Platelet-derived Growth Factor Stimulation of Monocyte Chemoattractant Protein-1 Gene Expression Is Mediated by Transient Activation of the Phosphoinositide 3-Kinase Signal Transduction Pathway*

(Received for publication, May 17, 1999, and in revised form, July 30, 1999)

John A. Alberta‡, Kurt R. Auger§¶, David Batt§, Palma Iannarelli‡, Grace Hwang‡, Heidi L. Elliott‡, Rebecca Duke‡, Thomas M. Roberts§, and Charles D. Stiles‡¶

From the ‡Department of Microbiology and Molecular Genetics, ¶Department of Pathology, Harvard Medical School and the Division of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Platelet-derived growth factor (PDGF) stimulates transcription of an immediate-early gene set in Balb/c 3T3 cells. One cohort of these genes, typified by c-fos, is induced within minutes following activation of PDGF receptors. A second cohort responds to PDGF only after a significant time delay, although induction is still a primary response to receptor activation as shown by “superinduction” in the presence of the protein synthesis inhibitor cycloheximide. PDGF-receptor activated signaling pathways for the “slow” immediate-early genes are poorly resolved. Using gain-of-function mutations together with small molecule inhibitors of kinase activity, we show that activation of PI 3-kinase is both necessary and sufficient for the induction of the prototype slow immediate-early gene, monocyte chemoattractant-1 (MCP-1). Following activation of PDGF receptors, MCP-1 mRNA does not begin to accumulate for at least 90 min. However, only a brief (10 min) interval of PI 3-kinase activity is required to trigger this delayed response. The serine/threonine protein kinase, Akt/PKB, likely functions as a downstream effector of PI 3-kinase for this induction.

In Balb/c 3T3 cells, platelet-derived growth factor (PDGF)* stimulates transcription of an “immediate-early” gene set. Included in the immediate-early gene class are numerous transcription factors such as c-myc and c-fos and cytokines such as M-CSF 1, KC/gro, and MCP-1 (see Ref. 1 for review by Herschman). By definition, transcription of immediate-early genes is induced even in the presence of drugs such as cycloheximide that inhibit protein synthesis. In fact transcription of immediate-early genes is more robust when PDGF is added together with cycloheximide: the “superinduction” response (2–7). The superinduction response shows that regulatory factors for immediate-early genes pre-exist within the cell. Thus, immediate-early genes define useful end points for analysis of PDGF-regulated signal transduction pathways.

Significant insights into PDGF signal transduction have been made using the c-fos gene as an experimental paradigm. Three functionally distinct cis-acting control elements are located within –350 nucleotides of the c-fos transcription start site. These elements, known as “SRE,” “CREB,” and “SIE,” respond to serum, cyclic AMP, and PDGF B B homodimers, respectively (8–12). Trans-acting proteins that interact with SRE, CREB, and SIE have been cloned and characterized (12–15). Comparative sequence analysis and promoter function studies show that several other immediate-early genes, notably those encoding zinc finger transcription factors, contain fos-like regulatory elements in their 5’-flanking sequences, and require these elements for induction in response to a stimulus (16–18).

The c-fos gene, however, does not stand as a prototype for all members of the immediate-early gene set. The MCP-1 gene, one of the first immediate-early genes to be cloned and characterized as such, contains no fos-like regulatory sequences within the MCP-1 promoter, 3’-flanking region, or within the coding sequence (19). The MCP-1 gene contains structurally distinct regulatory elements within both the 5’ and 3’-flanking regions (20–22). The differential structure of the c-fos and MCP-1 genes is further reflected at the level of gene regulation, where the temporal responses of c-fos and MCP-1 to PDGF are radically offset. In tandem nuclear run-on assays, transcription of c-fos is up-regulated within minutes following activation of PDGF receptors, while the transcription of MCP-1 is not up-regulated until at least 90 min following receptor activation (23). Despite the 90-min temporal offset, transcription of MCP-1 is a primary response to PDGF receptor activation, as treatment with cycloheximide does not prevent but augments transcriptional up-regulation (23, 24). The transcriptional nature of this delayed response can also be observed using reporter gene constructs (20).

