Platelet-derived Growth Factor Induction of the Immediate-early
gene MCP-1 Is Mediated by NF-κB and a 90-kDa Phosphoprotein
Coactivator* 

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A broad panel of agents including serum, interleukin-1, double-stranded RNA, and platelet-derived growth factor (PDGF) stimulate transcription of the “slow” immediate-early gene MCP-1. These disparate inducers act through a tight cluster of regulatory elements in the distal 5'-flanking sequences of the MCP-1 gene. We describe a 22-base element in this cluster which, in single copy, confers PDGF-inducibility to a tagged MCP-1 reporter gene. In mobility shift assays, the element binds a PDGF-activated form of NF-κB, and a 90-kDa protein (p90) which binds constitutively. Antibody cross-linking UV cross-linking experiments indicate that the PDGF-activated NF-κB species is a Rel A homodimer. The DNA binding form of p90 is a nuclear-restricted serine/threonine phosphoprotein. Mutagenesis of the 22-base element shows that the NF-κB and p90 binding sites overlap, but binding of the two species is mutually independent. Both sites, however, are required for optimum PDGF induction of MCP-1. Therefore, p90 appears to be a coactivator with NF-κB in PDGF-mediated induction of MCP-1.

Immediate-early genes are, by definition, induced at the transcriptional level by a stimulus, and induction is not blocked by drugs that inhibit protein synthesis (1–5). Currently there are approximately 100 members of the immediate-early gene set. Because all components of the transcriptional response pre-exist within cells, cis- and trans-acting regulatory elements for the immediate-early genes define convenient end points for analysis of cellular signal transduction pathways.

A potentially useful means of subclassifying the growing set of immediate-early genes, and one with apparent mechanistic implications, is to divide the group into genes with “fast” or “slow” kinetics of induction. A well characterized example of a “fast kinetics” immediate-early gene is c-fos. Platelet-derived growth factor (PDGF) added to quiescent 3T3 cells stimulates transcription of c-fos within 10 min. c-fos expression reaches peak levels within 30 min and returns to baseline levels within 2 h (6, 7). A cluster of three cis-acting regulatory elements, contained within the proximal 5'-flanking sequences of c-fos, mediate serum and growth factor-stimulated induction of c-fos and have proven to be of general interest in problems of growth factor signal transduction. The three functionally distinct c-fos elements include a 22-nucleotide region of dyad symmetry, termed the “serum response element,” a cyclic AMP response element, and an element responsive to PDGF B.B homodimers known as the SIE (“sis-inducible element”) (8–18). Nuclear trans-acting proteins interacting with these cis-acting regulatory elements have been isolated and characterized (19–27). By both sequence analysis and functional analysis, the regulatory elements defined initially within c-fos have also been detected within other fast kinetics immediate-early genes, and appear to be required for their induction (28–30).

The mechanisms underlying serum and growth factor-stimulated transcription of the c-fos gene, however, do not appear to be conserved among all members of the immediate-early gene set. Members of a second subset of immediate-early genes are induced with slower kinetics than c-fos (31, 32). Included in the “slow kinetics” subset of immediate-early genes are the c-myc proto-oncogene and the clinically important chemokine gene J E/MCP-1 (for monocyte chemotactant protein 1, hereafter referred to as MCP-1) (33–36). In contrast to the rapid but transient response exhibited by c-fos, slow kinetics immediate-early genes like c-myc and MCP-1 display a 60–90-min lag period before initiation of transcription in response to serum or growth factor stimulation (7, 37, 38). Nuclear run-off transcription analyses demonstrate that induction of MCP-1 RNA by PDGF and serum is primarily the result of increased transcription (31, 38–40). However, no fos-like regulatory elements are found within several kilobases of 5'- or 3'-flanking sequences of the MCP-1 gene or within its coding sequences. The distinct induction kinetics of the MCP-1 gene, and other slow immediate-early genes such as c-myc, might therefore reflect the action of cis-acting genomic elements distinct from the trio described for c-fos.

