Different pathways of colony-stimulating factor 1 degradation in macrophage populations revealed by wortmannin sensitivity

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Phosphatidylinositol 3′(OH)-kinase (PI 3-kinase) is activated on stimulation of macrophages with colony-stimulating factor 1 (CSF-1). We studied its potential role in the internalization and degradation of CSF-1 and its receptor in two primary populations of murine macrophages, namely bone marrow-derived macrophages (BMM) and resident peritoneal macrophages (RPM). Even though CSF-1 induced PI 3-kinase activity in both BMM and RPM, wortmannin, a potent inhibitor of PI 3-kinase activity, at concentrations that inhibited PI 3-kinase activity by 90 % in

these cells, had little or no effect on receptor internalization and degradation in either BMM or RPM or on CSF-1 degradation by BMM. Strong (more than 90 %) inhibition was, however, observed for CSF-1 degradation by RPM. These findings suggest that both wortmannin-sensitive and wortmannin-insensitive pathways of ligand degradation exist in macrophages and that, although CSF-1 and CSF-1 receptor share the same endocytic pathway initially, they might be targeted to different compartments at later stages of degradation.

INTRODUCTION

Macrophage colony-stimulating factor or colony-stimulating factor 1 (CSF-1) induces the non-covalent dimerization of its cognate receptor (c-Fms), which is a member of the protein tyrosine kinase receptor family and is found principally on cells of the monocyte/macrophage lineage [1]. The activation of the receptor initiates a cascade of signalling events leading to the phosphorylation and activation of a wide range of cytosolic and membrane proteins [2]. In parallel with the phosphorylation of proteins, the activated ligand-bound receptor is rapidly lost from the cell surface as a consequence of internalization via clathrin-coated pits [3].

The down-modulation of the CSF-1 receptor (CSF-1R) from the cell surface is presumed to serve an important biological role in attenuating the proliferative signal [4–7]; this concept is supported by an experiment with the FDC-P1 myeloid cell line in which a mutation in c-Fms that activates the CSF-1R, resulting in its accelerated degradation in the absence of ligand, led to cellular proliferation [8]. Once in clathrin-coated pits the ligand-bound receptor becomes sorted into vesicles eventually leading to its degradation in a chloroquine-sensitive lysosomal compartment [7,9]. The location of non-activated cell surface CSF-1R and the changes in compartmentalization of both the activated receptor and ligand along the pathway to internalization and endocytosis and subsequent sorting to the phagolysosome are therefore directly relevant to the biological response to CSF-1. Although some studies have suggested that growth factor receptors might remain active and continue to transduce a biological signal after a considerable period after internalization [10,11], the participation of such internalized receptors in transducing signals still remains unclear. These processes involving receptor endocytosis probably include the triggering of specific signals on the receptor itself [12] or might include the recruitment of other molecules to the receptor. One protein that has been shown to be involved in vesicle sorting is Vps 34, the yeast homologue of the phosphatidylinositol 3′-kinase (PI 3-kinase)

catalytic subunit, which targets newly synthesized hydrolases to yeast vacuoles [13]. We have previously shown that PI 3-kinase forms a stable complex with CSF-1R and other tyrosine-phosphorylated proteins on stimulation with ligands [14]. Given these observations, together with recent reports on the participation of PI 3-kinase in the degradative pathway of certain growth factor receptors [15], we decided to explore the possibility that PI 3-kinase might target CSF-1R and/or its ligand to vesicles in the endocytic pathway. These studies were performed with two populations of murine macrophages, namely bone marrow-derived macrophages (BMM) and resident peritoneal macrophages (RPM). The former population can proliferate strongly *in vitro* in response to CSF-1, whereas the latter proliferates poorly [1].

We found that, unlike PDGF-R, PI 3-kinase activity does not seem to be involved in CSF-1R endocytosis in macrophages. However, the results of the study revealed that the two macrophage populations possess ligand endocytic pathways that differ in their sensitivity to the PI 3-kinase inhibitor, wortmannin, and suggest that the signalling pathways that govern the intracellular compartmentalization of the CSF-1, but not of its receptor, might vary between different macrophage populations.

