The EMBO Journal Vol.16 No.19 pp.5880–5893, 1997

Sequential activation of phoshatidylinositol 3-kinase and phospholipase C-γ2 by the M-CSF receptor is necessary for differentiation signaling

Roland P.Bourette[1], Gary M.Myles, Jung-Lim Choi and Larry R.Rohrschneider[2]

Fred Hutchinson Cancer Research Center, Basic Sciences Division, Room B2-152, 1100 Fairview Avenue-N., Seattle, WA 98109-1024, USA

[1]Present address: Centre de Génétique Moléculaire et Cellulaire, UMR CNRS 5534, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

[2]Corresponding author
e-mail: lrohrsch@fhcrc.org

Binding of macrophage colony stimulating factor (M-CSF) to its receptor (Fms) induces dimerization and activation of the tyrosine kinase domain of the receptor, resulting in autophosphorylation of cytoplasmic tyrosine residues used as docking sites for SH2-containing signaling proteins that relay growth and development signals. To determine whether a distinct signaling pathway is responsible for the Fms differentiation signal versus the growth signal, we sought new molecules involved in Fms signaling by performing a two-hybrid screen in yeast using the autophosphorylated cytoplasmic domain of the wild-type Fms receptor as bait. Clones containing SH2 domains of phospholipase C-γ2 (PLC-γ2) were frequently isolated and shown to interact with phosphorylated Tyr721 of the Fms receptor, which is also the binding site of the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase). At variance with previous reports, M-CSF induced rapid and transient tyrosine phosphorylation of PLC-γ2 in myeloid FDC-P1 cells and this activation required the activity of the PI3-kinase pathway. The Fms Y721F mutation strongly decreased this activation. Moreover, the Fms Y807F mutation decreased both binding and phosphorylation of PLC-γ2 but not that of p85. Since the Fms Y807F mutation abrogates the differentiation signal when expressed in FDC-P1 cells and since this phenotype could be reproduced by a specific inhibitor of PLC-γ, we propose that a balance between the activities of PLC-γ2 and PI3-kinase in response to M-CSF is required for cell differentiation.

Keywords: differentiation/Fms/phosphatidylinositol 3-kinase/phospholipase C-γ/signal transduction

Introduction

Hematopoiesis is a developmental process in which totipotent bone marrow stem cells generate eight different blood cell lineages necessary for constant replacement of senescent mature cells or to respond to a stress, like hemorrhage or infection (Metcalf, 1989). This implies precise regulation of the balance between cell growth, survival and differentiation, regulated mainly by soluble or membrane-bound glycoproteins, called colony stimulating factors (CSF), growth factors or cytokines (Metcalf, 1984). In the monocytic lineage the principal regulator is the macrophage colony stimulating factor (M-CSF or CSF-1), synthesized by a variety of different cell types, including fibroblasts, endothelial cells and bone marrow stromal cells, and present at high concentration in serum (Roth and Stanley, 1992). *In vitro* soft agar assays demonstrate that M-CSF stimulates survival, proliferation and maturation of single bone marrow progenitors into macroscopic colonies of macrophages (Stanley *et al.*, 1978). M-CSF also stimulates survival and proliferation of mature monocytes and macrophages and enhances their differentiated functions (Stanley, 1981; Tushinski *et al.*, 1982). The key role of M-CSF in mononuclear phagocyte development has been demonstrated *in vivo* with osteopetrotic (*op*/*op*) mutant mice that lack functional M-CSF and are deficient in osteoclasts and macrophages. Both cell types are presumably derived from a common progenitor (Wiktor-Jedrzejczak *et al.*, 1990) and the *op*/*op* defects can be cured by injection of M-CSF (Wiktor-Jedrzejczak *et al.*, 1991).

All biological effects of M-CSF are mediated through a single high affinity receptor expressed on the cell surface of monocytes, macrophages and their progenitors (Byrne *et al.*, 1981) and encoded by the proto-oncogene c-*fms* (Sherr *et al.*, 1985; Woolford *et al.*, 1985). The protein product, Fms, is a member of a class of growth factor receptor tyrosine kinases (RTKs) that include the α and β platelet-derived growth factor (PDGF) receptors, Kit, a receptor of the stem cell factor and the Flt3/FLK2 receptor (Rosnet and Birnbaum, 1993). These transmembrane receptors transduce extracellular ligand messages into intracellular signals via pathways controlling cell survival, apoptosis, proliferation and differentiation, depending on cellular context (Ullrich and Schlessinger, 1990). Their common mechanism of activation is initiated by ligand binding that enables receptor dimerization, activation of the kinase domain and autophosphorylation of the cytoplasmic domain on specific tyrosine residues. Tyrosine autophosphorylation creates binding sites for Src homology 2 (SH2)-containing intracytoplasmic molecules that relay and perhaps amplify the signals (Koch *et al.*, 1991) along specific pathways leading to output through gene transcription.

The Fms receptor induces numerous biological effects when activated and we are interested in how these signals are integrated, transmitted and interpreted for the cellular output response. The process begins with at least five tyrosine (Y) autophosphorylation sites in the cytoplasmic domain. Y559 in the juxtamembrane region is a binding site for Src family members when phosphorylated (Alonso *et al.*, 1995). Three sites, Y697, Y706 and Y721, are

located in the kinase insert (KI) region that splits the functional tyrosine kinase domain of the receptor. Phosphorylated Y697 binds the SH2 domain of the Grb2 adaptor molecule, which is constituitively associated with the nucleotide exchange factor mSOS. Translocation of the Grb2–mSOS complex from the cytoplasm to the plasma membrane is sufficient for Ras activation (van der Geer and Hunter, 1993; Lioubin *et al.*, 1994). Phosphorylated Y706 binds the STAT1 transcription factor (Novak *et al.*, 1996) and phosphorylated Y721 binds the p85 subunit of phosphatidylinositol 3-kinase (PI3 kinase) (Reedijk *et al.*, 1992). Another autophosphorylation site, Y807, is located in the second half of the kinase domain (Tapley *et al.*, 1990) and corresponds to an autophosphorylation site conserved among most of the tyrosine kinases (Hanks and Quinn, 1991). Based on structural analysis of other RTKs, this latter tyrosine could act as a regulatory site rather than a direct binding site (Hubbard *et al.*, 1994), however, the Y807 site in v-Fms has recently been implicated in p120Ras GAP binding (Trouliaris *et al.*, 1995).

One of our principal interests is defining the mechanisms that control signaling pathways for growth, survival and differentiation. Do the existing pathways described above each regulate one or more biological functions of the receptor or do additional, as yet undefined, signaling pathways participate? To address these possibilities, a two-hybrid screen was initiated to look for additional signaling proteins interacting with activated Fms. The results identified several new interacting proteins and their analysis will be reported elsewhere (R.P.Bourette, G.M.Myles and L.R.Rohrschneider, in preparation). In addition, the SH2 domains of phospholipaseC-γ2 (PLC-γ2) were identified as Fms binding proteins in several screens, even though PLC-γ2 has been shown previously not to bind or be activated by Fms (Downing *et al.*, 1989). Further exploration of this potential interaction in more detail, however, demonstrated its existence both *in vitro* and *in vivo*. This report presents those data along with results on the reciprocal regulation of PLC-γ2 with PI3-kinase and its association with differentiation signaling.

Results

Identification of SH2-containing proteins interacting with activated Fms

A yeast two-hybrid assay (Fields and Song, 1989; Fields, 1993; Vojtek *et al.*, 1993) was used to identify new proteins that interact with the cytoplasmic domain of the murine Fms receptor. For the bait we constructed a LexA–Fms fusion protein containing the entire cytoplasmic domain of the wild-type murine Fms protein (Figure 1A). This construct was transformed into *Saccharomyces cerevisiae* L40 and expression of the bait fusion protein examined by immunoprecipitation and Western blotting. Yeast lysates were immunoprecipitated with antibodies to the cytoplasmic domain of Fms and Western blotting with anti-LexA, anti-Fms or anti-phosphotyrosine antibodies demonstrated that a tyrosine-phosphorylated fusion protein of the size expected for the LexA–Fms construct (~75 kDa) was produced (Figure 1B). The tyrosine phosphorylation of this bait protein in yeast demonstrates that the receptor cytoplasmic domain retains the ability to undergo autophosphorylation when expressed as a fusion protein with the LexA DNA binding domain, as previously shown with the insulin receptor (O'Neill *et al.*, 1994). Autophosphorylation also occurred at expected tyrosine residues (see below), suggesting that LexA–Fms autophosphorylation accurately recapitulated this activation step.

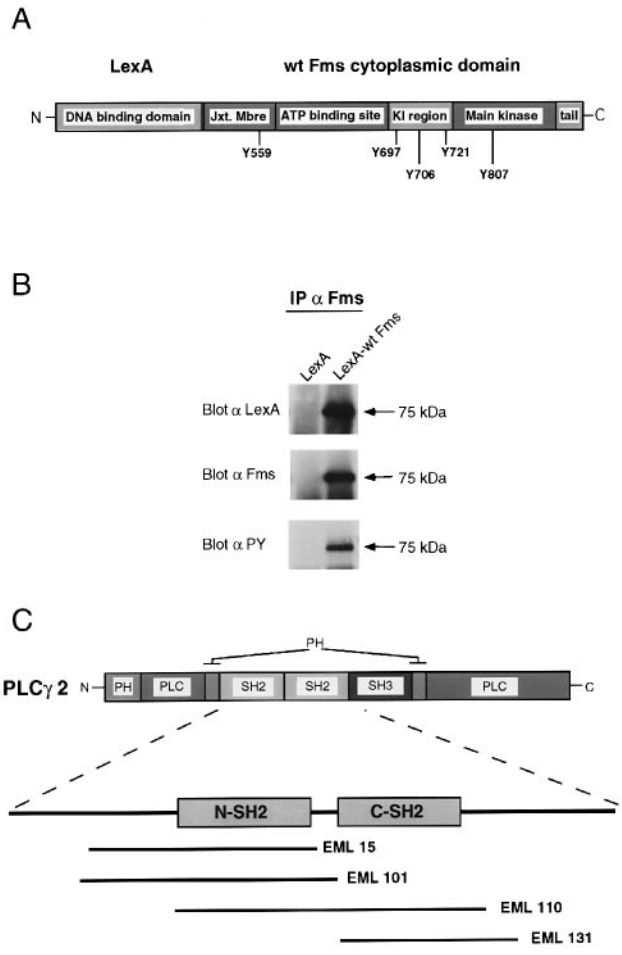

Fig. 1. Yeast two-hybrid system with murine wild-type Fms cytoplasmic domain. (**A**) Schematic representation of the LexA–wild-type Fms cytoplasmic domain fusion protein used as bait. The different tyrosine (Y) autophosphorylation sites are Y559 in the juxtamembrane region, Y697, Y706 and Y721 in the KI region and Y807 in the main kinase domain. (**B**) Expression and tyrosine phosphorylation of the bait in *S.cerevisiae* L40 strain. The L40 yeast strain was transformed and selected in medium lacking uracil and tryptophan (UW). A single colony was isolated and tested for expression and tyrosine phosphorylation of the LexA–wild-type Fms fusion protein. *Saccharomyces cerevisiae* lysates were immunoprecipitated with anti-murine Fms antibody 4599B prepared against the cytoplasmic domain. The proteins were run on a 7% polyacrylamide gel, blotted and probed sequentially with antibodies directed against LexA (αLexA), Fms cytoplasmic domain (αFms) and phosphotyrosine (αPY). (**C**) Schematic representation of PLC-γ2 clone segments obtained in the two-hybrid screen using the LexA–wild-type Fms cytoplasmic domain as bait. The domain structure of PLC-γ2 is shown with an expanded view of the SH2 domains. Representative clones from the EML VP16 library are aligned below the SH2 domains. Isolated clones containing PLC-γ2 N-SH2 domain (EML 15 and EML 101), C-SH2 domain (EML 131) or both SH2 domains (EML 110) are presented.