An obstacle to analysis of cis-acting regulatory elements for the slow immediate-early genes has been achieving regulated expression of these genes in transient transfections. This problem was solved recently for MCP-1 with the discovery of a 7-base motif, TTTTGTA, located in the proximal 3' MCP-1 untranslated sequences. This 7-mer is essential for regulated expression of MCP-1 in transient transfections (41). The discovery of the 3' 7-mer enabled detection of a 240-bp DNA enhancer fragment located 2.3 kb upstream of the MCP-1 transcription start site. The 240-bp fragment contains a cis-acting regulatory element(s) for serum, double-stranded RNA, interleukin-1α, and PDGF (41). When ligated to a truncated MCP-1 reporter gene, the 240-base fragment mediates a slow and sustained induction of the reporter gene, which parallels the response of the endogenous MCP-1 gene to serum and PDGF.

DNase I footprinting of the 240-bp MCP-1 enhancer fragment

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The abbreviations used are: PDGF, platelet-derived growth factor; bp, base pair(s); kb, kilobase(s); CIP, calf intestinal phosphatase; HMG, high mobility group.
highlights four discrete sequences protected by nuclear extracts prepared from PDGF-treated murine 3T3 fibroblasts. Two of these sequences were described previously (42). We now focus on a novel PDGF response element which binds NF-κB and a 90-kDa phosphoprotein.

**EXPERIMENTAL PROCEDURES**

**Growth Factors and Reagents—**Recombinant B:B isofrom of PDGF was obtained from Upstate Biotechnology, Inc. Ribonuclease A was from Pharmacia. Proteinase K, ribonuclease T1, calf testical phosphatase (CIP), and poly(dI-dC) were from Boehringer Mannheim Biochemicals. Hydrazinolysis 6,6'-dimethyl-2,2'-dithione-bridged platelet-poor plasma was prepared as described (43). Monodonal antibodies to phosphoserine, phosphothreonine, and phosphotyrosine were from Sigma. Polyclonal antibodies to Rel, Rel A, Rel B, p50, p52, and J unD, and the peptides these antibodies were raised against, were obtained from Santa Cruz Biotechnology. Synthetic oligonucleotides were generated by Macromolecular Resources.

**Cell Culture, DNA Transfections, Stimulation Assays, and RNA Preparation and Analysis—**NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum. NIH-3T3 cells were used for transient transfections because of their significantly greater transfection efficiency compared with Balb/c-3T3 cells. Twenty hours prior to transfection, the media were changed to a density of 3.8 × 10⁴ per 15-cm tissue culture dish. Calcium-phosphate-mediated DNA transfections were performed as described (10) with the following modifications. Cells were exposed to DNA-containing precipitates for 6 h followed by a 2-min 20% glycerin shock. Cells were then washed with Dulbecco's modified Eagle's medium and placed in Dulbecco's modified Eagle's medium plus 5% platelet-poor plasma. Because of variable background levels of MCP-1 transcripts in transfected cells made quiescent with 0.5% calf serum, we have found it more reliable to use 5% platelet-poor plasma for making transfected cells quiescent after the glycerin shock. Transfection mixtures included 35 μg of tagged MCP-1 reporter constructs together with 3.5 μg of α-globin reference construct (pSVu-1), a gift from M. Greenberg (Harvard Medical School), per 15-cm tissue culture plate. After 40–44 h in 5% platelet-poor plasma, quiescent cells were exposed to the B:B isofrom of PDGF (30 ng/ml) for the times indicated in the figure legends. Total RNA was prepared by the guanidinium isothiocyanate method and purified on CsCl gradients (44).

