

Signaling Pathways for Monocyte Chemoattractant Protein 1-Mediated Extracellular Signal-Regulated Kinase Activation

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ABSTRACT

G protein-coupled receptors (GPCRs) initiate diverse downstream signaling events in response to ligand stimulation, as rapid activation of the extracellular signal-regulated kinase ERK1 and ERK2. The chemokine monocyte chemoattractant protein-1 (MCP-1) is the agonist for several chemokine receptors that belong to the GPCR superfamily, CCR2 being the most important. Stimulation of mitogen-activated protein kinases (MAPKs) by MCP-1 has been implicated in integrin activation and chemotaxis, but the molecular pathways downstream of the receptors remain unclear. To dissect the cascade of events leading to MAPK activation upon CCR2 receptor stimulation, several specific inhibitors and mutants of signal transduction proteins were used in monocytic cells endogenously expressing CCR2 and/or in human embryonic kidney-293 cells transfected with CCR2B receptors and epitope-

tagged ERK1. We show that ERK activation by MCP-1 involves heterotrimeric G_i protein subunits, protein kinase C, phosphoinositide-3-kinase, and Ras. On the other hand, the activity of cytosolic tyrosine kinases, epidermal growth factor receptor transactivation, or variations in intracellular calcium levels are not required for the mitogenic activation elicited by MCP-1. In addition, we find that internalization of CCR2B itself is not necessary for efficient MCP-1-induced activation of ERK, although a dynamin mutant partially inhibits ERK stimulation. These results suggest that different parallel pathways are being activated that lead to the full activation of the mitogen-activated protein kinase cascade and that internalization of other signaling proteins but not of the receptor is required for complete ERK activation.

G protein-coupled receptors (GPCRs) constitute the largest family of integral membrane proteins involved in signal transduction. Multiple messengers acting through GPCRs lead to the activation of mitogen-activated protein kinase (MAPK) cascades, including extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Marinissen and Gutkind, 2001). MAPKs have been implicated in many cellular processes such as proliferation, differentiation, and apoptotic cell death. Whereas a relatively small number of common mechanisms are responsible for receptor tyrosine kinase (RTK)-mediated activation of ERKs, GPCRs activate these kinases by numerous and diverse signaling pathways whose nature and molecular mechanisms remain the subject of intense

investigation (Marinissen and Gutkind, 2001; Pierce et al., 2002).

The chemokine monocyte chemoattractant protein 1 (MCP-1) interacts with GPCRs to activate different MAPK cascades (Dubois et al., 1996) that are important for the physiological function of MCP-1 and that have recently been shown to be involved in integrin activation and chemotaxis triggered by MCP-1 (Ashida et al., 2001).

MCP-1 belongs to the large family of chemotactic cytokines involved in leukocyte migration, which is an essential process for the recruitment of these cell populations to sites of inflammation. MCP-1 interacts with the CCR2B receptor (Charo et al., 1994) although, because of the promiscuity of the chemokine receptors, this receptor can also respond to other members of the CC chemokine family. The CCR2B receptor is one of the few chemokine receptors that has a natural splice variant, CCR2A (Charo et al., 1994), that differs only in the cytoplasmic carboxyl tail. CCR2 has been

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ABBREVIATIONS: GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RTK, receptor tyrosine kinase; MCP-1, monocyte chemoattractant protein-1; HEK, human embryonic kidney; PTX, *Bordetella pertussis* toxin; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; DMSO, dimethyl sulfoxide; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester; IL, interleukin; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Ro 31-8220, 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide (bisindolylmaleimide IX), methanesulfonate; AG1478, [4-(3-chloroanilo)]-6,7-dimethoxyquinazoline.

shown to couple to pertussis toxin (PTX)-sensitive heterotrimeric G proteins of the G_i family (Myers et al., 1995), although it can transduce signals through other PTX-insensitive G proteins (Arai and Charo, 1996). Ligand binding triggers a number of signaling pathways that lead to inhibition of adenylyl cyclase, phospholipase activation, calcium mobilization, and increases in tyrosine phosphorylation (Myers et al., 1995; Arai and Charo, 1996; Mellado et al., 2001). As in the case of other GPCRs, the desensitization of the responses elicited by chemokine receptors after ligand challenge is achieved by the phosphorylation at residues of serine and threonine in the carboxyl terminus of the receptors by G protein-coupled receptors kinases (GRKs) that favor the recruitment of the cytosolic proteins called arrestins, leading to the subsequent uncoupling from heterotrimeric G proteins and loss of receptor responsiveness (Franci et al., 1996; Aragay et al., 1998; Krupnick and Benovic, 1998). The binding of arrestin proteins to the receptor is also important for the recruitment of the receptor to clathrin-coated vesicles. In fact, it has been shown that in some cases internalization of GPCRs and β -arrestin binding are important for MAPK activation (for review, see Ferguson, 2001). Nevertheless, there are contradictory results in the literature about the requirement of receptor internalization for MAPK activation (Budd et al., 1999; Whistler and von Zastrow, 1999; Yang et al., 1999). In addition, arrestin proteins can act as docking proteins bringing other kinases such as Src or JNK3 to the vicinity of the receptor complex (Luttrell et al., 2001; Pierce et al., 2002).

Although the activation of MAPK by MCP-1 has been reported in different cell types (Dubois et al., 1996; Arai et al., 1997), the molecular pathways and the precise signaling proteins involved in CCR2 receptor-mediated ERK stimulation have not been described in detail. In this report, we have used a variety of pharmacological inhibitors and mutants of signaling proteins to better understand MCP-1 mitogenic signaling in two different monocytic cell lines and in HEK-293 cells transfected with the CCR2B receptor. We show that MCP-1-mediated ERK stimulation involves the participation of different signal transduction pathways and is independent of receptor internalization, although it requires the endocytosis of other signaling proteins for efficient ERK activation. The identification of proteins implicated in MAPK activation promoted by MCP-1 could shed new light for understanding key physiological processes elicited by this and other chemokines such as cellular adhesion or chemotaxis.

Materials and Methods

Materials. HEK-293 cells were purchased from the American Type Culture Collection (Manassas, VA), and Mono Mac 6 and THP-1 cells were from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Culture media and LipofectAMINE Plus were from Invitrogen (Carlsbad, CA). Epidermal growth factor (EGF) was obtained from Upstate Biotechnology (Lake Placid, NY), and MCP-1 was purchased from PeproTech (Rocky Hill, NJ). PTX and M2-agarose beads were obtained from Sigma-Aldrich (St. Louis, MO). Inhibitors AG1478, Ro 31-8220, and LY 294002 were purchased from Calbiochem (San Diego, CA). All other reagents were of the highest grade commercially available.

Plasmids. The cDNA encoding *Gat* was provided by Dr. M. I. Simon (California Technology Institute, Pasadena, CA). The vectors encoding dominant negative Ras (Ras N17) and human hemaggluti-

nin-tagged ERK1 (HA-ERK1) were provided by Dr. J. Moscat (Centro de Biología Molecular, Madrid, Spain). The cDNA of wild type β -arrestin1 was a gift from Dr. V. V. Gurevich (Sun Health Research Institute, Sun City, AZ) and were subcloned in our laboratory in the pcDNA3-higro+ plasmid using *NotI* and *ApaI* sites. The cDNAs constructs for β -arrestin1 V53D and dynamin1 K44A were provided by Dr. M. G. Caron (Duke University, Durham, NC) and pcDNA3-CCR2BIX was generously provided by Dr. I. F. Charo (University of California, San Francisco, CA). All other plasmids were constructed in our laboratory.

