Association between phosphatidylinositol-3 kinase, Cbl and other tyrosine phosphorylated proteins in colony-stimulating factor-1-stimulated macrophages

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Colony stimulating factor-1 (CSF-1) stimulation of the macrophage cell line BAC1.2F5 and murine bone marrow-derived macrophages resulted in tyrosine phosphorylation of phosphatidylinositol-3 kinase (PI-3 kinase) p85α and its stable association with several tyrosine phosphorylated proteins, including CSF-1 receptor (p165), p120, p95 and p55–p60. p120 co-migrated with the product of the protooncogene c-*cbl* in anti-p85α immunoprecipitates, and associated with p85α in a rapid and transient manner. Reciprocal experiments confirmed the presence of p85α in anti-Cbl immunoprecipitates on CSF-1 stimulation of macrophages. PI-3 kinase immunoprecipitates from the myeloid FDC-P1 cell line expressing mutant CSF-1 receptor (Y721F), which does not associate with PI-3 kinase, still contained Cbl. The identity of the tyrosine phosphorylated protein p95 remains unknown. The interaction between p85α and the tyrosine phosphorylated proteins survived anion-exchange chromatography, suggesting perhaps the presence of a stable complex; furthermore, in CSF-1-treated BAC1.2F5 cell extracts, only one of the two pools of PI-3 kinase separated by chromatography was present in this putative complex. The association did not appear to correlate with proliferation, since a similar interaction between p85α and tyrosine phosphorylated proteins was also observed in poorly proliferating resident peritoneal macrophages stimulated with CSF-1. The possible significance of these findings for CSF-1-regulated macrophage functions is discussed.

INTRODUCTION

The receptor for colony stimulating factor-1 (CSF-1), encoded by the c-*fms* proto-oncogene [1], is expressed in cells of the monocyte/macrophage lineage and trophoblasts. It has been most extensively studied in the monocyte/macrophage lineage, where it appears that CSF-1 is required for survival, proliferation and differentiation to the mature phenotype [2]. In addition to these roles, mature cell functions of the macrophages are also activated by CSF-1 [3,4]. The CSF-1 receptor (CSF-1R) is structurally related to a family of growth factor receptors, which possess ligand-activated tyrosine kinase activity and which also contain a variable sequence of amino acids inserted within the kinase domain, members of which include the α and β platelet-derived growth factor (PDGF) receptors [5].

CSF-1 binding to the extracellular domain of the receptor results in receptor dimerization [6], thus activating the tyrosine kinase domain, a consequence of which is the transphosphorylation of specific tyrosine residues within the cytoplasmic domain [7–11]. Four such sites have been identified in the murine CSF-1R; three of these, Y697, Y706 and Y721, are located in the kinase insert domain, whereas Y807 is located in the C-terminal half of the kinase domain. Peptide mapping of [^{32}P]orthophosphate-labelled CSF-1R immunoprecipitates has shown that Y706 and Y697 (and the equivalent positions in the human receptor) are major sites of phosphorylation, whereas the extent of phosphorylation of Y721 and Y807 is less [7–11]. Phosphorylation of other tyrosines, e.g. Y561, which may be involved in the binding of Fyn to the activated CSF-1R [12], has been inferred, but not as yet demonstrated, by phosphopeptide mapping. These phosphorylated tyrosine residues serve as binding sites for proteins containing src homology 2 (SH2) domains. Two such proteins, the regulatory subunit p85α of phosphatidylinositol-3 kinase (PI-3 kinase) and Grb2, have been shown to bind to Y721 and Y697 respectively [9,11]. No specific proteins have been shown to interact with Y706 or Y807. The recent report demonstrating the association of the guanine nucleotide exchange factor Sos with CSF-1R, either via Grb2 or via tyrosine phosphorylated p150, has provided a possible pathway by which Ras may be activated in myeloid cells following activation by CSF-1 stimulation [13]. The signalling pathways, which are 'downstream' of Y721, and the activation of PI-3 kinase are not well understood.

PI-3 kinases are enzymes which phosphorylate inositol phospholipids in the D-3 position of the inositol ring [14]. The receptor tyrosine kinase-activated PI-3 kinase consists of a heterodimer of an 85 kDa regulatory subunit (p85), which can bind directly to specific phosphorylated tyrosine residues of receptors via its SH2 domains, and a 110 kDa catalytic subunit (p110). The presence of at least two isoforms of both p85 and p110, α and β [15–17], and more recently a γ isoform of the catalytic subunit, which is activated by G-proteins [18], extends the complexity of this family of kinases. A new regulatory subunit migrating at 55 kDa, having significant identity to p85α and p85β [19], has also recently been reported to associate with and activate p110, after binding to insulin receptor substrate-1 on growth factor stimulation. These findings suggest potentially unique means of regulating PI-3 kinase involved in diverse cellular processes after growth-factor stimulation.

PI-3 kinase has been shown, by *in vitro* assays, to be activated in macrophages following CSF-1 stimulation [20,21], while others have detected, *in vivo*, a transient increase in 3-phosphorylated

Abbreviations used: CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; PI-3 kinase, phosphatidylinositol-3 kinase; SH2 and SH3, Src homology regions 2 and 3; pTyr, phosphotyrosine; BMM, bone marrow-derived macrophages; RPM, resident peritoneal macrophages; PDGF, platelet-derived growth factor; LCM, L-cell conditioned medium; FCS, fetal calf serum; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; HRP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium.

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inositides [22]. One potential role for the 3-phosphorylated inositides is as second messengers [23,24]. Other possible diverse roles of PI-3 kinase, as either a lipid kinase or as a serine-specific protein kinase [25], are in cellular processes such as cytoskeletal reorganization [26,27] and vesicular protein sorting [28,29].

Having found that PI-3 kinase p85α was the major ^{35}S-labelled protein associated with activated CSF-1R, and given the potential roles of PI-3 kinase, we decided to investigate the association of PI-3 kinase with intracellular proteins on activation of the CSF-1R. We report that, following CSF-1 stimulation of different macrophage populations, PI-3 kinase p85α associates with several tyrosine phosphorylated proteins, including the proto-oncogene product Cbl. The kinetics of formation of the association in response to CSF-1 stimulation was investigated in proliferating and poorly proliferating macrophage populations. The association between PI-3 kinase p85α and tyrosine phosphorylated proteins is stable to ion-exchange chromatography, suggesting the formation of a complex, and may have significance for the function of PI-3 kinase in the various physiological processes accompanying CSF-1 stimulation of macrophages.

EXPERIMENTAL

Reagents

Polyclonal antibody raised to a peptide corresponding to amino acids 955–971 of the human CSF-1R, anti-CSF-1R$_1$, which recognized both the human and murine CSF-1R, was raised in our laboratory. A second polyclonal antibody to the kinase domain of the murine CSF-1R [15], anti-CSF-1R$_2$, was a gift from Dr. L. Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.). Rabbit polyclonal antibody against the p85 subunit of PI-3 kinase (anti-p85α) and the monoclonal anti-phosphotyrosine antibody 4G10, conjugated to horseradish peroxidase (HRP) (anti-pTyr), were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Rabbit polyclonal anti-Cbl was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Purified human recombinant CSF-1 was a gift from Chiron, Emeryville, CA, U.S.A.