**RESULTS**

**PDGF Induction of MCP-1 Involves Three Distinct DNA Elements—**DNase I footprinting of the 240-bp MCP-1 enhancer fragment highlighted four discrete elements protected by nuclear extracts prepared from PDGF-treated murine 3T3 fibroblasts (42). Initial experiments examined the contributions of each of the four elements compared to the PDGF induction observed with the intact 240-bp MCP-1 5' enhancer. The four elements were added back individually to a 5'-truncated non-inducible MCP-1 reporter gene and the resulting constructs tested for PDGF-inducibility in transient transfections. MCP-1 element constructs containing elements II, III, or IV were all PDGF-inducible in these experiments (Fig. 1A). Summing the inductions observed with the single element addback constructs at 1.5 or 3 h of PDGF treatment equaled the induction observed with the 240-bp addback construct at either time (Fig. 1). These latter findings suggest that synergistic interactions between the four elements do not occur to a significant degree in transient transfections. Second, the major elements conferring PDGF-inducibility onto the truncated MCP-1 gene are elements II and IV (Fig. 1).

**PDGF-inducible and Constitutive Proteins Bind Element IV—**Synthetic oligonucleotides corresponding to element IV were employed as double-stranded DNA probes in mobility shift assays using nuclear extracts prepared from quiescent and PDGF-stimulated fibroblasts. In these experiments both a strongly PDGF-inducible protein complex, and a more rapidly migrating constitutively present complex, bound labeled element IV (Fig. 2a, upper arrow and lower arrow, respectively). Both element IV binding complexes were also observed in nuclear extracts prepared from PDGF-stimulated fibroblasts concurrently treated with cycloheximide (data not shown). Binding of both complexes was specific as it was competed by unlabeled oligonucleotide corresponding to element IV but not competed by an unrelated oligonucleotide corresponding to MCP-1 element IV.
Rel A and p90 Involved in PDGF Induction of MCP-1

**Fig. 1. PDGF induction of MCP-1 involves three distinct elements within a 240-bp enhancer fragment.** A, shown in schematic form (construct T) is the structure of the 5' end of a non-inducible tagged MCP-1 reporter gene (pGMJE1) which includes 466 bp of 5'-flanking sequences and 1.9 kb of 3'-untranslated sequences. All additional constructs are derived from construct T by addition of the shown 240-bp fragment or double-stranded oligonucleotides. The 240-bp fragment is present in the distal 5' MCP-1 flanking sequences (with ends at -2537 and -2298 bp relative to the MCP-1 start of transcription) and is added back in the in vivo orientation. Sequences of MCP-1 elements I-IV are listed under "Experimental Procedures." The rightward-facing bold arrow above each element denotes readaddition in the in vivo orientation. B, RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 35 μg of the shown constructs, allowed to become quiescent, and then not exposed (−) or exposed to the B:B isofrom PDGF (30 ng/ml) for 1.5 or 3 h. The upper and lower arrows highlight the 305- and 241-nucleotide protected fragments corresponding to expression of transfected MCP-1 constructs and endogenous MCP-1, respectively. PhosphorImager images were designed to optimally demonstrate expression of the transfected constructs. Equal inductions of the 240-bp addback construct and endogenous MCP-1 were observed in all experiments. The experiment was performed three times with similar results. PDGF inductions at the 3-h time point were quantitated on a PhosphorImager. The inductions observed at 3 h with elements I, II, III, and IV were 4, 31, 19, and 54%, respectively, of the induction observed with the intact 240-bp addback construct (set at 100%). C, RNase protection assays on 15 μg of total cellular RNA from the transfections shown in panel B with an α-globin riboprobe.

