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# Signal Transduction by the Chemokine Receptor CXCR3

## ACTIVATION OF Ras/ERK, Src, AND PHOSPHATIDYLINOSITOL 3-KINASE/Akt CONTROLS CELL MIGRATION AND PROLIFERATION IN HUMAN VASCULAR PERICYTES*

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**Andrea Bonacchi‡, Paola Romagnani§, Roberto G. Romanelli‡, Eva Efsen‡¶, Francesco Annunziato‡, Laura Lasagni§, Michela Francalanci§, Mario Serio§, Giacomo Laffi‡, Massimo Pinzani‡, Paolo Gentilini‡, and Fabio Marra‡‖**

*From the ‡Dipartimento di Medicina Interna and §Dipartimento di Fisiopatologia Clinica, University of Florence, Florence I-50134, Italy*

**Hepatic stellate cells (HSC) and glomerular mesangial cells (MC) are tissue-specific pericytes involved in tissue repair, a process that is regulated by members of the chemokine family. In this study, we explored the signal transduction pathways activated by the chemokine receptor CXCR3 in vascular pericytes. In HSC, interaction of CXCR3 with its ligands resulted in increased chemotaxis and activation of the Ras/ERK cascade. Activation of CXCR3 also stimulated Src phosphorylation and kinase activity and increased the activity of phosphatidylinositol 3-kinase and its downstream pathway, Akt. The increase in ERK activity was inhibited by genistein and PP1, but not by wortmannin, indicating that Src activation is necessary for the activation of the Ras/ERK pathway by CXCR3. Inhibition of ERK activation resulted in a decreased chemotactic and mitogenic effect of CXCR3 ligands. In MC, which respond to CXCR3 ligands with increased DNA synthesis, CXCR3 activation resulted in a biphasic stimulation of ERK activation, a pattern similar to the one observed in HSC exposed to platelet-derived growth factor, indicating that this type of response is related to the stimulation of cell proliferation. These data characterize CXCR3 signaling in pericytes and clarify the relevance of downstream pathways in the modulation of different biologic responses.**

In different tissues, the wound healing response shares many similarities, involving the recruitment of inflammatory cells and the deposition of extracellular matrix, to fill the gap created by the dying cells. The concurrent presence of inflammation and extracellular matrix deposition is a characteristic of chronic tissue injury, where the persistence of a wound healing response may lead to permanent scarring and end-stage organ failure, such as in the case of glomerulosclerosis in the kidney, cirrhosis of the liver, atherosclerosis, or pulmonary fibrosis (1). The pivotal role played by vascular pericytes of different tissues in the process of wound healing has been clearly recognized in recent years. These cells become activated in the presence of damage to the specific tissue, proliferate, migrate, and acquire a myofibroblast-like phenotype, resulting in the production of extracellular matrix as part of the healing process. Hepatic stellate cells (HSC)[1] and renal glomerular mesangial cells (MC) represent tissue-specific pericytes, which are deeply involved in the development of the wound healing response and in the pathogenesis of tissue fibrosis in the setting of a chronic damage (2, 3). Understanding the biology of these cells may help to devise novel strategies for the treatment of chronic liver and kidney diseases.

The chemokine system has received considerable attention for its involvement in a number of biologic processes. Although members of this family have been initially recognized for their ability to recruit leukocytes to sites of injury, more recent investigation has shown that this system participates in the regulation of a wide number of conditions, including physiologic leukocyte homeostasis, development, angiogenesis, cancer, and the response to infection (4, 5). Activation of the chemokine system has been reported in the presence of chronic inflammation and fibrosis, two processes that are part of the wound healing response (6, 7). In addition, several studies have shown that the pericytes responsible for tissue fibrosis may both express chemokines and be targets of the action of chemokines (8–11). In fact, pericytes express chemokine receptors, which, upon activation, elicit biologic actions that favor the wound healing process, including proliferation, migration, and extracellular matrix synthesis (6, 12, 13).

The chemokine receptor CXCR3 is bound with high affinity by the chemokines interferon-inducible protein-10 (IP-10), monokine activated by interferon-γ (Mig), and interferon-inducible T cell α chemoattractant (14, 15). CXCR3 has been identified on several cells of hematopoietic lineage, including T and B lymphocytes, and natural killer cells (4, 16). In addition, expression of CXCR3 has been indicated as a marker of polarization of the T helper subset of T cells toward a Th1 phenotype (17). We have recently reported that CXCR3 is expressed by human MC in culture, where CXCR3 agonists induce an increase in cell proliferation, and the expression of CXCR3 on MC is up-regulated in conditions of chronic glomerular damage, indicating a possible involvement in wound healing and repair

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‖ To whom correspondence should be addressed: Dipartimento di Medicina Interna, University of Florence, Viale Morgagni 85, Florence I-50134, Italy. Tel.: 39-055-4296-475; Fax: 39-055-417-123; E-mail: f.marra@dfc.unifi.it.

[1] The abbreviations used are: HSC, hepatic stellate cells; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptors; GST-RBD, glutathione *S*-transferase-Ras-binding domain; IP-10, interferon-inducible protein-10; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MC, glomerular mesangial cells; Mig, monokine activated by interferon-γ; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PAGE, polyacrylamide gel electrophoresis.

(6). Despite the relevance of this system in a number of pathophysiologic conditions, little information is available on the signal transduction pathways activated by CXCR3 and their possible correlation with the biologic actions elicited by its agonists. In this study we have characterized CXCR3's signaling in HSC and in MC, as paradigm of vascular pericytes belonging to different tissues. We report that CXCR3 activates multiple signaling pathways, including the Ras/ERK pathway, Src, and the PI3K/Akt pathway, which correlate with the ability to induce biologic actions in target cells.

## EXPERIMENTAL PROCEDURES

*Reagents*—Human recombinant IP-10, Mig, EGF, and PDGF-BB were purchased from Peprotech (Rocky Hill, NJ). Monoclonal antibodies against CXCR3 were from R&D Systems (Minneapolis, MN). Phospho-specific antibodies against the activated form of ERK, MEK, Raf-1, and Akt (Ser-473), and anti-MEK antibodies were from New England BioLabs (Beverly, MA). Polyclonal Anti-ERK antibodies used for Western blotting, polyclonal anti-Akt antibodies, and rat monoclonal anti-Ras antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Agarose-conjugated anti-phosphotyrosine antibodies were from Oncogene Science (Uniondale, NY). Polyclonal anti-ERK antibodies used for immune complex kinase assay, phospho-specific antibodies against activated Src (Y416), and monoclonal anti-Src antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). For anti-phosphotyrosine immunoblotting, a mixture of PY99 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology Inc.) monoclonal antibodies was used. PD98059, AG1478, wortmannin, and genistein were purchased from Calbiochem (La Jolla, CA). PP1 was from Biomol (Plymouth Meeting, PA). Radionuclides were purchased from ICN (Costa Mesa, CA). All other reagent were of analytical grade.

*Cell Culture*—Human HSC were isolated from wedge sections of liver tissue unsuitable for transplantation by collagenase/pronase digestion and centrifugation on Stractan gradients. Procedures used for cell isolation and characterization have been extensively described elsewhere (18). All the experiments were conducted on cells cultured on uncoated plastic dishes (passage 3–6), showing an "activated" or "myofibroblast-like" phenotype.