A VP16 target library containing cDNA from the pluripotent hematopoietic EML cell line (Tsai *et al.*, 1994; Lioubin *et al.*, 1996) was used to screen for clones encoding Fms-interacting proteins. EML cells expressing

the wild-type murine Fms from a retroviral vector exhibit both proliferation and differentiation in response to M-CSF stimulation (R.P.Bourette, L.R.Rohrschneider and S.Tsai, unpublished data). This suggests that the EML VP16 cDNA library should contain most of the cytoplasmic substrates necessary for these two Fms signaling pathways.

A stable LexA–Fms-expressing L40 yeast clone was transformed with the EML VP16 cDNA library and between 10 and 20×10^6 transformants were screened, resulting in 166 clones positive for transcriptional activation of the *his3* and *lacZ* reporter genes. Using VP16 sense and antisense primers (Vojtek and Hollenberg, 1995), sequences of the interacting EML VP16 clones were determined and compared with existing cDNA sequence databases. The vast majority of the clones obtained contained SH2 domains, including the SH2 domains of the known Fms partners, Grb2, p85 and Fyn. New SH2-containing proteins were identified and some non-SH2-containing proteins were cloned. In addition, we also obtained multiple clones that contained one (N- or C-terminal) or both SH2 domains of PLC-γ2 (Figure 1C). PLC-γ was not believed to interact with Fms or to be activated by Fms (Downing *et al.*, 1989) yet we obtained multiple clones of each SH2 domain. The potential relevance of this interaction was therefore explored in more detail.

The specificity of the interaction between the tyrosine-phosphorylated Fms cytoplasmic domain and the SH2 domains of PLC-γ2 was tested in a mating experiment. Representative clones containing either the N-SH2 domain (clone EML 101), the C-SH2 domain (clone EML 131) or both SH2 domains of PLC-γ2 (clone EML 110) were cured of the LexA–wild-type Fms bait and mated with AMR70 yeast containing various control baits (Figure 2). These included LexA alone as a negative control, LexA–wild-type Fms as a positive control and LexA–kinase-dead (K614A) Fms to check for tyrosine kinase dependence of the interaction. Different single autophosphorylation site Fms mutations were used to determine the exact site of interaction and an Fms mutant with five known autophosphorylation sites mutated to phenylalanines (5F) was included as a general check for specificity. Both SH2 domains of the p85 subunit of PI3-kinase were fused to VP16 as a positive control and shown to interact, as expected (Reedijk *et al.*, 1992), with the tyrosine phosphorylated Y721 site (Figure 2, lower right panel). This demonstrated the specificity of the system and accurate autophosphorylation of the Y721 site. The Grb2 SH2 domain also demonstrated appropriate binding specificity for the phosphorylated Y697 site (data not shown).

PLC-γ2 SH2 domain clones, as VP16 fusions, were each retested against the panel of negative, positive and mutant LexA–Fms baits described above. In the mating experiment, no interactions were detected with LexA alone or with the LexA–kinase-dead (KD) Fms (Figure 2), indicating that the interactions between the LexA–wild-type Fms bait and the PLC-γ2 SH2 domain clones (fused to VP16) were dependent on the presence of the cytoplasmic domain of Fms and its tyrosine kinase activity. The EML clone (EML101) containing only the N-SH2 domain of PLC-γ2 did not interact with either the LexA–Fms mutant Y721F or the LexA–Fms 5F mutant (Figure 2), suggesting that phosphorylated residue Y721 is the binding site for

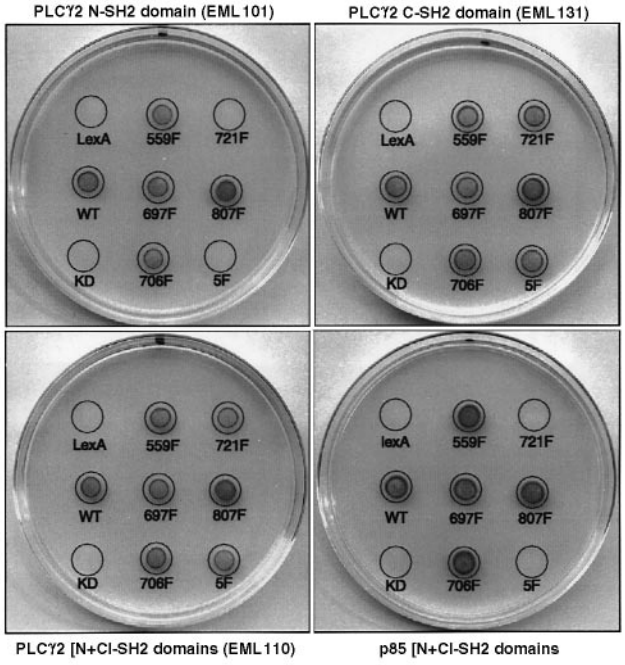

Fig. 2. Mating assays between EML VP16 clones containing PLC-γ2 SH2 domains and different wild-type or mutant Fms cytoplasmic baits to determine specificity of the interaction with the Fms receptor and autophosphorylation sites. Representative isolated EML VP16 yeast clones containing PLC-γ2 N-, C- or N- and C-SH2 domains (shown above or below each panel) were cured of the LexA–wild-type Fms bait on medium containing tryptophan and lacking uracil and leucine and mated with *S.cerevisiae* AMR70 strains containing different LexA fusion baits: LexA alone, LexA–wild-type Fms (WT), LexA–kinase-dead (KD) Fms, LexA–Y559F Fms, LexA–Y697F Fms, LexA–Y706F Fms, LexA–Y721F Fms, LexA–Y807F Fms and Lex–Y[559, 697, 706, 721, 807]F Fms (5F). Interactions between the baits and the EML VP16 SH2 domains were detected by spotting yeast on WHULK medium containing X-Gal and observation of blue colonies after 3 days. A clone containing N- and C-SH2 domains of p85 was added to this experiment as a control.

the N-SH2 domain of PLC-γ2. In contrast, the clones containing the C-SH2 domain (EML131) or both the N- and C-SH2 domains (EML110) of PLC-γ2 reacted with all Tyr→Phe mutants examined. Because the interaction with Fms was still tyrosine kinase dependent (i.e. negative on the KD mutant), these results indicate that the C-SH2 domain of PLC-γ2 is interacting with a tyrosine-phosphorylated site on Fms either non-specifically and/or with one not examined in this study.

The PLC-γ2 SH2 domains interact cooperatively with activated Fms in vitro

To confirm the interaction between PLC-γ2 SH2 domains and the Fms receptor, we constructed GST fusion proteins containing either N-, C- or N- and C-SH2 domains of murine PLC-γ2. As a control, similar fusion proteins with the SH2 domains of the p85 subunit of PI3-kinase were constructed. FDC-P1 cells expressing wild-type murine Fms (FD/wtFms) (Bourette *et al.*, 1995) were starved of growth factor and either unstimulated or stimulated with M-CSF (1 min at 37°C). Cell lysates were mixed with the different GST fusion proteins coupled to glutathione–Sepharose beads. Tyrosine-phosphorylated proteins binding strongly to the GST–SH2 domain fusion proteins were analyzed by Western blotting with an anti-phosphotyrosine

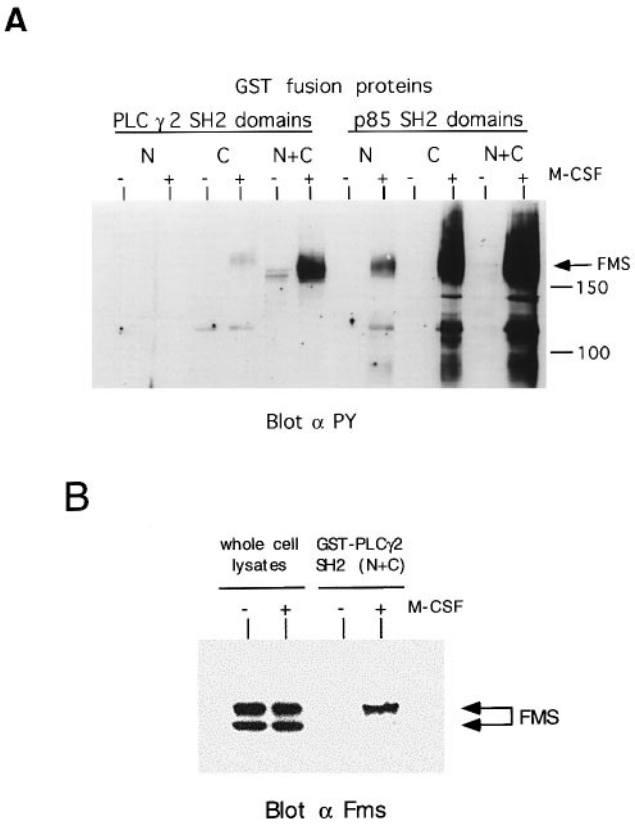

Fig. 3. The Fms receptor interacts with a GST fusion protein containing both (N- and C-) SH2 domains of PLC-γ2. Lysates of FD/wtFms, with (+) or without (–) M-CSF stimulation for 1 min at 37°C, were mixed with GST fusion proteins immobilized on glutathione–Sepharose. (**A**) The fusions contained N-, C- or both (N- and C-) SH2 domains of PLC-γ2 or p85. Bound proteins were run on a 7% polyacrylamide gel, blotted and probed with anti-phosphotyrosine antibody (αPY). (**B**) The fusion contained both (N- and C-) SH2 domains of PLC-γ2. Bound proteins were run on a 7% polyacrylamide gel, blotted and probed with anti-Fms antibody (αFms). The upper Fms band is the mature cell surface form.

antibody (Figure 3A). Under these conditions activated Fms could be detected as a 165 kDa phosphoprotein. Significant Fms association was not observed with single PLC-γ2 N- or C-SH2 domains, but when both PLC-γ2 SH2 domains in tandem were fused to the GST protein, a detectable amount of Fms receptor was precipitated from the M-CSF-stimulated cells (Figure 3A). In contrast, either the N- or C-SH2 domain alone of the p85 protein associated with Fms after M-CSF stimulation. Interaction of Fms with the N-SH2 domain was much weaker than interaction with the C-SH2 domain. When both N- and C-SH2 domains of p85 were fused to GST, a very large amount of M-CSF-stimulated Fms was observed. To ensure that the precipitated 165 kDa phosphoprotein was really Fms and not another tyrosine kinase receptor of the same size we performed a precipitation experiment using a GST–PLCγ2 (N- and C-)SH2 domain fusion, detecting precipitated Fms receptor with a polyclonal anti-Fms antibody (Figure 3B). Fms receptor was detected in precipitates only when the GST fusion protein was mixed with lysate of M-CSF-stimulated cells (Figure 3B). Consistent with the data in Figure 3A, this experiment demonstrates that PLCγ2 (N- and C-)SH2 domains bind to activated Fms receptor *in vitro*.

Synergistic binding of SH2 domains to receptors has been previously reported for p85 (Reedijk *et al.*, 1992), PLC-γ1 and GAP (Anderson *et al.*, 1990). These results demonstrate that SH2 domains of PLC-γ2 interact *in vitro* with intact M-CSF-activated Fms, confirming the observation made with the two-hybrid system. The interaction of PLC-γ2 SH2 domains with activated Fms, however, was much lower than that detected in the two-hybrid yeast system and required the presence of both SH2 domains. This is probably due to the fact that the two-hybrid system is much more sensitive than immunoprecipitation in detecting protein–protein interactions.