EXPERIMENTAL

Reagents

Purified human recombinant CSF-1 was a gift from Chiron (Emeryville, CA, U.S.A.). Other reagents were obtained from the following commercial sources: wortmannin and LY294002, Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.); iodoacetic acid, Sigma Chemical Co. (St. Louis, MO, U.S.A.); sulphosuccinimidobiotin (S-NHS-biotin), Pierce (Rockford, IL, U.S.A.); streptavidin–biotinylated horseradish peroxidase complex, enhanced chemiluminescence detection reagents, ‘Amplify’ fluorographic scintillant, Amersham (Little Chalfont, Bucks., U.K.); rabbit polyclonal antibody against the p85 α subunit of PI 3-kinase (anti-p85 α) and the monoclonal anti-phosphotyrosine

Abbreviations used: BMM, bone marrow-derived macrophages; CSF-1, colony-stimulating factor 1; CSF-1R, CSF-1 receptor; FBS, fetal bovine serum; S-NHS-biotin, sulphosuccinimidobiotin; PI 3-kinase, phosphatidylinositol 3-kinase; pTyr, phosphotyrosine; RPM, resident peritoneal macrophages; wtFms, wild-type murine CSF-1R.

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antibody 4G10 conjugated with horseradish peroxidase (anti-pTyr), Upstate Biotechnology Incorporated (Lake Placid, NY, U.S.A.). Rabbit polyclonal anti-(CSF-1R) was prepared in our laboratory as reported previously [14].

Cell culture conditions

Murine BMM from CBA mice were prepared from precursor cells in bone marrow by a procedure described previously [16] and grown as reported [14]. The cells were seeded at a density of 10^5 cells/ml and cultured until subconfluent. BMM were rendered quiescent by incubating in growth medium lacking L-cell-conditioned medium for 18–20 h before stimulation with growth factor. RPM from CBA mice were obtained as described previously [17] and cultured at 10^6 cells/ml as described [14]. FDC-P1 myeloid progenitor cell lines expressing wild-type murine CSF-1R (wtFms) and CSF-1R with a mutation (Y721F) in the autophosphorylation site were a gift from Dr. L. Rohrschneider. FDC-P1 cells were grown as described previously [14].

Metabolic labelling

Quiescent subconfluent BMM grown in 10 cm tissue culture dishes were washed twice with methionine-free RPMI (ICN Biochemicals, Irvine, CA, U.S.A.) and labelled for 4 h with $100 \mu\text{Ci/ml}$ [^{35}S]methionine/cysteine (ICN Biochemicals) at 37°C in 3 ml of methionine-free medium supplemented with glutamine and 1% (v/v) fetal bovine serum (FBS). FDC-P1 cells in 30 ml of medium were starved of growth factor by reculturing in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated FBS for 16 h at 37°C . FDC-P1 cells were then labelled for 4 h with $100 \mu\text{Ci/ml}$ of [^{35}S]methionine/cysteine at 37°C in 6 ml of methionine-free DMEM containing glutamine and 1% (v/v) FBS.

Immunoprecipitations

Subconfluent cultures of quiescent BMM or of RPM, grown in 10 cm tissue culture dishes, were stimulated with CSF-1 at 5000 units/ml at 37°C for the indicated times. Aliquots of 10^7 untreated and CSF-1-treated (5000 units/ml) FDC-P1 cells expressing wtFms and the Y721F mutant of the CSF-1R were removed at the indicated times and washed in ice-cold PBS before solubilization in lysis buffer. Immunoprecipitations from [^{35}S]methionine-labelled cell extracts were prepared as described previously [14]. Cell extracts containing approx. 0.5 mg of protein were precleared before immunoprecipitation with the specific primary antibody. The immunoprecipitates were resolved by SDS/PAGE [10% (w/v) gel] and detected by fluorography of the dried gels.