Antibodies. Polyclonal C-16 and C-14 antibodies that recognize ERK1 and ERK2 and polyclonal C-20 antibody against the C-terminal tail of CCR2B were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies phospho-ERK1/2 and phospho MEK1/2 were purchased from New England Biolabs (Beverly, CA). The monoclonal antibody against dynamin was obtained from Transduction Laboratories (Lexington, KY). The polyclonal Ab186 antibody that recognizes β -arrestin1 has been described by our laboratory (Penela et al., 2000).

Cell Culture and Transfection. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere. Transfections were performed on 70 to 80% confluent monolayers in 60-mm dishes for MAPK activation assays by using the LipofectAMINE reagent. Empty vector was added to transfections as needed to keep the total amount of DNA added per dish constant. Assays were performed 48 h after transfection. Transient expression was confirmed by immunoblot analysis of whole-cell lysates using specific antisera. Human Mono Mac 6 and THP-1 cells were maintained in RPMI 1640 medium supplemented with glutamine, nonessential amino acids, and fetal bovine serum (10%). Mono Mac 6 cells also require sodium pyruvate and bovine insulin.

Cell Treatments. MCP-1 and EGF stimulation of HEK-293 cells was performed 48 h after transfection at 37°C in culture medium without fetal bovine serum, during the indicated times. MCP-1 stimulation of Mono Mac 6 and THP-1 cells was performed 16 h after serum starvation. Cells were treated with 100 ng/ml PTX for 16 h before ligand stimulation. Treatments with 50 μ M LY 294002 (a specific PI3K inhibitor), 250 nM AG1478 (a highly selective inhibitor of tyrosine-dependent phosphorylation of EGF receptor) or with 5 μ M Ro 31-8220 (a specific inhibitor that blocks all PKC isoforms) were performed at 37°C for 15 or 30 min before ligand stimulation. The tyrosine kinase inhibitor herbimycin A (1 μ M), previously diluted in DMSO (125 μ M), was added for 20 h at 37°C. Control cells were treated with the same DMSO concentration. The inhibition of the c-Src tyrosine kinase was done treating the cells with 10 μ M PP2 for 15 min at 37°C. BAPTA-AM, which is a calcium chelator, was used at a concentration of 50 μ M for 15 min at 37°C. This inhibitor was previously diluted in DMSO and the same quantity of DMSO was added to the control cells. Cells were treated with 0.5 M sucrose for 20 min before ligand activation. All treatments were maintained during the stimulation periods. The cells were then subjected to lysis. Treatments of HEK-293 cells were done in a similar way.

Lysis, Immunoprecipitation, and Western Blotting. Before lysis, the cells were washed twice with ice-cold phosphate-buffered saline, solubilized in 300 μ l/60-mm dish of *N*-dodecyl- β -D-maltoside buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 1% *N*-D-maltoside, 10 mM NaF, 1 mM Na₃VO₄, and a cocktail of protease inhibitors). After gentle rocking for 90 min at 4°C, the lysates were clarified by centrifugation. The supernatants were resuspended in SDS sample buffer. All lysate samples were boiled for 5 min before resolution by 10% SDS-polyacrylamide gel electrophoresis and transference to nitrocellulose membranes. MAPK and MEK activation were assessed using anti-phospho ERK and anti-phospho MEK antibodies, respectively. The presence of MAPK, β -arrestin1, and dynamin proteins in the lysates was determined by using anti-ERK1/ERK2, Ab186, and anti-dynamin antibodies, respectively. To detect CCR2B expressed in HEK-293 cells, we subjected supernatants to

immunoprecipitation. Supernatant was diluted three times in the same buffer without *N*-D-maltoside, and 35 μ l of M2-agarose beads was added for 16 h at 4°C. The immunocomplexes were centrifuged (3,000g, 5 min) and washed four times with 15 ml of 0.05% *N*-D-maltoside buffer. The immunoprecipitated proteins were resuspended in 40 μ l of sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The nitrocellulose membranes were incubated with a polyclonal antibody (C-20) raised against the C-terminal CCR2B domain. Blots were developed using a chemiluminescent method (enhanced chemiluminescence; Amersham Biosciences Inc., Piscataway, NJ). The membranes were reprobed with other antibodies after stripping at 65°C.

Data Analysis. Quantitation of protein amounts in Western blots was achieved by densitometric analysis using 300A computing densitometer (Amersham Biosciences Inc.). Different exposures of the films were considered. A minimum of three different independent experiments were performed for each of the treatments, with similar results. The amount of active, phosphorylated-HA-ERK or endogenous ERKs in each experimental condition was normalized by the corresponding total levels of transfected or endogenous ERK. Fold activation was then calculated with respect to control conditions (no stimulation). All data are expressed as the mean \pm S.E.M. Student's *t* test was used to compare mean values as appropriate. *p* values <0.05 were considered to represent significant differences.

Results

G_{i/o} Proteins and G $\beta\gamma$ Subunits Participate in MCP-1-Mediated ERK Activation. To analyze the regulation of the MAPK cascade by MCP-1, we have used different cell lines: two monocytic cell lines endogenously expressing receptors for this chemokine (Mono Mac 6 and THP-1) and HEK-293 cells expressing the CCR2B receptor. The activation of the mitogenic cascade was determined using an antibody that solely recognize the dual phosphorylated and so activated forms of ERK1 and ERK2. In both THP-1 and Mono Mac 6 cell lines ERK1/2 phosphorylation was detected as soon as 1 min after 20 nM MCP-1 treatment, peaked between 3 and 5 min, and decreased thereafter (Fig. 1, A and B). No change was detected in the total amount of ERK as judged by the equal staining with antibodies that recognized ERK1 and ERK2 proteins. Similar experiments were performed in CCR2B-transfected HEK-293 cells. Expression of the recep-

tor was confirmed by Western blot of immunoprecipitated CCR2B (Fig. 2A). We have previously shown that the transfected receptor can promote Ca²⁺ signaling upon agonist stimulation, is phosphorylated, and forms a complex with GRK2 and arrestin proteins (Aragay et al., 1998; Mellado et al., 1998). The stimulation with 20 nM MCP-1 promoted a strong activation of both transfected HA-ERK1 and endogenous ERK1 and 2 in HEK-293 cells (Fig. 2B). All these results confirmed previous studies in other cell types showing that MCP-1 stimulation leads to the activation of the ERK/MAPK cascade (Dubois et al., 1996; Arai et al., 1997).