PLC-γ2 is rapidly and transiently tyrosine phosphorylated in response to M-CSF in vivo

To determine whether PLC-γ2 was tyrosine phosphorylated in response to M-CSF, starved FD/wtFms cells were stimulated at 37°C with M-CSF and cell lysates were prepared at times up to 10 min after stimulation. PLC-γ2 and p85 were immunoprecipitated from each lysate and the extent of tyrosine phosphorylation of each protein determined by Western blotting (Figure 4A). A PLC-γ2 tyrosine-phosphorylated band of 150 kDa was detected after 5 s stimulation. The extent of tyrosine phosphorylation reached a maximum after 30 s with a broad plateau to 2 min and complete disappearance after 3 min M-CSF stimulation. Tyrosine phosphorylation of p85 after M-CSF stimulation (Figure 4A) also occurred rapidly (within 5 s), but remained high for a longer period than observed for PLC-γ2. Tyrosine-phosphorylated p85 was still detected 10 min after M-CSF stimulation (Figure 4A). Both anti-phosphotyrosine blots (p85 and PLC-γ2) were stripped and reblotted with the same antibodies used for immunoprecipitation to show the amounts of each protein present (Figure 4). These results demonstrate that PLC-γ2 is rapidly and transiently phosphorylated on tyrosine after Fms activation by M-CSF. The existing protein is phosphorylated on tyrosine within 30 s of M-CSF stimulation and dephosphorylated and/or degraded 3 min later. As previously described (Varticovski *et al.*, 1989), p85 is also tyrosine phosphorylated in response to M-CSF, but the tyrosine-phosphorylated form persists for a longer period of time.

The effect of a phosphotyrosine phosphatase inhibitor (Na_3VO_4) on the extent and duration of PLC-γ2 tyrosine phosphorylation was examined. FD/wtFms cells were incubated for 15 min with a phosphatase inhibitor prior to M-CSF stimulation and transient tyrosine phosphorylation of PLC-γ2 was measured as above (Figure 4B). An increase in the duration of PLC-γ2 phosphorylation was seen (from 3 to 5 min) with an elevated peak of tyrosine phosphorylation at 3 min. This suggests that endogenous tyrosine phosphatases are indeed implicated in the decrease in PLC-γ2 phosphorylation after M-CSF stimulation. Tyrosine phosphatases have been shown to be activated by Fms (Yi and Ihle, 1993; Carlberg and Rohrschneider, 1997) and could participate in down-regulation of PLC-γ2. Alternatively, we cannot rule out the possibility that a minor fraction of PLC-γ2 is proteolytically degraded.

The Y721F and Y807F Fms mutations strongly decrease tyrosine phosphorylation of PLC-γ2 in FDC-P1 cells

The association of p85 with the phosphorylated Fms Y721 site is known (Reedijk *et al.*, 1992) and we next sought

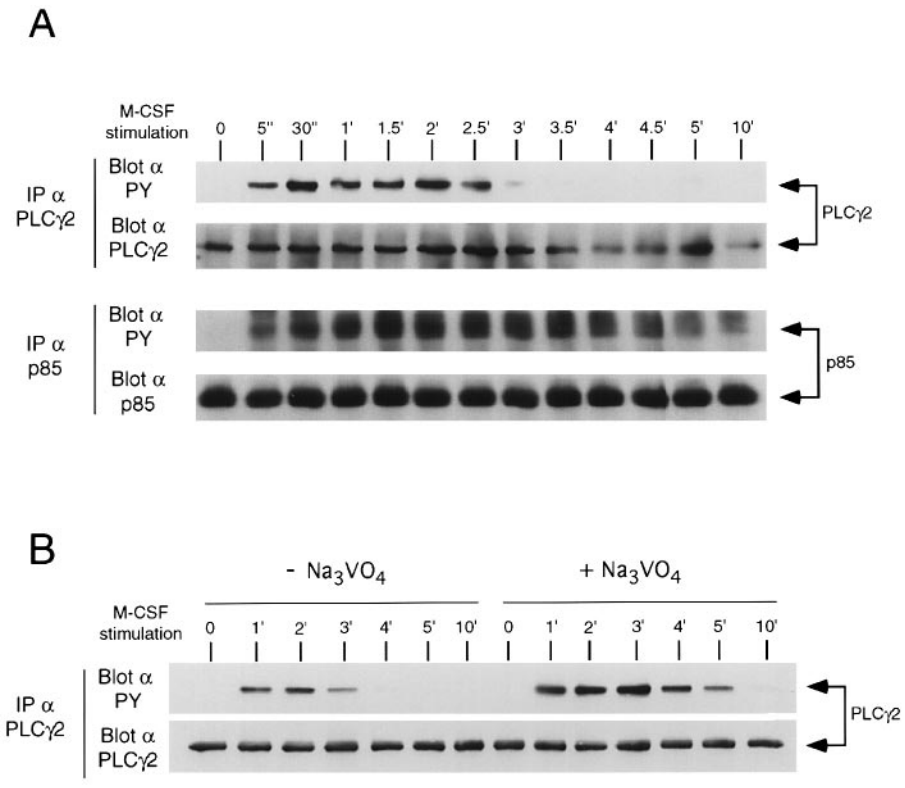

Fig. 4. PLC-γ2 is rapidly and transiently tyrosine phosphorylated in response to M-CSF in myeloid FDC-P1 cells. (**A**) Starved FD/wtFms cells were resuspended in PBS and stimulated with M-CSF for different times at 37°C. Cell lysates were immunoprecipitated with anti-PLC-γ2 or anti-p85 antibodies. Immunoprecipitates were run on a 7% polyacrylamide gel, blotted and probed with anti-phosphotyrosine antibody (αPY) or antibodies to PLC-γ2 or p85. (**B**) As above, but starved FD/wtFms cells were incubated at 37°C in the presence of 2 mM Na_3VO_4 before (15 min) and during M-CSF stimulation. Cell lysates were immunoprecipitated with anti-PLC-γ2 antibody and Western blots probed sequentially with anti-phosphotyrosine (αPY) and anti-PLC-γ2 (αPLC-γ2) antibodies.

a similar genetic link between phosphorylation of PLC-γ2 and the tyrosine-phosphorylated Fms 721 site. The FmsY721F mutant receptor was introduced into FDC-P1 cells and *in vivo* tyrosine phosphorylation of PLC-γ2 and p85 was examined 1 min after M-CSF stimulation and compared with that seen in FD/wtFms cells (Figure 5A). The Y721F mutation abolished p85 tyrosine phosphorylation as previously described (Reedijk *et al.*, 1992) and mature tyrosine-phosphorylated 165 kDa FmsY721F protein did not associate with p85. The SH2 domains of p85, however, still associate with a tyrosine-phosphorylated 100 kDa protein, as we have previously shown (Carlberg and Rohrschneider, 1997). Immunoprecipitation of PLC-γ2 from FD/wtFms cells revealed a single 150 kDa tyrosine-phosphorylated band after M-CSF stimulation and mutation of the Y721 autophosphorylation site resulted in a significant decrease in PLC-γ2 tyrosine phosphorylation (Figure 5B). A time course study of M-CSF stimulation of FD/FmsY721F cells confirmed that the strong decrease in PLC-γ2 tyrosine phosphorylation was not due to a delay in the response to M-CSF (Figure 5C).

A phosphorylated band of approximately the size of PLC-γ2 was visible on the p85 immunoprecipitate of FD/wtFms and this blot was stripped and reprobed with anti-PLC-γ2 antibody. PLC-γ2 was not detectable, suggesting that PLC-γ2 did not co-immunoprecipitate with p85 (not shown). Although not the most sensitive assay, the results suggest that PLC-γ2 and p85 do not simultaneously bind to the same Fms molecule.

We next investigated the effects of other Fms autophosphorylation site mutants on PLC-γ2 tyrosine phosphorylation. No effect of Y559F, Y697F or Y706F mutations could be detected on PLC-γ2 or p85 tyrosine phosphorylation (not shown). In contrast, the FmsY807F mutant dramatically and selectively affected tyrosine phosphorylation of PLC-γ2. Almost no detectable tyrosine-phosphorylated PLC-γ2 was observed after stimulation of FD/FmsY807F cells compared with FD/wtFms cells (Figure 6A). This effect was reproduced when PLC-γ2 was immunoprecipitated from FD/FmsY807F cells at various times after M-CSF stimulation and phosphotyrosine content detected by Western blotting (Figure 6B). Again, little or no tyrosine phosphorylation of PLC-γ2 was detectable. Binding of p85 to Fms in M-CSF-stimulated cells was high regardless of whether wild-type- or Y807F mutant-transfected cells were examined (Figure 6 bottom). The Y807F mutation also had no effect on M-CSF-dependent tyrosine phosphorylation of p85 (not shown). Interestingly, as we have noted elsewhere (K.Carlberg and L.R. Rohrschneider, manuscript in preparation), a significant amount of p85 was bound to the unstimulated FmsY807F mutant receptor (Figure 6C).

The ability of both SH2 domains from PLC-γ2 or p85 to bind either the wild-type, Y721F or Y807F Fms receptors was tested by measuring the ability of GST–SH2 fusion proteins to immunoprecipitate Fms. The content of tyrosine-phosphorylated proteins in lysates of control uninfected cells, wild-type, Y721F or Y807F FD/Fms

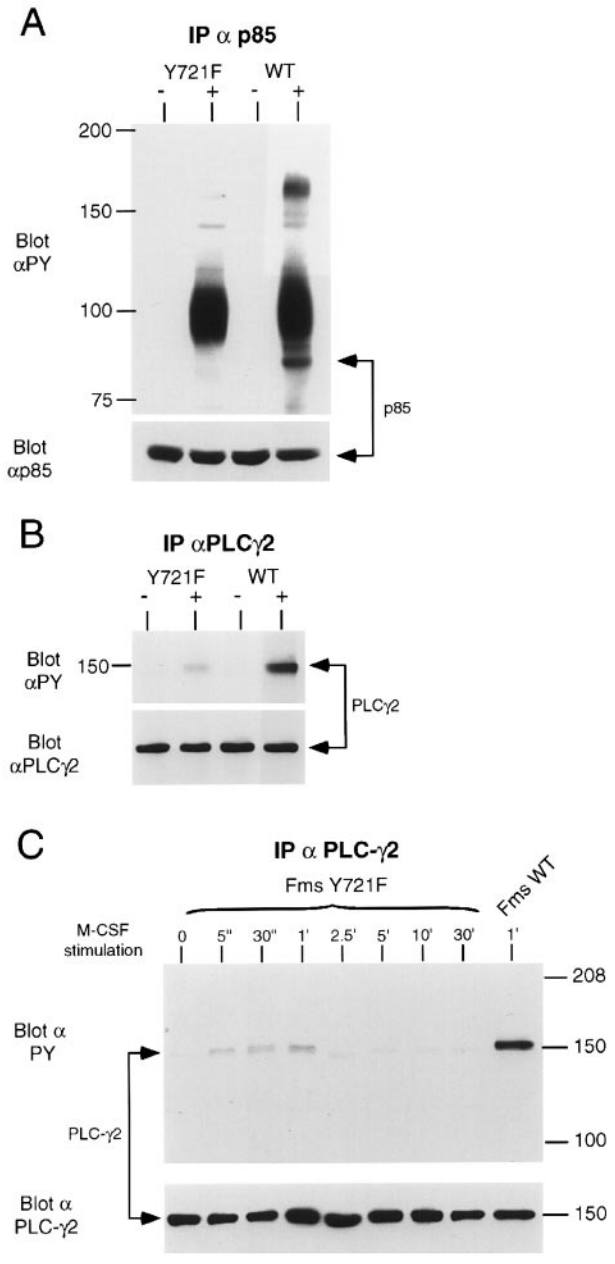

Fig. 5. Mutation of Fms Tyr721 to Phe (Y721F) decreases tyrosine phosphorylation of both p85 and PLC-γ2 by activated Fms *in vivo*. Starved FD/Y721F Fms and FD/wtFms cells were stimulated with M-CSF for 1 min at 37°C. Cell lysates were immunoprecipitated with (**A**) anti-p85 or (**B**) anti-PLC-γ2 antibodies. Immunoprecipitates were run on a 7% polyacrylamide gel, blotted and probed with anti-phosphotyrosine antibody (αPY) or with anti-p85 or anti- PLC-γ2 antibodies, respectively. (**C**) FD/Y721F Fms cells were stimulated with M-CSF for various times at 37°C. FD/wtFms cells were included as a positive control and Western blots were probed as indicated.