Cell culture conditions

The CSF-1-dependent murine macrophage cell line, BAC1.2F5, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS) and 30 % (v/v) L-cell conditioned medium (LCM) as a source of CSF-1. Murine bone marrow-derived macrophages (BMM) were prepared from precursor cells in bone marrow by a procedure described previously [30] and were grown in RPMI supplemented with 15 % (v/v) heat-inactivated FCS, 30 % (v/v) LCM, 5×10^{-5} M 2-mercaptoethanol, 20 mM Hepes and 50 units/ml penicillin. The cells were seeded at a density of 10^5 cells/ml and cultured until subconfluent. Both BAC1.2F5 cells and BMM were rendered quiescent by reculturing in growth media lacking LCM for 18–20 h before stimulation with growth factor [30]. Resident peritoneal macrophages (RPM) were obtained as previously described [31] and cultured at 10^6 cells/ml in RPMI supplemented with 15 % (v/v) heat-inactivated FCS and 20 mM Hepes. After 3 h at 37 °C, the non-adherent cells were removed by washing twice in sterile PBS. The adherent macrophages were recultured in fresh culture medium and incubated overnight before use.

FDC-P1 myeloid progenitor cell lines expressing wild-type murine CSF-1R (WTfms) and CSF-1R with a mutation in the autophosphorylation site, Y721F, were a gift from Dr. L. Rohrschneider. FDC-P1 cells were maintained in DMEM containing 10 % (v/v) heat-inactivated FCS and 2 % (v/v) WEHI 3B cell-conditioned medium as described previously [32]. Before use in experiments, cells were washed twice in PBS to remove interleukin 3 and recultured overnight in media containing DMEM and 10 % (v/v) heat-inactivated FCS.

Metabolic labelling

Subconfluent BAC1.2F5 cells and BMM were grown in 10 cm tissue-culture dishes as described above. Quiescent cells were washed twice with methionine-free RPMI medium or DMEM (ICN Biochemicals Inc., Irvine, CA, U.S.A.) as appropriate, and labelled for 4 h with 100 μCi/ml of [^{35}S]methionine (ICN Biochemicals Inc.) at 37 °C in a minimum volume of methionine-free medium supplemented with glutamine and 1 % (v/v) dialysed FCS.

Immunoprecipitations

Subconfluent cultures of BAC1.2F5 cells or BMM grown in 15 cm tissue-culture dishes were rendered quiescent as described above, and were stimulated with CSF-1 at 5000 units/ml at 37 °C for the indicated times. RPM, on the other hand, were grown in 10 cm tissue-culture dishes and stimulated with CSF-1. Cells were placed on ice after stimulation, and washed twice in ice-cold PBS before solubilization in lysis buffer [25 mM Hepes buffer (pH 7.5), 137 mM NaCl, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Nonidet P-40, 10 μg/ml aprotinin, 1 μM leupeptin, 1 μM pepstatin, 0.1 mM Pefabloc, 50 mM sodium fluoride, 50 mM β-glycerophosphate and 1 mM sodium vanadate]. After 5 min on ice, lysates were centrifuged at 15000 ***g*** for 5 min at 4 °C. Extract containing 2 mg of protein was precleared by incubation with Protein A–Sepharose before immunoprecipitating with specific antibodies. Incubation with anti-PI-3 kinase p85α was carried out for 3 h at 4 °C, while other antibodies required overnight incubation. Protein A–Sepharose was added and incubation continued for a further 1 h, after which the beads were washed three times in wash buffer [25 mM Hepes buffer (pH 7.5), 137 mM NaCl, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Nonidet P-40 and 1 mM sodium vanadate]. Immunoprecipitated proteins were resolved on a 10 % (w/v) SDS/polyacrylamide gel and transferred to nitrocellulose for immunoblotting. Proteins recognized by the primary antibody were visualized with HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents (Amersham Corporation). Blots were reprobed with other primary antibodies after removal of bound antibody by incubation in 62.5 mM Tris/HCl, pH 6.7/0.1 M 2-mercaptoethanol/2 % (w/v) SDS (60 °C, 30 min).

FDC-P1 cells expressing WTfms and the Y721F mutant of CSF-1R were starved of growth factor, as described previously, and were either untreated or stimulated with CSF-1 for 1 min at 37 °C. Cells were lysed and extracts were prepared for immunoprecipitation as described for macrophages.

Immunoprecipitations from [^{35}S]methionine-labelled macrophage extracts were prepared as descibed above, except that the detergent used for solubilization was 1 % (v/v) Triton X-100. Cell extracts containing approx. 0.5 mg of protein were precleared before immunoprecipitating with the specific primary antibody. The immunoprecipitates were resolved on a 10 % (w/v) SDS polyacrylamide gel and detected by autoradiography of the dried gels. Competition with a peptide corresponding to amino acids 955–971 of the human CSF-1R (CSF-1R$_1$) was carried out by incubating the pre-cleared extract with 150 μM peptide for 1 h, followed by the addition of the primary antibody which was generated from the peptide, anti-CSF-1R$_1$.

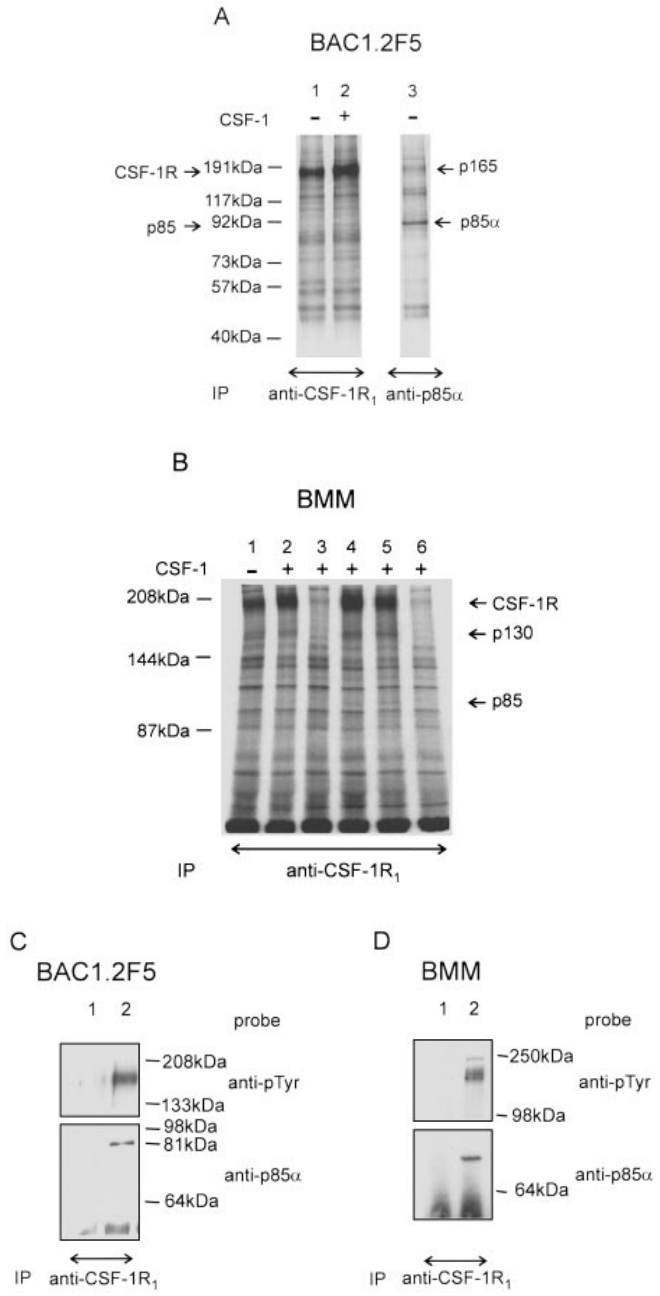

Figure 1 p85 protein is the only detectable protein that is enhanced in CSF-1R immunoprecipitates on CSF-1 stimulation