We next investigated the pathways leading to MAPK activation. We and others (Myers et al., 1995; Aragay et al., 1998) have previously shown that chemokine receptors are coupled

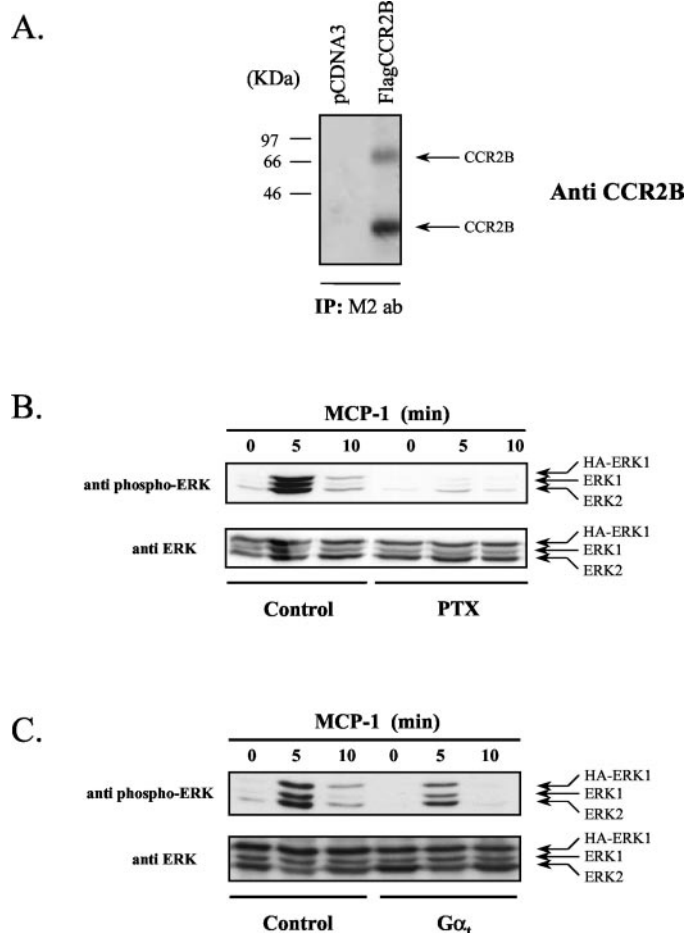


Fig. 2. Activation of ERK upon MCP-1 stimulation of CCR2B receptors in HEK-293 cells is mediated by G_i proteins. A, immunoprecipitation of CCR2B expressed in HEK-293. Lysates of HEK-293 cells transfected with pCDNA3 or FLAG-CCR2B were immunoprecipitated with anti-FLAG M2 agarose beads. The immunoprecipitated receptor was analyzed by immunoblotting with an anti-CCR2B antibody. B, blockage of ERK stimulation in the presence of PTX. HEK-293 cells transiently transfected with CCR2B and HA-ERK1 were pretreated with or without 100 ng/ml PTX for 16 h and then stimulated with MCP-1 (20 nM) for the indicated periods. Lysates were directly subjected to immunoblotting with antibody against phosphorylated ERK (top). The blots were stripped and reprobed with anti-ERK antibodies (bottom). Representative gels from two independent experiments are shown. C, ERK stimulation by MCP-1 is inhibited by overexpression of Gα_i subunits. HEK-293 cells transiently transfected with CCR2B and HA-ERK1 with or without (control) Gα_i were stimulated with MCP-1 for the indicated times. Analysis of active and total ERKs was carried out as described above. The blots are representative of two independent experiments with similar results.

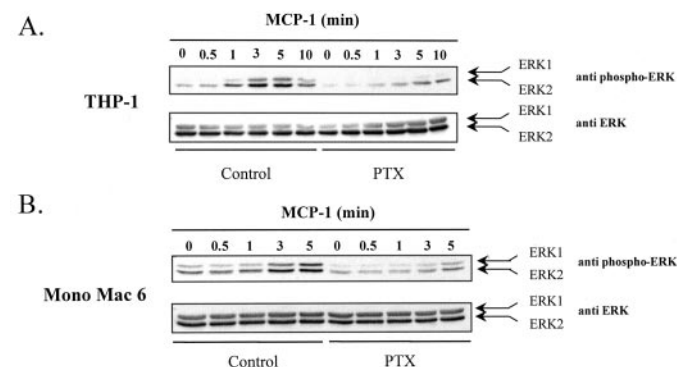


Fig. 1. Activation of the ERK pathway by MCP-1 in two monocytic cell lines is blocked in the presence of PTX. THP-1 (A) and Mono Mac 6 cells (B) were pretreated with or without 100 ng/ml PTX for 16 h and then stimulated with MCP-1 (20 nM) for the indicated periods. Lysates were directly subjected to immunoblotting with antibodies against phosphorylated and activated ERK (top). The blots were stripped and reprobed with anti-ERK antibodies (bottom). Representative gels from two independent experiments are shown.

to G proteins of the $G_{i/o}$ family, although their coupling to other members of the heterotrimeric G family, such as $G_{q/11}$ also has been described previously (Arai and Charo, 1996). To analyze the contribution of different G proteins to the stimulation of the ERK/MAPK cascade, we pretreated cells with 100 ng/ml PTX. As shown in Figs. 1, A and B, and 2B, this toxin produced a complete inhibition of ERK activation after MCP-1 addition in the three different cell lines, indicating that the activation of G_i proteins is essential for the stimulation of this pathway. The PTX treatment did neither modify ERK1 and ERK2 endogenous levels, nor expressed HA-ERK1 protein (bottom, Figs. 1, A and B, and 2B). Because signals elicited by CCR2B through G_i can be also mediated by $G\beta\gamma$ dimers (Myers et al., 1995; Arai et al., 1997), we investigated their participation in this process. Overexpression of the $G\alpha_t$ subunits has been shown to sequester free $G\beta\gamma$ dimers (Hung et al., 1992). Figure 2C shows the effect of overexpressing $G\alpha_t$ with the receptor and HA-ERK1 in HEK-293 cells. A clear decrease in ERK activation can be observed, suggesting that $G\beta\gamma$ subunits participate in the CCR2B-mediated mitogenic signaling.

Together, these results show that the activation of the ERK/MAPK cascade by MCP-1 in cells that endogenously express the CCR2 receptor and in CCR2B-transfected HEK-293 cells takes place by similar pathways involving G_i protein subunits. Therefore, we have used HEK-293 cells transfected with CCR2B and HA-ERK1 as a model for delineating the detailed molecular mechanisms of this signaling pathway. When feasible, similar experiments have been performed in the monocytic cell lines.

Role of PKC and PI3K in ERK Activation by CCR2B. CCR2B activation can lead to either G_i -mediated (via $G\beta\gamma$ and phosphoinositide phospholipase $C\beta 2/3$) or $G\alpha_q$ -mediated PKC stimulation. To study the role of this kinase in CCR2B-mediated ERK stimulation, we pretreated THP-1 (Fig. 3A) and HEK-293 cells expressing the receptor and HA-ERK1 (Fig. 3B) with 5 μ M Ro 31-8220, a specific inhibitor that blocks all PKC isoforms. Figure 3 indicates that this treatment caused a marked reduction (40% for ERK1 in THP-1 cells and 76% for HA-ERK1 in HEK-293 cells) in the activation of transfected and endogenous ERKs by MCP-1, without changes in overall ERK expression, suggesting the implication of PKC in this signaling pathway. Previous work demonstrated that MCP-1 also stimulates at least two separate PI3-kinase isoforms, namely, p85/p110 PI3K and PI3K-C2 α (Turner et al., 1998). To investigate whether isoforms of PI3K are involved in ERK activation after MCP-1 challenge, the selective PI3-kinase inhibitor LY 294002 was used at a concentration of 50 μ M. Treatment of THP-1 cells with LY 294002 reduces (by 59%) the activation of ERKs by MCP-1 (Fig. 4A). Figure 4B shows that cotransfected HA-ERK1 phosphorylation was reduced by 38% in MCP-1-stimulated HEK-293 cells treated with this inhibitor. These results suggest that PI3K also participates, to a different extent, in the modulation of this CCR2B-regulated pathway in both monocytes and HEK-293 cells. Interestingly, when PKC and PI3K inhibitors were added together in HEK-293 cells, activation of HA-ERK1 was reduced by 84% after 5 min of agonist challenge (data not shown). The extent of inhibition was higher than that observed in the presence of either inhibitor alone, thus suggesting that both PKC and PI3K participate in at least partially independent signaling pathways leading

to MAPK activation mediated by MCP-1 in CCR2B-HEK-293 cells.