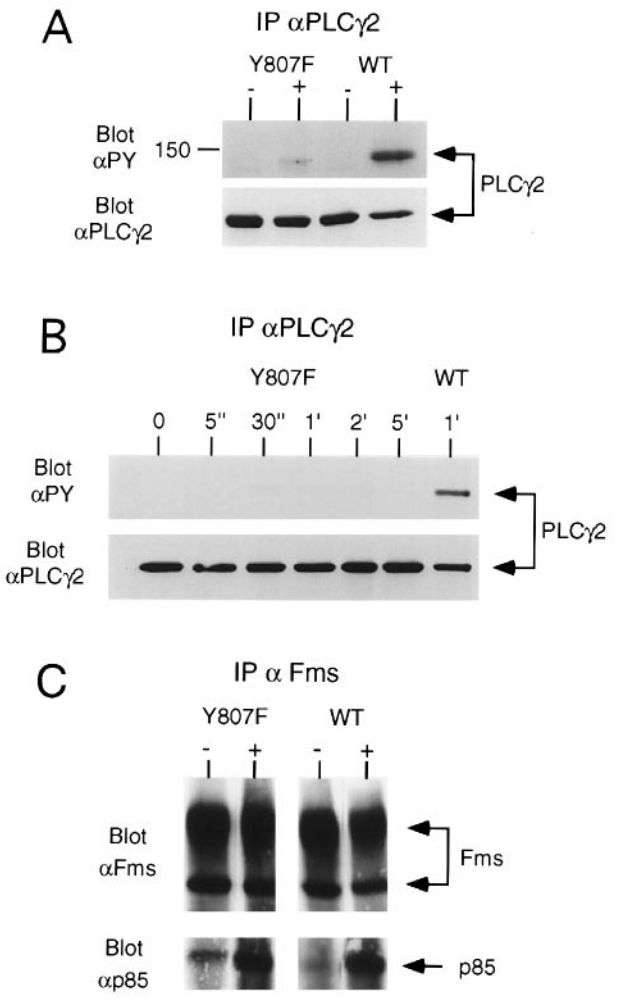

Fig. 6. Mutation of Fms Tyr807 to Phe (Y807F) strongly decreases PLC-γ2 tyrosine phosphorylation in M-CSF-stimulated FDC-P1 cells without affecting p85 association with Fms. Starved FD/Y807F Fms and FD/wtFms cells were stimulated with M-CSF at 37°C for 1 min (**A**) or different times (**B**). Cell lysates were immunoprecipitated with anti-PLC-γ2 antibody and Western blots were probed sequentially with anti-phosphotyrosine (αPY) and anti-PLC-γ2 (αPLC-γ2) antibodies. (**C**) As in (A), but lysates were immunoprecipitated with anti-Fms receptor antibody (αFms) and the filter was probed with anti-Fms receptor (αFms) and then anti-p85 subunit (αp85) antibodies.

cells stimulated with M-CSF was examined with anti-phosphotyrosine blots (Figure 7A). As we have shown before (Carlberg *et al.*, 1991; Bourette *et al.*, 1995), the extent of Fms tyrosine autophosphorylation is not dramatically different among the wild-type and mutant receptors, but the Y721F mutant was slightly lower in this experiment. The interaction of p85 SH2 domains with activated Fms was eliminated by the Y721F mutation, whereas the Y807F mutation had no effect on p85 SH2 domain binding. This result is consistent with previous publications and with our two-hybrid screen demonstrating specificity of the p85 SH2 domains for the Y721 site of Fms (Chen and Rohrschneider, 1992; Reedijk *et al.*, 1992). The PLC-γ2 SH2 domains, on the other hand, bound to tyrosine-phosphorylated wild-type Fms (Figures 3 and 7) and with weaker avidity to the mutant receptors. The binding of the PLC-γ2 SH2 domains to Y721F Fms is consistent with the two-hybrid data, showing that the N-SH2 domain is specific for the Y721 site, whereas the C-SH2 domain is promiscuous. Compared with the Y721F mutant, binding of both PLC-γ2 SH2 domains to the Y807F mutant Fms was higher, but it was still less than that seen with wild-type Fms. As a control we used a GST–Grb2 SH2 domain fusion protein, since Grb2 binds to phosphotyrosine 697 and its association with Fms is not altered by Y721F or Y807F mutations (van der Geer and Hunter, 1993; Lioubin *et al.*, 1994). Thus it was expected that this fusion protein would bind equally to

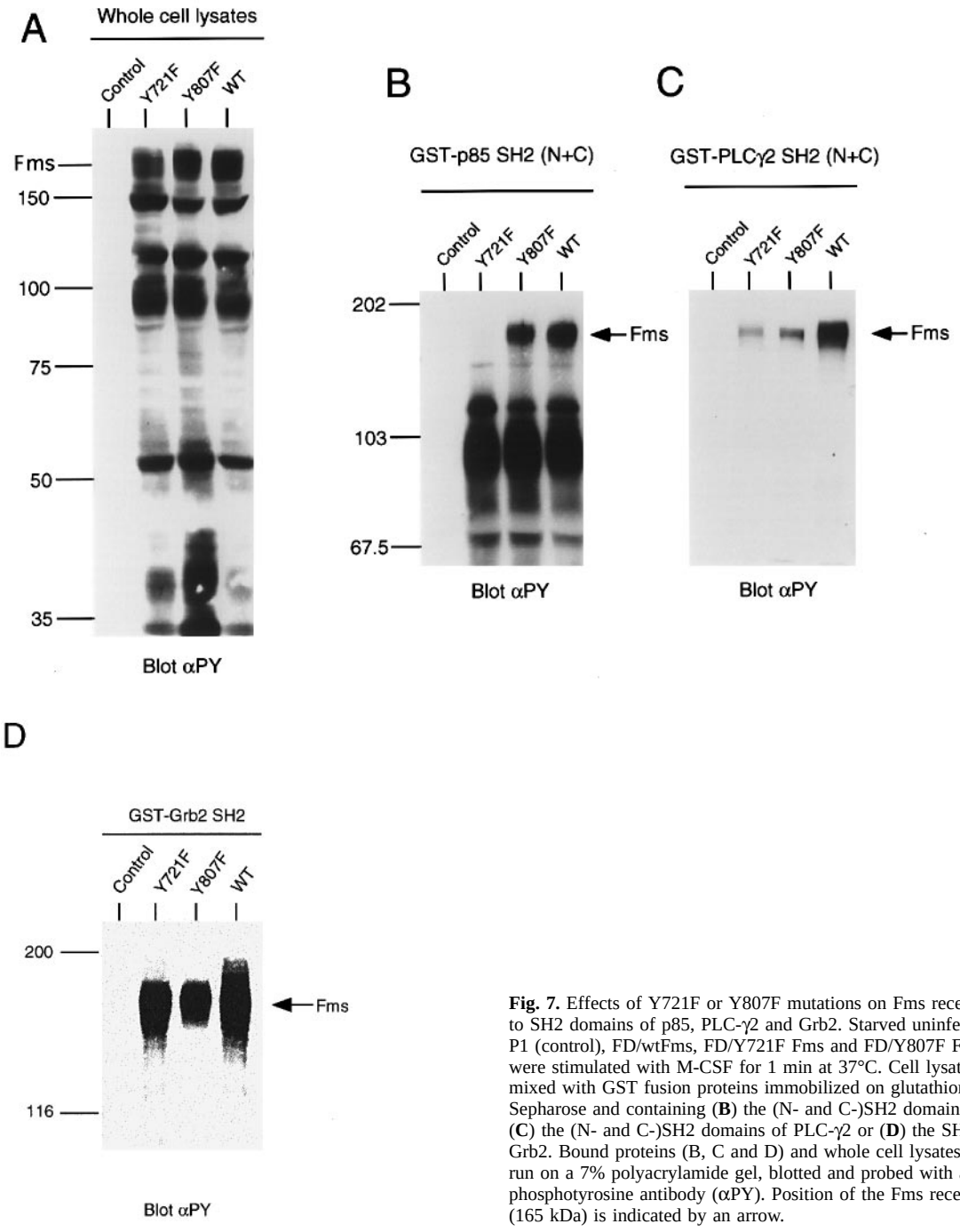

Fig. 7. Effects of Y721F or Y807F mutations on Fms receptor binding to SH2 domains of p85, PLC-γ2 and Grb2. Starved uninfected FDC-P1 (control), FD/wtFms, FD/Y721F Fms and FD/Y807F Fms cells were stimulated with M-CSF for 1 min at 37°C. Cell lysates were mixed with GST fusion proteins immobilized on glutathione–Sepharose and containing (**B**) the (N- and C-)SH2 domains of p85, (**C**) the (N- and C-)SH2 domains of PLC-γ2 or (**D**) the SH2 domain of Grb2. Bound proteins (B, C and D) and whole cell lysates (A) were run on a 7% polyacrylamide gel, blotted and probed with anti-phosphotyrosine antibody (αPY). Position of the Fms receptor (165 kDa) is indicated by an arrow.

wild-type, Y721F and Y807F Fms proteins. As shown in Figure 7D, binding of Y721F and Y807F Fms mutants with the fusion protein were only slightly affected compared with wild-type Fms. Accordingly, this demonstrates that low phosphotyrosine levels in Fms precipitates obtained after interaction of Y721F and Y807F Fms with GST–PLCγ2 SH2 (Figure 7C) could not be simply explained by a reduced phosphotyrosine content per receptor (due to the single tyrosine mutation).

These results indicate that both SH2 domains of p85 and the N-SH2 domain of PLC-γ2 specifically bind to the phosphorylated Y721 site and demonstrate that the lack of PLC-γ2 tyrosine phosphorylation by Fms mutant Y807F is not merely due a loss of the PLC-γ2 binding site.