PI 3-kinase assay

Cells were washed, solubilized in lysis buffer containing 1% (v/v) Nonidet P40 and 0.5 mg of lysate immunoprecipitated with anti-pTyr(4G10), as reported [14]. Wortmannin was added at 100 nM to those samples that were used to determine the inhibitory effect on PI 3-kinase. The immunoprecipitates were washed and assayed for PI 3-kinase activity *in vitro* as described previously [18].

Iodination of CSF-1

Iodination of CSF-1 was performed as described [19]. CSF-1 was iodinated with chloramine T as oxidant to between 1 and 2 mol of ^{125}I per mol of CSF-1, separated from the bulk of un-

incorporated label by chromatography on PD10 columns (Pharmacia) and used within 10 days of iodination. A small but variable amount of acid-soluble radioactivity remaining in the iodinated CSF preparation after gel-permeation chromatography (typically 1–2%) was subtracted from all measurements of acid-soluble counts described in the text.

Internalization and degradation of ^{125}I -CSF-1

Internalization and degradation of ^{125}I -CSF-1 were measured as follows: quiescent BMM or RPM, cultured to a density of 10^6 cells per well in six-well plates, were cooled on ice for 30 min; inhibitors were then added where indicated. Multiple cycles of ligand uptake were followed by the addition of saturating concentrations of ^{125}I -CSF-1 (300 pM) to cells in a volume of 1 ml of medium per well at 37°C . The uptake of ligand was stopped at times indicated by the transfer of cultures to 4°C and washing twice in 2 ml of buffer (α -minimal essential medium/0.2% BSA/0.02% sodium azide). Receptor-bound ^{125}I -CSF-1 was determined as radioactivity released after the incubation of cells at pH 4 for 60 s. The internalized ^{125}I -CSF-1 was determined after solubilization in 1.5 ml of lysis buffer [50 mM Tris/HCl (pH 7.5)/0.5% SDS].

Internalization in a single uptake of ^{125}I -CSF-1 was followed in a temperature-shift experiment as described [7]. Quiescent BMM and RPM, cultured to a density of 10^6 cells per well in six-well plates, were cooled on ice for 30 min, after which inhibitors were added where indicated. Cultures were then incubated at 4°C with ^{125}I -CSF-1 (300 pM) for a further 60 min. Unbound ^{125}I -CSF-1 was removed by washing three times in 2 ml of ice-cold RPMI/1% (v/v) heat-inactivated FBS; the cultures were then shifted to 37°C by the addition of 1 ml of prewarmed medium for the times indicated and in the continued presence of inhibitors where appropriate. Internalization and degradation were stopped by transfer of cultures to 4°C and washing twice in 2 ml of buffer (α -minimal essential medium/0.2% BSA/0.02% sodium azide) as described above. Receptor-bound ^{125}I -CSF-1 was determined as radioactivity released after incubation of cells at pH 4; the internalized ^{125}I -CSF-1 was determined after solubilization in 1.5 ml of lysis buffer as described above. Cells at the zero time point were maintained at 4°C after the removal of unbound ^{125}I -CSF-1; cell surface receptor-associated ^{125}I -CSF-1 was then measured. The surface-bound ligand and internalized ligand at a given time point are represented as percentages of the total ^{125}I -CSF-1 present at that time. Degradation of ^{125}I -CSF-1 was determined by measuring trichloroacetic acid-soluble radioactivity in the medium at the indicated times after the temperature shift to 37°C , and is expressed as a percentage of the total cell surface receptor-bound ^{125}I -CSF-1 measured at zero time.

Degradation of the biotinylated CSF-1R

Quiescent subconfluent BMM or RPM at a density of 10^7 cells per 10 cm tissue culture plate were labelled with sulpho-NHS-biotin at 4°C as described [20] and then incubated for a further 60 min in the presence or absence of recombinant human CSF-1 (5000 units/ml). The cultures were transferred to 37°C for the times indicated and then lysed in 1% (v/v) Triton X-100 buffer as reported previously [14]. Anti-(CSF-1R) immunoprecipitates of the lysates were separated by SDS/PAGE [7.5% (w/v) gel]; the proteins were electroblotted to nitrocellulose. The membrane was incubated in blocking solution containing streptavidin-biotinylated horseradish peroxidase complex and the biotinylated CSF-1R was detected by enhanced chemiluminescence.