Tyrosine Kinases and Intracellular Calcium Are Not Involved in CCR2B-Mediated ERK Activation. There are numerous evidences that demonstrate the activation of Src-family cytosolic tyrosine kinases after GPCR ligand stimulation. In the case of β -adrenergic and some chemokine receptors such as IL-8 receptors, this stimulation is important for ERK activation (Luttrell et al., 1999; Cao et al., 2000; Venkatakrishnan et al., 2000). To test this possibility, we examined the effect of PP2, a specific inhibitor of Src, in our system. Pretreatment of THP-1 or HEK-293 CCR2B-expressing cells with 10 μ M PP2 did not have any effect on ERK activation after MCP-1 stimulation, although this treatment caused a decrease in the total amount of tyrosine-phosphorylated proteins in the lysates (data not shown). This result indicates that Src-like tyrosine kinases are not necessary for MCP-1-mediated ERK activation in these cells. The treat-

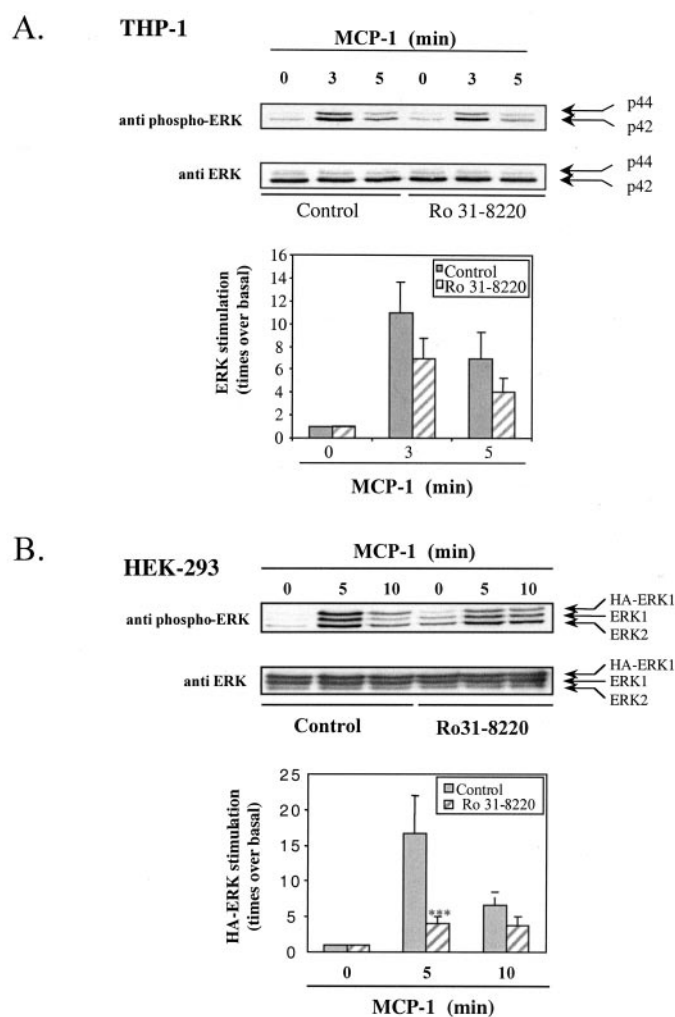


Fig. 3. Inhibition of PKC diminishes stimulation of ERK by CCR2B. THP-1 cells (A) or HEK-293 cells transiently transfected with CCR2B and HA-ERK1 (B) were pretreated with or without Ro 31-8220 (5 μ M) for 30 min and then stimulated with MCP-1 (20 nM) for different periods. Analysis of active (top) and total (bottom) ERKs was performed as in previous figures as detailed under *Materials and Methods*. After densitometric analysis of the gels, data were expressed as average \pm S.E.M fold-stimulation of endogenous ERK (A) or HA-ERK1 (B) phosphorylation over basal level in three to five independent experiments. Representative blots are shown. ***, $p < 0.001$ versus control activation.

ment with a more general inhibitor of cytosolic tyrosine kinases, such as herbimycin A (1 μ M) did not have any significant effect on the mitogenic stimulation promoted by MCP-1 (data not shown), confirming that cytosolic tyrosine kinase activity is not a critical step in mediating MAPK stimulation by CCR2B in our cellular system.

We also studied the possible occurrence of RTK transactivation. Previous results demonstrated that the mechanisms of GPCR-mediated activation of the ERK cascade closely parallel those used by RTKs and that EGF receptors become tyrosine phosphorylated after GPCR activation and may mediate GPCR-stimulated ERK activation (Marinissen and Gutkind, 2001). However, activation of EGF receptor was not necessary for MCP-1 stimulation of HA-ERK1 or endogenous ERKs in our systems. Figure 5 shows that the presence of 250 nM AG1478, which is a selective inhibitor of tyrosine-dependent phosphorylation of EGF receptor, did not have any effect on MCP-1-mediated ERK activation in both THP-1 cells (Fig. 5A) and HEK-293 cells expressing CCR2B (Fig. 5B), whereas clearly blocking EGF-mediated stimulation.

Another functional response promoted by MCP-1 acting through CCR2B is intracellular calcium mobilization (Charo et al., 1994; Aragay et al., 1998). To test whether the change in calcium intracellular levels was implicated in ERK activation after MCP-1 challenge, we used the calcium chelator BAPTA-AM. Treatment with 50 μ M BAPTA-AM of THP-1 or HEK-293 cells expressing CCR2B and HA-ERK1, did not produce any effect on the mitogenic stimulation promoted by MCP-1, whereas causing a 56% decrease in ERK activation elicited by 100 μ M epinephrine acting on HEK-293 cells transiently transfected with α_{2A} -adrenergic receptors (data not shown). Together, these results suggest that neither changes in intracellular calcium levels promoted by ligand-stimulated CCR2B, nor activation of cytosolic tyrosine kinases or EGF receptor transactivation are necessary for MCP-1-induced ERK activation.