With the structure of these “fast” (c-fos) and “slow” (MCP-1) genes so divergent, it is not surprising that they can respond to non-overlapping extracellular cues. For example in Balb/c 3T3 cells, MCP-1, but not c-fos, is induced by interleukin 1 (IL-1) (19). Likewise, MCP-1, but not c-fos, is induced by double-stranded RNA (23). Conversely, induction of c-fos in Balb/c 3T3 cells appears to be channeled through activation of protein kinase C, whereas MCP-1 induction is independent of protein kinase C activation (24). What is unclear is whether the common inducer of these genes, PDGF, regulates expression of c-fos and MCP-1 through common or distinct cytoplasmic signaling pathways.

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: PDGF, platelet-derived growth factor; PI 3-kinase, phosphoinositide 3-kinase; MCP-1, monocyte chemoattractant protein-1; PPP, platelet-poor plasma; IL, interleukin; MAP, mitogen-activated protein; MEK, MAP kinase/extracellular signal-regulated kinase kinase.

* This work was supported by Grant PO1 HD24826–10 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: DuPont Pharmaceuticals Co. Experimental Station E336/34B, Wilmington, DE 19880.
¶ To whom correspondence should be addressed: Dept. of Microbiology and Molecular Genetics, Dana-Farber Cancer Inst., 44 Binney St., Boston, MA 02115. Tel.: 617-632-3512; Fax: 617-632-4663; E-mail: charles_stiles@dfci.harvard.edu.

1 The abbreviations used are: PDGF, platelet-derived growth factor; PI 3-kinase, phosphoinositide 3-kinase; MCP-1, monocyte chemoattractant protein-1; PPP, platelet-poor plasma; IL, interleukin; MAP, mitogen-activated protein; MEK, MAP kinase/extracellular signal-regulated kinase kinase.
Activated PDGF receptors are known to drive the Ras/Raf/ MAP kinase cascade. PDGF receptors also activate src family non-receptor tyrosine kinases and Janus kinase kinases. Directly or indirectly via Janus kinase activation, PDGF receptors can phosphorylate and activate signal transducers and activators of transcription proteins. Activated PDGF receptors also generate two lipid second signals, diacylglycerol and phosphatidylinositol triphosphate. Which component(s) of this signal-generating repertoire is used by PDGF to stimulate expression of MCP-1? Are they identical to the ones that drive expression of c-fos? In studies presented here, we show that lipid signaling via the alternative lipid signaling pathway involving PI 3-kinase is necessary and sufficient for the stimulation of MCP-1 mRNA levels. Further, only a transient period of PI 3-kinase activity is sufficient to drive this response, and that response may involve protein kinase B/Akt.

MATERIALS AND METHODS

Growth Factors and Reagents—All reagents were of molecular biology grade or better. Recombinant platelet-derived growth factor BB and interleukin-1α were from Upstate Biotechnology, Inc., Lake Placid, NY. Wortmannin was from Sigma; anti-Akt and PD98059 were from New England Biolabs, Beverly, MA; LY294002, was a gift from Lilly Pharmaceuticals; STI571 was a gift from Novartis Pharmaceuticals; rapamycin was from Calbiochem, La Jolla, CA. Human deficient platelet-poor plasma (PPP) was prepared as described (26).