(**A**) Quiescent [^{35}S]methionine-labelled BAC1.2F5 cells were either untreated (lanes 1and 3) or stimulated with CSF-1 (5000 units/ml) for 2 min at 37 °C (lane 2). Lysates were immunoprecipitated with anti-CSF-1R$_1$ or anti-PI-3 kinase p85 as indicated, and samples were resolved by SDS/PAGE. The gels were exposed against Kodak XAR-5 for 3 days. Arrows indicate the positions of PI-3 kinase p85 and the CSF-1R. (**B**) Anti-CSF-1R$_1$ immunoprecipitates (IP) of [^{35}S]methionine-labelled BMM were either not stimulated (lane 1) or stimulated with CSF-1 for 30 s at 37 °C (lanes 2 and 3), 2 min at 37 °C (lane 4) and 30 min at 4 °C (lanes 5 and 6). Lysates were immunoprecipitated and analysed as described above. CSF-1-stimulated lysate of BMM was preincubated with the peptide used to raise the anti-CSF-1R$_1$ antibody before immunoprecipitating with the same antibody (lanes 3 and 6). Arrows indicate the positions of CSF-1R and major bands specifically competed by the peptide. (**C**) Lysates of untreated BAC1.2F5 cells (lane 1) and cells treated with CSF-1 for 2 min (lane 2) were immunoprecipitated with anti-CSF-1R$_1$. Immunoprecipitates were resolved by SDS/PAGE and immunoblotted with anti-pTyr (top panel) then stripped and reprobed with anti-PI-3 kinase p85 (bottom panel). (**D**) Lysates of untreated (lane 1) and CSF-1-stimulated (2 min) BMM (lane 2) were immunoprecipitated with anti-CSF-1R$_1$. Western blots of the immunoprecipitates were probed with anti-pTyr (top panel) and anti-PI-3 kinase p85 (bottom panel). The primary antibody was detected using HRP-coupled secondary antibodies and ECL. The positions of prestained molecular-mass markers are indicated.

Column chromatography

Untreated BAC1.2F5 cells and those treated with CSF-1 for 8 min at 37 °C were lysed in solubilization buffer containing 25 mM Tris/HCl, pH 7.5, 137 mM NaCl, 1 mM EDTA, 1 % (v/v) Triton X-100, 10 μg/ml aprotinin, 1 μM leupeptin, 1 μM pepstatin, 0.1 mM Pefabloc, 50 mM sodium fluoride, 50 mM β-glycerophosphate and 1 mM sodium vanadate at 4 °C. Subsequent steps, including the column chromatography, were carried out at 4 °C. Approx. 3 mg of total protein extract was dialysed against 100 ml of buffer A (10 mM Tris/HCl, pH 7.5/ 40 mM β-glycerophosphate/1 mM dithiothreitol/1 mM EGTA/ 0.1 mM sodium vanadate). The extract was then centrifuged at 15000 ***g*** for 5 min to remove unsolubilized material and loaded onto a 1 ml Mono Q anion-exchange Econo column (Bio-Rad) equilibrated in buffer A. The column was washed in 10 vol. of buffer A at a flow rate of 1 ml/min. The unadsorbed fractions were pooled for analysis. A gradient of 0–400 mM NaCl was then applied and 1 ml fractions were collected over 30 min for analysis.

The unadsorbed material and every third fraction collected over the NaCl gradient were analysed by SDS/PAGE, followed by immunoblotting with anti-PI-3 kinase p85α. PI-3 kinase immunoreactivity was present in the unadsorbed fractions and in a single peak at 50 mM NaCl (following CSF-1 stimulation). Fractions containing PI-3 kinase p85α were pooled, then equal volumes of the unadsorbed fraction (1.5 ml) and pooled eluted fractions were immunoprecipitated with anti-PI-3 kinase p85α. Immunoprecipitates were analysed on a 10 % (w/v) SDS polyacrylamide gel and the Western transfer of the proteins immunoblotted with specific antibodies.

RESULTS

An 85 kDa protein is the major [^{35}S]methionine-labelled protein associated with CSF-1R in CSF-1-treated murine macrophages

Very few effector proteins which bind directly to the CSF-1R have been identified, although there is evidence for the binding of PI-3 kinase [9], Grb2 [11] and Src family members [12] to certain cytoplasmic phosphorylated tyrosine residues. We therefore decided to use [^{35}S]methionine-labelling as a sensitive method to detect proteins in anti-CSF-1R$_1$ immunoprecipitates. This method potentially allows us to detect proteins that associate with the CSF-1R independently of their phosphorylation status, and gives some idea of the relative amounts of binding. Lysates were prepared from the [^{35}S]methionine-labelled murine macrophage cell line BAC1.2F5, and murine BMM, after stimulation with CSF-1 for various lengths of time. Proteins immunoprecipitated from the lysates with anti-CSF-1R$_1$ antibody were analysed by SDS/PAGE. By using this approach, a protein migrating at approx. 85 kDa was the only detectable protein, albeit weakly, whose levels increased consistently in anti-CSF-1R$_1$ immunoprecipitates after CSF-1 stimulation of murine macrophages (Figures 1A and 1B). This protein co-migrated with the regulatory subunit of PI-3 kinase p85α (Figure 1A). Studies using the peptide to which the CSF-1R$_1$ antibody was raised showed that most of the bands seen in Figure 1 are non-specifically associated with the immunoprecipitates, except for the 165 kDa CSF-1R itself, a 130 kDa protein which is most

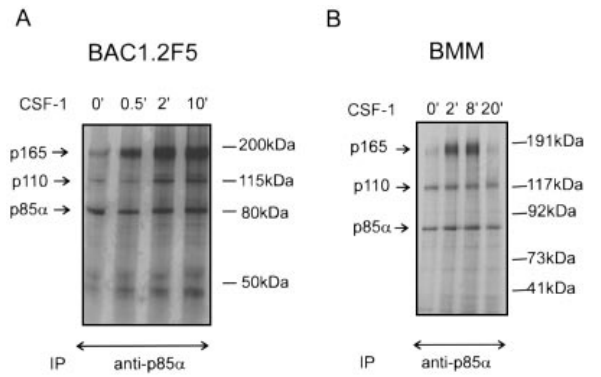

Figure 2 Association of proteins with PI-3 kinase p85

Immunoprecipitates of anti-PI-3 kinase p85 from [^{35}S]methionine-labelled BAC1.2F5 (**A**) or BMM (**B**), stimulated with CSF-1 for the indicated times, were analysed. Immunoprecipitates (IP) were resolved by SDS/PAGE and visualized by fluorography. Arrows indicate the major proteins immunoprecipitating with the antibody. Positions of molecular-mass markers are indicated on the right.

likely the immature glycosylated form of the CSF-1R [1], and the protein migrating at 85 kDa (Figure 1B).