Ras Participates in ERK Activation Promoted by MCP-1. Several of the mechanisms reported to activate ERK/MAPK cascade by GPCRs need Ras stimulation, which will lead to subsequent Raf and MEK activation, to reach

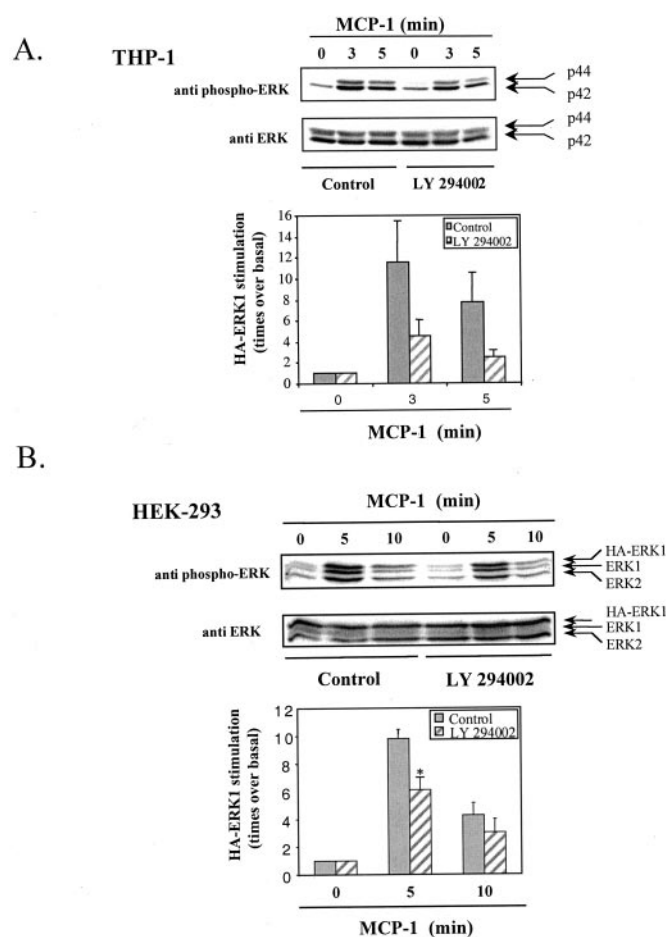


Fig. 4. Effects of PI3K inhibition on MCP-1-induced ERK stimulation. THP-1 cells (A) or HEK-293 cells transiently transfected with CCR2B and HA-ERK1 (B) were pretreated with vehicle or 50 μ M LY 294002, for the times indicated under *Materials and Methods* and then stimulated with MCP-1 (20 nM). Analysis of active (top) and total (bottom) ERKs was performed as in previous figures as detailed under *Materials and Methods*. After densitometric analysis of the blots, data were expressed as average \pm S.E.M. fold-stimulation of endogenous ERK (A) or HA-ERK1 (B) phosphorylation over basal level in three independent experiments. Representative blots are shown. *, $p < 0.05$ versus control activation.

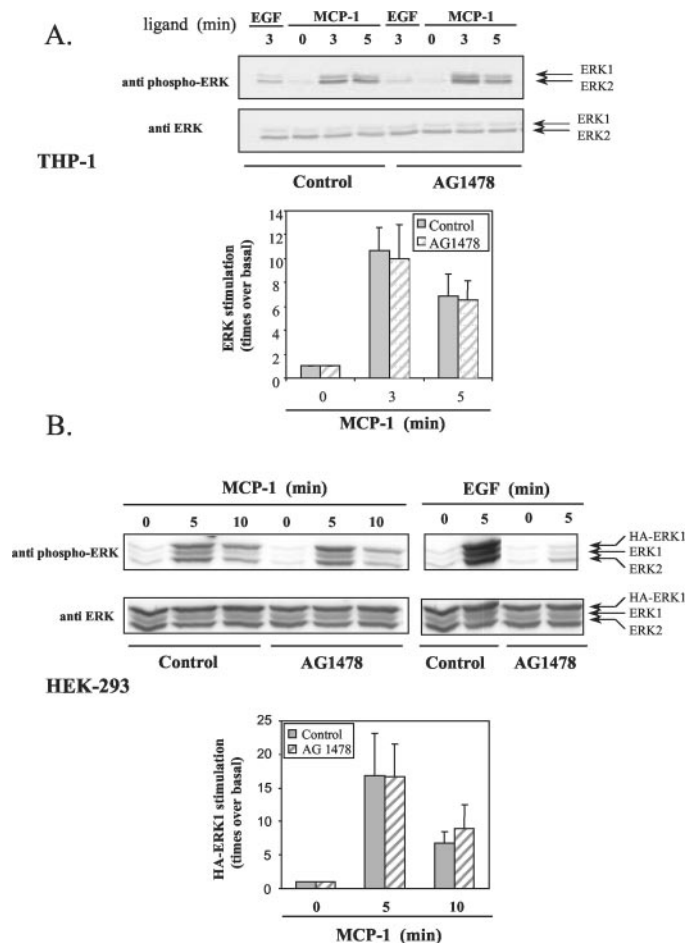


Fig. 5. Inhibition of EGF-receptor kinase activity does not interfere with MCP-1-mediated ERK activation. THP-1 cells (A) or HEK-293 cells transiently transfected with CCR2B and HA-ERK1 (B) were pretreated with vehicle or the AG1478 inhibitor (250 nM) for 30 min before stimulation with 20 nM MCP-1 or 100 ng/ml EGF for the indicated times. Analysis of active (top) and total (bottom) ERKs was performed as in previous figures as detailed under *Materials and Methods*. After densitometric analysis of the blots, data were expressed as average \pm S.E.M. fold-stimulation of endogenous ERK (A) or HA-ERK1 (B) phosphorylation over basal level in three independent experiments. Representative blots are shown.

ERK (Marinissen and Gutkind, 2001). On the other hand, PKC-mediated Raf phosphorylation can also lead to Ras-independent ERK activation. Because we have demonstrated that PKC is involved in MCP-1-mediated MAPK activation, it could be possible that activation of Ras was not necessary in our system. To test this, a dominant negative form of Ras, Ras N17, was cotransfected into HEK-293 cells. Figure 6 shows that the presence of this mutant partially inhibited (by 49%) ERK activation by MCP-1, suggesting a role for Ras in this mitogenic pathway elicited by CCR2B. Therefore, activation of Raf by both Ras and PKC seem to be required for the full activation of MAPK.

CCR2B Receptor Internalization Is Not Required for MCP-Induced ERK Activation. Receptor internalization seems to be required for agonist-mediated MAPK stimulation of some, but not all, GPCRs. To investigate the role of CCR2 receptor internalization in activation of ERK phosphorylation cascade, we first evaluated the ability of 0.5 M sucrose to modulate ERK1/2 phosphorylation after MCP-1 stimulation. Hypertonic sucrose prevents the recruitment of clathrin and interferes with normal coated pit formation and endocytosis (Hansen et al., 1993). The results obtained in THP-1 cells showed that the presence of sucrose produces an increased basal MAPK activity, but allowed agonist-induced ERK1/2 activation (Fig. 7A), although receptor internalization was completely blocked (data not shown). Similar results were obtained in HEK-293 cells (Fig. 7B).

β -Arrestin proteins are implicated in the regulation of signals elicited by GPCRs, acting as a mediator of receptor internalization. Indeed, our group has shown the existence of

a multimolecular complex with the activated CCR2, β -arrestin, and GRK2 (Aragay et al., 1998). More recently, it was shown that β -arrestins can also act as scaffolding proteins to regulate several pathways that result in the activation of mitogen-activated protein kinases (Miller and Lefkowitz, 2001). To provide further support for the lack of involvement of receptor internalization in ERK activation, we examined the possible role of β -arrestin and of CCR2B receptor internalization in ERK activation by MCP-1 in HEK-293 cells. Coexpression with CCR2B and HA-ERK1 of similar levels of wild-type β -arrestin1 or of the β -arrestin1V53D dominant negative mutant (which blocks receptor internalization) did not promote any significant effect on ERK activation by MCP-1 in HEK-293 cells (Fig. 8A). Similar results were obtained when β -arrestin 2-GFP and the corresponding dominant negative mutant were coexpressed with CCR2B in Cos-7 cells (data not shown). The expression of β -arrestin1 V53D caused a clear decrease in the level of internalization of CCR2B after MCP-1 stimulation, as assessed by confocal microscopy (M. C. Jimenes-Sainz, S. Butt, A. M. Aragay, unpublished observations). This result suggests that CCR2B endocytosis is not an essential step for ERK signaling. To corroborate this finding, we used a mutant of CCR2B, named CCR2BIX, that is deficient in phosphorylation and internalization (Franci et al., 1996). Stimulation with MCP-1 of cells that expressed this mutant receptor and HA-ERK1 promoted a strong activation of ERK (Fig. 8B), confirming the result found with the dominant-negative β -arrestin mutant. Together, these results suggest that receptor internalization is not necessary for the stimulation of the ERK cascade by MCP-1 both in THP-1 and in HEK-293 cells.