Cell Culture, mRNA Isolation, and Analysis—Balb/c 3T3 clone A31 cells as well as the stably transfected cell lines were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum (HyClone Laboratories, Logan, UT). Cells were made quiescent by replacement of the culture media with Dulbecco’s modified Eagle’s medium containing 5% platelet-poor plasma for 24 h. Except where noted, all compounds were added to the media 15 min prior to PDGF BB (30 ng/ml final) or IL-1 (1 ng/ml) stimulation. Transient exposure of cells to PDGF was performed by treating the cultures with PDGF BB (30 ng/ml) for the indicated periods of time followed by washing the cells three times with prewarmed media containing 5% PPP. Cells were then maintained in 5% PPP/Dulbecco’s modified Eagle’s medium for the duration of culture. mRNA was isolated by the guanidinium isothiocyanate method and purified through a modified Eagle’s medium for the duration of culture. mRNA was iso-specific for quiescent Balb/c 3T3 fibroblasts with a 15-min pretreatment of the cultures with 0, 5, or 50 μM STI571. Cells were quiesced in 5% platelet-poor plasma + Dulbecco’s modified Eagle’s medium for 24 h, and then treated as indicated. RNA analysis was performed as described under “Materials and Methods.”

RESULTS

Enzyme/Receptor Activity Assays—Nonidet P-40 lysates were generated for routine examination of PDGF receptor activation after washing a 100-mm plate containing cells twice with ice-cold Tris-buffered saline (10 mM Tris, pH 7.4, 0.9% NaCl), and then scraping the cells into 250 μl of lysis buffer (20 mM Tris, 1% Nonidet P-40, 10% glycerol, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), containing 0.5 mM sodium orthovanadate. Extracts were gently mixed for 10 min at 4 °C, and then spun 5 min in a microcentrifuge (10,000 × g) to remove cellular debris. Soluble fractions were then subjected to either immunoblot analyses or in activity assays. For monitoring PDGF receptor activation (tyrosine phosphorylation), we employed an antibody, anti-pY751, generated against a phosphopeptide corresponding to the Tyr-751 site in the human PDGF b-receptor (29). This antibody recognizes the PDGF receptor phosphorylated at the PI 3-kinase binding site(s) corresponding to Tyr740 and Tyr751. Immunoblot analyses were performed as described previously (30). In vitro PI 3-kinase activation assays were performed as described previously (31). In vitro MAP kinase assays using myelin basic protein as substrate were performed as described previously (32). All assays were performed at least three times. Error bars reflect standard deviation of the results.

Small Molecule Kinase Inhibitors Map the PDGF-activated Signal Generator(s) for MCP-1 Gene Induction Downstream of PDGF Receptor Kinase but Upstream of MAP Kinase—STI571 is a small molecule kinase inhibitor of the 2-phenylaminopyrimidine class originally developed as an inhibitor of the abl oncprotein (33). However, STI571 is also a potent and reasonably selective inhibitor of subgroup III receptor tyrosine kinases including the PDGF receptors α and β. With the exception of Abl, a broad range of other growth factor receptors, non-receptor tyrosine kinases, and serine/threonine protein kinases are refractory to inhibition by STI571 at concentrations 100–1000-fold in excess of those needed to inhibit PDGF receptor activation (33).

We treated Balb/c 3T3 cells with PDGF in the presence or absence of STI571 (34). As shown, STI571 inhibited both PDGF receptor autophosphorylation (Fig. 1A) and MCP-1 mRNA accumulation (Fig. 1B). STI571 did not, however, prevent the induction of MCP-1 when cells were treated with a different stimulator of MCP-1 transcription, IL-1. Thus, STI571 blocks PDGF receptor activation and PDGF-mediated gene induction. However, the drug does not interfere with the basic transcriptional machinery needed for MCP-1 gene induction by other agents. This result also demonstrates that IL-1-mediated induction does not involve the PDGF receptor via any feedback mechanism.

One of the best described pathways for transmitting a signal from a growth factor receptor to the nucleus is the Ras/Raf/ MEK-MAP kinase pathway. However, the well characterized MEK inhibitor PD98059 (35) was unable to inhibit the accumulation of MCP-1 mRNA (Fig. 2B) at levels that inhibit MAP kinase activation as monitored by autophosphorylation (Fig. 2A). Collectively, these data with small molecule kinase inhibitors map the PDGF-activated signal generator(s) for MCP-1 gene induction downstream of PDGF receptor kinase but other than MAP kinase.

