The CC Chemokine Monocyte Chemotactic Peptide-1 Activates both the Class I p85/p110 Phosphatidylinositol 3-Kinase and the Class II PI3K-C2α

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The cellular effects of MCP-1 are mediated primarily by binding to CC chemokine receptor-2. We report here that MCP-1 stimulates the formation of the lipid products of phosphatidylinositol (PI) 3-kinase, namely phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P₃) in THP-1 cells that can be inhibited by pertussis toxin but not wortmannin. MCP-1 also stimulates an increase in the in vitro lipid kinase activity present in immunoprecipitates of the class Ia p85/p110 heterodimeric PI 3-kinase, although the kinetics of activation were much slower than observed for the accumulation of PI 3,4,5-P₃. In addition, this in vitro lipid kinase activity was inhibited by wortmannin (IC₅₀ = 4.47 ± 1.88 nM, n = 4), and comparable concentrations of wortmannin also inhibited MCP-stimulated chemotaxis of THP-1 cells (IC₅₀ = 11.8 ± 4.2 nM, n = 4), indicating that p85/p110 PI 3-kinase activity is functionally relevant. MCP-1 also induced tyrosine phosphorylation of three proteins in these cells, and a fourth tyrosine-phosphorylated protein co-precipitates with the p85 subunit upon MCP-1 stimulation. In addition, MCP-1 stimulated lipid kinase activity present in immunoprecipitates of a class II PI 3-kinase (PI3K-C2α) with kinetics that closely resembled the accumulation of PI 3,4,5-P₃. Moreover, this MCP-1-induced increase in PI3K-C2α activity was insensitive to wortmannin but was inhibited by pertussis toxin pretreatment. Since this mirrored the effects of these inhibitors on MCP-1-stimulated increases in D-3 phosphatidylinositol lipid accumulation in vivo, these results suggest that activation of PI3K-C2α rather than the p85/p110 heterodimer is responsible for mediating the in vivo formation of D-3 phosphatidylinositol lipids. These data demonstrate that MCP-1 stimulates protein tyrosine kinases as well as at least two separate PI 3-kinase isoforms, namely the p85/p110 PI 3-kinase and PI3K-C2α. This is the first demonstration that MCP-1 can stimulate PI 3-kinase activation and is also the first indication of an agonist-induced activation of the PI3K-C2α enzyme. These two events may play important roles in MCP-1-stimulated signal transduction and biological consequences.

Chemokines are a rapidly growing superfamily of 8–10 kDa peptides that selectively attract and activate leukocyte populations (1, 2). Monocyte chemotactic peptide-1 (MCP-1) (1) is a member of the CC chemokine family (1, 2), is a potent inducer of monocyte and CD45RO⁺ lymphocyte chemotaxis (3, 4), and also activates host defense mechanisms such as superoxide release (5). In vivo studies suggest that MCP-1 recruits monocytes to sites of inflammation in a variety of pathological conditions including atherosclerosis (6) and rheumatoid arthritis (7) as well as pulmonary fibrosis and granulomatous lung disease (8). MCP-1 has also been demonstrated to augment cytotoxic lymphocyte and natural killer cell activity in vitro, suggesting a novel role for chemokines as costimulators of T cell activation (9). Support for MCP-1’s importance in the physiology of inflammation comes from demonstrations in transgenic mice that it functions as a monocyte chemoattractant in vivo (10–12). Moreover, abnormalities in monocyte recruitment and cytokine expression are observed in MCP-1-deficient mice (13).

The effects of chemokines are mediated by a family of closely related G protein-coupled receptors (1). MCP-1 binds to CC chemokine receptor 2 (CCR2), which exists in A or B forms that arise via alternative splicing of the carboxyl-terminal tail (14). CCR2 is activated by multiple agonists, including MCP-2 (15), MCP-3 (16, 17), MCP-4 (18, 19), and MCP-5 (20). In addition, only chemokines of the MCP family (MCP-1, -2, -3, -4, and -5) appear to activate CCR2, although CCR2 agonists can also bind and activate other receptors, since MCP-3 activates CCR1 (16, 17) and MCP-3 and MCP-4 activate CCR3 (18, 19). Studies with CCR2⁻/⁻ mice have recently revealed, however, that MCP-1 initiates cellular responses primarily through binding to CCR2 (21). Analysis of the signal transduction pathways activated by MCP-1 has revealed pertussis toxin-sensitive phospholipase C activation (22), elevation of intracellular calcium (14, 23), and inhibition of adenyl cyclase (24). Interestingly, both CCR2A and CCR2B can both couple to the Gα₄5β PLCβ2 pathway, but these receptors demonstrate an interesting specificity in their coupling to the α-subunits of the Gq class. Hence, CCR2B couples to both Ga16 and Ga14, whereas CCR2A cannot couple to either Ga14 or Ga16 (22). Other signaling events downstream of MCP-1 remain relatively poorly defined. In this respect, it is interesting to note that the related CC chemokine RANTES has been demonstrated to stimulate the tyrosine phosphorylation of a number of proteins in a T-cell clone (25, 26) and to activate the protein-tyrosine kinase (PTK-src homology domain 2-coupled phos-