Cultures of MC were obtained from macroscopically normal kidneys of patients with localized renal tumors undergoing nephrectomy, as described previously (6, 19). The cortex was separated from the medulla and minced; glomeruli were isolated by a standard sieving technique through graded mesh size screens (60, 80, 150 mesh). The glomerular suspension was collected, washed with RPMI 1640 (Sigma), and incubated with 750 units/ml collagenase type IV at 37 °C for 30 min. The glomeruli were then cultured in RPMI 1640 with 17% fetal calf serum and other supplements to obtain MC (6). Cultured glomeruli were maintained in a humidified environment of 5% $CO_2$/95% air at 37 °C, and the medium was changed three times a week. MC were used between passages 4 and 7.

*Flow Cytometry*—After saturation of nonspecific binding sites with total rabbit IgG, cells were incubated for 20 min on ice with specific or isotype control antibody. In the indirect staining, this step was followed by a second incubation on ice with an appropriate anti-isotype-conjugated antibody (Southern Biotechnology Associates, Birmingham, AL). Finally, cells were washed and analyzed on a FACSCalibur cytofluorimeter using CellQuest software (Becton Dickinson, San Jose, CA). In all cytofluorimetric analyses, a total of $10^4$ events for each sample was acquired.

*Measurement of DNA Synthesis*—Confluent HSC or MC were washed with phosphate-buffered saline and incubated in serum-free medium for 48 h. The cells were incubated with agonists for 24 or 36 h and then pulsed with [$^3$H]thymidine. DNA synthesis was measured as the incorporation of tritiated thymidine, as described elsewhere (20).

*Cell Migration*—Confluent HSC or MC were serum-starved for 48 h and then washed, trypsinized, and resuspended in serum-free medium containing 1% albumin at a concentration of $3 \times 10^5$ cells/ml. Chemotaxis was measured in modified Boyden chambers equipped with 8-$\mu$m pore filters (Poretics, Livermore, CA) coated with rat tail collagen (Collaborative Biomedical Products, Bedford, MA), as previously described (21). When inhibitors were used, cultured cells were incubated with the drugs to be tested or with their vehicle for 15 min before trypsinization, and equal concentrations were added in the Boyden chamber.

*Preparation of Cell Lysates and Western Blotting*—Confluent, serum-starved HSC or MC were treated with the appropriate conditions, quickly placed on ice, and washed with ice-cold phosphate-buffered saline. Except for the analysis of Ras activation (see below), the monolayer was lysed in radioimmune precipitation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, 0.05% (w/v) aprotinin). Insoluble proteins were discarded by high speed centrifugation at 4 °C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (Pierce, Rockford, IL).

Equal amounts of total cellular proteins were separated by SDS-PAGE and analyzed by Western blot as previously described (21). Immunoblot analysis of EGF receptor tyrosine phosphorylation was conducted after immunoprecipitation. Briefly, 150 $\mu$g of total cellular proteins were incubated with anti-EGF receptor antibodies and protein A-Sepharose for 2 h at 4 °C. The Immunobeads were washed twice in lysis buffer and once in 20 mM Tris-HCl, pH 7.4, 1 mM $Na_3VO_4$, resuspended in Laemmli buffer, and analyzed by Western blot as described above.

*ERK Assay*—ERK was immunoprecipitated from 25 $\mu$g of total cell lysate using polyclonal anti-ERK antibodies and protein A-Sepharose. After washing, the immunobeads were incubated in a buffer containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM $Na_3VO_4$, 25 $\mu$M ATP, 1 $\mu$Ci of [$\gamma$-$^{32}$P]ATP, and 0.4 mg/ml myelin basic protein for 30 min at 30 °C. At the end of the incubation, the reaction was stopped by addition of Laemmli buffer and run on 15% SDS-PAGE. After electrophoresis, the gel was dried and autoradiographed.

*Phosphatidylinositol 3-Kinase (PI3K) Assay*—This assay was performed after immunoprecipitation with anti-phosphotyrosine antibodies, as described elsewhere (22, 23). Radioactive lipids were separated by thin-layer chromatography, using chloroform/methanol/30% ammonium hydroxide/water (46/41/5/8, v/v). After drying, the plates were autoradiographed. The radioactive spots were then scraped and counted in a $\beta$-counter.

*Ras Assay*—Activation of Ras in response to CXCR3 ligands was determined exactly as described by deRooij and Bos (24). The plasmid encoding for the GST-RBD fusion protein (kindly provided by Dr. J. L. Bos) was expressed in bacteria and used to obtain recombinant GST-RBD, as described elsewhere (24). Serum-deprived HSC exposed to different conditions were lysed in a buffer containing 50 mM Tris, 150 mM NaCl, 0.5% deoxycholase, 1% Nonidet P-40, 0.1% SDS, and protease inhibitors. One milligram of protein was incubated with recombinant GST-RBD and glutathione-agarose beads (Sigma), washed, and analyzed by 15% SDS-PAGE followed by anti-Ras immunoblotting.

*Src Kinase Assay*—Activation of Src was analyzed by immune complex kinase assay as described by Ishida *et al.* (25), with minor modifications. Protein (200 $\mu$g) in radioimmune precipitation buffer was incubated with monoclonal anti-Src antibodies and precipitated by the addition of rabbit anti-mouse IgG and protein A-Sepharose. After washing with radioimmune precipitation buffer, the immunobeads were incubated with a buffer containing 20 mM Hepes, pH 7.0, 10 mM $MnCl_2$, 20 $\mu$g of enolase (Sigma), and 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP for 10 min at 30 °C. At the end of the incubation, the reaction was stopped with Laemmli buffer and analyzed by 10% SDS-PAGE. The gel was stained with Coomassie Blue, and the band corresponding to enolase was cut and counted in a $\beta$-counter.

*Akt Assay*—An immune complex kinase assay of Akt activity was performed as described elsewhere (26). Briefly, 100 $\mu$g of protein was immunoprecipitated using anti-Akt antibodies and protein G-agarose. The Immunobeads were washed three times with washing buffer (20 mM HEPES (pH 7.5), 40 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 20 mM $\beta$-glycerophosphate, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml pepstatin, and 10 $\mu$g/ml aprotinin). The assay was performed by resuspending the beads in kinase buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 mM $\beta$-glycerophosphate, and 0.5 mM sodium orthovanadate) in the presence of 1 $\mu$M protein kinase A inhibitor peptide, 50 $\mu$M unlabeled ATP, and 6 $\mu$Ci of [$\gamma$-$^{32}$P]ATP, using exogenous histone H2B (1.5 $\mu$g/assay tube) as the substrate and incubating for 20 min at room temperature. The proteins in the samples were resolved by 12% SDS-PAGE, and the gel was stained with Coomassie Blue and subjected to autoradiography.

*Data Presentation*—Data presented herein are representative of at least three experiments with comparable results. Unless otherwise indicated, bar graphs show the mean ± S.D. of data from a representative experiment.

