphorylation of PLC γ1 correlates with increased activity toward PIP_2 [8]. Despite structural similarities of CSF1R and PDGFR, CSF1R does not phosphorylate PLCγ1 [9]. Proliferation of mouse bone marrow-derived macrophages and other receptor-bearing cells in response to CSF1 is not accompanied by inositol lipid breakdown or rapid release of Ca^{2+} from intracellular stores [10,11]. Recently, CSF1 has been shown to stimulate phosphatidylcholine hydrolysis and protein kinase C activation in human peripheral blood monocytes [12], suggesting that the receptor utilizes alternative signalling pathways compared with PDGFR. We showed recently that CSF1 treatment of a mouse myeloid progenitor line expressing transfected human CSF1R cDNA stimulates mitogenicity and partial macrophage differentiation [13]. To distinguish between pathways generally activated by CSF1R from those that might be macrophage-specific, mouse NIH 3T3 fibroblasts transfected with wild-type and mutant human CSF1R cDNAs were examined in this report. We show that CSF1R can couple to PC hydrolysis in fibroblasts which do not normally express endogenous

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Abbreviations: CSF1, colony stimulating factor-1; CSF1R, colony stimulating factor-1 receptor; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; IP_3, inositol 1,4,5-trisphosphate; KI, kinase insert; PC, phosphatidylcholine; PDGFR, platelet-derived growth factor receptor; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PMSF, phenylmethylsulfonylfluoride; Protein A, *Staphylococcus aureus* membrane Protein A; PtdIns 3-kinase, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; U, units.

CSF1R and also that the KI domain, which may affect receptor–substrate interactions [3,4], is dispensable for CSF1R-induced PC hydrolysis.

2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant human CSF1 (lot no. 38322A, activity 100 U/μl) was from Cellular Products, (Buffalo, N.Y.). Genistein was from Aldrich. [^{3}H]glycerol and [^{3}H]phosphatidylcholine were from New England Nuclear. All other reagents were analytical grade.

2.2. *Cell lines*

Mouse NIH 3T3 fibroblasts (a kind gift of R.A. Weinberg) were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Mouse NIH 3T3 cells transfected with human CSF1R cDNA (2–9 cells) have been described [3,14]. A 67 amino acid KI domain deletion mutant (Gly-684) was constructed by in vitro site directed mutagenesis as described [15]. This mutated cDNA was transfected into NIH 3T3 cells to establish the mutant receptor expressing line (1A1A, [3]). All transfected cell lines were grown in DMEM plus 10% fetal bovine serum in the presence of 0.8 mg/ml G-418, to select for the *neo* gene present in the expression vector. The cells were made quiescent by incubating confluent monolayers in medium containing 0.5% serum for 20 h. Quiescent cells were stimulated with 1000 U/ml CSF1.

2.3. *Analysis of in vivo DAG production*

The cells were labeled in [^{3}H]glycerol (8 μCi/ml) for 48 h and then starved for serum in medium containing 0.5% dialyzed fetal bovine serum plus [^{3}H]glycerol for 20 h. The cells were stimulated with 1000 U/ml CSF1. At indicated time points the cell monolayer was extracted with 540 μl of 1 N HCl/MeOH/$CHCl_3$ (1:1:1). The aqueous layer was extracted with the same solvent mixture. The pooled organic phase was dried under a stream of nitrogen and resuspended in 15 μl of $CHCl_3$ containing cold DAG standard and analyzed on silica gel 60 thin layer chromatography plates using a benzene/ethyl acetate (7:3) solvent [16]. The DAG was visualized by spraying the plates with *p*-2-toluidinylnaphthalene-6-sulphonic acid and UV irradiation. The corresponding DAG spots in experimental samples were scraped and counted in aquasol.