PLC-γ2 tyrosine phosphorylation is regulated by the activity of PI3-kinase

The data indicate that two enzymes, sharing a common substrate (phosphatidylinositol 4,5-bisphosphate), both bind to the same tyrosine-phosphorylated site on Fms. We therefore examined potential regulatory mechanisms for their mutually exclusive binding. We determined whether the enzymatic activity of PI3-kinase was necessary for tyrosine phosphorylation of PLC-γ2 in M-CSF-stimulated cells. Wortmannin, a relatively specific inhibitor of PI3-kinase activity (Ui *et al.*, 1995; Wymann *et al.*, 1996), was used to treat FD/wtFms cells at 37°C for 30 min before M-CSF stimulation and tyrosine phosphorylation of PLC-γ2 was examined by immunoprecipitation and

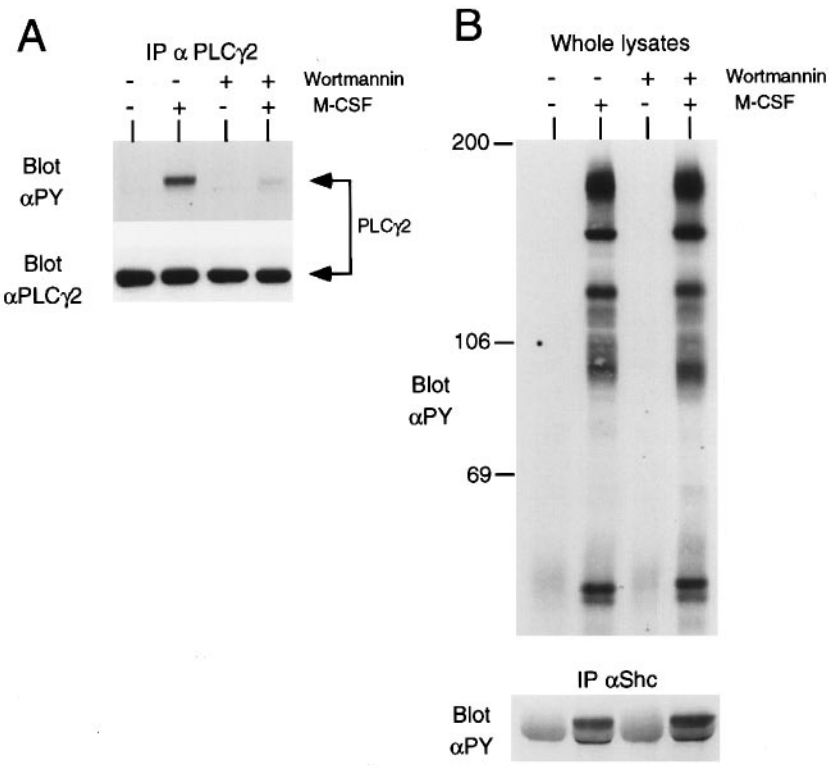

Fig. 8. Effect of the PI3-kinase specific inhibitor wortmannin on tyrosine phosphorylation of PLC-γ2 in FD/wtFms cells. Starved FD/wtFms cells were incubated in the presence or absence of 100 nM wortmannin for 30 min at 37°C. Cells were then stimulated with M-CSF for 1 min at 37°C. Cell lysates were immunoprecipitated with anti-PLC-γ2 antibody (αPLC-γ2) and the Western blot probed sequentially with anti-phosphotyrosine antibody (αPY) and αPLC-γ2 (**A**). Whole cell lysates and anti-Shc (αShc) immunoprecipitates (**B**) were blotted and probed with αPY.

blotting (Figure 8A). Almost no tyrosine-phosphorylated PLC-γ2 could be detected following wortmannin incubation. On a gross scale, the effect appeared to be specific to PLC-γ2 because tyrosine-phosphorylated proteins in total cell lysates did not change after treatment with wortmannin (Figure 8B). Tyrosine phosphorylation of immunoprecipitated Shc also did not change with wortmannin treatment. In separate experiments, no significant change in tyrosine phosphorylation of p85 could be detected and no significant modulation of p85 bound to Fms was detectable (not shown). These results suggest that the activity of PI3-kinase is required for tyrosine phosphorylation and activation of PLC-γ2 by the Fms receptor.

PLC-γ2 activity is required for differentiation of M-CSF-stimulated FD/wtFms cells

The potential role of PLC-γ2 in the biological effects of M-CSF was tested by treating FD/wtFms cells with the PLC-γ (both 1 and 2 isotypes) inhibitor U73122 (Chen *et al.*, 1996) before M-CSF stimulation. Cell proliferation in liquid cultures with or without U73122 (0.1 μM) was measured in the presence of either 5% WCM, as a source of IL-3, or with 2500 U/ml M-CSF. The presence of the PLC-γ inhibitor had no effect on IL-3-dependent exponential proliferation of FD/wtFms cells (Figure 9A). When FD/wtFms cells were cultivated in the presence of M-CSF but in the absence of the inhibitor the growth rate was lower than in the presence of IL-3 and gradually decreased over the 6 day period. This is due to terminal macrophage differentiation in the population, as we have shown previously (Bourette *et al.*, 1995). When the inhibitor was present in the M-CSF grown cultures no such decrease in the rate of M-CSF-dependent proliferation was observed and the cells exhibited exponential growth, as observed in cultures grown in the presence of IL-3 (Figure 9B). These results suggest that PLC-γ2 activity is connected with the growth inhibition (and differentiation) occurring in FD/wtFms cells stimulated with M-CSF (Bourette *et al.*, 1995).

The M-CSF-dependent macrophage differentiation of FD/wtFms cells was also monitored by morphological criteria in the presence or absence of the PLC-γ inhibitor (U73122). The results in Figure 9C–E demonstrate the morphology of cells grown under various conditions and stained with May–Grünwald/Giemsa. FD/wtFms cells maintained in the presence of IL-3 had an immature myeloid cell morphology with a large nucleus and a basophilic cytoplasm. These cells resembled parental FDC-P1 cells and did not change morphology in the presence of U73122 (Figure 9C). Morphological differentiation was apparent in FD/wtFms cells grown with M-CSF for 3 days without inhibitor (Figure 9D), as previously demonstrated (Bourette *et al.*, 1995). In the presence of the inhibitor, however, M-CSF grown cells failed to demonstrate a major morphological change (Figure 9E). Instead, most cells resembled those grown in IL-3, with a minority acquiring monocytic morphology. These results indicate that PLC-γ activation is involved in the M-CSF-dependent pathway for signaling terminal differentiation of FD/wtFms cells along the macrophage lineage.

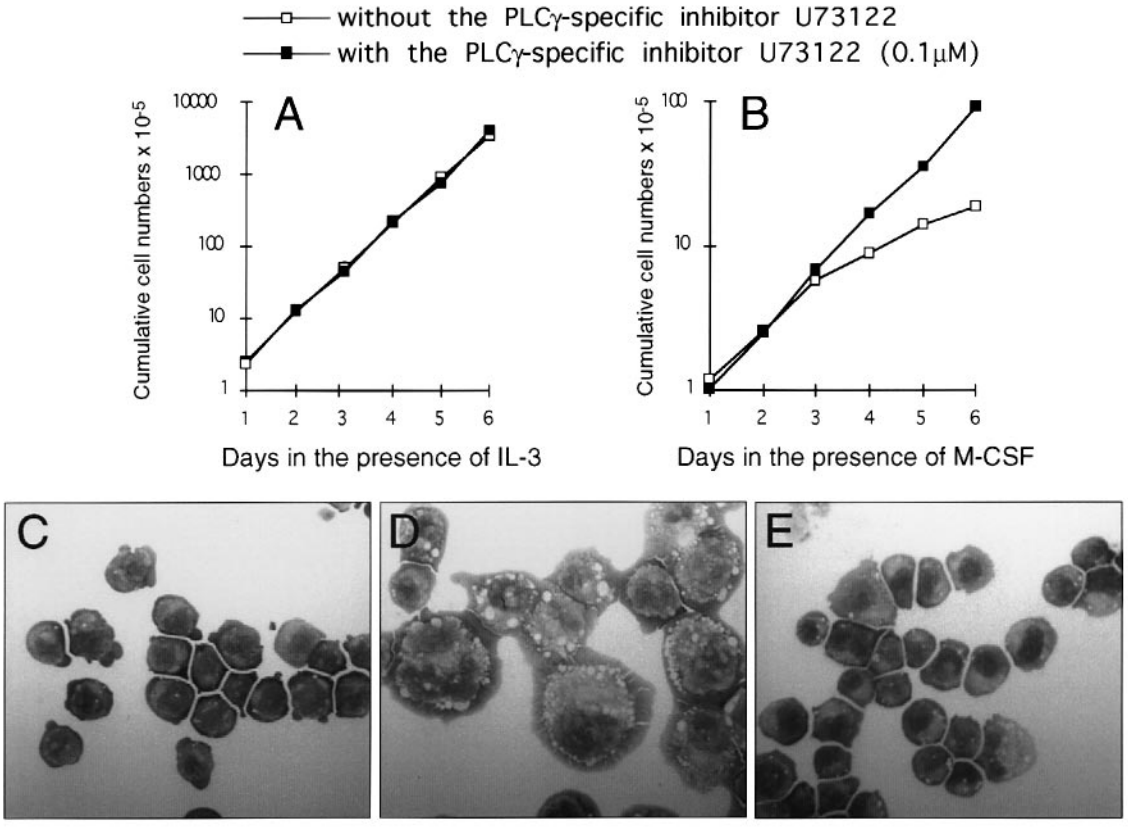

Fig. 9. The PLC-γ inhibitor U73122 blocks differentiation of M-CSF-stimulated myeloid FD/wtFms cells. FD/wtFms cells were washed free of IL-3 and seeded at 5×10^4 cells/ml in DMEM, 10% FBS containing (**A**) 5% WCM as a source of IL-3, with or without 0.1 μM U73122, and (**B**) 2500 U/ml M-CSF with or without 0.1 μM U73122. Viable cell number was determined daily, cultures were split and the complete medium was changed. The cumulative cell numbers are presented. Cellular morphology was examined after 3 days in the presence of (**C**) IL-3 without U73122, (**D**) M-CSF without U73122 and (**E**) M-CSF with U73122. Cells were cytocentrifuged onto glass slides, air dried and visualized with May–Grünwald/Giemsa stain.

Discussion

The two-hybrid system was used to isolate proteins interacting with the cytoplasmic domain of the murine Fms/M-CSF receptor (Fields and Song, 1989; Chien *et al.*, 1991; Vojtek *et al.*, 1993; Fields and Sternglanz, 1994; Kikuchi *et al.*, 1994; O'Neill *et al.*, 1994; Wang,T. *et al.*, 1994; Lioubin *et al.*, 1996). A LexA–Fms fusion protein, containing the entire cytoplasmic tyrosine kinase domain of wild-type murine Fms protein, was expressed as a bait. Because the LexA portion of the fusion protein binds to DNA as a dimer, it is assumed that co-dimerization and transphosphorylation of the attached Fms tyrosine kinase domain is facilitated, as previously proposed for the insulin receptor tyrosine kinase domain (O'Neill *et al.*, 1994). This is analogous to the c-Met receptor, whose activation results from substitution of a dimerization motif for the extracellular and transmembrane domains (Rodrigues and Park, 1993). Introduction of the LexA–Fms construct into yeast resulted in expression of the fusion protein and tyrosine autophosphorylation on three known sites (Y559, Y697 and Y721), as judged by the fact that molecules known to bind these phosphorylated sites were isolated during the library screen. In addition, mutation of the Fms Y807 site, although not a known binding site for signaling proteins, exhibited identical properties in the yeast system as the LexA fusion protein compared with expression of the full-length mutant protein in FDC-P1 cells (see below). Thus the LexA fusion protein with the Fms domain in yeast appeared to recapitulate the initial autophosphorylation process characterized in mammalian cells.

PLC-γ2 is a substrate of the Fms/M-CSF receptor and binds to phosphorylated Y721

The yeast two-hybrid screen identified several PLC-γ2 cDNA clones interacting with tyrosine-phosphorylated Fms (Figure 1 and data not shown). In all cases the PLC-γ2 clones encompassed one or both of the SH2 domains. The N-SH2 domain exhibited absolute specificity for the phosphorylated Fms site Y721, whereas the C-SH2 domain bound equally well to phosphorylated wild-type and Y721F Fms, indicating a much more promiscuous interaction. Neither N- nor C-SH2 domains bound to KD Fms. This same specificity for binding activated Fms at the phosphorylated Y721 site was demonstrated by immunoprecipitation experiments utilizing the PLC-γ2 SH2 domains fused to GST and by tyrosine phosphorylation and activation (Nishibe *et al.*, 1990) of PLC-γ2 in M-CSF-stimulated FD/wtFms cells. Activation of PLC-γ2 was greatly decreased in M-CSF-stimulated FD/FmsY721F cells. Therefore, we proposed that PLC-γ2 interacts with the phosphorylated Y721 site on activated Fms and is tyrosine phosphorylated in the process. However, we could not detect any significant association of Fms receptor and PLC-γ2 *in vivo* by co-immunoprecipitation (data not

shown). This could reflect the transient activation of PLC-γ2 and competition with p85 for binding to phosphotyrosine 721 (see below). On the other hand, hepatocyte growth factor/scatter factor (HGF/SF) receptor was shown to induce tyrosine phosphorylation of PLC-γ in two different cell lines, expressing 5000 and 100 000 receptors/cell respectively, but complexes of PLC-γ and receptors could be detected only in the latter cell line (Ponzetto *et al.*, 1994). Since FD/wtFms cells express ~5000 receptors/cell (Novak *et al.*, 1996), complexes of Fms receptor and PLC-γ2 in FD/wtFms cells might be below the limit of detection by a co-immunoprecipitation experiment.