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The abbreviations used are: MCP-1, monocyte chemotactic peptide-1; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; PI, phosphatidylinositol; PI 3-P, phosphatidylinositol 3-monophosphate; PI 3,4-P₂, phosphatidylinositol 3,4-bisphosphate; PI 3,4,5-P₃, phosphatidylinositol 3,4,5-trisphosphate; PTK, protein-tyrosine kinase; RANTES, regulated on activation normal T cell expressed and secreted; CCR2, CC chemokine receptor 2.
phosphatidylinositol (PI) 3-kinase (27), a member of the class IA family of the phosphatidylinositol 3-kinases (28).

The prototypical class IA PI 3-kinase consists of an 85-kDa regulatory subunit responsible for protein-protein interactions via protein tyrosine phosphate-binding src homology domains and a catalytic 110-kDa subunit (28). PI 3-kinase is now regarded as an important intracellular signal that is upstream of a variety of responses including insulin-stimulated glucose uptake (29), membrane ruffling (30), superoxide production (31), activation of p70 S6 kinase (32), and activation of Akt/protein kinase B (33). A G protein-coupled PI 3-kinase, namely PI 3-kinase γ has also been identified (34, 35). PI 3-kinase γ is, to date, the only characterized member of the class IA G protein-coupled PI 3-kinase family and consists of a unique 101-kDa regulatory subunit and a distinct 110-kDa catalytic subunit termed p110γ (28, 33, 34). Nevertheless, there is some evidence that G protein-coupled receptors, such as mILP receptors, are able to activate the p85/p110 PI 3-kinase (36, 37). In this study, therefore, we have investigated the possible involvement of PI 3-kinase(s) in MCP-1 signal transduction (41), and RANTES-stimulated chemotaxis of T-lymphocytes (4), and neutrophil peroxide release (31), interleukin-8-stimulated neutrophil chemotaxis (4), and MCP-1 signal transduction (41). In this report, we describe the novel human member of the class II PI 3-kinase family that, in vitro, PI and phosphatidylinositol 4-monophosphate as substrates are phosphorylated (40).

To date, the only characterized member of the class IA PI 3-kinases is the 190-kDa PI3K-C2α that potentially generates three lipid products, namely phosphatidylinositol 3-monophosphate (PI 3-P), phosphatidylinositol 3,4-bisphosphate (PI 3,4-P2), and phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P3), which are collectively known as D-3 phosphatidylinositol lipids (reviewed in Refs. 38 and 39). To date, PI 3-P and PI 3,4-P2 and PI 3,4,5-P3 are regarded as signaling molecules, whereas PI 3-P is thought to regulate membrane trafficking (38, 39). The PI 3-kinase family is completed by the class II C2 domain-containing PI 3-kinases and the class III PtdIns-specific 3-kinases (28). The 190-kDa PI3K-C2α is a novel human member of the class II PI 3-kinase family that, in contrast to members of the other PI 3-kinase classes, is refractory to inhibition by the PI 3-kinase inhibitors wortmannin and LY294002 (40). In addition, PI3K-C2α utilizes predominantly PI and phosphatidylinositol 4-monophosphate as substrates in vitro, but when presented with phosphatidylinerine, it can also phosphorylate PI 4,5-bisphosphate (40).

Given the functional role of MCP-1 on superoxide generation and chemotaxis (4, 5), it is interesting to note that the PI 3-kinase inhibitor wortmannin inhibits fMLP-stimulated superoxide release (31), interleukin-8-stimulated neutrophil chemotaxis (41), and RANTES-stimulated chemotaxis of T-lymphocyte (28). In this study, therefore, we have investigated the possible involvement of PI 3-kinase(s) in MCP-1 signal transduction and chemotaxis using the THP-1 monocytic cell line.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant MCP-1 was purchased from Peprotech (Rocky Hill, NJ). Mouse p85α monoclonal antibody (mAb) was a generous gift from Doreen Cantrell (Imperial Cancer Research Fund, London). 4G10 anti-phosphotyrosine mAb was purchased from Upstate Biotechnology. All cell culture reagents and pertussis toxin were purchased from Life Technologies, Inc. Wortmannin and standard phosphatidylinositol lipids were purchased from Sigma as was the PY20 anti-phosphotyrosine antibody. [γ-32P]ATP (3000 Ci/mmol) and [32P]orthophosphate (8500–9120 Ci/mmol; NEN Life Science Products) as described (42).