RESULTS

Wortmannin inhibits CSF-1R associated PI 3-kinase activity but not the binding of p85 α with CSF-1R following CSF-1 treatment of macrophages

PI 3-kinase associates with the CSF-1R after the stimulation of macrophages with CSF-1, resulting in an increase in its activity [18,21,22]; this activation is accompanied by tyrosine phosphorylation of the p85 α regulatory subunit [14]. Anti-pTyr immunoprecipitates of lysates prepared from both BMM and RPM showed an increase in the formation of PtdIns3P in a lipid kinase assay *in vitro* after stimulation with CSF-1 (Table 1); however, the extent of stimulation of PI 3-kinase activity in BMM was higher (10-fold) than in RPM (4-fold). This difference is correlated with the relative level of receptor expressed in the two macrophage populations, with BMM expressing approx. 3-fold that of RPM [1,7] (results not shown). Anti-(CSF-1R) immunoprecipitates showed the same relative degree of stimulation in PI 3-kinase activity after CSF-1 treatment of the macrophages (results not shown). The anti-pTyr-immunoprecipitable lipid kinase activity was blocked by almost 90 % on pretreatment of the macrophages with wortmannin (100 nM), a potent inhibitor of PI 3-kinase activity (Figure 1). Another specific PI 3-kinase inhibitor, LY294002, was less effective, with approx. 40 % inhibition of the PI 3-kinase activity after pretreatment with 10 μ M LY294002.

Table 1 PI 3-kinase activity is stimulated in macrophages after treatment with CSF-1

Lysates derived from quiescent and CSF-1-treated (2 min) BMM and RPM were immunoprecipitated with anti-pTyr (4G10) antibody; the immunoprecipitates were assayed for PI 3-kinase activity. The PtdIns3P product was quantified by densitometric scanning of the autoradiogram after exposure of the TLC plate; the values are expressed as fold stimulation of the untreated immunoprecipitates. Results are means $\pm$ S.E.M. for triplicate samples.

Condition	PI 3-kinase activity (fold stimulation)	
	BMM	RPM
Without CSF-1	1.0	1.0
With CSF-1	9.8 $\pm$ 3.6	4.4 $\pm$ 1.5

Figure 1 Wortmannin blocks PI 3-kinase activity in CSF-1-stimulated macrophages

Lysates prepared from CSF-1-treated (2 min) BMM and RPM, preincubated with 100 nM wortmannin where indicated, were immunoprecipitated with anti-pTyr (4G10) antibody and the immunoprecipitates assayed for PI 3-kinase activity. The arrow indicates the position of the 32 P-labelled PtdIns3P [PI-(3)P] product separated on a TLC plate.

Figure 2 PI 3-kinase p85 α association with CSF-1R is not affected by wortmannin

[35 S]Methionine-labelled BMM were treated with 100 nM wortmannin or left untreated before stimulation with CSF-1 (5000 units/ml) for 2 min. Lysates were immunoprecipitated with anti-(PI 3-kinase) p85 α and resolved by SDS/PAGE. Proteins were detected by fluorography. The positions of the prestained molecular mass markers are indicated. Arrows indicate the positions of the CSF-1R, and the p85 α and p110 α subunits of PI 3-kinase (PI-3K) [14].

In addition to the CSF-1R, PI 3-kinase has recently been demonstrated to form an association with a number of tyrosine-phosphorylated proteins in CSF-1-treated BMM and RPM [14]. We initially investigated the effect of wortmannin on the stability of the association between PI 3-kinase and the CSF-1R. Analysis of PI 3-kinase p85 α immunoprecipitates from lysates prepared from 35 S-labelled BMM showed that the significant association of p85 α with CSF-1R (p165) on CSF-1 stimulation, identified previously [14], was unaffected by pretreatment with wortmannin (Figure 2). Although the PI 3-kinase activity present in anti-(CSF-1R) immunoprecipitates of CSF-1-stimulated BMM lysates was abolished on pretreatment with wortmannin, the association of the CSF-1R and other tyrosine-phosphorylated proteins with PI 3-kinase p85 α did not seem to be significantly affected (results not shown). From these experiments we conclude that the stability of the complex between PI 3-kinase, the CSF-1R and other tyrosine-phosphorylated proteins is not affected by inhibition of PI 3-kinase activity.