Similar findings were made if immunoprecipitates were prepared and analysed from cells treated at 4 °C with CSF-1 (Figure 1B). These conditions have been shown to facilitate the detection of tyrosine phosphorylated proteins by slowing down the rate of CSF-1R internalization [6,13]. Hence, the only protein present in sufficient quantity, and which forms sufficiently stable association with activated CSF-1R to enable detection by this technique, is p85. As shown below, this protein is the regulatory subunit of PI-3 kinase but the catalytic subunit (p110) is not visible in these experiments. This is probably due to the presence of overlapping non-specific proteins co-immunoprecipitating with anti-CSF-$1R_1$ antibody (Figures 1A and 1B).

Similar experiments were conducted using milder detergent lysis conditions, both in the presence and absence of a range of chemical cross-linkers, in order to stabilize any transient interactions that might be present. Cross-linking was carried out both *in vivo*, following CSF-1 stimulation, as well as *in vitro*, following lysis of cells. In all these trials the only consistent interaction that was enhanced on stimulation compared with untreated cells was that of p85, as observed in the experiment described in Figure 1.

PI-3 kinase activity has been shown to increase in anti-pTyr immunoprecipitates of CSF-1-stimulated macrophages [20,21] and fibroblasts expressing CSF-1R [21,22]. The 85 kDa protein which co-immunoprecipitated with CSF-1R was shown to be the regulatory subunit of PI-3 kinase after immunoblotting of anti-CSF-$1R_1$ immunoprecipitates with anti-PI-3 kinase p85α (Figures 1C and 1D). This observation supports previous reports that PI-3 kinase p85α binds to the activated CSF-1R [9,33]. We decided to investigate other proteins that may interact with PI-3 kinase p85α upon activation of BAC1.2F5 cells and BMM. Immunoprecipitates of anti-PI-3 kinase p85α, prepared from [^{35}S]methionine-labelled macrophages, showed, apart from the putative 110 kDa catalytic subunit of PI-3 kinase, a major protein of molecular mass 165 kDa co-immunoprecipitating with p85 (Figures 2A and 2B). This protein migrated with the same mobility as CSF-1R. Figures 2(A) and 2(B) illustrate also that this association, though minor before activation of the receptor, increased upon the addition of CSF-1 and remained associated for at least 8–10 min after stimulation in macrophages. We show in Figure 2(B) for BMM that the association was not visible after 20 min of CSF-1 stimulation at 37 °C. The interaction observed between p85α and CSF-1R was also present in lysate prepared using RIPA lysis buffer containing 0.1 % (w/v) SDS. Apart from the CSF-1R, a 125 kDa unidentified protein, migrating with a slower mobility relative to the putative PI-3 kinase p110 catalytic subunit, was found to be associated with p85α on CSF-1 stimulation of BAC1.2F5.

PI-3 kinase p85α co-immunoprecipitates with tyrosine phosphorylated proteins on CSF-1 stimulation

We, and others, have previously observed that PI-3 kinase activity increases in anti-pTyr immunoprecipitates following CSF-1 stimulation of macrophages [20,21]. We verified that PI-3 kinase immunoreactivity increased in these immunoprecipitates (results not shown). These findings suggest that PI-3 kinase is itself phosphorylated on tyrosine following CSF-1 stimulation, and/or that it associates with tyrosine phosphorylated proteins on stimulation. Since there are differing statements in the literature regarding the degree of tyrosine phosphorylation of PI-3 kinase p85α following CSF-1 stimulation [33,34], we examined both of these possibilities. BAC1.2F5 lysates were immunoprecipitated with anti-PI-3 kinase p85α antibody, and the co-immunoprecipitated proteins were analysed by immunoblotting with anti-pTyr antibody. Several major protein bands (p165, p120, p95, p85 and p55) were apparent on stimulation (Figure 3A), with some minor tyrosine phosphorylated proteins appearing in the range p65–p75 (see also Figure 4A discussed below).

The p85 protein recognized by the anti-PI-3 kinase p85α antibody (Figure 3B) comigrates with the strong tyrosine phosphorylated band in the p85α immunoprecipitates (Figure 3A). These observations provide evidence of extensive tyrosine phosphorylation of PI-3 kinase p85α upon CSF-1 stimulation, and confirms and extends a recent report that the pool of PI-3 kinase bound to CSF-1R in human monocytes is tyrosine phosphorylated [33]. This situation is different to that in fibroblasts expressing human CSF-1R in which it is difficult to demonstrate the tyrosine phosphorylation of PI-3 kinase [34]. The level of tyrosine phosphorylation of PI-3 kinase and associated proteins increased significantly after 2 min of CSF-1 stimulation, and remained higher than basal levels even after 20 min of CSF-1 stimulation. The kinetics of association of PI-3 kinase p85α with CSF-1R, however, differs between BAC1.2F5 cells (Figure 3A) and BMM (Figure 2B) (discussed below).

Consistent with the metabolic labelling data in Figures 2(A) and 2(B), the tyrosine phosphorylated p165 band was identified as CSF-1R, with its association only detectable following CSF-1 stimulation and, though reduced beyond 2 min after CSF-1 stimulation, it persisted over a period of 20 min (Figure 3C). The kinetics of tyrosine phosphorylation of p165 followed similar kinetics to this association, with the initial increase in the phosphorylation on CSF-1 stimulation being followed by a slow down-modulation in signal intensity when stimulation was continued (Figure 3A).