P-Labeled THP-1 cells were aliquoted at 107/120 μl and stimulated as described in the figure legends, and the phospholipids were extracted with 700 μl of chloroform:methanol:H2O (32.6, 65.3, and 2.1% (v/v/v), respectively) (42). The samples were desalted and analyzed by anion exchange HPLC analysis (Partisil SAX column (Whatman) (42). The eluate was fed into a Canberra Packard A-500 Flo-One on-line radiodetector, and the results were analyzed by the Flo-One data program (Radiomatic). Eluted peaks were compared with retrieval times for standards prepared from 32P-labeled phosphatidylinositol lipids (Amersham Pharmacia Biotech) and 32P-labeled D-3 phosphatidylinositols described elsewhere (42).

**Cell Lysis and in Vitro Lipid Kinase Assays**—2 × 107 cells/ml were equilibrated for 10 min at 37 °C and then stimulated in RPMI 1640 medium as described in the figure legends. Reactions were terminated by pelleting cells in a microcentrifuge for 10 s and aspirating supernatant, followed by the addition of 0.5 ml of ice-cold lysis buffer (1% (v/v) Nonidet P-40, 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM iodoacetamide, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml β-glycerophosphate, and 1 mM sodium orthovanadate). Lysates were rotated at 4 °C for 15 min, followed by centrifugation at 14,000 rpm. The supernatants were precleared, and immunoprecipitation was performed as described (43, 44) using either P85α mAb (1 μg/ml) precoated to protein G-Sepharose beads (Pharmacia Biotech Inc.) or PI3K-C2α polyclonal antibody precoated to protein A-Sepharose beads. Immunoprecipitates were washed and subjected to in vitro lipid kinase assays as described (43, 44) using a lipid mixture of 100 μl of 0.1 mg/ml PI and 0.1 mg/ml phosphatidylinerine dispersed by sonication in 20 ml HEPES, pH 7.0, and 1 ml EDTA. The reaction was initiated by the addition of 10 μCi of [γ-32P]ATP (3000 Ci/mm; NEN Life Science Products) and 100 μl ATP to the immunoprecipitates suspended in 80 μl of kinase buffer (5 mM MgCl2, 0.25 mM EDTA, 20 mM HEPES, pH 7.4). The reaction was terminated after 15 min, and the resulting phospholipids were then separated by TLC (43, 44). The TLC plates were stained with iodine to confirm extraction of substrate lipid between individual samples, and 32P-labeled PtdIns(3,4,5)P3 was visualized by autoradiography (43, 44).

**Immunoblotting**—2 × 107 cells/ml resuspended in RPMI 1640 were equilibrated at 37 °C for 10 min and stimulated as described in the figure legends, and cell lysates were prepared (43, 44). Aliquots of cell lysate (20 μl) or immunoprecipitates generated using either anti-phosphotyrosine mAb PY20 (1 μg/ml) or anti-P85 (1 μg/ml) antibodies were boiled in Laemmli buffer and electrophoresed through 7.5% (v/v) acrylamide gels by SDS-PAGE, and the proteins were transferred by electroblotting onto nitrocellulose (Schleicher & Schuell) as described previously (44). The blots were probed with the anti-PI3K-C2α polyclonal antibody (1500 dilution) or anti-phosphotyrosine mAb 4G10 (0.1 μg/ml) as indicated in the appropriate figure legends, and the proteins were visualized by chemiluminescence detection system (Amersham Pharmacia Biotech) with goat anti-rabbit or goat anti-mouse IgG (0.1 μg/ml) horseradish peroxidase conjugate, respectively, as a secondary antibody (Dako).

**Chemotaxis Assays**—THP-1 cell chemotaxis was examined using a...
96-well chemotaxis chamber (Neuro Probe, Cabin John, MD). The wells of the 96-well plate were filled with 380 μl of chemoattractant diluted in RPMI 1640 containing 0.1% bovine serum albumin and covered with an adhesive polyvinylpyrrolidine-free polycarbonate membrane (5-μm pore size). 5 × 10^5 cells were added to each upper well in a volume of 200 μl, and the chamber was incubated at 37 °C for 2 h. The cell suspension was subsequently aspirated off, and 200 μl of Versene (Life Technologies Inc., Paisley, UK) was added to each well. After a 20-min incubation at 4 °C, the 96-well plate and membrane were centrifuged at 1200 rpm for 10 min, the supernatant was removed, and the cells were resuspended in 100 μl of RPMI containing 0.1% bovine serum albumin. THP-1 cell migration was assessed by adding 20 μl of Cell Titer 96 AQueous solution (Promega, UK) to each well. After a 2-h incubation at 37 °C, the plate was read at a wavelength of 490 nm, subtracting the readings at a reference wavelength of 650 nm to reduce the background contributed by nonspecific absorbance.