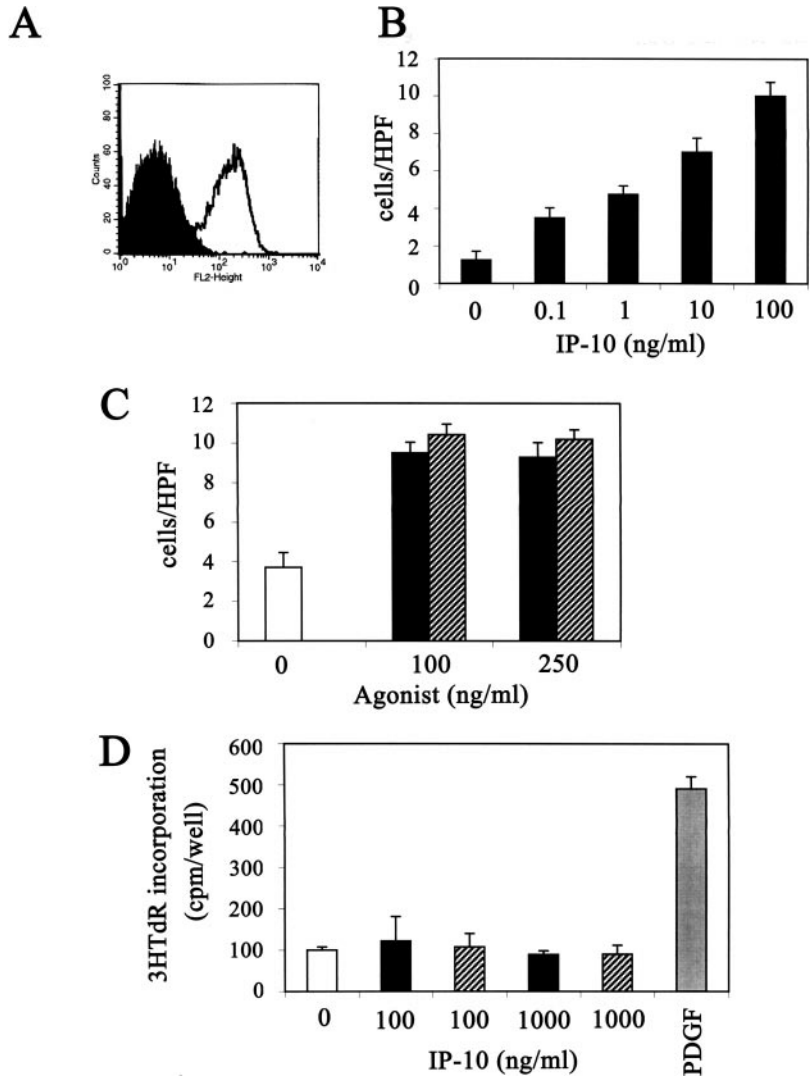


FIG. 1. **Expression and biologic actions of CXCR3 in human HSC.** *A*, cultured HSC were analyzed by fluorescence-activated cell sorter using monoclonal anti-CXCR3 antibodies (*white area*) or isotype-specific control mouse IgG (*black area*). *B*, migration of serum-starved HSC in response to increasing concentrations of IP-10 was measured in Boyden chambers as described under "Experimental Procedures". *C*, migration of HSC was measured in response to the indicated concentrations of IP-10 (*black bars*) or Mig (*cross-hatched bars*). *D*, serum-starved HSC were incubated with the indicated concentrations (ng/ml) of IP-10 (*black bars*) or Mig (*cross-hatched bars*) or with 10 ng/ml PDGF. After 24 h the cells were pulsed with [$^3$H]thymidine, and DNA synthesis was measured as described under "Experimental Procedures."

## RESULTS

*Expression of CXCR3 and Effects of CXCR3 Ligands on HSC*—Because CXCR3 expression has been previously reported in MC (6), we first investigated by flow cytometry whether human HSC, as liver-specific pericytes, express this chemokine receptor. As compared with the cells treated with isotype-specific control antibody, anti-CXCR3 antibodies induced a clear shift in fluorescence, indicating CXCR3 expression on the cell surface (Fig. 1*A*). Expression of the receptor by human HSC was also confirmed by Western blotting (data not shown). To test whether CXCR3 expressed by HSC is functional, we assessed the effects of IP-10 on HSC migration, because activation of chemokine receptors is often associated with the induction of chemotaxis. Exposure of HSC to increasing concentrations of IP-10 resulted in a dose-dependent increase in cell migration, which was 3- to 4-fold greater than that observed in unstimulated cells (Fig. 1*B*). When IP-10 (100 ng/ml) was added to both the upper and lower chamber of the Boyden system, HSC migration was similar to that of unstimulated cells, indicating that the effects of IP-10 are dependent on chemotaxis rather than on chemokinesis (data not shown). CXCR3 may be activated by three ligands, including Mig, therefore, we compared the effects of IP-10 and Mig to confirm the role of CXCR3 in the IP-10-induced cell migration. Both agonists were equally effective in their ability to induce cell migration (Fig. 1*C*). In addition, the chemotactic activity was shown to peak at a concentration of 100 ng/ml for both ligands (Fig. 1*C* and data not shown).

We and others have previously reported that ligands of CXCR3 stimulate proliferation of vascular pericytes, including MC (6) and smooth muscle cells (9). Therefore, we examined the effects of CXCR3 ligands on DNA synthesis in HSC. However, neither IP-10 nor Mig, when tested at concentrations as high as 1 $\mu$g/ml, resulted in an increase in DNA synthesis, whereas the cells were clearly responsive to known mitogens, such as PDGF (Fig. 1*D*). These results indicate that HSC express functional CXCR3 receptors on the cells surface and that activation of this chemokine receptor induces migration, but not proliferation, of this cell type.

*Ligands of CXCR3 Activate the Ras/ERK Pathway*—Despite the relevance of CXCR3 in many pathophysiologic processes, no information on the signal transduction pathways activated by this chemokine receptor is presently available. Using HSC as a model system of cells constitutively expressing this receptor, we explored the intracellular signaling pathways activated by CXCR3. We first focused on the Ras/ERK pathway, which is activated in response to many soluble factors, including chemokines (27), and regulates different cellular functions. Exposure of HSC to IP-10 for different periods of time resulted in increased phosphorylation of both ERK isoforms, p44$^{ERK-1}$ and p42$^{ERK-2}$, as assessed by using antibodies that specifically recognize the phosphorylated forms of ERK (Fig. 2*A*). Peak acti-

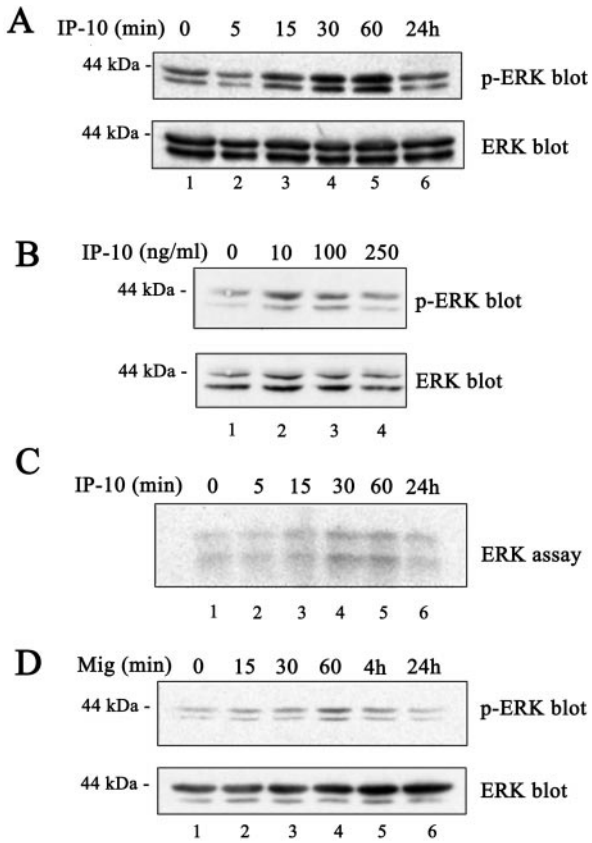


FIG. 2. **CXCR3 ligands activate ERK.** *A*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points. Total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of ERK (*top panel*) or total ERK (*bottom panel*). Migration of molecular weight markers is indicated on the *left*. *B*, HSC were incubated with different concentrations of IP-10, as indicated, for 30 min. Western blot analysis for the phosphorylated form of ERK was carried out as in *A*. *C*, HSC were treated exactly as described in *A*. Cell lysates were subjected to immune complex kinase assay of ERK activity as described under "Experimental Procedures." *D*, the experiment was conducted exactly as the one shown in *A*, except for the fact that Mig (100 ng/ml) was used as an agonist.