Stimulation by multiple tyrosine kinase receptors, including receptors for PDGF, epidermal growth factor, nerve growth factor, fibroblast growth factor, Kit and the insulin receptor, all induce increased phosphorylation and activation of PLC-γ (Noh *et al.*, 1995). Even though Fms shares structural features with some of these growth factor receptors, several reports have indicated that Fms does not bind nor activate PLC-γ (Whetton *et al.*, 1986; Downing *et al.*, 1989; Hartmann *et al.*, 1990). There are several possible explanations for the discrepancies between our present results and previous failures to detect PLC-γ activation by Fms. Many of the previous studies were done on PLC-γ1, which may give different results than PLC-γ2. Also, we show here that PLC-γ2 is rapidly phosphorylated and dephosphorylated after M-CSF stimulation (see Figure 4) and after 5 min, when measurements were previously made (Downing *et al.*, 1989), our results indicate that PLC-γ2 is already dephosphorylated. Another possibility is that PLC-γ2 activation by Fms is cell type specific. Fms may not activate PLC-γ2 in mature macrophages (Whetton *et al.*, 1986; Downing *et al.*, 1989) and human Fms may not function properly in hamster fibroblasts (Hartmann *et al.*, 1990). This may not be surprising because two examples of myeloid-specific interactions of Fms have already been reported (Lioubin *et al.*, 1994, 1996; Carlberg and Rohrschneider, 1997).

p85/PI3-kinase and PLC-γ2 compete for binding to the Fms Y721 site

The phosphorylated Y721 of the Fms receptor is a known binding site for the p85 subunit of PI3-kinase (Reedijk *et al.*, 1992), suggesting that PLC-γ2 and p85 may compete for binding to the same site. Both could occupy the same Y721 site on different subunits of the dimer receptor, however, we feel this is unlikely because we have not detected PLC-γ2 in immunoprecipitates of p85 bound to M-CSF-stimulated Fms. Two signaling molecules sharing a common phosphotyrosine-containing site has been reported for the PDGF receptor, where Nck and p85 both bind to phosphorylated Y751 (Nishimura *et al.*, 1993), and for the HGF/SF receptor (Ponzetto *et al.*, 1994). The recognition sequence of the Fms receptor that binds p85 SH2 domains is YVEM (Songyang *et al.*, 1993), but the recognition sequence for the PLC-γ2 SH2 domains has not yet been described and it is difficult to extrapolate from the known binding motif of PLC-γ1 SH2 domains because the amino acid homology between the respective SH2 domains of PLC-γ1 and PLC-γ2 is ~60% (Emori *et al.*, 1989; Koch *et al.*, 1991). Interestingly, substitution of amino acids surrounding Tyr1021 on the PDGF receptor, which binds only PLC-γ1, creates a site accommodating binding of either PLC-γ1 or the p85 subunit of PI3-kinase (Larose *et al.*, 1993). Clearly, binding of PLC-γ2 and p85 to a single tyrosine-phosphorylated site is a possibility. However, based on our results with the GST–SH2 domain fusions (Figure 3), the affinity for Fms appears to be much higher with p85 SH2 domains than with PLC-γ2 SH2 domains.

The yeast mating data and the immunoprecipitations with the GST–SH2 fusion proteins (Figures 2 and 3) indicated that both SH2 domains of p85 were specific for the phosphorylated Y721 site of Fms. In contrast, only the N-SH2 domain of PLC-γ2 bound to the Y721 site, but the C-SH2 domain bound equally well to wild-type Fms or to Fms containing five mutated known sites of tyrosine autophosphorylation. This suggests that an as yet unknown autophosphorylation site may bind the C-SH2 domain of PLC-γ2. The C-SH2 domain of PLC-γ2 may anchor the protein to Fms allowing the N-SH2 domain to exchange with the p85 SH2 domain(s) under appropriate conditions. A similar situation has been described for the PDGF receptor, where the C-SH2 domain of GAP specifies binding to Y771, the C-SH2 domain of p85 binds to either Y740 or Y751 and each N-SH2 domain of the proteins binds to unidentified phosphorylation sites (Cooper and Kashishian, 1993).

The experiments with the PI3-kinase inhibitor wortmannin demonstrated that PI3-kinase activity was required for activation of PLC-γ2. This suggests, perhaps, that some product in the PI3-kinase pathway may negatively influence binding of p85 to phosphorylated Y721 and/or positively affect PLC-γ2 binding to the same site. If the N-SH2 domain of PLC-γ2 exchanges with the p85 SH2 domains for binding to the phosphorylated Y721 site on activated Fms, how does this occur if the affinity of p85 for Fms is much higher? One possibility is that a feedback mechanism removes the higher affinity binder (p85), allowing the lower affinity molecule (PLC-γ2) to bind. Phosphatidylinositol 3,4,5-trisphosphate (PtdIns[3,4,5]P_3) is the lipid product of PI3-kinase acting on the substrate phosphatidylinositol 4,5-bisphosphate. PtdIns[3,4,5]P_3 has been shown to bind to the SH2 domains of p85 (Rameh *et al.*, 1995) and, as a product in the PI3-kinase pathway, would be a logical molecule decreasing p85 binding at the Fms Y721 site and permitting the N-SH2 domain of PLC-γ2 to occupy this site.

We also investigated possible negative feedback along the PLC-γ2 pathway. Treatment of FD/wtFms cells with the specific PLC-γ inhibitor U73122 before and during M-CSF stimulation had no effect on tyrosine phosphorylation of PLC-γ2 (not shown), suggesting that the products of PLC-γ2 enzymatic activation do not feed back to influence PLC-γ2 binding to Fms. Therefore, alternative mechanisms, such as phosphorylation or dephosphorylation of PLC-γ2, could influence its association with Fms, and this will require further investigation.

Relationship between differentiation and activation of both of PLC-γ2 and PI3-kinase by the Fms receptor

The Fms autophosphorylation site Y807 is located in the second half of the kinase domain (Tapley *et al.*, 1990) and corresponds to an autophosphorylation site conserved among most tyrosine kinases (Hanks and Quinn, 1991).

It is presently uncertain whether this site interacts with specific SH2-containing signaling proteins or whether signal transduction is changed by an altered protein conformation induced by phosphorylation at this site (Hubbard *et al.*, 1994). In favor of the first hypothesis, Tyr807 of the v-Fms oncogene product has been described as a possible binding site of p120Ras GAP in fibroblasts (Trouliaris *et al.*, 1995), however, direct evidence that GAP binds to this site is lacking. Alternatively, interaction of Fms with GAP could occur through a conformational change in the Fms kinase domain as a result of tyrosine phosphorylation at Y807. Other proteins also bind to M-CSF-stimulated Fms in a Y807-dependent manner. Association of Src-like proteins with the human Fms receptor occurs at Tyr561 (559 of the murine receptor) (Alonso *et al.*, 1995), but mutation of Tyr809 (807 of the murine receptor) reduces binding (Courtneidge *et al.*, 1993). Using Fms receptor mutants and phosphopeptides, it was shown that the STAT1 binding site on the murine Fms receptor is Tyr706, but both Y706F and Y807F mutations abrogate M-CSF-dependent STAT1 activation in FDC-P1 cells (Novak *et al.*, 1996). Based on our data and the literature, we suggest that tyrosine phosphorylation of FmsY807 controls the conformation of the receptor and its differential interaction with signaling molecules. Lack of phosphorylation at the Fms Y807 (i.e. FmsY807F) site would result in decreased binding of Src family members, the STAT1 transcription factor and PLC-γ2 (Bishayee *et al.*, 1988; Keating *et al.*, 1988).

We have previously shown that M-CSF-induced differentiation of FD/wtFms cells toward macrophages was eliminated by the single Y807F mutation (Bourette *et al.*, 1995). Proliferation of the cells was not decreased by the Y807F mutation, but rather increased, consistent with elimination of terminal differentiation. The effects on Fms signaling of the specific inhibitor of PLC-γ U73122 are very similar to those observed with the Y807F mutation (Figure 9), suggesting that the lack of differentiation observed with the Y807F mutation was due to its inability to activate the PLC-γ pathway. Wortmannin, a specific inhibitor of PI3-kinase activity, also prevented PLC-γ activation. Together, these data suggest a complex mechanism for regulating cellular differentiation by controlling reciprocal activation of PI3-kinase and PLC-γ. We propose that upon M-CSF dimerization and activation of Fms one conformation of the autophosphorylated cytoplasmic tyrosine kinase domain binds signal transduction proteins including p85/PI3-kinase at the phosphorylated Y721 site. Subsequent trans-autophosphorylation of Fms at the Y807 site induces a second conformation in the Fms tyrosine kinase domain, favoring release of p85/PI3-kinase and binding of PLC-γ2 at the now available Y721 site. Rapid tyrosine phosphorylation and activation of PLC-γ2 triggers its release and the more abundant p85/PI3-kinase again assumes its higher affinity interaction with the phosphorylated Y721 site subsequent to dephosphorylation of the Y807 site. Thus one cycle activates PI3-kinase and PLC-γ2 sequentially and regulates temporal activation of downstream targets leading to differentiation. Although we have focused on the reciprocal interactions of p85/PI3-kinase and PLC-γ2 at the Fms Y721 site, other signaling proteins may also interact preferentially with each conformation. Further discussion and illustration of this mechanism can be found on our laboratory Web page (http://www.fhcrc.org/~lrr).

It is not clear whether such a mechanism alone regulates differentiation signaling, but the U73122 inhibitor and Y807F mutant studies effectively block PLC-γ2 activation, indicating that PLCγ activity is required for differentiation, although, as noted above, other signaling proteins may also participate. Surprisingly, however, mutation of Fms Tyr721 has only a minor effect on differentiation signaling in FDC-P1 cells (Bourette *et al.*, 1995). This suggests that the PLC-γ2 activated through Y721 may have only a minor role in differentiation signaling or accounts for only a portion of the mechanism. However, the Fms Y721F mutant could simply represent a 'leaky' mutation, because some PLC-γ2 is still tyrosine phosphorylated in the mutant-transfected cells (Figure 5). Alternatively, other Fms signaling molecules, such as Cbl and Src-like proteins, also may contribute to PI3-kinase and PLC-γ activation (Hartley *et al.*, 1995; Lee and Rhee, 1995; Takata and Kurosaki, 1996). Redundant neuronal differentiation signaling through the Trk receptor has been described (Obermeier *et al.*, 1994; Stephens *et al.*, 1994) and Fms may also utilize multiple signals for myeloid differentiation.

The opposing effects of PI3-kinase and PLC-γ2 reported in the literature are consistent with our proposed mechanism for Fms signaling. The role of PI3-kinase in the mechanism of cell transformation and mitogenic signaling has been extensively documented (for a review see Carpenter and Cantley, 1996). In contrast, PLC-γ has been described as a negative feedback regulator of the proliferation signal transduced by tyrosine kinase receptors (Kerr *et al.*, 1996; Obermeier *et al.*, 1996) and neurite outgrowth can be blocked by inhibitors of PLC-γ (Kimura *et al.*, 1994; Hall *et al.*, 1996). Moreover, several studies have recently elucidated the role of protein kinase C (PKC) family members in monocytic differentiation (Mischak *et al.*, 1993; Whetton *et al.*, 1994; Kiley and Parker, 1995; Rossi *et al.*, 1996). PKC is a downstream element of the PLC-γ pathway and therefore regulated activation of PLC-γ2 by Fms is a critical component of the monocyte/macrophage differentiation pathway.