**RESULTS**

**MCP-1 Stimulates the Generation of PI 3,4-P_2 and PI 3,4,5-P_3 in THP-1 Cells**—MCP-1 induces a rapid and transient activation of phospholipase C in THP-1 cells (23, 45). In this study, we have used 32P-labeled THP-1 cells to investigate the effect of MCP-1 stimulation on the activation of another signaling pathway, namely PI 3-kinase. Accordingly, treatment of THP-1 cells with MCP-1 resulted in the concentration-dependent formation of PI 3,4,5-P_3 (Fig. 1A). The MCP-1-induced increase in PI 3,4,5-P_3 exhibited bell-shaped characteristics with the maximum response observed in the presence of 60 nM MCP-1 (Fig. 1A). Furthermore, the MCP-1-stimulated formation of PI 3,4,5-P_3 was extremely rapid and transient, since it was detectable 5 s after stimulation and had returned to basal levels 2 min after MCP-1 treatment (Fig. 1B). MCP-1 also stimulated the concentration-dependent formation of PI 3,4-P_2 (Fig. 1C), which occurred with slightly slower but more sustained kinetics than those observed for the formation of PI 3,4,5-P_3 (Fig. 1D).

**MCP-1 Enhances the in Vitro Lipid Kinase Activity Present in p85 Immunoprecipitates Derived from THP-1 Cells**—To investigate the possible effects of MCP-1 on the prototypical p85/p110 heterodimer, we performed experiments to measure the lipid kinase activity present in p85 immunoprecipitates derived from MCP-1-stimulated THP-1 cells. Accordingly, MCP-1 stimulated a notable increase above basal levels of in vitro PI 3-kinase activity in THP-1 cells. This effect was detectable after 60 s, and the lipid kinase activity continued to increase up to 300 s post MCP-1 stimulation (Fig. 2A). The increase in in vitro PI 3-kinase activity following MCP-1 stimulation was also concentration-dependent (Fig. 2B).

**Effects of Pertussis Toxin or Wortmannin Pretreatment on MCP-1-stimulated PI 3-Kinase Activation in THP-1 Cells**—To further examine the PI 3-kinase activity stimulated by MCP-1, we used the G protein inhibitor pertussis toxin and the PI 3-kinase inhibitor wortmannin. Pretreatment of THP-1 cells for 16 h with 100 ng/ml pertussis toxin almost completely abrogated the MCP-1-induced increase in PI 3,4,5-P_3 (Fig. 3A).
MCP-1 Stimulates the Protein Tyrosine Phosphorylation of Multiple Substrates in THP-1 Cells—Following our observations that MCP-1 stimulated an increase in in vitro lipid kinase activity of the class Iα p85/p110 PI 3-kinase, it was important to determine whether MCP-1 could modulate tyrosine phosphorylation of cellular proteins. Indeed, immunoblotting PY20 anti-phosphotyrosine immunoprecipitates derived from resting and stimulated THP-1 cells with another anti-phosphotyrosine mAb (4G10) revealed that three proteins were consistently tyrosine-phosphorylated following MCP-1 treatment (Fig. 5A). The MCP-1 stimulated protein tyrosine phosphorylation occurred predominantly on proteins of approximately 50 and 80 kDa and to a much lesser extent on a 120-kDa protein. Maximum phosphorylation of the 80- and 50-kDa proteins in THP-1 cells was observed at 30 s post-MCP-1 stimulation, and the levels of phosphorylation had returned to basal levels by 120 s. In contrast, phosphorylation of the 120-kDa protein was only detectable 30 s poststimulation. Pertussis toxin (100 ng/ml) totally abrogated the MCP-1-induced phosphorylation of these proteins (Fig. 5B). However, a 5-min pretreatment of the cells with 100 nM wortmannin had no effect on the tyrosine phosphorylation of the 50-, 80-, and 120-kDa proteins (Fig. 5C). To determine whether any of these proteins could be interacting with the p85/p110 PI 3-kinase, we immunoblotted p85 immunoprecipitates (derived from unstimulated or MCP-1-stimulated THP-1 cells) with the 4G10 anti-phosphotyrosine mAb. As shown in Fig. 5D, a tyrosine phosphoprotein of approximately 55 kDa was observed to co-precipitate with the p85 subunit of PI 3-kinase. This 55-kDa tyrosine phosphoprotein differed from those detected in the anti-phosphotyrosine immunoprecipitates in both its phosphorylation kinetics and migration distance. The 55-kDa tyrosine phosphoprotein was barely visible at 30 and 60 s but was greatly increased by 120 s and was maximally phosphorylated at 300 s. So far, the identities of these tyrosine phosphoproteins are not known, but they are under further investigation.