2.4. *Phospholipase C assay*

CSF1-stimulated cells were lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF and 0.25% aprotinin) at 4°C for 30 min. The cell extract was centrifuged at 10000 × *g* for 30 min. Protein concentration of the supernatant was determined using BCA reagent (Pierce Chemical Co.). Equal amounts of protein were used in a PLC assay employing [^{3}H]PC (choline-methyl [^{3}H]PC) as described by Katan and Parker [17]. Briefly, the RIPA lysate was incubated with [^{3}H]PC in PLC assay buffer (100 mM NaCl, 0.6% Na-deoxycholate, 2 mM $CaCl_2$, 4 mM EGTA, 5 mM 2-mercaptoethanol and 20 mM Tris-maleate pH 6.0) at 37°C for 30 min. The reaction was extracted with 0.5 ml of $CHCl_3$/MeOH/11.6 N HCl (100:100:0.6) and 0.15 ml of 1 N HCl. The aqueous layer was routinely counted. Measured amounts of the aqueous layer were dried under vacuum and resuspended in cold phosphorylcholine standard solution and analyzed on TLC using 0.5% NaCl/MeOH/NH_4OH [18] as a solvent. The phosphorylcholine standard was visualized by spraying the plates with Dragendorff's reagent, scraped off the plate and counted.

2.5. *Western blot analysis*

Cell lysates were analyzed by SDS polyacrylamide gel electrophoresis. The separated proteins were transferred onto nitrocellulose membrane and probed with a rabbit anti-*c-fms* antibody. The bound antibody was detected by [^{125}I]-protein A essentially as described [13].

3. RESULTS AND DISCUSSION

Activation of PI-specific phospholipase C, observed after stimulation of many cell surface receptors [7,8], does not accompany CSF1-induced mitogenesis in bone marrow-derived macrophages [10]. Chinese hamster lung cells and NIH 3T3 cells expressing human CSF1R also do not show significant PI hydrolysis in response to CSF1 although CSF1 is mitogenic in these cells [3,11] (Ghosh Choudhury, unpublished). The observations are consistent with the inability of CSF1R to phosphorylate PI-specific PLCγ1 [9], an event correlated with stimulation of PLCγ1 activity in other systems [8]. In the absence of PI hydrolysis several agonists can elicit their effects through hydrolysis of other membrane phospholipids [19]. To determine if transfected CSF1R can couple to another phospholipid breakdown pathway, we studied DAG production in 2–9 cells. The cells were metabolically labelled with [^{3}H]glycerol, stimulated with human recombinant CSF1 and intracellular levels of DAG were measured at different time points (Fig. 1A). CSF1-stimulated DAG production in vivo was significantly elevated at the earliest time point examined (80 s). The intracellular DAG level peaked at 4 min of human CSF1 stimulation and remained elevated at 12 min. To determine if DAG production was associated with PC hydrolysis, we stimulated clone 2–9 cells with human CSF1 for varying periods of time and the cleared cell extracts were assayed for PC hydrolyzing activity in vitro (Fig. 1B). Human CSF1 stimulated PC hydrolysis in 2–9 cells with kinetics similar to those obtained for in vivo DAG production (Fig. 1A). These studies provide evidence that, in the absence of PI turnover, CSF1 can stimulate the PC hydrolysis to produce second messengers. The DAG produced in this reaction might stimulate protein kinase C as a CSF1 induced signalling event in transfected fibroblasts, as has been shown for peripheral blood monocytes [12].

Recently the isoflavone derivative, genistein, has been shown to inhibit the tyrosine kinase activity of EGFR and PDGFR and to block their biological activity [20–22]. Like these receptors, the biological responses mediated by CSF1R also depend upon its intrinsic tyrosine kinase activity. We tested if genistein could inhibit CSF1-induced PC hydrolysis in 2–9 cells. The cells were treated with 100 μM genistein and stimulated with human CSF1 for 4 min. The cleared cell lysate was tested for PLC activity in vitro using [^{3}H]PC as a substrate. Genistein significantly inhibited CSF1-induced phosphorylcholine production from PC (Fig. 2), suggesting that the tyrosine kinase activity of CSF1R is necessary for CSF1-induced PC hydrolysis.