Interestingly, we find that the presence of the dynamin K44A mutant partially inhibits CCR2B-mediated ERK activation in HEK-293 cells. Dynamin is a GTPase that regulates the formation of clathrin-coated vesicles (Hinshaw and

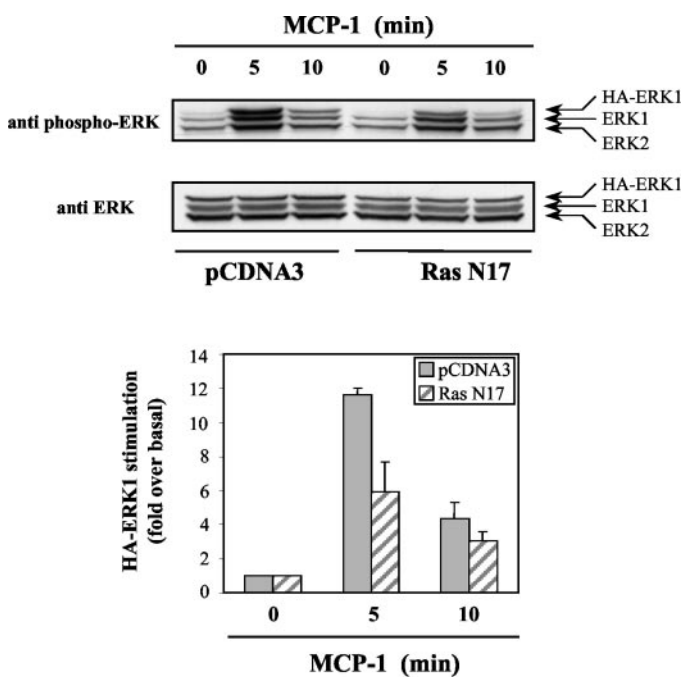


Fig. 6. Dominant negative Ras partially inhibits MCP-1-mediated ERK activation. HEK-293 cells transiently transfected with CCR2B and HA-ERK1, a control vector (pCDNA3) or a vector encoding the Ras N17 mutant were stimulated with 20 nM MCP-1 for the times indicated. Analysis of active (top) and total (bottom) ERKs was performed as in previous figures as detailed under *Materials and Methods*. After densitometric analysis of the blots, data were expressed as average \pm S.E.M. fold-stimulation of HA-ERK1 phosphorylation over basal level in three independent experiments. A representative blot is shown.

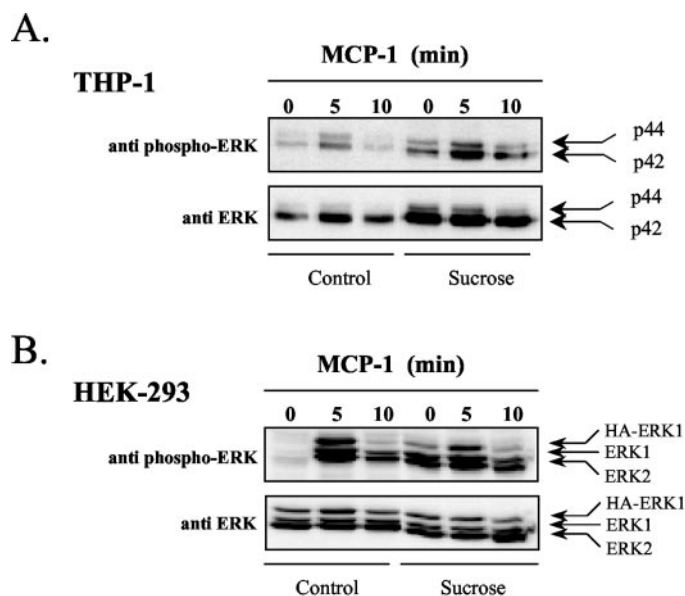


Fig. 7. Internalization of CCR2B is not necessary for MCP-1-mediated ERK activation. THP-1 cells (A) or HEK-293 cells (B) were pretreated with vehicle or 0.5 mM sucrose for 20 min and then stimulated with 20 nM MCP-1 for different times as indicated. Analysis of active (top) or total (bottom) ERKs was performed as in previous figures as detailed under *Materials and Methods*. Representative blots from at least three independent experiments are shown.

Schmid, 1995). The dynamin K44A mutant is defective in GTP binding, and acts as a dominant negative mutant, blocking endocytosis after the initiation of coat assembly and preceding the endocytosis of receptors into the cell. The co-expression of dynamin K44A together with CCR2B and HA-ERK1 significantly reduced (51%) the MCP-1-induced ERK activation (Fig. 9A). Because the results with the dominant negative β -arrestin and the CCR2B receptor mutants indicate that receptor internalization is not necessary for ERK activation, this result suggests that dynamin could affect this process in a receptor internalization-independent way. Consistently, the increase in phosphorylation/activation of the upstream kinase MEK after MCP-1 challenge is comparable between cells expressing or not dynamin K44A (Fig. 9B). Together, these results indicate that the signaling cascade leading to MEK activation is not inhibited by the dynamin mutant and that dynamin K44A exerts its inhibitory effect at the step between MEK and ERK, probably by blocking MEK internalization.

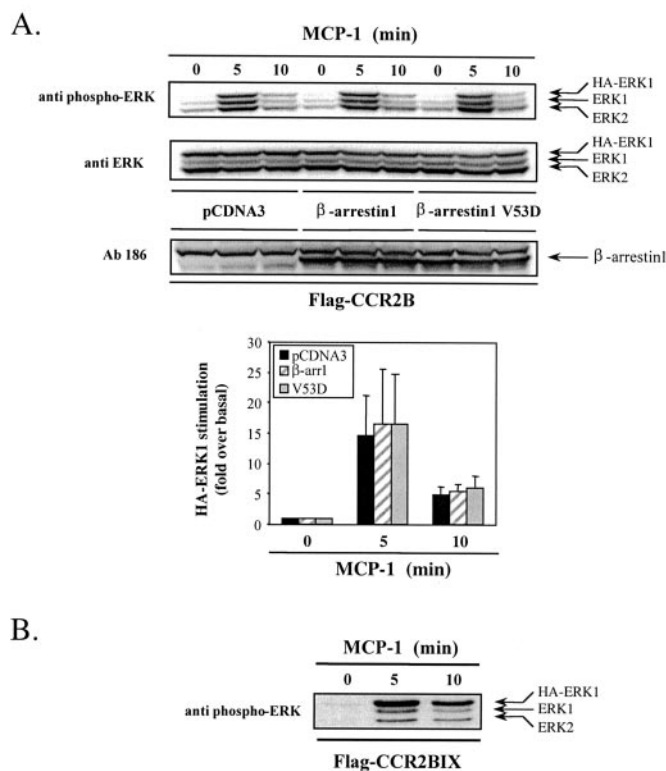
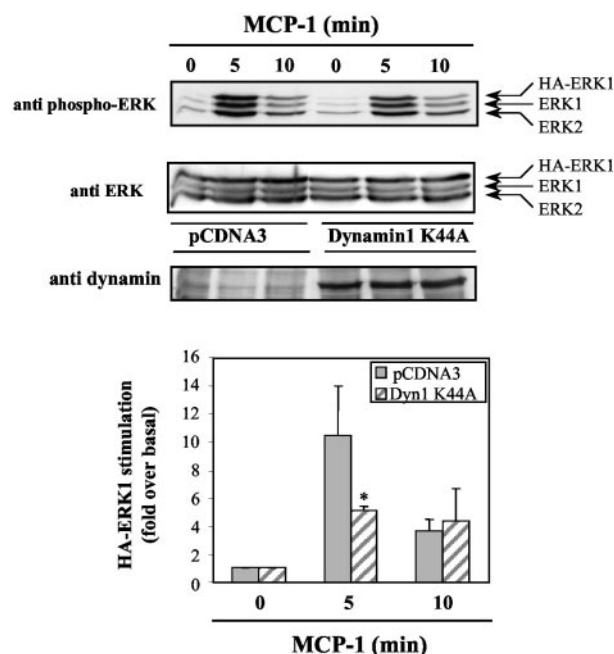