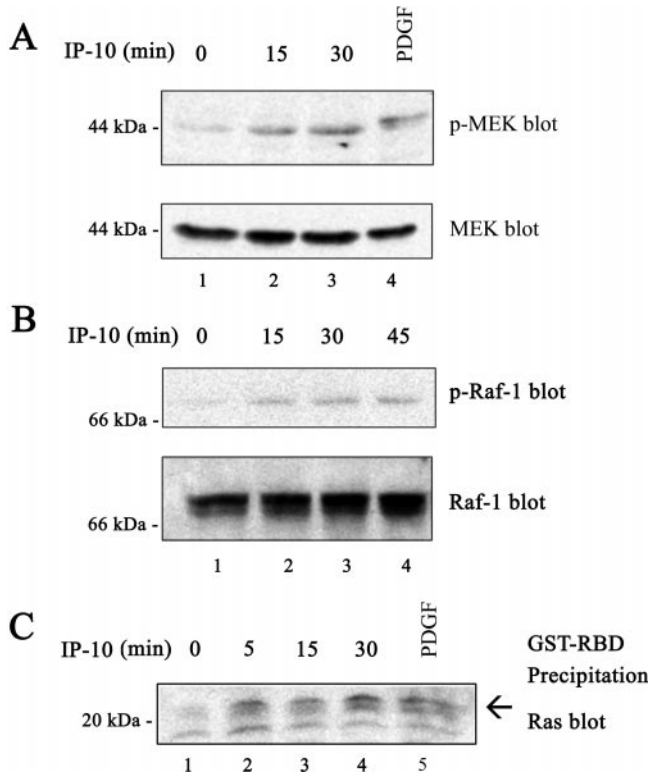


FIG. 3. **CXCR3 activate the Ras/MEK/ERK cascade.** *A*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points or with 10 ng/ml PDGF for 10 min. Total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of MEK (*top panel*) or total MEK (*bottom panel*). *B*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points, and total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of Raf-1 (*top panel*) or total Raf-1 (*bottom panel*). *C*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points, or with 10 ng/ml PDGF for 10 min. One milligram of total cellular proteins was incubated with a GST-RBD fusion protein and glutathione-agarose beads as described under "Experimental Procedures." After washing, the beads were analyzed by immunoblotting with anti-pan Ras antibodies. Specific Ras bands are indicated by the *arrow*. Migration of molecular weight markers is indicated on the *left*.

vation was observed at 15–30 min after addition of IP-10 and returned to basal levels within 4 h after stimulation. At later time points, no ERK phosphorylation could be detected (Fig. 2*A*, and data not shown). Concentrations of IP-10 higher than 100 ng/ml did not result in a further increase in ERK phosphorylation (Fig. 2*B*). We also tested whether increased phosphorylation of ERK was indeed associated with increased catalytic activity using an immune complex kinase assay. Increased phosphorylation of the substrate myelin basic protein was observed at the same time points where increased ERK phosphorylation was detected (Fig. 2*C*). To demonstrate that the effects of IP-10 on ERK are due to CXCR3 activation, similar experiments were conducted using Mig as an agonist. Also in this case, a transient activation of ERK was observed, with a similar time course as that observed in cells exposed to IP-10 (Fig. 2*D*). Taken together, these data indicate that interaction between CXCR3 and its ligands leads to activation of ERK.

Similar to other members of the mitogen-activated protein kinase (MAPK) family, ERK activation results from the sequential activation of a small G protein of the Ras superfamily, and a kinase cascade involving a MAPK kinase kinase and a MAPK kinase (28). MEK-1/2 is the MAPK kinase responsible for ERK activation. Therefore we explored the effects of IP-10 on the phosphorylation of MEK. At the same time points in which activation of ERK was observed, IP-10 caused a clear increase in the amount of phosphorylated MEK, similarly to PDGF, a known activator of the Ras/ERK pathway (Fig. 3*A*). Of note, activation of MEK was associated with activation of the upstream kinase Raf-1, which acts as a MAPK kinase kinase in the Ras/ERK cascade (Fig. 3*B*). Finally, we tested whether exposure of HSC to a CXCR3 ligand was associated with increased activation of Ras. We used a fusion protein (GST-RBD) comprising the Ras-binding domain of Raf-1 to selectively precipitate active Ras, which physically interacts with this domain of Raf-1 (24). In unstimulated cells, active Ras was barely detectable as a doublet migrating at 21 kDa (Fig. 3*C*). Exposure of the cells either to IP-10 or to PDGF, used as a positive control, resulted in a greater amount of precipitated Ras, indicating increased activation.

*Src and PI3K/Akt as Downstream Pathways of CXCR3 Activation*—Upon activation of G protein-coupled receptors, several mechanisms may be involved in the activation of the Ras/ERK pathway, including nonreceptor tyrosine kinases and phosphatidylinositol 3-kinase (PI3K). Because the nonreceptor tyrosine kinase Src has been implicated in the downstream signaling of different G protein-coupled receptors, we explored the ability of IP-10 to activate Src. CXCR3 activation by IP-10 was accompanied by a marked increase in Src phosphorylation on the activation-specific tyrosine 416 (Fig. 4*A*) (29), indicating

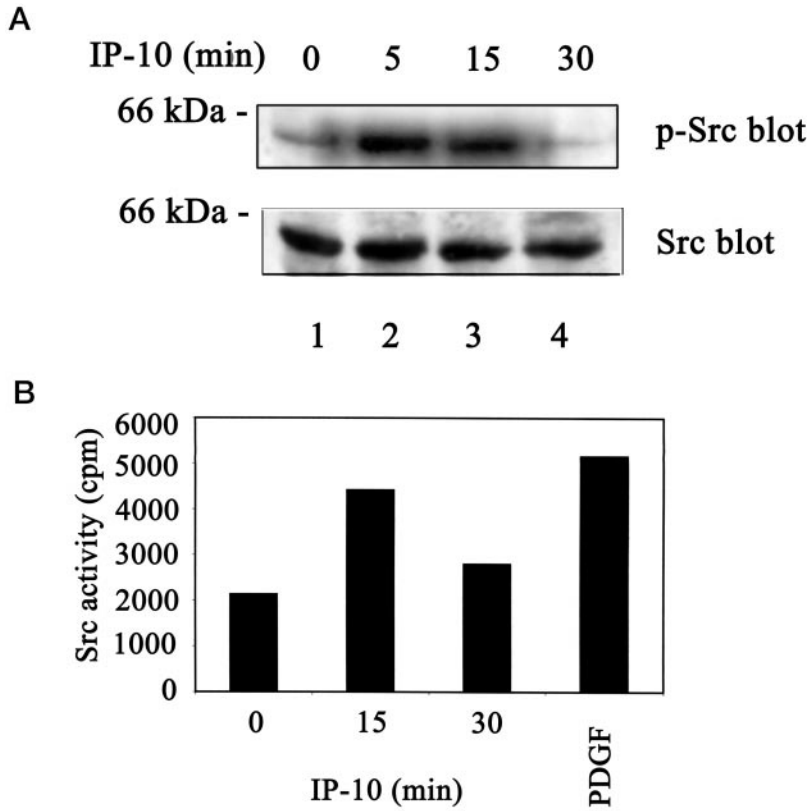


FIG. 4. **CXCR3 ligands activate Src.** *A*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points. Total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of Src (Y416) (*top panel*) or total Src (*bottom panel*). Migration of molecular weight markers is indicated on the left. *B*, HSC were treated with IP-10 for the indicated time points or with 10 ng/ml PDGF. Assay of Src kinase activity was performed as described under "Experimental Procedures." Data are from a representative experiment.

that this receptor activates Src. To confirm that increased phosphorylation of Src was associated with enhanced kinase activity, we performed immune complex kinase assays using immunoprecipitated Src and enolase as a substrate. Also in this condition, an increase in Src activity was observed, which was of the same magnitude as that induced by PDGF, a known activator of this pathway (Fig. 4*B*).