The highly hydrophilic KI domains of PDGFRB and CSF1R appear to be important in receptor function, perhaps in substrate recognition [3,4,23–25]. In the case of PDGFRB a KI deletion mutant is dramatically im-

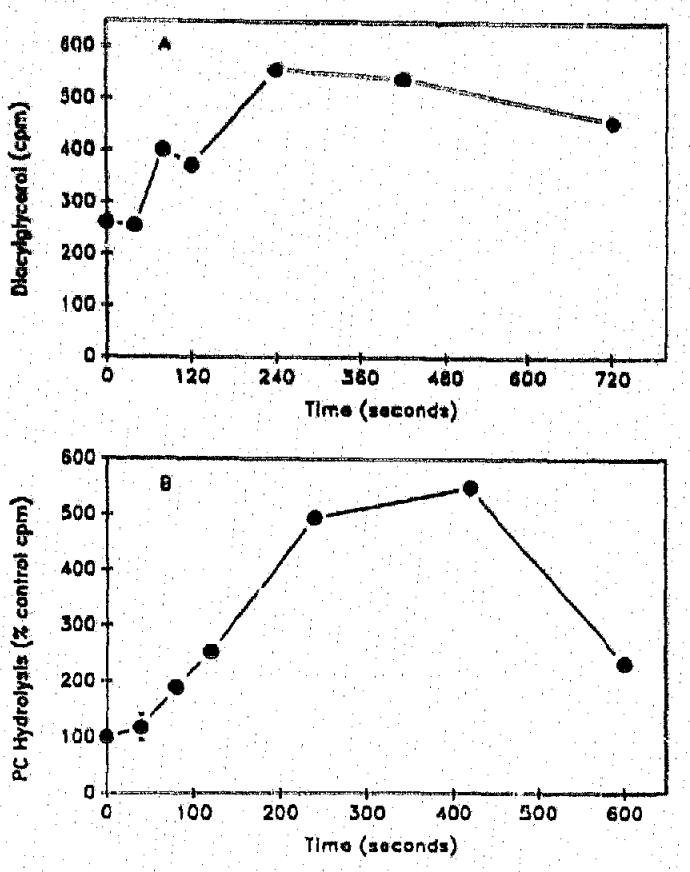

Fig. 1. CSF1-induced PC hydrolysis in 2-9 cells. (A) [^{3}H]glycerol labeled quiescent 2-9 cells were stimulated with 1000 U/ml human recombinant CSF1. At indicated times the cell monolayer was extracted with $CHCl_3$/MeOH/1 N HCl (1:1:1) and the DAG level was determined as described in section 2. Each point is a mean of duplicate determination. (B) Quiescent cell monolayers were stimulated with 1000 U/ml CSF1. At indicated time points the cells were lysed and 32 μg of cell extract was assayed for PLC activity using [^{3}H]PC as a substrate as described in section 2. The data are plotted as percent of total cpm in the aqueous extract. The basal value of PLC activity at time zero was 381 ± 14 cpm.

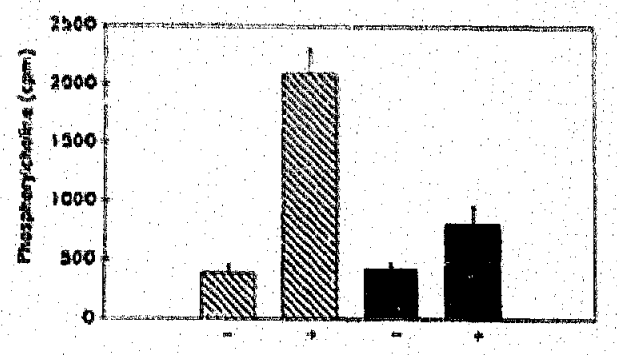

Fig. 2. Effect of genistein on PC hydrolysis in 2-9 cells. Quiescent 2-9 cells were treated with 100 μM genistein for 2 h and stimulated with CSF1 for 4 min, lysed in RIPA buffer and 32 μg of cell lysate was assayed for PLC activity using [^{3}H]PC as a substrate. After organic extraction the aqueous layer was analyzed on TLC to detect phosphorylcholine as described in section 2. Hatched and dark bars are in the absence and presence of genistein, respectively. '–' and '+' are in the absence and presence of CSF1, respectively.