The major tyrosine phosphorylated protein migrating at 95 kDa (Figure 3A) runs as a broad band and shows a gradually decreasing mobility on stimulation with CSF-1. A protein showing these characteristics is a major tyrosine phosphorylated protein evident in anti-pTyr immunoblots of whole cell lysates prepared from CSF-1-stimulated macrophages (results not shown). This 95 kDa protein co-immunoprecipitating with PI-3 kinase p85α showed no immunoreactivity with anti-vav or anti-dynamin which migrate during SDS/PAGE with a similar

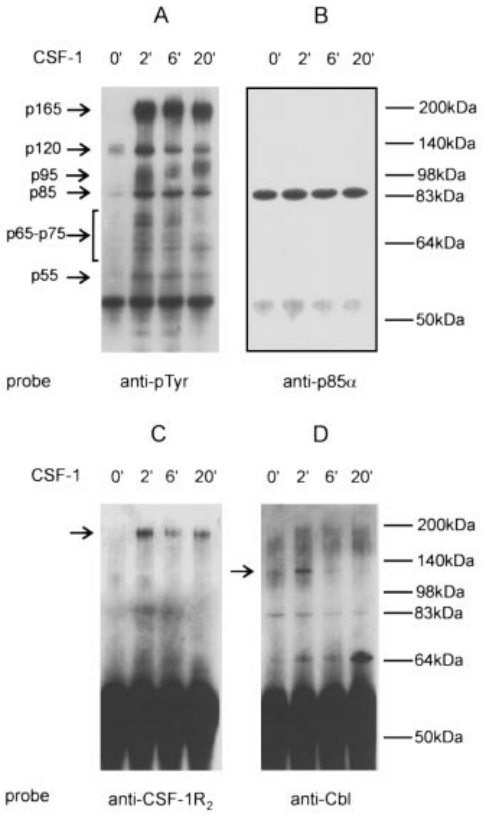

Figure 3 PI-3 kinase p85α associates with tyrosine phosphorylated proteins on CSF-1 stimulation

Lysates of quiescent BAC1.2F5 or of cells treated with CSF-1 for the indicated times were immunoprecipitated with anti-PI-3 kinase p85 and resolved by SDS/PAGE. Proteins transferred to nitrocellulose were probed with anti-pTyr(4G10)–HRP conjugate and detected by ECL (**A**). The same blot was stripped and then reprobed with anti-PI-3 kinase p85α (**B**), anti-CSF-1R_2 (**C**) and anti-Cbl (**D**). The arrows show the positions of major tyrosine phosphorylated proteins immunoprecipitating with anti-PI-3 kinase p85α (**A**), or the major protein species recognized by the primary antibody (**C** and **D**). The positions of prestained molecular-mass markers are indicated on the right.

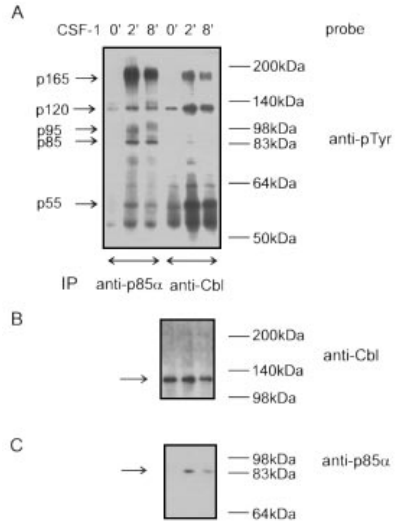

Figure 4 Cbl associates with PI-3 kinase

Lysates from unstimulated and from BAC1.2F5 cells treated with CSF-1 for the indicated times were immunoprecipitated with either anti-PI-3 kinase p85 or anti-Cbl. Immunoprecipitates were resolved by SDS/PAGE and transferred to nitrocellulose. The membrane was probed with anti-pTyr (4G10)–HRP conjugate (**A**), and the same blot was stripped and reprobed with anti-Cbl (**B**) and anti-PI-3 kinase p85 (**C**). Arrows indicate the major protein species recognized by the primary antibodies. The positions of prestained molecular-mass markers are indicated on the right.

mobility. Shc and members of the Src family kinases [35] are obvious candidates for the p55 tyrosine phosphorylated protein; verification of this possibility is currently underway.

Cbl associates with PI-3 kinase and is tyrosine phosphorylated after CSF-1 stimulation

Recent reports have found that the 120 kDa product of the proto-oncogene c-*cbl* is rapidly tyrosine phosphorylated in response to activation of various receptor signalling systems [35–38]. These findings led us to examine whether the p120 tyrosine phosphorylated protein co-immunoprecipitating with PI-3 kinase p85α in CSF-1-stimulated BAC1.2 F5 extracts was in fact this protein. The presence of Cbl in PI-3 kinase immunoprecipitates was confirmed (Figure 3D): Cbl was shown to be present in immunoprecipitates prepared from unstimulated extracts, and to increase transiently on CSF-1 stimulation. The p120 band immunoreacting with anti-Cbl antibody comigrated with the tyrosine phosphorylated protein of the same molecular mass observed in Figure 3A. Furthermore, the kinetics of association of Cbl with PI-3 kinase paralleled the transient increase in the level of tyrosine phosphorylation of p120 observed on stimulating BAC1.2F5 cells (Figure 3A).

We decided to seek further confirmation of the association of Cbl with PI-3 kinase p85 by examining anti-Cbl immunoprecipitates. Probing with anti-pTyr antibody in these immunoprecipitates showed a dramatic increase in the tyrosine phosphorylation of Cbl, after CSF-1 stimulation (Figures 4A and 4B), and apparent loss of anti-Cbl reactive protein at 8 min of CSF-1 stimulation. A protein of the same molecular mass was also observed to be one of the major tyrosine phosphorylated proteins in CSF-1-stimulated crude lysates (results not shown). Similar kinetics of tyrosine phosphorylation of Cbl were observed as for Cbl present in PI-3 kinase immunoprecipitates (see also Figure 3A). The immunoprecipitates of anti-Cbl prepared from CSF-1-stimulated cells also contained PI-3 kinase p85α (Figure 4C), which was tyrosine phosphorylated (Figure 4A). These observations support the results obtained from reciprocal experiments with anti-PI-3 kinase p85α immunoprecipitates illustrated in Figure 3D. The kinetics of PI-3 kinase p85α association with Cbl were similar in Figures 4(C) and 3(D).

In addition to p120 being heavily tyrosine phosphorylated on CSF-1 stimulation, the immunoprecipitates of anti-Cbl also contained other tyrosine phosphorylated proteins, some of which were also detectable in the PI-3 kinase p85α immunoprecipitates (Figure 4A). The p165 protein is most likely to be CSF-1R, and it comigrated with CSF-1R in the anti-PI-3 kinase immunoprecipitates (Figure 3A). These experiments, however, do not reveal whether the association between CSF-1R and Cbl is direct (see below).

The complex of tyrosine phosphorylated proteins with PI-3 kinase p85α is stable to chromatography

Even though the association of PI-3 kinase with tyrosine phosphorylated proteins was observed after stringent washing of

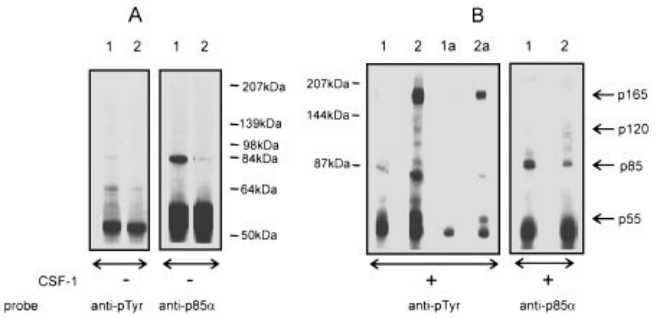

Figure 5 Analysis of PI-3 kinase-associated proteins after column chromatography

Quiescent and CSF-1-treated BAC1.2 F5 cells (8 min at 37 °C) were lysed and the solubilized lysate was applied to a MonoQ anion-exchange column (Bio-Rad). Protein fractions were separated using a 0–0.4 M NaCl gradient and resolved by SDS/PAGE. The proteins were transferred to a nitrocellulose membrane which was then immunoblotted with anti-PI-3 kinase p85 and fractions containing PI-3 kinase were immunoprecipitated with the same antibody. Immunoprecipitations of fractions containing PI-3 kinase p85 from the separation of untreated (**A**) and CSF-1-stimulated (**B**) cell extracts were analysed by SDS/PAGE and transferred to nitrocellulose. The immunoblot was probed with anti-pTyr(4G10)–HRP followed by anti-PI-3 kinase p85 as indicated. Lane 1 corresponds to immunoprecipitates obtained from unadsorbed fractions, whereas lane 2 represents those from fractions eluted at 50 mM NaCl. Lanes 1a and 2a in (**B**) represent lower exposures of lanes 1 and 2 respectively, probed with anti-pTyr antibody. Arrows in (**B**) indicate the positions of the major tyrosine phosphorylated proteins co-immunoprecipitating with PI-3 kinase p85 in lane 2. The positions of prestained molecular-mass markers are indicated.