Because cell migration may be critically regulated by activation of PI3K, we also tested whether the activity of this kinase could be modified by CXCR3 ligands. PI3K activity associated with anti-phosphotyrosine immunoprecipitates was increased by 3-fold in cells exposed to IP-10 (Fig. 5*A*) and declined thereafter. Activation of c-Akt, also known as protein kinase B, has been recognized as a pathway that lies downstream of PI3K activation (30). Akt phosphorylation on the activation-specific residue Thr-473 (30), was markedly increased in HSC exposed to IP-10, similarly to cells treated with PDGF, a known activator of Akt (Fig. 5*B*). Moreover, activation of CXCR3 increased the catalytic activity of Akt, as assessed by immune complex kinase assay using PDGF as a positive control (Fig. 5*C*) (31). Taken together, these data indicate that CXCR3 activation leads to increased activity of Src and of the PI3K/Akt pathway.

*Upstream Signaling Pathways Required for ERK Activation in Response to IP-10*—To establish which pathways are critical for the CXCR3-mediated activation of ERK, we treated HSC with specific inhibitors of different signaling pathways before adding IP-10. Tyrosine kinases, including Src, and PI3K have both been shown to be implicated in the activation of Ras and the ERK cascade by GPCR (32–34). To assess the relative importance of these pathways, we pretreated the cells with genistein, a tyrosine kinase inhibitor, or with wortmannin, a PI3K inhibitor. In the presence of genistein, ERK phosphorylation in IP-10-stimulated cells was completely inhibited, indicating that tyrosine kinase activity is required for CXCR3-induced ERK activation (Fig. 6*A*). In contrast, wortmannin, at concentrations that completely block PI3K activation, was ineffective on ERK activation (Fig. 6*A*). As expected on the basis of the ability of IP-10 to activate MEK, PD98059, an inhibitor of MEK, completely blocked ERK phosphorylation. Because Src activation is induced by IP-10 (Fig. 4) and a tyrosine kinase inhibitor blocks ERK activation, we sought to confirm the involvement of Src using PP1, a specific inhibitor of tyrosine kinases of the Src family (35). A marked reduction in ERK activation was observed in cells preincubated with PP1 before addition of IP-10 (Fig. 6*B*), thus providing additional evidence for a role of Src in the transduction of CXCR3 signals to the ERK pathway. Interestingly, PP1 also reduced ERK phosphorylation in unstimulated cells.

It has been previously shown that ERK activation by GPCR may be related to transactivation of receptor tyrosine kinases, such as the epidermal growth factor (EGF) receptor (36, 37), and this mechanism has been recently shown to be involved in the activation of ERK mediated by the chemokine receptors CXCR1/2 in ovary cancer cells (27). To assess whether transactivation of the EGF receptor could be involved in CXCR3 signaling, HSC were pretreated with tyrphostin AG1478, a specific inhibitor of the EGF receptor tyrosine kinase activity (38). The ability of AG1478 to effectively block EGR receptor signaling was confirmed by the complete inhibition of EGF-induced activation of ERK (Fig. 7*A*). However, no effects of AG1478 were observed on ERK activation in cells exposed to IP-10. To provide additional evidence that EGF receptor activation was not involved in IP-10-stimulated HSC, cell lysates were immunoprecipitated with anti-EGF receptor antibodies and analyzed by anti-phosphotyrosine immunoblotting. Again, no changes in the phosphorylation status of this receptor tyrosine kinase were induced by IP-10 (Fig. 7*B*). Taken together, these findings indicate that activation of ERK by CXCR3 is dependent on Src kinase activity, but independent of EGF receptor or PI3K activation.

*Effects of Inhibitors of ERK or PI3K on IP-10-induced Cell Migration*—In HSC, activation of CXCR3 leads to cell migra-

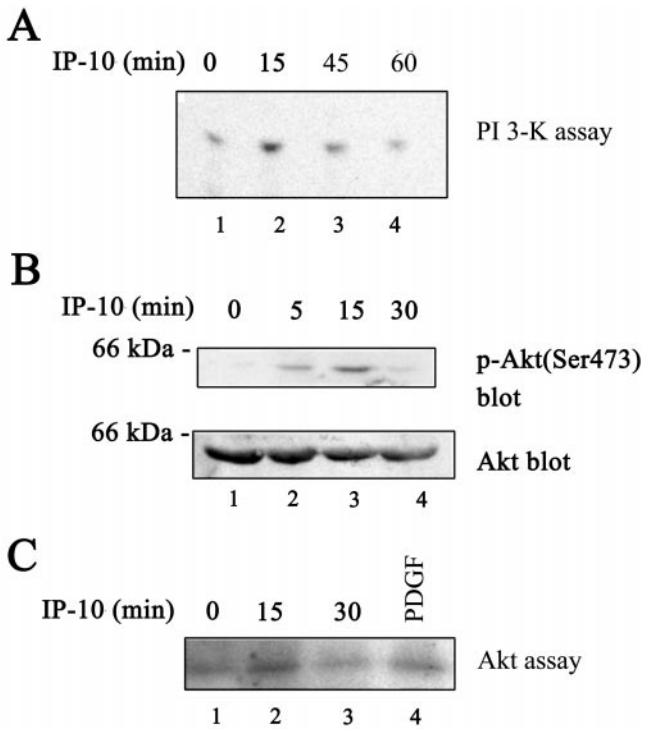


FIG. 5. **Activation of the PI3K/Akt pathway by CXCR3 ligands.** *A*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points, and the PI3K activity associated with anti-phosphotyrosine immunoprecipitates was measured as described under "Experimental Procedures." The radioactive spots correspond to radioactive PI(3)P, the product of PI3K activity. *B*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points. Total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of Akt (Ser-473, *top panel*) or total Akt (*bottom panel*). Migration of molecular weight markers is indicated on the *left*. *C*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points or with 10 ng/ml PDGF for 10 min, and Akt activity was measured by immune complex kinase assay as described under "Experimental Procedures."