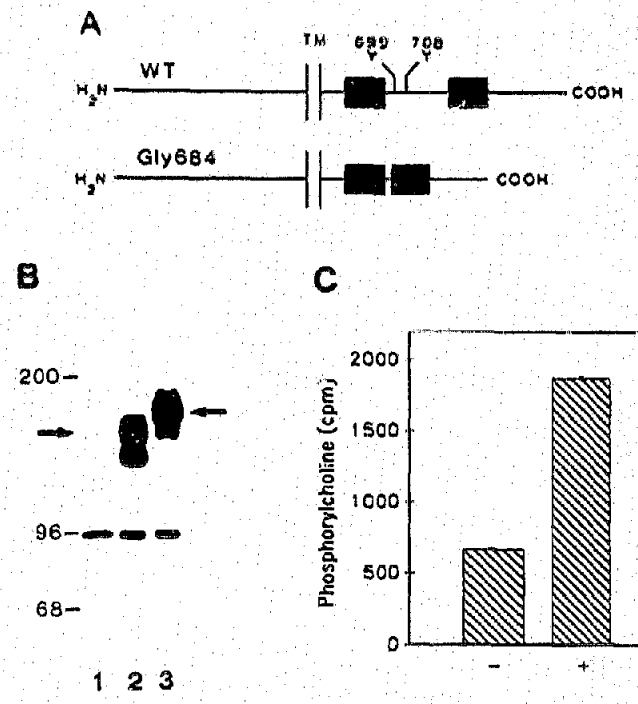

Fig. 3. Western blot analysis of wild-type and mutant CSF1R protein, and coupling of mutant to PC hydrolysis. (A) Structure of the wild-type and KI domain deletion mutant receptors. 'TM' is the transmembrane domain; dark bars are the tyrosine kinase domain. The major in vivo tyrosine autophosphorylation sites are indicated (residues 699 and 708). (B) Equal amounts of cell lysate were separated on a 7.5% SDS polyacrylamide gel, transferred onto a nitrocellulose filter and probed with a rabbit anti-*c-fms* antibody as described in section 2. Lane 1, NIH 3T3 cells; Lane 2, cell line 1A1A expressing CSF1R KI domain deletion mutant; Lane 3, wild type receptor expressing line (2-9). Arrows indicate the mature forms of mutant receptor protein (142 kDa) and wild-type receptor protein (150 kDa) (C) PC hydrolysis in clone 1A1A cells. The quiescent cell monolayer was stimulated with 1000 U/ml CSF1 for 4 min, lysed in RIPA buffer, then 32 μg of cell extract was assayed for PLC activity in vitro using [^{3}H]PC as substrate. After organic extraction the aqueous layer was analyzed for phosphorylcholine as described in section 2. '–' and '+' are in the absence and presence of CSF1, respectively.

paired in stimulating mitogenesis and in its association with at least two cytoplasmic proteins, PtdIns-3 kinase and *ras* GTPase activating protein [23,24]. Mouse and human CSF1R mutants devoid of the KI domain do not associate well with PtdIns-3 kinase and are mitogenically impaired in transfected fibroblasts [3,4,25].

Furthermore, two major tyrosine autophosphorylation sites have been mapped in the KI domain of the mouse receptor (the positions of the homologous residues in human CSF1R are Y699 and Y708) [26,27] (Fig.3A). We expressed a 67 amino acid KI domain deletion mutant (Gly-684), spanning from glycine 684 to leucine 750, in NIH 3T3 cells [3]. Fig. 3B compares the mutant CSF1R protein with wild-type CSF1R (indicated by arrows), and as expected, the fully processed form of the mutant protein is 8 kDa smaller than the wild-type receptor (compare lanes 2 and 3, Fig. 3B). This cell line (clone 1A1A) expressing the KI domain

deletion mutant was stimulated with human CSF1, and the cell lysate was tested for PC hydrolysis in vitro. The data (Fig. 3C) show that CSF1 stimulates PLC activity cleaving PC in clone 1A1A cells. The extent of PC hydrolysis elicited by the Gly-684 deletion mutant was consistently lower than that obtained with wild-type receptor. In 5 experiments the mean fold-increase in CSF1-induced PC hydrolysis over basal activity in 2-9 cells was 4.97, while that for Gly-684 was 3.20. The decrease obtained with the Gly-684 deletion mutant might be due in part to the slightly lower expression of receptor protein compared to the wild-type receptor expressing line (Fig. 3B, compare lanes 2 and 3; and [3]). It is presumed, however, that despite the likely effect on the conformation of the cytoplasmic domain, deletion of the CSF1R KI segment has not abrogated the ability of the receptor to stimulate the PC hydrolysis pathway. The mechanism(s) coupling CSF1R to PC hydrolysis and the role(s) of this phospholipid hydrolysis pathway in CSF1-induced growth and differentiation are currently under investigation.

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