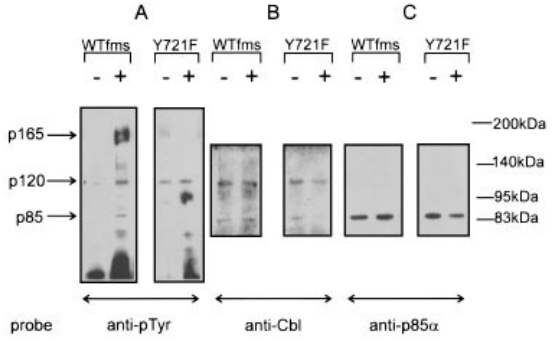

Figure 6 Cbl associates with PI-3 kinase in FDC-P1 myeloid cell line expressing the wild-type CSF-1R (Wtfms) and the Y721F mutant CSF-1R (Y721F)

Lysates from untreated or CSF-1-treated (1 min, 37 °C) FDC-P1 cells expressing either wild-type CSF-1R (WTfms) or a mutant CSF-1R (Y721F) were immunoprecipitated with anti-PI-3 kinase p85 and resolved by SDS/PAGE. A Western transfer of the immunoprecipitates was probed with anti-pTyr (4G10)–HRP (**A**), anti-Cbl (**B**) and anti-PI-3 kinase p85 (**C**). Arrows in (**A**) indicate the positions of the major tyrosine-phosphorylated proteins co-immunoprecipitated with PI-3 kinase.

immunoprecipitations with 1 % (v/v) Nonidet P-40 lysis buffer, the possibility remains that the co-immunoprecipitations are non-specific. We therefore determined whether the association observed was stable to chromatography. Lysates prepared from BAC1.2F5 cells were resolved by anion-exchange chromatagraphy, and fractions containing PI-3 kinase were identified by immunoblotting with anti-PI-3 kinase p85α. Anti-PI-3 kinase immunoprecipitates were prepared from these fractions only and immunoblotted with anti-pTyr and anti-PI-3 kinase p85α (Figures 5A and 5B).

Chromatographic separation of lysate from unstimulated BAC1.2F5 cells showed a single major pool of PI-3 kinase to be present in the unadsorbed fraction, which did not associate with tyrosine phosphorylated proteins to any detectable extent (Figure 5A, lane 1). However, two main pools of PI-3 kinase p85α were separated by anion-exchange chromatography of CSF-1-stimulated lysate, one present in the unadsorbed fraction (Figure 5B, lane 1), corresponding to that from unstimulated lysate (Figure 5A, lane 1) and the second eluted with approx. 50 mM NaCl (Figure 5B, lane 2). PI-3 kinase immunoprecipitates prepared from the former pool did not contain other tyrosine phosphorylated proteins, while those prepared from the second pool contained similar tyrosine phosphorylated proteins to PI-3 kinase immunoprecipitates prepared from whole cell lysates (Figure 5B, lane 2; cf. Figure 4A, lane 3), specifically p165, p120, p95, p85, p65–p75 and p55. However, there are some differences in the relative detectabilities of the tyrosine phosphorylated proteins, in particular p75 and p55. This may reflect the differences in the stability of interaction of various members of the complex under the conditions of column chromatography, or differences in loss of phosphotyrosine on different members of the complex during chromatography. Fractions separated from unstimulated lysate, corresponding to the position of elution of the second pool of PI-3 kinase from stimulated lysate, contained only very minor levels of PI-3 kinase (Figure 5A, lane 2). The results indicate that the association observed in the PI-3 kinase p85α immunoprecipitates of CSF-1-stimulated crude lysate were maintained after anion-exchange chromatography (see the Discussion section).

Association of PI-3 kinase with tyrosine phosphorylated proteins in cell lines expressing wild-type and mutant murine CSF-1R

In order to determine whether Cbl and PI-3 kinase p85α form an association regardless of the interaction of the CSF-1R, PI-3 kinase immunoprecipitates were prepared from a myeloid progenitor cell line, FDC-P1, expressing either murine wild-type CSF-1R (WTfms) or CSF-1R containing a mutation in the autophosphorylation site (Y721F) to which PI-3 kinase is known to bind [9]. Analysis of the anti-PI-3 kinase p85α immunoprecipitates from cells expressing WTfms by immunoblotting with anti-pTyr showed increased tyrosine phosphorylation of the CSF-1R (p165) on CSF-1 stimulation, which was not evident in the Y721F mutant (Figure 6A), consistent with the fact that the activated mutant CSF-1R (Y721F) does not bind PI-3 kinase p85α [9]. The level of tyrosine phosphorylation of p120 and p85 itself in the immunoprecipitates prepared from FDC-P1/WTfms cells was also enhanced following stimulation with CSF-1. However, FDC-P1/Y721Ffms cells showed a high basal level of tyrosine phosphorylation of p120, but little if any increase on CSF-1 stimulation, while tyrosine phosphorylation of p85 was not visible.

Probing the immunoblots with anti-Cbl verified that the 120 kDa protein, Cbl, was present in the PI-3 kinase p85α immunoprecipitates prepared from FDC-P1 cells expressing both WTfms and the Y721F mutant receptor (Figure 6B). Cbl was also found to be present in PI-3 kinase p85 immunoprecipitates from FDC-P1 cells expressing other CSF-1R mutations, including Y706F, Y697F and Y807F (results not shown). In contrast to macrophages (Figure 3D), the myeloid cells expressing all of the above CSF-1R constructs showed a higher basal level of Cbl in PI-3 kinase p85α before CSF-1 stimulation (the results for Wtfms and Y721F are given in Figure 6B). The tyrosine phosphorylated p120 protein (Figure 6A) comigrated with the band recognized by anti-Cbl. The findings suggest that the association between PI-3 kinase and Cbl can occur as a separate