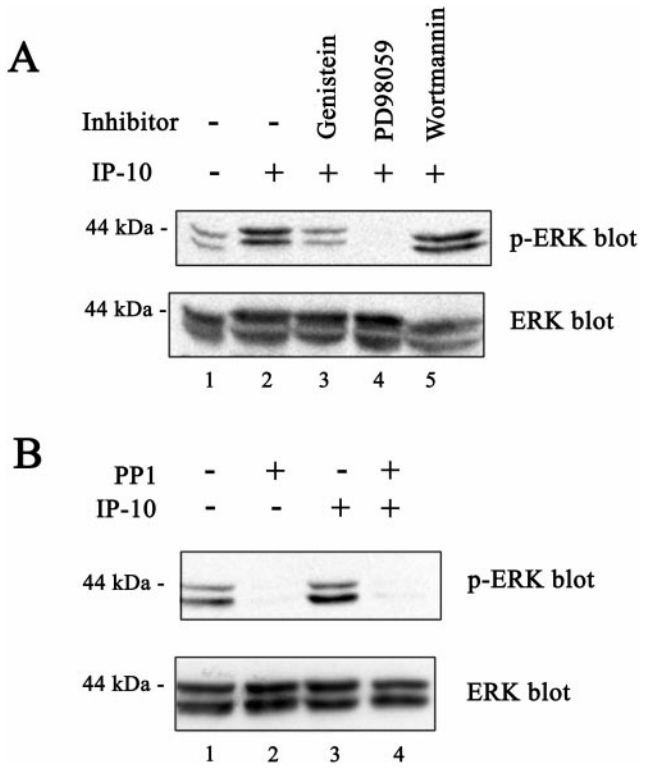


FIG. 6. **Differential requirement of signaling pathways for ERK activation and migration induced by IP-10.** *A*, serum-starved HSC were preincubated with 10 $\mu$M genistein, 30 $\mu$M PD98059, or 100 nM wortmannin, as indicated, before exposure to 100 ng/ml IP-10 for 15 min. ERK phosphorylation was analyzed by immunoblotting. *B*, serum-starved HSC were preincubated in the presence or absence of 5 $\mu$M PP1 and then exposed to 100 ng/ml IP-10. ERK phosphorylation was analyzed by immunoblotting.

tion (see Fig. 1). To test the relative importance of ERK in transducing the chemotactic signals of CXCR3, HSC were preincubated with the MEK inhibitor PD98059 before measuring the effects of IP-10 on cell migration. Preincubation with this compound was associated with a reduction of the chemotactic effect of IP-10 (Fig. 8*A*), although the inhibition was not complete at a concentration that completely blocked ERK activation (Fig. 6*A*). Exposure of HSC to the PI3K inhibitors wortmannin or LY294002 also inhibited the increase in cell migration, and their effects were more marked than those produced by the inhibitor of MEK (Fig. 8*B*).

*Different Time Kinetics of ERK Activation and Biologic Actions of IP-10 in MC*—In MC, IP-10 and Mig have been previously shown to increase cell proliferation, a biologic action not observed in HSC (see Fig. 1). To establish whether the mitogenic activity of IP-10 or Mig in MC was associated with a different behavior of CXCR3 signaling, we analyzed the time kinetics of ERK activation in MC. Similar to the effects induced in HSC, exposure to IP-10 caused a rapid increase in ERK phosphorylation (Fig. 9*A*). However, a late peak of ERK phosphorylation was also observed around 24 h following the beginning of stimulation, a finding that was not observed in HSC (Fig. 9*A*). An extended time course carried out at late time points indicated that the second peak of activation appeared between 14 and 24 h following exposure to IP-10 (Fig. 9*B*). In addition, both peaks of ERK phosphorylation were associated with increased enzymatic activity, as shown by immune complex kinase assay (Fig. 9*C*). Thus, ERK activation in response to IP-10 shows a biphasic temporal pattern in cells that respond to CXCR3 activation with increased DNA synthesis. Activation of PI3K also occurred in response to IP-10 stimulation of MC, similarly to what observed in HSC (data not shown). Moreover, no evidence of EGF receptor transactivation was present also in this cell type, and AG1478 had no effects on either the early or the delayed peaks of ERK activity (data not shown).

We next tested whether activation of ERK was required for cell proliferation induced by IP-10. Exposure of MC to IP-10 was associated with increased incorporation of tritiated thymidine, and incubation with PD98059 completely blocked this event, thus demonstrating that ERK activation is required for increased DNA synthesis mediated by CXCR3 activation (Fig. 10*A*). Because HSC and MC behave differently with respect to cell proliferation, we also tested the effects of CXCR3 ligands on chemotaxis of MC. In agreement with the results observed in HSC, MC migration was increased in the presence of IP-10 or Mig, indicating that the biologic effects of CXCR3 activation on vascular pericytes are partially overlapping (Fig. 10*B*).

*Biphasic ERK Activation Occurs in HSC in Response to Effective Mitogenic Stimulation*—The observation of a biphasic increase in ERK activity in MC exposed to IP-10 raises the issue of whether this phenomenon is associated with the ability of a soluble mediator to induce mitogenesis in a given cell type. To further address this point, we tested the effects of PDGF, a known mitogen for HSC (20) on ERK activation at late time points. As previously reported, a rapid increase in ERK phosphorylation was evident after exposure of HSC to PDGF (39). Within 6 h, ERK activation was markedly reduced, but clearly

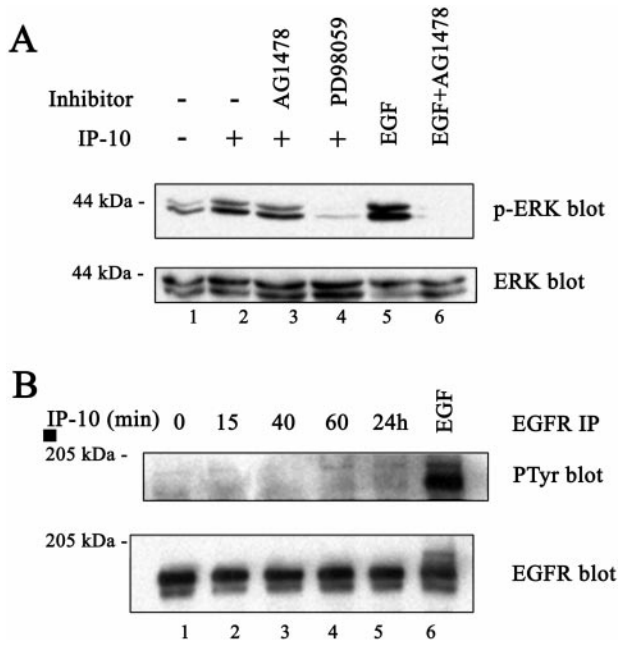


FIG. 7. **Activation of ERK by CXCR3 ligands does not involve transactivation of the EGF receptor.** *A*, serum-starved HSC were preincubated in the presence or absence of 250 nM AG1478 and then exposed to 100 ng/ml IP-10 or 100 ng/ml EGF, as indicated. ERK phosphorylation was analyzed by immunoblotting. *B*, serum-starved HSC were incubated with 100 ng/ml IP-10 for the indicated time points or with 100 ng/ml EGF for 10 min. One hundred micrograms of protein was immunoprecipitated with anti-EGF receptor antibodies and analyzed by anti-phosphotyrosine blotting. Migration of molecular weight markers is indicated on the *left*.