Fig. 8. β -Arrestin-mediated internalization of CCR2B is not necessary for MCP-1-mediated ERK activation in HEK-293 cells. **A**, ERK activation is not affected by the expression of different β -arrestin1 constructs. HEK-293 cells transiently transfected with CCR2B and HA-ERK1 and either a control vector (pCDNA3) or plasmids encoding β -arrestin1 or the β -arrestin1 V53D dominant negative mutant were stimulated with 20 nM MCP-1 for the indicated times. Analysis of active (top) and total (middle) ERKs was performed as in previous figures as detailed under *Materials and Methods*. Levels of β -arrestin1 and the mutant β -arrestin1 V53D in lysates were controlled by Western blotting with a specific antibody (bottom). After densitometric analysis of the blots, data were expressed as average \pm S.E.M. fold-stimulation of HA-ERK1 phosphorylation over basal level in three independent experiments. A representative blot is shown. **B**, internalization deficient CCR2B-mutant CCR2BIX promotes a marked ERK activation upon MCP-1 stimulation. HEK-293 cells were transiently transfected with CCR2BIX and HA-ERK1 and stimulated with 20 nM MCP-1 for the indicated times. Analysis of active ERK was carried out as described above. The blot is representative of five independent experiments with similar results.

Discussion

In the present study, we have analyzed the signaling pathways involved in the stimulation of the ERK/MAPK cascade upon activation of CCR2B receptors by the chemokine MCP-1. CCR2B is one of the numerous receptors that belongs to the GPCR family. The G proteins activated by these receptors, as well as other cellular components, are capable of transmitting signals from the receptor to MAPK cascades using different pathways, which often form an elaborate network of signaling cascades and that can vary depending on the particular receptor activated. In many cases, the precise molecules that promote the activation of these pathways are unknown. The chemokine MCP-1 has an essential role in the migration of monocyte and other leukocyte subpopulations,

A.



B.

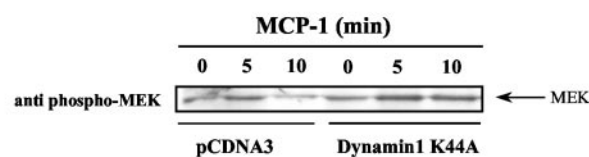


Fig. 9. Effect of dynamin K44A on ERK activation by CCR2B. **A**, dynamin K44A decreases MCP-1-mediated ERK activation. HEK-293 cells were transiently transfected with CCR2B and HA-ERK1, a control vector (pCDNA3) or a vector encoding dynamin K44A, and stimulated with 20 nM MCP-1 for the indicated times. Analysis of active (top) and total (middle) ERKs was performed as in previous figures as detailed under *Materials and Methods*. Levels of dynamin K44A in lysates were controlled by Western blotting with a specific antibody (bottom). After densitometric analysis of the blots, data were expressed as average \pm S.E.M. fold-stimulation of HA-ERK1 phosphorylation over basal level in three independent experiments. A representative blot is shown. *, $p < 0.05$ versus control activation. **B**, presence of dynamin K44A does not alter MCP-1-mediated activation of MEK. The same lysates from the experiments shown in **A** were analyzed for the presence of activated MEK using a phospho-specific antibody as detailed under *Materials and Methods*. The blot is representative of two independent experiments with similar results.

and it has been implicated in diverse pathological processes, such as rheumatoid arthritis, multiple sclerosis, or atherosclerosis. The detailed knowledge of the biochemical pathways elicited by MCP-1 is essential to understand the physiological and pathological events in which this chemokine is involved. In this report, we provide novel information about the mechanisms leading to ERK activation by MCP-1 upon stimulation of endogenous receptors in monocytic cell lines or of transfected CCR2B receptors in HEK-293 cells (see scheme in Fig. 10).

We have previously reported that intracellular calcium changes induced by MCP-1 are sensitive to PTX and that the activated CCR2B receptor could be found associated to G_{α_i} proteins in monocytes and HEK-293 cells (Aragay et al., 1998; Mellado et al., 1998). G_i proteins are necessary for the chemotaxis promoted by MCP-1 (Arai et al., 1997). We find that the activation of ERK by MCP-1 is also sensitive to pertussis toxin in both monocytes and HEK-293 cells, indicating the participation of G_i proteins. This is consistent with previous reports showing that ERK activation by CCR2B in T cells (Dubois et al., 1996) and by other chemokine receptors (Jones et al., 1995) is also PTX-sensitive.

Previous studies have shown that several receptors, included CCR2B, are able to activate different G protein families, such as G_q and G_i (Myers et al., 1995; Arai and Charo, 1996). Thus, the activation of inositol-1,4,5-trisphosphate/calcium levels as well as the stimulation of different isoforms of PKC after MCP-1 challenge of CCR2B in HEK-293 cells, could be mediated by G_q family proteins and/or $G\beta\gamma$ subunits liberated from G_i , acting through several phosphoinositide phospholipase $C\beta$ isoforms. Our data suggest that $\beta\gamma$ subunits of G proteins participate, at least partially, in the mitogenic activation pathway elicited by MCP-1 in HEK-293 cells, because of the fact that ERK stimulation is decreased in the presence of overexpression of G_{α_t} , that sequesters the $\beta\gamma$ dimers. However, the possibility that overexpression of G_{α_t} has an indirect effect uncoupling CCR2 receptors from G_i as consequence of sequestering $\beta\gamma$ cannot be ruled out. Our attempts to demonstrate the possible participation of G_q

proteins in this process, by expressing blocking peptides or overexpressing G_{α_q} subunits, have been unsuccessful. Nevertheless, we can not exclude the possibility that G_q proteins are involved in this signaling pathway, although G_i activation is strictly necessary, as demonstrated by the effects of pretreatment with pertussis toxin.