MCP-1 Stimulates PI3K-C2α in THP-1 Cells—Since the MCP-1-induced elevations of PI 3,4-P_2 and PI 3,4,5-P_3 are both refractory to wortmannin treatment (unlike the MCP-1-stimulated activation of the in vitro lipid kinase activity present in p85 immunoprecipitates, which also occurs with slower kinetics), we examined whether accumulation of these D-3 phosphatidylinositol lipids could be explained by MCP-1-stimulated activation of PI3K-C2α, which is relatively insensitive to wortmannin (40). In Western blotting studies with an anti-PI3K-C2α polyclonal antibody, a single p190 polypeptide could be detected in 1.5 × 10^6 and 5 × 10^6 cell equivalents of total Nonidet P-40 cell lysates prepared from nonactivated THP-1 cells, thus indicating expression of PI3K-C2α in THP-1 cells and confirming that the anti-PI3K-C2α Ab did not cross-react with other undefined proteins (Fig. 6). MCP-1 induced an increase in PI3K-C2α activity that was both rapid and transient, with the maximal response being detected at 5 s post-MCP-1 stimulation, and by 30 s this response had declined back down to basal levels (Fig. 7A). MCP-1 induced a concentration-dependent increase in the lipid kinase activity in anti-PI3K-C2α

**FIG. 2.** MCP-1 stimulates increased in vitro lipid kinase activity in p85 immunoprecipitates derived from THP-1 cells. A × 10^7 THP-1 cells were stimulated at 37 °C for various times with 180 nM MCP-1 (A) or stimulated with various concentrations of MCP-1 for 120 s (B). Cells were lysed, and lysates were subjected to immunoprecipitation with an anti-p85 mAb. The washed immunoprecipitates were analyzed for PI kinase activity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under “Experimental Procedures.” Lipids were detected by exposure to film at −70 °C (top parts of A and B) and quantitated by densitometry (bottom parts). The data are representative of at least three separate experiments. PtdIns, phosphatidylinositol.

In contrast, a 5-min pretreatment with the potent PI 3-kinase inhibitor wortmannin had no effect on the MCP-1-induced PI 3,4,5-P_3 accumulation (Fig. 3B). Interestingly, pretreatment of the cells with various concentrations of pertussis toxin (0.1–100 ng/ml) had no notable effects on the MCP-1-induced increases in in vitro lipid kinase activity (Fig. 3C). Furthermore, 5-min pretreatment of the cells with various concentrations of wortmannin potently inhibited the MCP-1-induced increases in lipid kinase activity in p85 immunoprecipitates (Fig. 3D) with an IC_{50} of 4.47 ± 1.88 nM (n = 4).

**Effects of Wortmannin on the MCP-1-induced Chemotactic Response in THP-1 Cells**—The functional relevance of activation of the p85/p110 PI 3-kinase was assessed by the use of the PI 3-kinase inhibitor wortmannin. MCP-1 stimulated the chemotaxis of THP-1 cells in a bell-shaped concentration-dependent manner that is characteristic of chemokine-dependent chemotaxis (Fig. 4A) (27). THP-1 cell chemotaxis in response to a concentration of MCP-1 sufficient to elicit optimal chemotaxis was inhibited by pretreatment of the cells with wortmannin (Fig. 4B). The IC_{50} for wortmannin inhibition of MCP-1-stimulated chemotaxis was 11.8 ± 4.2 nM (n = 4).