FIG. 1. PDGF receptor activation is necessary for PDGF-mediated MCP-1 mRNA accumulation. A, immunoblot analysis of protein extracts generated from untreated cells or cells treated with STI571 (5 μM) following PDGF (30 ng/ml) stimulation for 10 min. The immunoblot was probed with an antibody (anti-pY751) that recognizes the PDGF receptor when phosphorylated at tyrosine 740 or 751 (human β-receptor numbering), a canonical site for the binding of the p85 regulatory subunit of PI 3-kinase to the PDGF receptor. B, Northern blot examination of cellular levels of MCP-1 mRNA following treatment of quiescent Balb/c 3T3 fibroblasts with a 15-min pretreatment of the cultures with 0, 5, or 50 μM STI571. Cells were quiesced in 5% platelet-poor plasma + Dulbecco’s modified Eagle’s medium for 24 h, and then treated as indicated. RNA analysis was performed as described under “Materials and Methods.”

PDGF Receptor Kinase but Upstream of MAP Kinase—STI571
a reversible competitor of ATP binding to the enzyme. As shown in Fig. 3A, these PI 3-kinase inhibitors suppress PDGF induction of MCP-1 mRNA. Good correlation between inhibition of PI 3-kinase activity and the reduction in MCP-1 mRNA levels was observed (Fig. 3B). It should be pointed out that, while 100 μM LY294002 was sufficient to completely abolish MCP-1 expression, 100 nM wortmannin was not. Larger doses of wortmannin were more effective in inhibiting MCP-1 mRNA expression, but the specificity of the compound at such high doses is somewhat suspect.

Within the human PDGF β-receptor subunit, tyrosines 740 and 751 serve, in the phosphorylated state, as binding sites for the p85 adapter subunit of PI 3-kinase (38). As shown by immunoblot analysis with phosphorylation state-specific antibodies (29), neither wortmannin nor LY294002 interfered with phosphorylation of the p85 recognition sites (Fig. 4). Moreover, as shown by immunoblot using a generic antibody to phosphotyrosine, neither wortmannin nor LY294002 diminish the overall level of phosphorylation on PDGF receptors (data not shown). Finally, neither compound had an affect on the IL-1-mediated induction of MCP-1 mRNA (Fig. 3A), indicating the independence of at least the primary steps in the mechanisms behind these two stimuli.

**Transient Activation of the PDGF Receptor and PI 3-Kinase Is Sufficient for Stimulating MCP-1 mRNA Levels**—Previous studies using nuclear run-on assays have shown a 60–90-min lag between activation of PDGF receptors and enhanced transcription of the MCP-1 gene (23). However, as shown in Fig. 5A, only a brief (10 min) pulse of PDGF is necessary to fully stimulate MCP-1 mRNA accumulation. Since a brief exposure of fibroblasts to growth factor is sufficient to stimulate MCP-1 mRNA accumulation, we wondered if the activation of PI 3-kinase need only be transient as well. We treated fibroblasts with wortmannin at various times with respect to the time of PDGF stimulation, and examined the accumulation of MCP-1 mRNA 2 h following growth factor addition. Stimulation for 2 h resulted in approximately maximal accumulation of MCP-1, and was used as a standard accumulation period. Fig. 5B shows a Northern blot analysis of MCP-1 mRNA levels in these cells. Pretreatment of these cells with wortmannin for 15 min greatly inhibited the accumulation of MCP-1 mRNA. However, if the inhibitor was added as little as 5 min following stimulation of the cells by PDGF, little or no effect was observed on the mRNA levels. To ensure that we were not missing a delayed stimulation of PI 3-kinase activity following breakdown of wortmannin or a reversibility problem with LY294002, we measured activity of PI 3-kinase after 2 h of stimulation with PDGF. Only basal levels of activity could be observed at the 2 h time point (data not shown). This is in good agreement with the results of Domin et al. (39) for the examination of PI 3-kinase activity following growth factor treatment.