Degradation of the CSF-1R in CSF-1-treated macrophages

PI 3-kinase has been reported to be involved in the endocytosis of other growth factor receptors, in particular that of the platelet-derived growth factor receptor [15]. This receptor, together with Flt2/flk3, is structurally related to the CSF-1R in that it possesses a short polypeptide sequence inserted within the kinase domain, the so-called kinase insert domain. To determine whether PI 3-kinase has a role in CSF-1R-mediated internalization and degradation, we measured the effect of wortmannin on its degradation in both BMM and RPM. We initially attempted to measure the rate of decrease of immunoprecipitable CSF-1R from 35 S-labelled BMM lysates after stimulation with CSF-1 in pulse-chase experiments. We did not observe any significant degradation of CSF-1R over 3 h in BMM, which is presumably because, in the BMM at any given time, a considerable proportion of the receptor is present in the 'cryptic pool' and not participating in ligand binding at the surface [7]. We therefore

Figure 3 Degradation of the CSF-1R in CSF-1-treated cells expressing CSF-1R

Quiescent BMM (**A**) or RPM (**B**) were biotinylated at 4 °C for 30 min; after removal of excess S-NHS-biotin the macrophages were incubated at 37 °C with medium in the presence or absence of CSF-1 (5000 units/ml) for the indicated times. Lysates of the cells were immunoprecipitated with anti-(CSF-1R) antibody and the immunoblots were probed with streptavidin–horseradish peroxidase conjugate. (**C**) [³⁵S]Methionine-labelled FDC-P1 cells expressing wtFms and Y721F mutant CSF-1R were treated with CSF-1 (5000 units/ml) at 37 °C for the indicated times. Lysates were immunoprecipitated with anti-(CSF-1R) antibody and the proteins were resolved by SDS/PAGE. Proteins were detected by fluorography. The arrow in each panel indicates the position of the CSF-1R (p165).

measured the degradation of the receptor located specifically at the cell surface by labelling this pool with biotin before stimulation of cells with CSF-1.

The amount of intact biotinylated CSF-1R in BMM lysates decreased by 45 % within 3 h of exposure to CSF-1 (Figure 3A). Although time points between 0 and 3 h were assayed there was minimal degradation of the CSF-1R (0 % at 1 h and 24 % at 2 h). Pretreatment with 100 nM wortmannin did not significantly inhibit the rate of degradation (Figure 3A). The CSF-1R in RPM degraded more rapidly (Figure 3B) (more than 99 % within 90 min); this was also apparent when the time courses for CSF-1R degradation in the two macrophage populations were compared. Pretreatment of RPM with wortmannin seemed to have only a minor effect on CSF-1R degradation at the time point shown (Figure 3B, compare lanes 3 and 2) and at earlier time points (results not shown). These results suggest that PI 3-kinase activity does not have a significant role in the CSF-1-stimulated

Figure 4 Internalization of CSF-1 in macrophage populations

Quiescent BMM (**A**, **B**) or RPM (**C**) were treated with 100 nM wortmannin for 10 min, 8 mM iodoacetic acid for 15 min at 37 °C or left untreated. The internalization of ¹²⁵I-CSF-1 in BMM under multiple turnover conditions was measured by incubating cells with ¹²⁵I-CSF-1 (300 pM) at 37 °C (**A**) after the various treatments. The surface-bound ¹²⁵I-CSF-1 was determined as radioactivity dissociable at pH 4, and ¹²⁵I-CSF-1 present as internalized ligand was estimated from total radioactivity in the lysate. The internalization of ¹²⁵I-CSF-1 in BMM (**B**) or RPM (**C**) in a temperature-shift experiment was measured as radioactivity remaining in lysate after dissociation at pH 4 of cells preincubated with ¹²⁵I-CSF-1 (300 pM) at 4 °C and after removal of unbound ligand, warming the cells to 37 °C. Internalized ¹²⁵I-CSF-1 is represented as a proportion of the total radioactivity (representing both the surface-bound and internalized ¹²⁵I-CSF-1): untreated (□), wortmannin-treated (■), iodoacetic-treated (●) BMM and untreated (◇), wortmannin-treated (◆) RPM. Results are means ± S.E.M. for triplicate samples and are representative of four experiments. S.E.M. is not indicated when below the scale represented.