MCP-1 Stimulates PI3K-C2α in THP-1 Cells—Since the MCP-1-induced elevations of PI 3,4-P_2 and PI 3,4,5-P_3 are both refractory to wortmannin treatment (unlike the MCP-1-stimulated activation of the in vitro lipid kinase activity present in p85 immunoprecipitates, which also occurs with slower kinetics), we examined whether accumulation of these D-3 phosphatidylinositol lipids could be explained by MCP-1-stimulated activation of PI3K-C2α, which is relatively insensitive to wortmannin (40). In Western blotting studies with an anti-PI3K-C2α polyclonal antibody, a single p190 polypeptide could be detected in 1.5 × 10^6 and 5 × 10^6 cell equivalents of total Nonidet P-40 cell lysates prepared from nonactivated THP-1 cells, thus indicating expression of PI3K-C2α in THP-1 cells and confirming that the anti-PI3K-C2α Ab did not cross-react with other undefined proteins (Fig. 6). MCP-1 induced an increase in PI3K-C2α activity that was both rapid and transient, with the maximal response being detected at 5 s post-MCP-1 stimulation, and by 30 s this response had declined back down to basal levels (Fig. 7A). MCP-1 induced a concentration-dependent increase in the lipid kinase activity in anti-PI3K-C2α
immunoprecipitates, with the maximum being observed at 12.5 nM MCP-1 (Fig. 7B). Further characterization of the MCP-1-induced activation of PI3K-C2α was performed in the presence of pertussis toxin and wortmannin. Pretreatment with pertussis toxin almost completely abrogated the MCP-1-induced PI3K-C2α activity (Fig. 7C). In contrast, 5-min pretreatment of the cells with various concentrations of wortmannin (1–300 nM) had no noticeable effects on the MCP-1-induced PI3K-C2α activity (Fig. 7D). Since there are no currently available inhibitors to PI3K-C2α and use of pertussis toxin could potentially disrupt other G protein-dependent signaling events such as calcium mobilization, it is not currently feasible to assess the functional relevance of the PI3K-C2α activation by MCP-1 using a pharmacological approach.

**DISCUSSION**

This study has demonstrated that MCP-1 induces a concentration- and time-dependent accumulation of both PI 3,4-P₂
and PI 3,4,5-P_3 in THP-1 cells. This MCP-1-stimulated generation of D-3 phosphatidylinositol lipids was not affected by pretreatment of the cells with wortmannin but was greatly reduced by pertussis toxin. Further characterization of the PI 3-kinase family members that act downstream of the MCP-1-stimulated receptor, demonstrated that MCP-1 activates the p85/p110 heterodimeric PI 3-kinase, but this activation was very sensitive to wortmannin and insensitive to pertussis toxin. Concentrations of wortmannin sufficient to inhibit p85/p110 lipid kinase activity also inhibited MCP-1-stimulated THP-1 cell chemotaxis. Investigation of the MCP-1-induced tyrosine phosphorylation of various proteins demonstrated that three proteins were rapidly and transiently phosphorylated by MCP-1 in THP-1 cells, although a fourth tyrosine phosphoprotein was found to co-precipitate with the p85 subunit of PI 3-kinase. Moreover, following the relative insensitiv-

**FIG. 4.** Effects of wortmannin on the MCP-1-induced chemotactic response in THP-1 cells. 5 × 10^5 THP-1 cells were incubated with increasing concentrations of MCP-1 (0.125–12.5 nM) (A) or pretreated with increasing concentrations of wortmannin (0.1–100 nM) (B) for 10 min at 37 °C and then incubated with 1.25 nM MCP-1 in a 96-well chemotaxis chamber at 37 °C for 2 h. THP-1 cell migration was assessed using Cell Titer 96 AQsa solution as described under “Experimental Procedures.” Results are expressed as a mean chemotactic index ± S.E., which is the ratio of OD readings of the stimulated samples against the OD readings of the control samples incubated with medium alone, from quadruplicate wells. The data are representative of at least three separate experiments.

**FIG. 5.** Effects of pertussis toxin and wortmannin on the MCP-1-induced tyrosine phosphorylation of various proteins. 1 × 10^7 THP-1 cells were stimulated with 180 nM MCP-1 for various times, and cell lysates were prepared. The cell lysates were subsequently immunoprecipitated with either the anti-phosphotyrosine mAb PY20 (A–C) or anti-p85 mAb (D). THP-1 cells were either pretreated for 16 h with 100 ng/ml pertussis toxin (B) or pretreated for 5 min with 100 nM wortmannin (C) prior to stimulation with MCP-1. The immunoprecipitates were washed, and the proteins were then separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with the 4G10 anti-phosphotyrosine mAb as described under “Experimental Procedures.” The data are from a single experiment representative of at least three others. The tyrosine phosphoproteins are indicated by the arrows on the right, and the molecular weight standards are shown by the arrows on the left.
MCP-1 appears to have differential effects on the accumulation of PI3,4-P_2 and PI3,4,5-P_3. For instance, MCP-1 stimulates a rapid and transient increase in PI3,4,5-P_3 compared with the slower, sustained increase in PI3,4-P_2. This may be explained by the concomitant activation of a PI3,4,5-P_3 5-phosphatase, which has been identified in other systems (46–48) and which converts PI3,4,5-P_3 to PI3,4-P_2. The elevation of D-3 phosphatidylinositol lipids observed in response to MCP-1 may be the result of activation of more than one PI3-kinase (e.g. the p85/p110 PI3-kinase, PI3-kinase γ, and/or other classes of PI3-kinase such as PI3K-C2α). However, the use of pharmacological inhibitors revealed some interesting and important points concerning the in vivo accumulation of D-3 phosphatidylinositol lipids stimulated by MCP-1. First, the accumulation of D-3 phosphatidylinositol lipids in THP-1 cells