**PI 3-Kinase Activation Is Sufficient to Stimulate MCP-1 mRNA Levels**—We examined several fibroblast cell lines that were stably transfected with a myristoylated form of the cata-

---

**FIG. 2.** The MEK/MAP kinase pathway is not involved in the PDGF induction of MCP-1 mRNA. A, myelin basic protein phosphorylation in MAP kinase immunoprecipitates from fibroblasts pretreated with the indicated amounts of the MAP kinase inhibitor, PD98059, for 15 min, followed by stimulation with PDGF (30 ng/ml) for 15 min. Cells were lysed and extracts generated as described under "Materials and Methods." B, Northern blot analysis of MCP-1 mRNA levels in cells that were quiesced in 5% platelet-poor plasma + Dulbecco’s modified Eagle’s medium for 24 h, and then pretreated with the indicated amounts of PD98059 for 15 min. Cells were then stimulated with PDGF as indicated for 2 h, and total RNA was isolated and analyzed as described under "Materials and Methods."

**FIG. 3.** PI 3-kinase mediates the PDGF induction of MCP-1 mRNA. A, Northern blot analyses of MCP-1 mRNA levels in cells pretreated for 15 min with various concentrations of either LY294002 or wortmannin, and then stimulated with PDGF (+, 30 ng/ml) or IL-1 (1 ng/ml) for 2 h as indicated. B, graphical representation of phosphatidylinositol triphosphate levels and MCP-1 mRNA following treatment with indicated amounts of wortmannin. PI 3-kinase activity was assayed in anti-phosphotyrosine (4G10) immunoprecipitates from quiescent cells pretreated for 15 min with the indicated amounts of wortmannin, and then harvested following 10 min of PDGF (30 ng/ml) treatment. Filled bars, PI 3-kinase activity; hatched bars, MCP-1 RNA levels. RNA levels were measured in six independent experiments; enzymatic activity was measured in three independent experiments. Error bars denote standard deviation.

**FIG. 4.** Wortmannin or LY294002 do not affect phosphorylation of the PDGF receptor at the PI 3-kinase signaling site. Quiescent cells were pretreated with the indicated amounts of compound for 15 min, and then stimulated with PDGF (30 ng/ml) for 10 min. Extracts were prepared as indicated in the text and analyzed by immunoblot using anti-pY751, the antibody that detects the phosphorylation status of the PDGF receptor at tyrosines 740 and 751 (human β-receptor numbering).
Northern blot analysis of MCP-1 mRNA accumulation following treatment of quiescent Balb/c 3T3 cells treated with PDGF (30 ng/ml, +) for the indicated times, followed by washing the growth factor from the cells three times with medium not containing PDGF. Cells were harvested 2 h following treatment. Samples for the zero time of washout were treated with PDGF and immediately washed and harvested as described. B, Northern blot analysis of quiescent Balb/c 3T3 cells treated with wortmannin (100 nM) at time points indicated relative to PDGF (30 ng/ml, +) stimulation. Cells were incubated for 2 h following PDGF administration, and then processed for mRNA analysis as indicated in the text.

PI 3-Kinase Mediates PDGF Induction of MCP-1

**DISCUSSION**

Receptors for PDGF activate multiple signal generating pathways in fibroblasts (46, 47). We have evaluated several of these pathways for their involvement in the regulation of MCP-1 gene expression by PDGF. MCP-1 is a member of the immediate-early gene family whose growth factor-stimulated RNA expression mirrors that of c-myc in that accumulation of MCP-1 mRNA occurs with slow kinetics. The data presented here clearly define a role for PI 3-kinase in PDGF-mediated induction of the MCP-1 gene. The involvement of PI 3-kinase in this process is transient in nature. Activity for 10 min is sufficient to stimulate the downstream cascade of events that culminate in MCP-1 stimulation. This temporal window for PI 3-kinase function in MCP-1 gene expression fits very well with previous studies from other workers showing that the association of PI 3-kinase with activated PDGF receptors and the accumulation of in vivo lipid products is transient in nature (39).