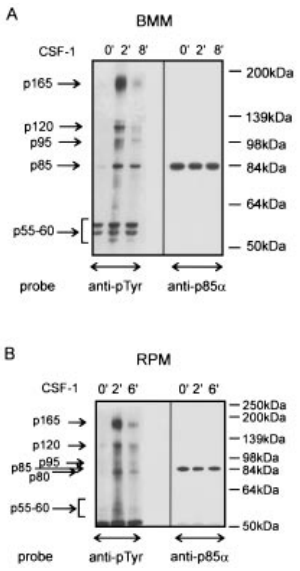

Figure 7 PI-3 kinase associates with tyrosine-phosphorylated proteins on CSF-1 stimulation of proliferating and poorly proliferating macrophages

BMM (**A**) and RPM (**B**) were stimulated with CSF-1 for the indicated times at 37 °C. Lysates prepared from these cells were immunoprecipitated with anti-PI-3 kinase p85 and proteins were separated by SDS/PAGE. A Western blot of the immunoprecipitates was probed with anti-pTyr (4G10)–HRP (left panel) and anti-PI-3 kinase p85 (right panel). Arrows indicate the positions of the main tyrosine-phosphorylated proteins. The positions of prestained molecular-mass markers are indicated on the right.

complex independent of the CSF-1R, at least in FDC-P1 cells. The tyrosine-phosphorylated p85 observed in CSF-1-stimulated FDC-P1/WTfms cells (Figure 6A) comigrated with p85 on reprobing the blot with anti-PI-3 kinase p85α (Figure 6C).

Association of PI-3 kinase with tyrosine phosphorylated proteins in proliferating and poorly proliferating macrophage populations

Since the observation that PI-3 kinase associates with tyrosine phosphorylated proteins was made using extracts prepared from a murine macrophage cell line, we decided to investigate whether similar associations could be demonstrated in primary cultures of macrophages, and whether there is a difference between proliferating BMM and the poorly proliferating murine RPM. Anti-PI-3 kinase p85α immunoprecipitates prepared from BMM contained similar tyrosine phosphorylated proteins to those prepared from BAC1.2F5 cells (Figure 7A; cf. Figure 3A); however, there appear to be significant differences in the kinetics of tyrosine phosphorylation of these proteins, particularly of p120 and the CSF-1R (p165) where dephosphorylation occurred for the BMM more rapidly after 2 min of CSF-1 stimulation. This observation was found in five separate experiments and may be a consequence of a faster rate of CSF-1R internalization or dephosphorylation in BMM compared with BAC1.2F5. There were also other differences between complexes present in BMM and BAC1.2F5 cells. In contrast to BAC1.2F5 cells (Figure 3A), a group of three proteins in the range p55–p60 co-immunoprecipitated with PI-3 kinase p85α from BMM. As discussed previously, this set of proteins may belong to the Src family kinases or Shc.

Poorly proliferating RPM also showed a similar pattern of tyrosine phosphorylated proteins associating with PI-3 kinase p85α (Figure 7B); however, tyrosine phosphorylation of p85, which comigrated with PI-3 kinase p85α, appeared to be only weakly stimulated relative to the other tyrosine phosphorylated proteins on treatment with CSF-1. Its detection was complicated by the presence of a protein, which was more intensely phosphorylated, migrating at a slightly higher mobility. The identity of this protein is not known but Figure 7(B) shows that it is immunologically unrelated to PI-3 kinase p85α.

DISCUSSION

We, and others, have shown that the lipid kinase activity of PI-3 kinase present in anti-pTyr immunoprecipitates increases following CSF-1 stimulation of macrophages [20,21] and fibroblasts [21,22] expressing CSF-1R. We have demonstrated above that PI-3 kinase p85α is tyrosine phosphorylated to a significant extent on stimulation with CSF-1 in primary cultures of macrophages and in the BAC1.2F5 macrophage cell line, which is in agreement with a recent report on studies with human monocytes [33]. These findings seem to differ from observations made in fibroblasts engineered to express the CSF-1R [34], as well as in haemopoietic cells stimulated with other cytokines [39] where there is little if any tyrosine phosphorylation of PI-3 kinase following receptor activation. The significance of this difference is not known but may be related either to a difference in phosphatase activity in fibroblasts and macrophages or to different amounts of CSF-1R–PI-3 kinase complexes being present. This observation is another example of differences in the downstream events from the CSF-1R depending on whether the particular response is measured in myeloid cells or fibroblasts; obviously it is important to understand the situation in monocyte/macrophages, the cell lineage where CSF-1R is normally expressed. Though the lipid kinase activity of PI-3 kinase present in anti-pTyr immunoprecipitates increases following CSF-1 stimulation, how tyrosine phosphorylation of p85α may regulate PI-3 kinase activity itself is not clear. The significant increase in tyrosine phosphorylation of p85α observed in macrophages, apart from its association with other tyrosine phosphorylated proteins, could be an important factor in regulating PI-3 kinase activity. Several structural motifs within the p85 regulatory subunit enable PI-3 kinase to couple potentially to multiple 'downstream' signalling proteins, such as the N-terminal src homology 3 (SH3) domain, a proline-rich motif, a bcr-homology region, and two SH2 domains.

The studies presented here also indicate that the interaction of p85α with the CSF-1R, after activation, is quite stable, surviving relatively stringent lysis conditions using a lysis buffer containing 0.1 % (w/v) SDS. Results from metabolically labelled macrophages presented in Figure 1 indicate that only a minor signal from PI-3 kinase was present in anti-CSF-1R immunoprecipitates. This observation could be consistent with that of Downing et al. who showed that in CSF-1-stimulated fibroblasts expressing CSF-1R, only a small proportion of the total pool of the receptor interacts with PI-3 kinase p85α [40]; in addition, a large cryptic pool of CSF-1R exists [41] which is not located at the cell surface and which presumably does not participate in cell signalling.

In the present study we have shown that the tyrosine phosphorylation of PI-3 kinase p85α is accompanied by the association of PI-3 kinase with other tyrosine phosphorylated proteins, including, as expected, the CSF-1R (p165). These include proteins migrating at 120 kDa, 95 kDa and 55–60 kDa, as well as proteins in the range 65–75 kDa, which are not as extensively phosphorylated. Our data do not allow us to say whether these proteins are constitutively associated with PI-3 kinase before CSF-1 stimulation. Analysis of immunoprecipitates prepared from metabolically labelled cells suggests that the amount of these

proteins associating with PI-3 kinase is much less than the level of CSF-1R; whether some of these proteins interact indirectly via CSF-1R is unknown. Given that non-specific interactions can develop during immunoprecipitation we determined whether the associations with PI-3 kinase survived anion-exchange chromatography. The positive findings of such an interaction surviving column chromatography have not been reported before for PI-3 kinase, and suggest the presence of a stable multimeric protein complex containing PI-3 kinase. The significance of the two pools of PI-3 kinase on CSF-1 stimulation (Figure 5) is not clear, but may reflect different states of activation of the PI-3 kinase population, since the p85α in the pool from which the complex was isolated showed an increased level of tyrosine phosphorylation. The two pools may also be contained in separate cellular compartments with the possibility that they could be involved in different cellular functions of macrophages following CSF-1 stimulation.