![Figure 6. Expression of PI3K-C2α in THP-1 cells. Cytoplasmic extracts from 1.5 × 10⁶ and 5 × 10⁶ cell equivalents of total Nonidet P-40 cell lysates prepared from THP-1 cells were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with the anti-PI3K-C2α polyclonal antibody as described under "Experimental Procedures." The data are from a single experiment representative of at least three others.](image1)

![Figure 7. MCP-1 stimulates increases in PI3K-C2α immunoprecipitates derived from THP-1 cells. 1 × 10⁷ THP-1 cells were stimulated at 37 °C for various times with 12.5 nM MCP-1 (A and C) or stimulated with various concentrations of MCP-1 for 15 s (B and D). THP-1 cells were pretreated for 16 h with 100 ng/ml pertussis toxin (C) or for 5 min with 1–300 nM wortmannin (D), and then 1 × 10⁷ cells were stimulated at 37 °C with 12.5 nM MCP-1 for various times (C) or with 12.5 nM MCP-1 for 15 s (D). Cells were lysed, and lysates were subjected to immunoprecipitation with an anti-PI3K-C2α polyclonal antibody. The washed immunoprecipitates were analyzed for PI kinase activity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under "Experimental Procedures." Lipids were detected by exposure to film at 70 °C (top parts of A–D) and quantitated by densitometry (bottom parts). The data are representative of at least three separate experiments. PtdIns, phosphatidylinositol.](image2)
stimulated by MCP-1 could be completely inhibited by pretreatment with pertussis toxin, strongly indicating that D-3 phosphatidylinositol lipid accumulation occurs via a pertussis toxin-sensitive G-protein-coupled PI 3-kinase. Crucially however, the lack of effect of wortmannin on the MCP-1-induced D-3 phosphatidylinositol lipid generation indicated that it is likely that MCP-1 activates a PI 3-kinase distinct from PI 3-kinase γ, since PI 3-kinase γ is known to be inhibited by the concentrations of wortmannin used in this study (28, 49). Second, although MCP-1 activates a wortmannin-sensitive (but pertussis toxin-resistant) p85/p110 heterodimer in vitro, the slower kinetics of p85/p110 activation and the lack of effect of wortmannin on D-3 phosphatidylinositol lipid generation in vivo suggest that the formation of D-3 phosphoinositide lipids resulting from p85/p110 activation does not significantly contribute to the overall detectable D-3 phosphatidylinositol lipid pool. Nevertheless, activation of the p85/p110 heterodimer appears to be functionally relevant, since wortmannin inhibits MCP-1-stimulated THP-1 chemotaxis. Our data are similar to previous findings of other groups who have reported that concanavalin A-stimulated generation of D-3 phosphatidylinositol lipids in THP-1 cells is completely inhibited by pertussis toxin, while the increased PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates is unaffected by pertussis toxin (50).

Our surprising observations using wortmannin and pertussis toxin led us to look at the effects of MCP-1 on the novel PI3K-C2α, which has recently been identified as displaying reduced sensitivity to wortmannin (40). MCP-1 stimulation of THP-1 cells did activate PI3K-C2α, and this activation was resistant to pretreatment with wortmannin but inhibited by pertussis toxin. The effects of wortmannin and pertussis toxin on PI3K-C2α in vitro mirror the effects of these inhibitors on the MCP-1-stimulated increases in D-3 phosphatidylinositol lipid generation. Thus, the pertussis toxin-sensitive MCP-1-induced activation of PI3K-C2α may account for the detectable changes in PI 3,4-P2 and PI 3,4,5-P3 observed in vivo. From analogy with what is known about receptor coupling to PI 3-kinase γ, there are at least two putative mechanisms by which MCP-1 can activate PI3K-C2α in a pertussis-sensitive manner: (i) direct interaction of Gβγ subunits with PI3K-C2α, as is known to occur for PI 3-kinase γ activation (51); (ii) Gβγ subunits may activate PI3K-C2α via PTK(s) given that there are several lines of evidence to support involvement of PTKs in cell signaling mediated by pertussis toxin-sensitive G proteins (36, 52–54). In this respect, it is interesting to note that the MCP-1-stimulated tyrosine phosphorylation of proteins is inhibited by pertussis toxin pretreatment. However, until effective PI3K-C2α mutants and or pharmacological inhibitors of PI3K-C2α are available, the precise functional role of this PI3K-C2α activation in mediating MCP-1-induced responses cannot be determined. Our data do not, however, exclude the possibility that MCP-1 activates a unique wortmannin-resistant, class Iα G protein-coupled PI 3-kinase that is distinct from p110, which might also contribute (along with PI3K-C2α) to the observed accumulation of PI 3,4-P2 and PI 3,4,5-P3.