degradation of CSF-1R. To support this conclusion further, we have also measured receptor degradation in the FDC-P1 myeloid cell line engineered to express c-Fms carrying a mutation (Y721F) in the PI 3-kinase-binding site and found the mutation not to affect CSF-1R degradation (Figure 3C). Densitometric scanning of the 165 kDa protein in anti-(CSF-1R) immunoprecipitates of ³⁵S-labelled cell lysates showed that a significant level of CSF-1R in FDC-P1 cells expressing wtFms (63 % of the initial level at time 0) as well as cells expressing mutant Y721F CSF-1R (60 % of the initial level at time 0) was degraded after treatment of cells with CSF-1 for 60 min at 37 °C.

Wortmannin shows a differential effect on the degradation of CSF-1 in different populations of macrophages without significant effect on internalization

Given the possibility that receptor and ligand could be targeted to different vesicles during the process of endocytosis, we extended the study by examining the effect of wortmannin on the rates of internalization and degradation of CSF-1. Initially we measured the uptake of ¹²⁵I-CSF-1 under conditions of excess ligand (300 pM), allowing multiple cycles of uptake to occur [7]. The results presented in Figure 4(A) show that treatment with wortmannin at levels that substantially inhibit PI 3-kinase activity did not affect the amount of cell-associated radioactivity in BMM

Figure 5 CSF-1 degradation in macrophage populations

Quiescent BMM (A) and RPM (B) were pretreated with wortmannin for 15 min at 37 °C or left untreated. The cells were chilled and ^{125}I -labelled CSF-1 (300 pM) was allowed to bind for 60 min on ice. After the removal of unbound ligand, cells were replaced with medium at 37 °C in the continued presence or absence of wortmannin. Cells at the zero time point were replaced with medium at 4 °C and the radioactivity in the lysate was estimated to determine the total surface-associated counts before internalization. Degradation of the ^{125}I -CSF-1 was estimated by counting the trichloroacetic acid-soluble fraction of the media. Degradation is shown as a proportion of the initial surface-associated ^{125}I -CSF-1. (C) Data from (A) and (B) were plotted as a first-order degradation of ^{125}I -CSF-1, where D_0 and D_t are the trichloroacetic acid-soluble radioactivity at zero time and at the indicated times respectively, minus the degradation at time infinity: untreated ($\square$), wortmannin-treated ($\blacksquare$) BMM and untreated ($\diamond$), wortmannin-treated ($\blacklozenge$) RPM. Results are means $\pm$ S.E.M. for triplicate samples and are representative of six independent experiments. S.E.M. is not indicated when below the scale represented.

present after dissociating surface-bound radioactivity in acid. This result implies that PI 3-kinase activity is not required for ligand internalization under these conditions. In contrast, 8 mM iodoacetic acid used as a control significantly inhibited the internalization of ^{125}I -CSF-1, a result consistent with studies of Li and Stanley [23], who have shown that carboxymethylation of the CSF-1R by iodoacetic acid prevents its covalent dimerization and thereby its internalization. Although pretreatment with IAA showed ligand to remain at the surface for a longer period than with untreated control, the inhibition was reversible as observed by the eventual increase in the level of the internalized receptor on further incubation at 37 °C.