Materials and methods

Yeast two-hybrid system

Plasmids, *S.cerevisiae* strains, selective media and the transformation protocol have been described previously (Vojtek and Hollenberg, 1995). For construction of the LexA–wild-type Fms bait the cDNA encoding the entire Fms cytoplasmic domain (amino acids 536–976) was inserted into the BTM116 vector, resulting in production of a LexA–wild-type Fms fusion protein. The *S.cerevisiae* L40 strain was transformed and selected in a medium lacking uracil and tryptophan (UW). A single colony, selected for expression of the LexA–wild-type Fms fusion protein, was tested for Fms autophosphorylation. For this analysis yeast lysates were made using glass beads in RIPA lysis buffer (Carlberg and Rohrschneider, 1994) and proteins immunoprecipitated with rabbit polyclonal antiserum against the murine Fms cytoplasmic domain. Immunoprecipitates were run on a 7% polyacrylamide gel, blotted and probed sequentially with anti-Fms, anti-LexA and anti-phosphotyrosine antibodies. A colony containing the LexA–wild-type Fms bait was then transformed with the VP16 cDNA library derived from the multipotential hematopoietic cell line EML (Tsai *et al.*, 1994; Lioubin *et al.*, 1996). Transformants were selected on medium lacking uracil, tryptophan and leucine (UWL). Colonies containing a VP16 fusion protein interacting with the bait were then selected on the basis of transactivation of the

*His*3 reporter gene after 3 days growth on medium lacking uracil, tryptophan, leucine, lysine and histidine (WHULK) and containing 50 mM 3-amino-1,2,4-triazole (Sigma). Purified DNA isolated from positive clones was sequenced by PCR using VP16-specific sense and antisense primers (Vojtek and Hollenberg, 1995). Sequence comparisons were done with the Genetics Computer Group (GCG) programs and different DNA sequence databases using the FASTA program. Mating assay experiments were performed as previously described (Vojtek and Hollenberg, 1995) and interactions between the new bait and the protein of the VP16 library were visualized by spotting cells on WHULK plates containing X-Gal to detect transactivation of the β-galactosidase reporter gene and observation of blue colonies after 3 days (Vojtek and Hollenberg, 1995).

Cells and culture conditions

FDC-P1 clone 19 cells and derivatives expressing wild-type or mutant Fms have been described previously (Bourette *et al.*, 1995). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and murine interleukin 3 (IL-3) from conditioned medium of WEHI cells (WCM) or X63-IL-3 cells (Karasuyama and Melchers, 1988). For culture with M-CSF, cells were washed and resuspended in DMEM, 10% FBS supplemented with 2500 U/ml recombinant murine M-CSF partially purified from a conditioned medium from Sf9 insect cells expressing M-CSF from a baculovirus vector (Wang *et al.*, 1993).

Antibodies and inhibitors

Rabbit polyclonal antiserum against LexA protein was a gift from M.Chen (FHCRC) and R.Brent (Harvard). Rabbit polyclonal antiserum 5674, anti-mouse Fms extracellular domain and FITC-conjugated F(ab′)2 fragment donkey anti-rabbit IgG second antibody (Jackson Immunoresearch Laboratories) were used for Fms-positive cell sorting. Rabbit polyclonal antiserum 4599B anti-murine Fms cytoplasmic domain was used for immunoprecipitation and Western blotting. Monoclonal anti-phosphotyrosine antibody (αPY) was from Upstate Biotechnology. Rabbit polyclonal anti-PLC-γ2 antibody (αPLC-γ2) reacts specifically with PLC-γ2, but not other PLC isozymes (Q20, catalog No. sc-407; Santa Cruz Biotechnology). Rabbit polyclonal anti-PI3-kinase subunit p85 antibody (αp85) was from Upstate Biotechnology. Rabbit polyclonal antiserum to the murine Shc SH2 domain was raised to the GST fusion protein (Lioubin *et al.*, 1996). PLC-γ-specific inhibitor U73122 was from BIOMOL and the PI3-kinase-specific inhibitor wortmannin was from Sigma.

Binding assays using GST–SH2 domain fusion proteins

The murine SH2 domains of PLC-γ2, p85 or Grb2 were expressed from pGEX1 in *Escherichia coli* XL-1 Blue (Stratagene) and purified using glutathione–Sepharose beads (Pharmacia) as described (Lowenstein *et al.*, 1992). FDC-P1 cells and derivatives were starved of growth factor by washing in phosphate-buffered saline (PBS) and resuspending in DMEM supplemented with 1% FBS for 3 h. Starved cells were then resuspended in PBS containing 2 mM Na_3VO_4 and stimulated or not with M-CSF (5000 U/ml) for 1 min at 37°C. Cells were lysed in ice-cold NP40 lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM Na_3VO_4 and whole cell lysates, equalized for protein content, were mixed for 18 h at 4°C with GST–SH2 domains fusion proteins immobilized on glutathione–Sepharose beads. The beads were washed three times with 1 ml lysis buffer, bound proteins were released by boiling in Laemmli buffer (Laemmli, 1970) for 10 min and separated on a 7% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and blotted with anti-phosphotyrosine or anti-Fms antibodies. For the binding assays using GST–PLC-γ2 SH2 domain fusion protein dithiothreitol (10 mM) was added during incubation with the cell lysates.

Immunoprecipitation and immunoblotting

Cells were washed in PBS and resuspended in DMEM supplemented with 1% FBS for 3 h. Starved cells were then resuspended in PBS and stimulated or not with M-CSF (5000 U/ml) for different times at 37°C. In one experiment cells were incubated in the presence of 2 mM Na_3VO_4 added 15 min prior to M-CSF stimulation. Cells were lysed in ice-cold NP40 lysis buffer containing 1 mM PMSF and 2 mM Na_3VO_4. Equalized whole cell lysates were mixed for 18 h with various antibodies as specified in the text and protein G coupled to agarose beads (Pharmacia) for immunoprecipitation. Proteins from whole cell lysates and immunoprecipitates were separated on a 7% SDS–polyacrylamide gel, then transferred to a nitrocellulose membrane and blotted with various antibodies as specified in the text. Composition of NP40 lysis buffer, techniques of Western transfer to nitrocellulose and immunoblotting have been described previously (Carlberg and Rohrschneider, 1994; Bourette *et al.*, 1995).

Morphological studies

Cells were cytocentrifuged onto glass slides, air dried, stained with May–Grünwald/Giemsa stain (Sigma) and mounted in Permount (Fisher).

Acknowledgements

We thank Hajime Karasuyama and Astar Winoto for the gift of X63-IL-3 cells, Michelle Chen for the anti-LexA antibody, Schickwann Tsai for the EML VP16 cDNA library, Andrew Berger for expert technical assistance with flow cytometry analysis, Ruth White for secretarial assistance, all the members of L.Rohrschneider's laboratory, R.Eisenmam's laboratory and J.Cooper's laboratory for generous gifts of reagents and helpful discussions, especially Paul Algate, Kristen Carlberg, Carol Laherty, Mario Lioubin, Christophe Quéva, Anne Vojtek, Sue Geier and Ingrid Wolf. This work was supported by a fellowship from the Leukemia Research Foundation (R.P.B.) and PHS grants CA20551 and CA40987 from the National Cancer Institute.

References

Alonso,G., Koegl,M., Mazurenko,N. and Courtneidge,S.A. (1995) Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors. *J. Biol. Chem.*, **270**, 9840–9848.

Anderson,D., Koch,C.A., Grey,L., Ellis,C., Moran,M.F. and Pawson,T. (1990) Binding of SH2 domains of phospholipase Cγ1, GAP, and Src to activated growth factor receptors. *Science*, **250**, 979–982.

Bishayee,S., Majumdar,S., Scher,C.D. and Khan,S. (1988) Characterization of a novel anti-peptide antibody that recognizes a specific conformation of the platelet-derived growth factor receptor. *Mol. Cell. Biol.*, **8**, 3696–3702.

Bourette,R.P., Myles,G.M., Carlberg,K. and Rohrschneider,L.R. (1995) Uncoupling of the proliferation and differentiation signals mediated by the murine macrophage colony-stimulating factor receptor expressed in myeloid FDC-P1 cells. *Cell Growth Differ.*, **6**, 631–645.

Byrne,P.V., Guilbert,L.J. and Stanley,E.R. (1981) Distribution of cells bearing receptors for a colony-stimulating factor (CSF-1) in murine tissues. *J. Cell Biol.*, **91**, 848–853.

Carlberg,K. and Rohrschneider,L.R. (1994) The effect of activating mutations on dimerization, tyrosine phosphorylation, and internalization of the macrophage colony stimulating factor receptor. *Mol. Biol. Cell*, **5**, 81–95.

Carlberg,K. and Rohrschneider,L.R. (1997) Characterization of a novel tyrosine phosphorylated 100-kDa protein that binds to SHP-2 and phosphatidylinositol 3′-kinase in myeloid cells. *J. Biol. Chem.*, **272**, 15943–15950.

Carlberg,K., Tapley,P., Haystead,C. and Rohrschneider,L.R. (1991) Role of kinase activity and the kinase insert region in ligand-induced internalization and degradation of the c-*fms* protein. *EMBO J.*, **10**, 877–883.

Carpenter,C.L. and Cantley,L.C. (1996) Phosphoinosotide 3-kinase and the regulation of cell growth. *Biochim. Biophys. Acta Rev. Cancer*, **1288**, 11–16.

Chen,A. and Rohrschneider,L.R. (1992) Mechanism of differential inhibition of factor dependent cell proliferation by transforming growth factor β1: selective uncoupling of *fms* from *myc*. *Blood*, **81**, 2539–2546.

Chen,P., Xie,H. and Wells,A. (1996) Mitogenic signaling from the EGF receptor is attenuated by a phospholipase C-γ/protein kinase C feedback mechanism. *Mol. Biol. Cell*, **7**, 871–881.

Chien,C.-T., Bartel,P.L., Sternglanz,R. and Fields,S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl Acad. Sci. USA*, **88**, 9578–9582.

Cooper,J.A. and Kashishian,A. (1993) *In vivo* binding properties of SH2 domains from GTPase-activating protein and phosphatidylinositol 3-kinase. *Mol. Cell. Biol.*, **13**, 1737–1745.

Courtneidge,S.A., Dhand,R., Pilat,D., Twamley,G.M., Waterfield,M.D. and Roussel,M.F. (1993) Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor. *EMBO J.*, **12**, 943–950.

Downing,J.R., Margolis,B.L., Zilberstein,A., Ashmun,R.A., Ullrich,A., Sherr,C.J. and Schlessinger,J. (1989) Phospholipase C-gamma, a substrate for PDGF receptor kinase, is not phosphorylated on tyrosine during the mitogenic response to CSF-1. *EMBO J.*, **8**, 3345–3350.

Emori,Y., Homma,Y., Sorimachi,H., Kawasaki,H., Nakanishi,O., Suzuki,K. and Takenawa,T. (1989) A second type of rat phosphoinositide-specific phospholipase C containing a *src*-related sequence not essential for phosphoinositide-hydrolyzing activity. *J. Biol. Chem.*, **264**, 21885–21890.

Fields,S. (1993) The two-hybrid system to detect protein–protein interactions. *Methods Companion Methods Enzymol.*, **5**, 116–124.

Fields,S. and Song,O.-K. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.

Fields,S. and Sternglanz,R. (1994) The two hybrid system: an assay for protein–protein interactions. *Trends Genet.*, **10**, 286–292.

Hall,H., Williams,E.J., Moore,S.E., Walsh,F.S., Prochiantz,A. and Doherty,P. (1996) Inhibition of FGF-stimulated phosphatidylinositol hydrolysis and neurite outgrowth by a cell-membrane permeable phosphopeptide. *Curr. Biol.*, **6**, 580–587.