Coupling of receptors to the class 1α p85/p110 PI 3-kinase is known to require interaction of src homology 2 domains within the p85 regulatory subunit with specific phosphotyrosine-containing binding motifs (pYXXM; where pY represents phosphotyrosine) located in several growth factor receptors or adapter molecules such as the insulin receptor substrate-1 (55, 56). The mechanisms by which the known G protein-coupled MCP-1 receptor couples to class 1α PI 3-kinase are unclear, since there is no recognized consensus binding motif for the p85 src homology 2 domains contained within the CCR2 sequence. Since pertussis toxin had no effect on the MCP-1-induced activation of the p85/p110 PI 3-kinase, the coupling of the MCP-1 receptor to a class 1α PI 3-kinase would have to involve pertussis toxin-insensitive G-proteins. In this respect, it is interesting to note that CCR2A and CCR2B are differentially coupled to the pertussis toxin-insensitive α-subunits of the Gq class of G proteins (22). Given the tyrosine phosphorylation of proteins after MCP-1 stimulation, it appears that MCP-1 stimulation of CCR2 can result in activation of PTK(s). Indeed, other G-protein-coupled receptors have also been shown to stimulate PTKs such as bombesin and vasopressin (57). Similarly, additional reports indicate that MCP-1 and the related CC chemokine RANTES can induce the protein tyrosine phosphorylation of a number of substrates (25, 26). Furthermore, MCP-1-induced tyrosine phosphorylation of p42/p44 mitogen-activated protein kinases in a murine T-cell hybridoma expressing human MCP-1 receptors has previously been reported (58). While we were unable to detect tyrosine phosphorylation of comparable proteins in THP-1 cells, MCP-1 treatment did stimulate protein tyrosine phosphorylation of 50-, 80-, and 120-kDa proteins. Our findings that MCP-1-induced tyrosine phosphorylation of these proteins was inhibited by pertussis toxin demonstrates that this is downstream of pertussis toxin-sensitive Gαi and/or Gαs G proteins. In contrast, the use of wortmannin indicates that PI 3-kinase activation is not a requirement for MCP-1-stimulated tyrosine phosphorylation. In addition to the above mentioned tyrosine-phosphorylated proteins, a 55-kDa phosphoprotein co-associates with p85 immunoprecipitates upon MCP-1 stimulation. The 55-kDa phosphoprotein was tyrosine-phosphorylated with slower kinetics than the other three proteins that were detected in anti-phosphotyrosine immunoprecipitates. This delayed appearance of the p85-associated 55-kDa phosphoprotein generally correlated with the kinetics of p85/p110 PI 3-kinase activation, which were slightly slower than the elevation of D-3 phosphatidylinositol lipids and PI3K-C2α activation. Therefore, it is possible that these tyrosine phosphoproteins, in particular the 55-kDa protein, could be involved in the coupling of the MCP-1 receptor to the p85/p110 PI 3-kinase. In this respect, activation of p85/p110 PI 3-kinase following binding of tyrosine phosphopeptides (59, 60) and synergistic activation of p85/p110 by tyrosine-phosphorylated peptides and βγ subunits of GTP binding proteins have both been reported (37, 61).

This investigation has demonstrated that MCP-1 activates at least two independent PI 3-kinase isoforms that may differentially regulate monocyte functions. For instance, although both the p85/p110 PI 3-kinase and PI 3-kinase γ have been shown to be activated by thrombin, studies with wortmannin indicated that only the p85/p110 PI 3-kinase complex was involved in regulating the conversion of the platelet integrin αIIbβ3 into a fibrinogen binding form required for platelet aggregation (62). Indeed, it has previously been proposed that phosphotyrosine-linked activation of PI 3-kinase is responsible for phagocytosis, whereas G protein-mediated activation of PI 3-kinase gives rise to the respiratory burst (50). PI 3-kinase activation has been implicated in a variety of cellular responses such as adhesion molecule up-regulation (63), superoxide release (32), and chemotaxis (27, 41). The MCP-1-induced activation of a wortmannin-sensitive PI 3-kinase appears to be an important signal required for MCP-1-stimulated chemotaxis. Our data indicate that the p85/p110 heterodimer is activated by MCP-1 and may be responsible for mediating the MCP-1 effects on chemotaxis. However, until a suitable inhibitor of PI3K-C2α becomes available, the role of this class II PI 3-kinase in MCP-1-induced monocyte functional responses will remain elusive.