Our results show that the activation of ERK upon MCP-1 stimulation is partially dependent on Ras in HEK-293 cells, as demonstrated with the dominant negative mutant Ras N17, as well as on PKC in both monocytic and HEK-293 cells, as indicated by the use of the specific Ro 31-8220 inhibitor. This data suggest that simultaneous pathways would be needed for the full activation of MAPK. In fact, it has been suggested that PKC-mediated phosphorylation of Raf leads to its activation and constitutes a Ras-independent mechanism for ERK activation (Marinissen and Gutkind, 2001), although the participation of Ras in PKC-mediated MAPK activation has been described previously (Marais et al., 1998). Furthermore, it has been observed that Ras does not activate completely Raf when both are expressed in Sf9 cells (Williams and Roberts, 1994). All these data suggest that MCP-1 activation of PKC could act on Raf to favor its complete activation after the interaction with Ras.

We also show that there is a reduction in ERK activation when PI3K activity is inhibited in either THP-1 or HEK-293 cells, thus suggesting that this protein also plays a role in MCP-1-mediated ERK modulation through CCR2B. Other chemokine receptors, such as IL-8 and stromal-derived factor-1 α receptors activate MAPK by a PI3K-dependent pathway (Knall et al., 1996; Sotsios et al., 1999). Nevertheless, there are contradictory data, because in a preB cell line transfected with CXCR-4, ERK activation by stromal-derived factor-1 α does not require PI3K participation (Ganju et al., 1998). The cellular system used as well as the expression levels of PI3K isoforms could be the reason for these differences, which could also explain the differences in the effect of PI3K inhibitors that we observe between THP-1 and HEK-293 cells. The PI3K γ isoform can be directly activated by $G\beta\gamma$ dimers and is highly expressed in cells of the immune system, but poorly in other cell types as fibroblasts (Bernstein et al., 1998). It has been recently described that $G\beta\gamma$ subunits may also mediate the activation of class 1 isoforms of PI3K (Murga et al., 2000), leading to Ras and ultimately to MAPK stimulation. It is tempting to speculate that a similar mechanism would explain the participation of a LY 294002-sensitive PI3K in CCR2B-mediated ERK activation. Because the inhibition of cytosolic tyrosine kinases with PP2 or herbimycin A does not have any effect in the level of ERK activation after MCP-1 challenge, PI3K-mediated ERK activation seems to be independent of these kinases, although the participation of other tyrosine kinases insensitive to the compounds used cannot be ruled out. Interestingly, it has been recently described a wortmannin- and LY 294002-sensitive inhibition of Ras GAP proteins, what leads to Ras activation independent of tyrosine kinases (Rubio and Wetzker, 2000), as seems to be the case for CCR2B.

Several GPCRs activate ERKs via transmodulation of receptor-tyrosine kinases and the chemokine receptors for IL-8, CXCR1, and CXCR2, promote ERK stimulation by EGF receptor transactivation, that in addition needs mobilization of intracellular calcium (Venkatakrishnan et al., 2000). However, CCR2B does not require either of these events for ERK

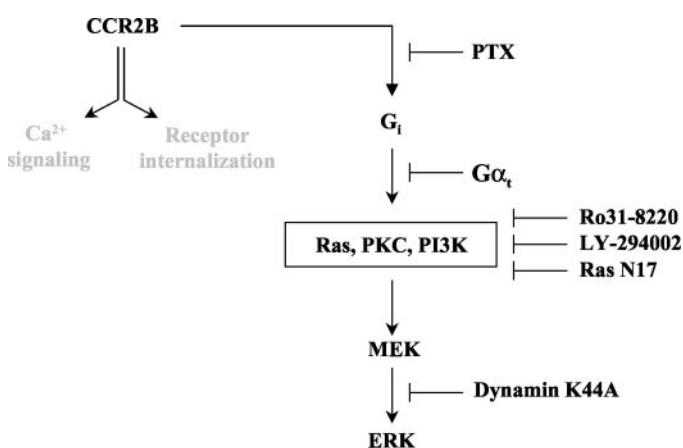


Fig. 10. Participation of different signaling pathways elicited by agonist stimulation of CCR2B on ERK activation in HEK-293. The diagram depicts the suggested level of action of the different pharmacological inhibitors and mutant proteins used in this study that are able to modulate MCP-1-mediated ERK activation. Other cellular processes triggered by CCR2B receptor activation, such as intracellular calcium mobilization or receptor internalization, do not seem to be required for stimulation of the ERK cascade.

stimulation elicited in HEK-293 or monocytic cells. Although the possible transactivation of other tyrosine kinase receptors cannot be ruled out, our data favor the hypothesis that MCP-1 activation of ERK is receptor transactivation-independent.

Recent studies have discussed the role of GPCR internalization and β -arrestin scaffold function in the activation of the ERK pathway (Ignatova et al., 1999; Luttrell et al., 2001). Several lines of evidence support that CCR2 receptor activates ERK independently of receptor internalization. Hypertonic sucrose, an experimental condition known to prevent GPCR internalization (Hansen et al., 1993), did not inhibit MAPK activation by MCP-1 in either THP-1 or HEK-293 cells, whereas inhibiting internalization of the CCR2B in these systems. On the other hand, overexpression of β -arrestins 1 or 2 or the dominant negative V53D mutant does not affect CCR2B-mediated ERK activation in HEK-293 cells, although the arrestin mutant, is able to abolish CCR2B receptor internalization. Moreover, CCR2BIX, an endocytosis-defective receptor mutant is able to promote a marked ERK activation upon agonist stimulation. These data are in agreement with other recent results indicating that the internalization of different GPCRs is not essential for ERK activation (Budd et al., 1999; Yang et al., 1999). However, it is worth noting that the dynamin mutant K44A (which impairs clathrin-mediated endocytosis) is able to produce a clear reduction in ERK activation elicited by CCR2B after its stimulation with MCP-1 in HEK-293 cells. Interestingly, this type of dynamin dependence has been described for μ - and δ -opioid receptors, whose endocytosis is not required for the MAPK activation elicited after their stimulation (Whistler and von Zastrow, 1999). The effect of this dynamin mutant on ERK activation promoted by other GPCRs has been explained by the blockage of the internalization of transmodulated RTKs (Pierce et al., 2000), which does not seem to be the case for CCR2B. Our finding that MEK kinase phosphorylation and activation is not altered in the presence of the dynamin K44A mutant, indicates that this protein is needed in the step between MEK and ERK stimulation. This is consistent with the hypothesis that MEK internalization is the critical step for ERK activation (Kranenburg et al., 1999). Dominant negative dynamin would be expected to block conversion of coated pits to coated vesicles. Therefore, it is possible that the further processing of internalization of vesicles is needed for MEK activation of MAPK. Interestingly, it has been shown that inhibitors of PI3K prevent the recruitment of dynamin to endocytic vesicles (Gold et al., 1999), which could be related to the effect of PI3K inhibitors on ERK activation in our experimental system. Together, our results suggest that the necessary step for ERK activation is the internalization of MEK, whereas receptor internalization is not required.

In summary, our data indicate that a variety of biochemical pathways elicited by MCP-1-stimulation of CCR2 receptors participate in the activation of the ERK cascade in transfected HEK-293 cells (Fig. 10). We show that a number of these pathways also play a role upon stimulation of native receptors in monocytic cells, by approaches that do not rely on protein overexpression. The detailed knowledge of the molecular interactions involved would allow a better understanding of how signal transduction pathways cooperate in

key physiological process mediated by chemokine-stimulated MAPK activation, such as leukocyte chemotaxis.

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