PI 3-kinase activity appears to be sufficient for MCP-1 mRNA accumulation. Increased levels of MCP-1 mRNA were readily observed in cell lines that expressed a myristoylated, constitutively active p110 catalytic subunit of PI 3-kinase. Treatment of the transfected cells with either of the PI 3-kinase inhibitors wortmannin or LY294002 for an extended period of time did decrease the amount MCP-1 mRNA in these cells, indicating that the accumulation of MCP-1 mRNA was indeed PI 3-kinase-dependent rather than an artifact of the generation of the stable cell lines. Thus, we conclude that PI 3-kinase is necessary and sufficient for the induction of the immediate-early gene MCP-1 in fibroblasts. This induction does not ap-
pears to involve the rapamycin-sensitive pp70S6 kinase, nor the MAP kinase cascade.

Glucose-6-phosphate dehydrogenase (48) and hexokinase II (49) are examples of genes whose stimulated expression is PI 3-kinase-dependent. The stimulation of these genes by insulin is rapamycin-sensitive, indicating the involvement of pp70S6K in this stimulatory mechanism. Fatty acid synthase is an example of a gene that is activated through a PI 3-kinase-dependent mechanism that is not sensitive to rapamycin (50). These genes are not, however, members of the immediate-early gene set as MCP-1 clearly is. There have been reports of immediate-early genes induced in a PI 3-kinase-dependent manner. The response of c-fos to EGF stimulation of HeLa cells has been shown to involve PI 3-kinase via the serum response element (51). Growth hormone stimulation of c-fos, egr-1, and jun B in fibroblasts has been shown to involve PI 3-kinase (52), as has vascular endothelial growth factor stimulation of c-fos in endothelial cells (53). In all of these cases, the stimulation of the target gene was also sensitive to PD98059, indicating involvement of the MAP kinase cascade.

The finding that MCP-1 expression is mediated by a PI 3-kinase-dependent, rapamycin-insensitive, and MAP kinase-independent mechanism suggests that other effectors are involved in this pathway. One excellent candidate is the serine threonine kinase Akt/PKB. This kinase is thought to be activated by a phospholipid product of PI 3-kinase, specifically phosphatidylinositol 3,4,5-trisphosphate (42, 54). Activation of the fatty acid synthase promoter has been shown to be Akt/PKB-dependent (50). Transcription factors of the forkhead family (FKHR and FKHRL1) have been shown to be substrates of PKB-dependent (50). Transcription factors of the forkhead family (FKHR and FKHRL1) have been shown to be substrates of phorbol esters (21, 22). Further studies have implicated accessory serine/threonine phosphoproteins in the stimulatory complex (21, 22). Further studies will be necessary to identify the role of PI 3-kinase and Akt/PKB in the inducible binding of these proteins to the regulatory sequence upstream of MCP-1.

Does the PI 3-kinase/Akt/PKB signaling axis account for induction of all slow immediate-early genes? Unification theories break down immediately upon analysis of another PDGF-responsive slow immediate-early gene, c-myc. As shown by Barone and Courtenidge (61), the induction of c-myc by PDGF appears to be channeled through activation of Src family kinases. Utilization of two distinct signaling pathways by these two slow immediate-early genes may, in part, explain a striking difference in mRNA abundance levels. In quiescent, PDGF-starved 3T3 cells, there are approximately 100 copies of MCP-1 mRNA per cell. Within 3–4 h following exposure to PDGF, the abundance level rises to approximately 3,000 copies of MCP-1 mRNA per cell. For c-myc, the corresponding abundance levels are approximately 0.1 and 5–10 copies of mRNA per cell in the absence and presence of PDGF. The c-myc and MCP-1 genes exhibit non-coordinate control in multiple other contexts. For example, in 3T3 cells IL-1 and double-stranded mRNA are potent and powerful inducers of MCP-1. The c-myc gene shows little or no response to these agents (19, 23). More recently, it has been shown that c-myc expression is suppressed by the adenomatous polyposis coli gene product and activated by β-catenin in colorectal cancer cells (62). The role of this signaling pathway, if any, in MCP-1 gene expression is unknown.