The co-immunoprecipitation experiments revealed that one such protein, Cbl, comigrating with the tyrosine phosphorylated, p120, associated with PI-3 kinase p85α in a transient manner. The [^{35}S]methionine-labelled 125 kDa band seen in Figure 2(A) in association with p85α did not migrate with Cbl (results not shown). While this manuscript was in preparation, a report showing the transient tyrosine phosphorylation and ubiquitination of Cbl was described in macrophages in response to CSF-1 [38], which supports our observations and accounts for the loss of Cbl in immunoprecipitates beyond 8 min of CSF-1 stimulation (Figure 4). The immunoprecipitates of anti-Cbl prepared from CSF-1-stimulated cells also contained PI-3 kinase p85α, which was tyrosine phosphorylated and is probably the tyrosine phosphorylated p80 protein found in anti-Cbl immunoprecipitates described in the previous report [38]. At present nothing is known about the role of Cbl in cell signalling, except that overexpression of cellular Cbl in NIH 3T3 cells, unlike that of viral Cbl, did not induce transformation but allowed cells to grow to a higher cell density [42]. The sequence of Cbl reveals several tyrosine residues and numerous proline-rich motifs that serve as possible binding sites for SH2- and SH3-binding domains respectively [43]. As mentioned, the Y721F mutation in CSF-1R abolishes PI-3 kinase binding, yet Cbl is present in the PI-3 kinase p85α immunoprecipitates of FDC-P1 cells containing this CSF-1R mutant; we propose that Cbl is most likely binding directly to the SH2/SH3 domains of p85α, and that Cbl found previously in CSF-1R immunoprecipitates reported by others [37] could bind via PI-3 kinase p85α. Given the presence of a large number of tyrosine residues in Cbl, and that Cbl tyrosine phosphorylated to a significant extent upon receptor activation (Figure 4) and also contains proline-rich motifs, this protein may serve as a multifunctional SH2/SH3 binding protein, recruiting potential signalling proteins involved in cell responses following receptor activation.

The identities of the other tyrosine phosphorylated proteins which co-immunoprecipitate with PI-3 kinase are not known. It is unlikely that the 95 kDa protein described in this work is the same as the recently cloned 93 kDa protein phosphorylated in response to CSF-1-activation of BAC1.2F5 cells [44], since the latter protein is not phosphorylated on tyrosine to any appreciable extent. One possibility is that it is a member of the immunoglobulin supergene family expressed in macrophages, since the β isoform of PI-3 kinase has been shown to associate with Cbl in activated B-cells, in complexes which contain the 95 kDa B-cell-specific cell surface antigen, CD19 [45]. Complexes of Cbl with PI-3 kinase, Grb2 and Fyn have been reported in T-cells stimulated with anti-CD3 [35] but, in this case, the antibody used to detect PI-3 kinase cross-reacted with both the α and β isoforms. This latter result suggests that members of the Src family, as well as Shc, are also likely candidates for the 55–60 kDa protein observed in our experiments to co-immunoprecipitate with PI-3 kinase. We are currently investigating the identities of the other, as yet unknown, proteins forming a novel multimeric complex with PI-3 kinase on CSF-1 stimulation of macrophages.

Mutating Tyr-721 in CSF-1R that associates with PI-3 kinase affected the cell density that Rat 2 fibroblasts, transfected with murine c-*fms*, could attain at confluence in response to CSF-1 [11], although the initial growth rate of cultures did not appear to be impaired. The fact that these cells were not completely impaired in growth suggests that CSF-1 is transmitting mitogenic signals via other sites on the receptor, such as the activation of Ras via Y697 [13]. In agreement with these results, Roche et al. [46] have shown that microinjection of a blocking antibody to p110α does not affect CSF-1-dependent proliferation of fibroblasts expressing CSF-1R, although similar treatments completely block PDGF and epidermal growth factor (EGF)-dependent proliferation of these same cells [46]. These observations, of course, do not rule out the possibility that other isoforms of PI-3 kinase are involved in proliferation in the fibroblasts. For FDC-P1 myeloid cells, in contrast to CSF-1R mutants in fibroblasts [11], mutations in Y721F and in the other kinase-insert phosphorylation sites did not alter the CSF-1-dependent proliferation [32]. Our results show that PI-3 kinase appears to be more weakly tyrosine phosphorylated in the poorly cycling RPM when compared with BAC1.2F5 cells and BMM; in spite of this apparent difference, p85α in the RPM associated with tyrosine phosphorylated proteins, including p120 and p165 (CSF-1R), although there were some differences in the associated proteins (Figure 7). It should be noted that a low percentage of RPM cells do proliferate in response to CSF-1 [47], and therefore it cannot be ruled out that these cells could be contributing to the effects observed. The lack of a detectable increase in PI-3 kinase activity in anti-pTyr immunoprecipitates previously reported in CSF-1-treated RPM [20] could be a reflection of the lower degree of tyrosine phosphorylation of PI-3 kinase p85α found above. In summary, the significance of PI-3 kinase for CSF-1-dependent macrophage proliferation awaits resolution, although it is relevant that Cbl is tyrosine phosphorylated when macrophages are treated with non-mitogens [37]. These findings may suggest that the association observed between PI-3 kinase and tyrosine phosphorylated proteins in response to CSF-1 may be more relevent to general CSF-1-mediated cellular function rather than to proliferation.

Similarity between PI-3 kinase and the yeast protein Vps34, and the role of the latter protein in vesicle sorting, has led to suggestions that PI-3 kinase may be involved in similar processes in higher organisms [28,29]. Since vesicle-mediated protein transport has been shown to occur at the Golgi, it is perhaps of relevance that it has been found, using confocal microscopy, that tyrosine phosphorylation of Cbl, following Fc receptor stimulation of macrophages and EGF stimulation of cells overexpressing the EGF receptor, is accompanied by a relocalization of Cbl to structures consistent with the Golgi network [37]. Hence, the association observed between PI-3 kinase and the tyrosine phosphorylated proteins, including Cbl, may have a role in vesicle sorting. The association of PI-3 kinase with cytoskeletal proteins may also suggest a role for this enzyme in the physical processes within the cell involving cytoskeletal reorganization following receptor activation, such as membrane ruffling, cell adhesion, cell spreading and migration [26,27]. In this connection, fibroblasts expressing the CSF-1R Y721F mutation have been shown to have a reduced ability to change cell morphology in response to CSF-1 compared with cells expressing the wild-type

receptor [11]. These postulated roles of PI-3 kinase in protein trafficking and endocytosis are consistent with the recent observation that the PDGF receptor, which contains mutations in the tyrosines involved in PI-3 kinase binding, is poorly degraded [48], and studies using the PI-3 kinase inhibitor, wortmannin, suggest that this requires PI-3 kinase catalytic activity. We are currently examining whether PI-3 kinase and its associated proteins may also play a similar role involving the CSF-1R.

We gratefully acknowledge the gift of the polyclonal antibody to the kinase domain of the murine CSF-1R, anti-CSF-1R$_2$, and the FDC-P1 myeloid progenitor cell line expressing wild-type murine CSF-1R (WTfms) and mutant CSF-1R, Y721F, from Dr. L. Rohrschneider, Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A. In addition, we thank Elizebeth Christie for technical assistance.

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Received 9 April 1996/26 June 1996; accepted 12 July 1996