We extended this observation by measuring the effect of wortmannin on the internalization of surface-bound ligand in the two different macrophage populations BMM and RPM. For these experiments we preloaded the cells with ^{125}I -CSF-1 at 4 °C, and then shifted to 37 °C to measure the internalization in a single uptake [7]. The proportion of surface-bound and internalized ^{125}I -CSF-1 were then measured over 20 min [7] as described in the Experimental section. At zero time, more than 98 % of surface-bound radioactivity was acid-dissociable and the remainder could be accounted for as non-specifically bound radioactivity because it was correlated with the amount of radioactivity that did not appear as trichloroacetic acid-soluble counts on prolonged incubation (see below). Pretreating macrophages with wortmannin did not significantly affect the initial binding of

Table 2 Effect of LY294002 on CSF-1 degradation in RPM

RPM were pretreated with LY294002 (10 μM) for 15 min at 37 °C or left untreated. ^{125}I -labelled CSF-1 (300 pM) was added to cells at 4 °C and left to bind for 60 min on ice. After the removal of unbound ligand, cells were replaced with medium at 37 °C for the indicated times in the continued presence or absence of the inhibitor. Cells at the zero time point were replaced with medium at 4 °C and the radioactivity in the lysate estimated to determine the total surface-associated counts before internalization. Degradation of the ^{125}I -CSF-1 was estimated by counting the trichloroacetic acid-soluble fraction of the media. The degradation is shown as a percentage of the initial surface-associated ^{125}I -CSF-1. Results are means $\pm$ S.E.M. for triplicate samples and are representative of three independent experiments.

Time (min)	CSF-1 degradation (% of initial surface CSF-1)	
	Control	LY294002
0	0.6 $\pm$ 0	0.6 $\pm$ 0
30	21.8 $\pm$ 2.0	14.0 $\pm$ 0.6
60	70.8 $\pm$ 0.7	45.9 $\pm$ 3.4

^{125}I -CSF-1 at 4 °C, as indicated by the number of moles of surface-bound ligand measured as radioactivity dissociable at pH 4 at the start of the experiment (results not shown) and had no effect on the rate of internalization of ^{125}I -CSF-1 on warming the cultures to 37 °C (Figures 4B and 4C). The lag in the initial rate of internalization in CSF-1 observed in BMM compared with RPM might be a consequence of retroendocytosis, perhaps contributed by the large cryptic pool of CSF-1R in BMM [7].

Having shown that PI 3-kinase activity is not involved in receptor-mediated internalization of CSF-1, we next investigated its potential role in the ligand degradative pathway given that, as mentioned above, Vps 34, the yeast homologue of PI 3-kinase catalytic subunit, is involved in intracellular vesicle sorting [13]. After its receptor-mediated uptake, ^{125}I -CSF-1 is degraded; most of the radioactivity appears as acid-soluble ^{125}I -tyrosine, which accumulates into the medium as degradation proceeds [7]. We measured the rate of degradation of cell surface-bound ^{125}I -CSF-1 in BMM and RPM over a time course of 60 min (Figures 5A and 5B). Over this period the formation of acid-soluble radioactivity was effectively abolished by the addition of a 100-fold excess of non-radiolabelled CSF-1, which implies that degradation of the bulk of ligand occurs after a receptor-mediated uptake. In both BMM and RPM approx. 50 % of surface-bound ligand was degraded after 50 min. Replotting the data (Figure 5C) showed that, after a lag period of approx. 25 min, degradation followed first-order kinetics, as shown previously by others in both BMM and murine peritoneal exudate macrophages with the use of L-cell-purified CSF-1 instead of recombinant human CSF-1 [7]. Pretreating BMM with wortmannin reduced the extent of degradation of ^{125}I -CSF-1 by only 15–20 % when measured at 60 min (Figure 5A). However, the degree of inhibition in RPM was significantly greater (over 90 % when measured at 60 min; Figure 5B), suggesting that there are differences in the pathways of ligand degradation between these two macrophage populations. Experiments with thioglycollate-induced peritoneal exudate macrophage cultures showed that ligand degradation was inhibited by 100 nM wortmannin to a similar extent to that in RPM (results not shown).