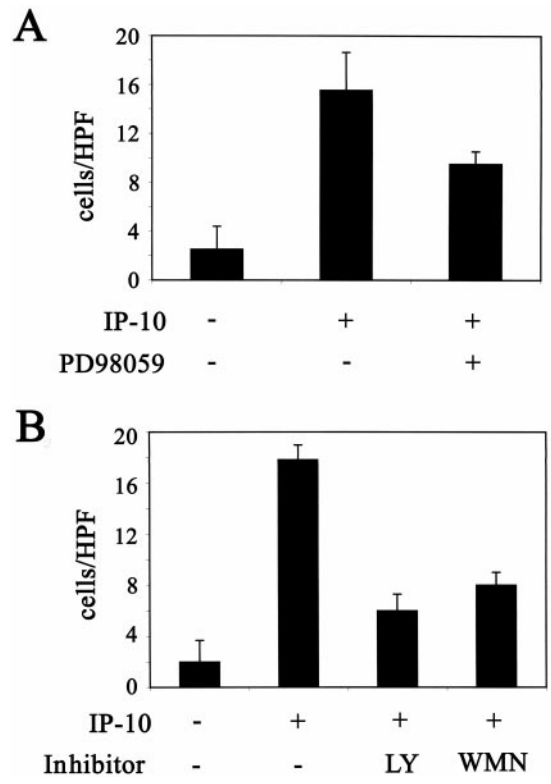


FIG. 8. **Effects of inhibitors of ERK or PI3K on IP-10-induced cell migration.** *A*, serum-starved HSC were preincubated in the presence or absence of 30 $\mu$M PD98059, and migration in response to IP-10 (100 ng/ml) was measured as described under "Experimental Procedures." *B*, migration induced by IP-10 (100 ng/ml) was measured in the presence or absence of 10 $\mu$M LY294002 (*LY*) or 100 nM wortmannin (*WMN*).

increased at 15–24 h from the beginning of stimulation (Fig. 11). An identical behavior was observed when MC were stimulated with PDGF, which is the most potent mitogen also for this cell type (data not shown). Thus, a biphasic activation of ERK appears to be associated with the ability of a certain agonist to elicit a mitogenic response in vascular pericytes.

## DISCUSSION

The biology of chemokines and their receptors has received considerable attention over the past few years. Dissecting the molecular events triggered by activation of chemokine receptors may help to understand the basis for different pathologic processes and to devise better therapeutic strategies. CXCR3 and its ligands have been linked to different processes, including atherosclerosis, glomerular diseases, and recruitment of T lymphocytes to sites of inflammation (6, 9, 40). Accordingly, CXCR3 has been found to be expressed on different cell types, such as T cells, B cells, natural killer cells, and vascular pericytes, including MC (4, 6, 14). In the present study we have characterized the intracellular signaling downstream of CXCR3 in HSC and MC, tissue-specific pericytes which constitutively express CXCR3 and are involved in tissue repair similarly to pericytes of other tissues. Using this approach, we report that CXCR3 ligands activate Ras/ERK, Src, and the PI3K/Akt pathway, thereby regulating critical cellular functions such as cell proliferation and migration. Activation of ERK has been observed in response to a wide number of agonists in different cell types, and chemokine receptors other than CXCR3 have recently been associated with activation of this pathway (27, 41). In this study, we provide evidence that CXCR3 activates all the components of the ERK cascade, including Ras, Raf-1, and MEK, and that activation of ERK plays an important role in the modulation of the biologic actions by CXCR3. Interestingly, CXCR3 ligands have previously been shown to inhibit ERK activation by granulocyte-macrophage colony-stimulating factor and Steel factor in hematopoietic MO7e cells (42). In addition, exposure of activated T lymphocytes to IP-10 induces only a very early and transient activation of ERK or Akt (41). These findings indicate that cell specificity is critical and that cross-talk among various receptors for soluble mediators may result in different patterns of signaling response.

Data from the present study also indicate that CXCR3 activates other signaling pathways with cross-talk with the Ras/ERK cascade. PI3K is a critical enzyme for multiple cellular functions and may be activated by several pathways, depending on the isoform involved and the regulatory molecule implicated (43). Following phosphotyrosine immunoprecipitation, we have found that PI3K activity is clearly increased in cells exposed to IP-10. These findings identify an additional target of CXCR3 signaling and underscore the possible importance of tyrosine phosphorylation for CXCR3-mediated downstream events. We extended these findings by testing the involvement of Akt, a kinase that is activated downstream of PI3K, with the participation of other signaling molecules such as PDK-1 (30). A transient increase in Akt phosphorylation and kinase activity was induced by IP-10, thus confirming that the PI3K/Akt axis is a target of CXCR3 signaling. The involvement of tyrosine phosphorylation in CXCR3 signaling also prompted us to investigate whether CXCR3 ligands activate the nonreceptor tyrosine kinase Src, because this molecule has been shown to be the target of several GPCRs, including chemokine receptors (27). Remarkably, Src enzymatic activity and phosphorylation on activation-specific residues were transiently induced by IP-10, thus identifying an additional element of the signaling

cascade activated by CXCR3. Moreover, ERK, Src, and PI3K may exhibit cross-talk in different ways, because Src has been shown to effectively activate the ERK cascade via Ras and PI3K may be either upstream of the Ras/ERK pathway or an effector of Ras (44, 45). Experiments using pharmacologic inhibitors of specific signaling pathways allowed us to establish that PI3K is not involved in the activation of the ERK cascade by CXCR3, because wortmannin had no effects on ERK phosphorylation. However, the observation that a tyrosine kinase inhibitor blocks ERK activation induced by IP-10 indicated that Src is likely implicated in the activation of this pathway. We confirmed this hypothesis by testing the effects of PP1, a specific inhibitor of protein tyrosine kinases of the Src superfamily (35). PP1 completely blocked the effects of IP-10 on ERK phosphorylation, thus indicating that Src kinase activity is necessary for the activation of this pathway by CXCR3. Receptor tyrosine kinases, in particular the EGF receptor, have been indicated as alternative effectors of ERK activation by GPCR, and may cooperate with Src to induce ERK activation (27, 36, 37). However, in HSC or MC exposed to IP-10, no increase in EGF receptor tyrosine phosphorylation could be observed, and a specific inhibitor of the EGF receptor tyrosine kinase activity did not modify ERK activation. Taken together, these findings are compatible with the hypothesis that activation of CXCR3 results in Src activation, which in turn leads to recruitment of Ras and activation of the ERK cascade. In parallel, activation of PI3K and Akt is also achieved, although the relation between this pathway and activation of Src remains to be established. This proposed picture is in agreement with data reported on the intracellular signaling activated by other GPCR, showing that activation of Src and Src-like kinases is responsible for the activation of Ras (34).

Migration and proliferation of vascular pericytes are critical biologic actions for wound healing and repair in different tissues. Despite the fact that stimulation of cell migration is a known action of chemokine receptors, the fact that cells of nonhematopoietic lineage, such as vascular pericytes, also exhibit increased chemotaxis when exposed to CXCR3 ligands adds further evidence for a role of this receptor in the regulation of tissue repair after wounding. Moreover, data from this study indicate that activation of both ERK and PI3K contrib-

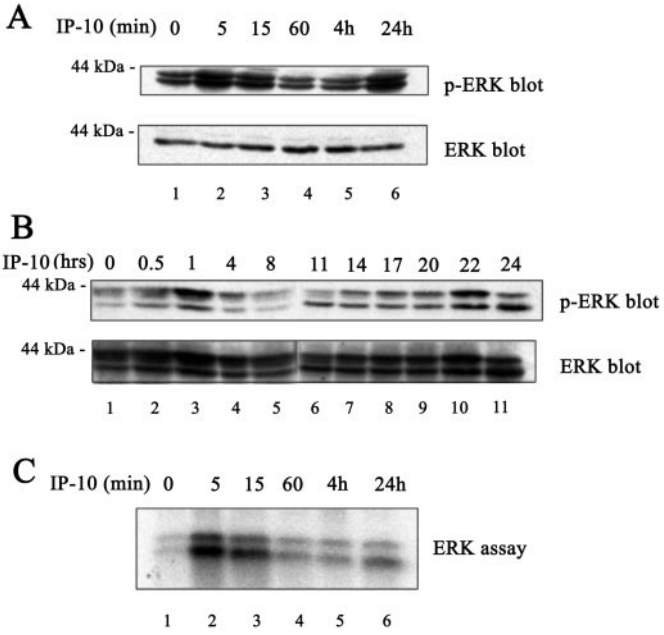


FIG. 9. **IP-10 induces a biphasic activation of ERK in MC.** *A*, serum-starved MC were incubated with 100 ng/ml IP-10 for the indicated time points. Total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of ERK (*top panel*) or total ERK (specific for the $p42^{ERK-2}$ isoform, *bottom panel*). Migration of molecular weight markers is indicated on the left. *B*, serum-starved MC were incubated with 100 ng/ml IP-10 for the indicated time points. Total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of ERK (*top panel*) or total ERK (*bottom panel*). *C*, MC were treated exactly as described in *A*. Cell lysates were subjected to immune complex kinase assay of ERK activity as described under "Experimental Procedures."