Hanks,S.K. and Quinn,A.M. (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods Enzymol.*, **200**, 38–62.

Hartley,D., Meisner,H. and Corvera,S. (1995) Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-*cbl*. *J. Biol. Chem.*, **270**, 18260–18263.

Hartmann,T., Seuwen,K., Roussel,M.F., Sherr,C.J. and Pouyssegur,J. (1990) Functional expression of the human receptor for colony-stimulating factor 1 (CSF-1) in hamster fibroblasts: CSF-1 stimulates Na^+/H^+ exchange and DNA-synthesis in the absence of phosphoinositide breakdown. *Growth Factors*, **2**, 289–300.

Hubbard,S.R., Wei,L., Ellis,L. and Hendrickson,W.A. (1994) Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature*, **372**, 746–754.

Karasuyama,H. and Melchers,F. (1988) Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4, or 5, using modified cDNA expression vectors. *Eur. J. Immunol.*, **18**, 97–104.

Keating,M.T., Escobedo,J.A. and Williams,L.T. (1988) Ligand activation causes a phosphorylation-dependent change in Platelet-Derived Growth Factor receptor conformation. *J. Biol. Chem.*, **263**, 12805–12808.

Kerr,W.G, Heller,M. and Herzenberg,L.A. (1996) Analysis of lipopolysaccharide-response genes in B-lineage cells demonstrates that they can have differentiation stage-restricted expression and contain SH2 domains. *Proc. Natl Acad. Sci. USA*, **93**, 3947–3952.

Kikuchi,A., Demo,S.D., Ye,Z.-H., Chen,Y.-W. and Williams,L.T. (1994) ralGDS family members interact with the effector loop of *ras* p21. *Mol. Cell. Biol.*, **14**, 7483–7491.

Kiley,S.C. and Parker,P.J. (1995) Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation. *J. Cell Sci.*, **108**, 1003–1016.

Kimura,K. *et al.* (1994) Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.*, **269**, 18961–18967.

Koch,C.A., Anderson,D., Moran,M.F., Ellis,C. and Pawson,T. (1991) SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science*, **252**, 668–674.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Larose,L., Gish,G., Shoelson,S. and Pawson,T. (1993) Identification of residues in the β platelet-derived growth factor receptor that confer specificity for binding to phospholipase C-γ1. *Oncogene*, **8**, 2493–2499.

Lee,S.B. and Rhee,S.G. (1995) Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr. Biol.*, **7**, 183–189.

Lioubin,M.N., Myles,G.M., Carlberg,K., Bowtell,D. and Rohrschneider, L.R. (1994) SHC, GRB2, SOS1 and a 150-kilodalton tyrosine-phosphorylated protein form complexes with Fms in hematopoietic cells. *Mol. Cell. Biol.*, **14**, 5682–5691.

Lioubin,M.N., Algate,P.A., Tsai,S., Carlberg,K., Aebersold,R. and Rohrschneider,L.R. (1996) $p150^{Ship}$, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev.*, **10**, 1084–1095.

Lowenstein,E.J. *et al.* (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell*, **70**, 431–442.

Metcalf,D. (1984) *The Hemopoietic Colony Stimulating Factors*. Elsevier, New York.

Metcalf,D. (1989) The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature*, **339**, 27–30.

Mischak,H., Pierce,J.H., Goodnight,J.A., Kazanietz,M.G., Blumberg, P.M. and Mushinski,J.F. (1993) Phorbol ester-induced myeloid differentiation is mediated by protein kinase C-alpha and -delta and not by protein kinase C-betaII, -epsilon, -zeta, and -eta. *J. Biol. Chem.*, **268**, 20110–20115.

Nishibe,S., Wahl,M.I., Hernández-Sotomayor,S.M.T., Tonks,N.K., Rhee,S.G. and Carpenter,G. (1990) Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. *Science*, **250**, 1253–1256.

Nishimura,R., Li,W., Kashishian,A., Mondino,A., Zhau,M., Cooper,J. and Schlessinger,J. (1993) Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. *Mol. Cell. Biol.*, **13**, 6889–6896.

Noh,D.-Y., Shin,S.H. and Rhee,S.G. (1995) Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochem. Biophys. Acta*, **1242**, 99–114.

Novak,U., Nice,E., Hamilton,J.A. and Paradiso,L. (1996) Requirement for Y706 of the murine (or Y708 of the human) CSF-1 receptor for STAT1 activation in response to CSF-1. *Oncogene*, **13**, 2607–2613.

Obermeier,A., Bradshaw,R.A., Seedorf,K., Choidas,A., Schlessinger,J. and Ullrich,A. (1994) Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLCγ. *EMBO J.*, **13**, 1585–1590.

Obermeier,A., Tinhofer,I., Grunicke,H.H. and Ullrich,A. (1996) Transforming potentials of epidermal growth factor and nerve growth factor receptors inversely correlate with their phospholipase Cγ affinity and signal activation. *EMBO J.*, **15**, 73–82.

O'Neill,T.J., Craparo,A. and Gustafson,T.A. (1994) Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. *Mol. Cell. Biol.*, **14**, 6433–6442.

Ponzetto,C., Bardelli,A., Zhen,Z., Maina,F., dalla Zonca,P., Giordano,S., Graziani,A., Panayotou,G. and Comoglio,P.M. (1994) A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell*, **77**, 261–271.

Rameh,L.E., Chen,C.-S. and Cantley,L.C. (1995) Phosphatidylinositol $(3,4,5)P_3$ interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell*, **83**, 821–830.

Reedijk,M., Liu,X., van der Geer,P., Letwin,K., Waterfield,M.D., Hunter,T. and Pawson,T. (1992) Tyr721 regulates specific binding of the CSF-1 receptor kinase insert to PI 3′-kinase SH2 domains: a model for SH2-mediated receptor–target interactions. *EMBO J.*, **11**, 1365–1372.

Rodrigues,G.A. and Park,M. (1993) Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. *Mol. Cell. Biol.*, **13**, 6711–6722.

Rosnet,O. and Birnbaum,D. (1993) Hematopoietic receptors of class III receptor-type tyrosine kinases. *Crit. Rev. Oncogen.*, **4**, 595–613.

Rossi,F., McNagny,K.M., Smith,G., Frampton,J. and Graf,T. (1996) Lineage commitment of transformed haematopoietic progenitors is determined by the level of PKC activity. *EMBO J.*, **15**, 1894–1901.

Roth,P. and Stanley,E.R. (1992) The biology of CSF-1 and its receptor. *Curr. Topics Microbiol. Immunol.*, **181**, 141–167.

Sherr,C.J., Rettenmier,C.W., Sacca,R., Roussel,M.F., Look,A.T. and Stanley,E.R. (1985) The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell*, **41**, 665–676.

Songyang,Z. *et al.* (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell*, **72**, 767–778.

Stanley,E.R. (1981) Methods for the purification, assay, characterization and target cell binding of a colony stimulating factor (CSF-1). *J. Immunol. Methods*, **42**, 253–284.

Stanley,E.R., Chen,D.M. and Lin,H.S. (1978) Induction of macrophage production and proliferation by a purified colony stimulating factor. *Nature*, **274**, 168–170.

Stephens,R.M., Loeb,D.M., Copeland,T.D., Pawson,T., Greene,L.A. and Kaplan,D.R. (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. *Neuron*, **12**, 691–705.

Takata,M. and Kurosaki,T. (1996) A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-γ2. *J. Exp. Med.*, **184**, 31–40.

Tapley,P., Kazlauskas,A., Cooper,J.A. and Rohrschneider,L.R. (1990) Macrophage colony-stimulating factor-induced tyrosine phosphorylation of c-fms proteins expressed in FDC-P1 and BALB/c-3T3 cells. *Mol. Cell. Biol.*, **10**, 2527–2537.

Trouliaris,S., Smola,U., Chang,J.-H., Parsons,S.J., Niemann,H. and Tamura,T. (1995) Tyrosine 807 of the v-Fms oncogene product controls cell morphology and association with p120RasGAP. *J. Virol.*, **69**, 6010–6020.

Tsai,S., Bartelmez,S., Sitnicka,E. and Collins,S. (1994) Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. *Genes Dev.*, **8**, 2831–2841.

Tushinski,R.J., Oliver,I.T., Guilbert,L.J., Tynan,P.W., Warner,J.R. and Stanley,E.R. (1982) Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. *Cell*, **28**, 71–81.

Ui,M., Okada,T., Hazeki,K. and Hazeki,O. (1995) Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem. Sci.*, **20**, 303–307.

Ullrich,A. and Schlessinger,J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**, 203–212.

van der Geer,P. and Hunter,T. (1993) Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. *EMBO J.*, **12**, 5161–5172.

Varticovski,L., Druker,B., Morrison,D., Cantley,L. and Roberts,T. (1989) The colony stimulating factor-1 receptor associates with and activates phosphatidylinositol-3 kinase. *Nature*, **342**, 699–702.

Vojtek,A. and Hollenberg,S.M. (1995) Ras–Raf interaction: two-hybrid analysis. In Balch,W.E., Der,C.J. and Hall,A. (eds), *Methods in Enzymology.* Academic Press, New York, Vol. 255, pp. 331–342.

Vojtek,A.B., Hollenberg,S.M. and Cooper,J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell*, **74**, 205–214.

Wang,T., Donahoe,P.K. and Zervos,A.S. (1994) Specific interaction of Type I receptors of the TGF-β family with the immunophilin FKBP-12. *Science*, **265**, 674–676.

Wang,Z., Myles,G., Brandt,C.S., Lioubin,M.N. and Rohrschneider,L.R. (1993) Identification of the ligand-binding regions in the macrophage colony-stimulating factor receptor extracellular domain. *Mol. Cell. Biol.*, **13**, 5348–5359.

Whetton,A.D., Monk,P.N., Consalvey,S.D. and Downes,C.P. (1986) The haemopoietic growth factors interleukin 3 and colony stimulating factor-1 stimulate proliferation but do not induce inositol lipid breakdown in murine bone-marrow-derived macrophages. *EMBO J.*, **5**, 3281–3286.

Whetton,A.D., Heyworth,C.M., Nicholls,S.E., Evans,C.A., Lord,J.M., Dexter,T.M. and Owen-Lynch,P.J. (1994) Cytokine-mediated protein kinase C activation is a signal for lineage determination in bipotential granulocyte macrophage colony-forming cells. *J. Cell Biol.*, **125**, 621–659.

Wiktor-Jedrzejczak,W., Bartocci,A., Ferrante,A.W.,Jr, Ahmed-Ansari,A., Sell,K.W., Pollard,J.W. and Stanley,E.R. (1990) Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc. Natl Acad. Sci. USA*, **87**, 4828–4832.

Wiktor-Jedrzejczak,W., Urbanowska,E., Aukerman,S.L., Pollard,J.W., Stanley,E.R., Ralph,P., Ansari,A.A., Sell,K.W. and Szperl,M. (1991) Correction by CSF-1 of defects in the osteopetrotic op/op mouse suggests local, developmental, and humoral requirements for this growth factor. *Exp. Hematol.*, **19**, 1049–1054.

Woolford,J., Rothwell,V. and Rohrschneider,L. (1985) Characterization of the human c-*fms* gene product and its expression in cells of the monocyte–macrophage lineage. *Mol. Cell. Biol.*, **5**, 3458–3466.

Wymann,M.P., Bulgarelli-Leva,G., Zvelebil,M.J., Pirola,L., Vanhaesebroeck,B., Waterfield,M.D. and Panayotou,G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.*, **16**, 1722–1733.

Yi,T. and Ihle,J.N. (1993) Association of hematopoietic cell phosphatase with c-Kit after stimulation with c-Kit ligand. *Mol. Cell. Biol.*, **13**, 3350–3358.

Received on March 10, 1997; revised on July 2, 1997