Despite being generally recognized as a specific inhibitor of PI 3-kinase in the nanomolar range, wortmannin is known to have other intracellular targets. We therefore tested the effect of a second inhibitor of PI 3-kinase, LY294002, on CSF-1 degradation in RPM (Table 2). At a concentration of 10 μM , LY294002 was able to block degradation of cell-surface-associated ^{125}I -CSF-1

by 30 %, which is consistent with the lower level of inhibition of PI 3-kinase achieved at this concentration of LY294002 when compared with wortmannin.

Guilbert and Stanley [7] showed that the rate of degradation of ligand in macrophages is a function of the extent of internalized ligand at the time of measurement. To verify that the different sensitivities of RPM and BMM to wortmannin with respect to ligand degradation were not due to a difference in the amount of internalized ligand, we measured the sensitivity to wortmannin by preloading cells at two concentrations of ligand (300 and 3 pM) corresponding to saturating and subsaturating levels of receptor occupancy [7]. Our results showed comparable levels of inhibition by wortmannin for both of these situations (results not shown), suggesting this is not the reason for the difference between BMM and RPM in their wortmannin sensitivity to ligand degradation.

DISCUSSION

PI 3-kinase has been implicated in several aspects of intracellular membrane trafficking, including down-regulation of growth factor receptors [13,15]. Although the mechanism by which PI 3-kinase exerts such effects remain unclear, analogies have been made with the product of the yeast *Vps 34* gene. The gene encodes a homologue of the p110 catalytic subunit of mammalian PI 3-kinase, and a mutation in the gene results in defective targeting of soluble proteins to yeast vacuoles, a process paralleling vesicle transport from the trans-Golgi network to lysosomes in mammalian cells [13].

We have shown that, at a concentration at which it drastically decreases the PI 3-kinase activity in CSF-1-treated macrophages, wortmannin does not affect CSF-1R degradation. Therefore it is unlikely that PI 3-kinase activity is involved in CSF-1-mediated endocytosis of its receptor in both BMM and RPM. Thus this situation is similar to that for Flt2/Flk3 receptor [24] but different from that for platelet-derived growth factor receptor [15] in other cell systems. These three receptors are grouped as a family of tyrosine kinase receptors with a conserved so-called kinase insert domain and are often considered likely to have similar properties and to interact with similar signal transduction molecules [25,26]. However, it cannot be assumed that this will always be true and our findings above support this conclusion. Deletion of this kinase insert domain in murine c-Fms, which contains among other sites the PI 3-kinase-binding site, decreased CSF-1-mediated receptor degradation in FDC-P1 cells and in Rat-2 fibroblasts [27]. On the basis of our results reported here it would seem that any altered PI 3-kinase activity in this mutant receptor system would not account for the altered CSF-1R degradation.

In contrast with the receptor, we did find that the two PI 3-kinase inhibitors wortmannin and LY294002 inhibited CSF-1 degradation (Figure 5 and Table 2), particularly in RPM. Interestingly, the effect of the wortmannin was less pronounced for the CSF-1 degradation by BMM. The results of this study suggest that there are differences in the cellular pathways governing ligand degradation in RPM and BMM and, assuming inhibitor specificity, would suggest that PI 3-kinase activity is important but with a different contribution for each of the two macrophage populations. BMM, in contrast with RPM, are a rapidly cycling population in the presence of CSF-1 [1] and our findings might relate to the differentiation state of the macrophage. The results suggest that CSF-1 and its receptor are degraded in separate compartments that show different

sensitivities to wortmannin in different populations of macrophages.

A recent study in a murine macrophage cell line has revealed that endosomal proteolysis of a ligand can occur in both a late endosome as well as in the expected lysosomal compartment [28]. PI 3-kinase has also been implicated in endosome fusion via its activation of Rab5 in experiments with wortmannin [29]. These reports, taken together with our observations, support both PI 3-kinase-dependent and PI 3-kinase-independent pathways of endocytosis and post-endocytic trafficking that might be used by different receptors or by the same receptor in different cell types. The surprising observation that in RPM the degradation of CSF-1 and the CSF-1R show different sensitivities to wortmannin suggests that although they share the same endocytic pathway initially they might be targeted to different compartments at later stages of degradation.

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