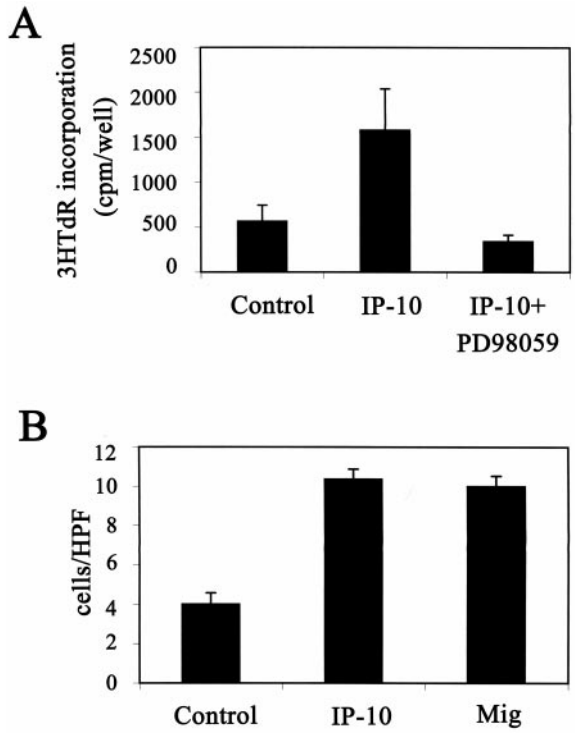


FIG. 10. **Induction of cell migration and proliferation by IP-10 in MC.** *A*, serum-starved MC were preincubated in the presence or absence of 50 $\mu$M PD98059 and then exposed to 10 ng/ml IP-10. DNA synthesis was measured as described under "Experimental Procedures." *B*, migration of MC was measured in response to 100 ng/ml IP-10 (*black bars*) or Mig (*cross-hatched bars*).

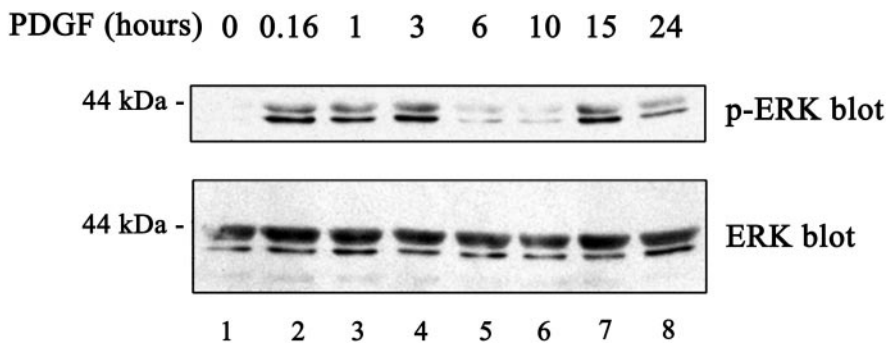


FIG. 11. **Biphasic activation of ERK in HSC exposed to PDGF.** Serum-starved HSC were incubated with 10 ng/ml PDGF for the indicated time points. Total cell lysates were immunoblotted with antibodies specifically recognizing the phosphorylated form of ERK (*top panel*) or total ERK (*bottom panel*). Migration of molecular weight markers is indicated on the *left*.

utes to the chemotactic effect of CXCR3. In fact, an inhibitor of MEK reduced, although not completely, CXCR3-dependent chemotaxis, and the effects of PI3K inhibitors were even more marked. Similar observations were previously obtained in PDGF-stimulated HSC or MC, where ERK inhibition was always less effective than inhibitors of PI3K, which reduced cell migration by 70–85% at concentrations similar to the ones used in this study (39, 46, 47). Interestingly, CXCR3 ligands were unable to induce cell proliferation in HSC, despite the fact that these molecules activate ERK and PI3K. On the other hand, different time kinetics of ERK activation were observed in MC, which proliferate in response to CXCR3 ligands, with the presence of a late peak, appearing 15–24 h following stimulation. Remarkably, a biphasic activation of ERK had been previously reported in cells synchronized in the M phase and subsequently released, compatible with the presence of peaks of ERK activation in the $G_1$ and $G_2$/M phases, respectively (48). Along these lines, interfering with ERK activation has been recently shown to cause a delay of cycling cells to progress through $G_2$ (49). These data indicate that, upon stimulation with CXCR3 ligands, cells that progress through the cell cycle, such as MC, show a second peak of ERK activation, whereas the response of those that do not proliferate is characterized by a single, early peak. This interpretation is supported by the observation that, when HSC were incubated with PDGF, a known mitogen for these cells, a similar pattern of ERK activation was observed. The different spectrum of biologic actions elicited by CXCR3 ligands in HSC or MC also indicates that pericytes of different tissues maintain specific features that allow them to respond more appropriately to the local events associated with injury. Along these lines, in conditions associated with proliferation of MC *in vivo*, polarization of immune response toward a Th1 phenotype has been reported, compatible with a role of IP-10 in MC proliferation in this setting (6, 50). The relevance of cell specificity for the effects of CXCR3 ligands is underscored by our recent observation of the presence of functional CXCR3 receptors on microvascular endothelial cells (51). In this cell type, exposure to IP-10 or Mig leads to inhibition of cell migration and proliferation, which results in a block of angiogenesis, in keeping with the reported antineoplastic effect of CXCR3 ligands. Information on CXCR3 signaling in these cells is likely to provide further information on the relation between signaling events and the biologic actions linked to activation of this receptor.

In conclusion, the present study provides the first characterization of the signaling pathways activated by CXCR3 in a model system of vascular pericytes, such as HSC and MC, involved in wound healing and repair. Activation of CXCR3 signaling is associated with activation of ERK, Src, and PI3K/Akt and results in stimulation of cell proliferation and migration. Interestingly, these pathways have also been shown to mediate cell survival signals and the possibility that chemokines activating CXCR3 regulate pericyte apoptosis will deserve further investigation. The identification of the activity and signaling of CXCR3 in the wound healing process may help to develop future strategies for the treatment of conditions associated with excessive fibrogenesis in conditions of chronic injury.

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# Signal Transduction by the Chemokine Receptor CXCR3: ACTIVATION OF Ras/ERK, Src, AND PHOSPHATIDYLINOSITOL 3-KINASE/Akt CONTROLS CELL MIGRATION AND PROLIFERATION IN HUMAN VASCULAR PERICYTES

Andrea Bonacchi, Paola Romagnani, Roberto G. Romanelli, Eva Efsen, Francesco Annunziato, Laura Lasagni, Michela Francalanci, Mario Serio, Giacomo Laffi, Massimo Pinzani, Paolo Gentilini and Fabio Marra

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