To better delineate the precise sequences required for protein binding to the 22-base element IV, we performed mobility shifts using a series of mutant competitors (i.e., seven, 22-base long, double-stranded oligonucleotides containing non-overlapping, two to five base, mutations spanning the length of element IV). Sequences required for binding of the PDGF-inducible and/or constitutive protein complexes to element IV, when altered in a mutant oligonucleotide, should result in diminished or absent competition of one or both complexes by the given mutant oligonucleotide competitor. Mutants 1, 5, 6, and 7 quantitatively competed all protein binding to element IV (Fig. 2b), suggesting that the sequences altered in these four mutants are not required for protein binding to element IV. In contrast, mutants 3 and 4, corresponding to mutations in the first half and second half of the decameric kB motif (GGGAGATTTCC) contained within element IV, respectively, did not compete the PDGF-inducible complex binding element IV, and only partially competed the constitutive complex binding element IV (Fig. 2b). Mutant 2, containing three altered nucleotides immediately 5' to the kB motif, quantitatively competed the PDGF-inducible complex binding element IV. Mutant 2, however, only partially competed constitutive binding to element II (Fig. 2a). An oligonucleotide consisting of two tandem NF-kB binding sequences also competed PDGF-inducible binding to element IV. In contrast, the NF-kB oligonucleotide did not compete the constitutively bound complex (data not shown), suggesting that the latter is distinct in nature from the PDGF-inducible complex. These data also demonstrate that binding of the constitutive complex occurs independently of PDGF-induced binding to element IV.

**Fig. 2. PDGF-inducible and constitutive proteins bind element IV.** a, radiolabeled double-stranded oligonucleotide probe, corresponding to element IV, was used in mobility-shift assays with 15 μg of nuclear extracts prepared from quiescent fibroblasts (−) or fibroblasts treated with the B:B isofrom PDGF (30 ng/ml) for 1.75 h (−). Increasing amounts of each of the shown unlabeled double-stranded oligonucleotides were used as competitors in 100-, 250-, and 1000-fold excess. Upper and lower arrows show the positions of the PDGF-inducible and constitutive element IV binding complexes, respectively. Free probe is not shown in this experiment. No complexes were observed with probe alone in the absence of extract (data not shown). b, radiolabeled double-stranded oligonucleotide probe, corresponding to element IV, was used in mobility-shift assays with 15 μg of nuclear extracts prepared from fibroblasts treated with the B:B isofrom PDGF (30 ng/ml) for 1.75 h. The shown double-stranded oligonucleotides, corresponding to unlabelled wild type element IV (wt), 7 non-overlapping mutants of element IV (M1-M7), element II (II), or element III (III) were used as competitors in 250-fold excess. Sequences of the oligonucleotide competitors are listed under "Experimental Procedures." Upper and lower arrows show the positions of the PDGF-inducible and constitutive element IV binding complexes, respectively. Free probe is not shown in this experiment. No complexes were observed with probe alone in the absence of extract (data not shown). c, A, shown in schematic form (construct T) is the structure of the 5' end of a non-inducible tagged MCP-1 reporter gene (pGMJE1) which includes 466 bp of 5'-flanking sequences and 1.9 kb of 3'-untranslated sequences. All additional constructs are derived from construct T by addition of the shown 240-bp fragments or double-stranded oligonucleotides corresponding to wild type element IV (wt) or element IV mutants 3 and 4 (mut 3 and mut 4, respectively). Sequences of the oligonucleotides are listed under "Experimental Procedures." The rightward-facing bold arrow above each element denotes readaddition in the in vivo orientation. B, RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 35 μg of the shown constructs, allowed to become quiescent, and then not exposed (−) or exposed to the B:B isofrom PDGF (30 ng/ml) for 1.5 or 3 h. The upper and lower arrows highlight the 305- and 241-nucleotide protected fragments corresponding to expression of transfected MCP-1 constructs and endogenous MCP-1, respectively. PhosphorImager images were designed to optimally demonstrate differences in expression of the transfected constructs. C, RNase protection assays on 15 μg of total cellular RNA from the transfections shown in panel B with an α-globin riboprobe.
mediated induction of correlation between protein binding to element IV and PDGF-
creasing times. A low basal level of the PDGF-activable com-
quiescent fibroblasts or fibroblasts treated with PDGF for in-
ment IV was determined using nuclear extracts prepared from
Kinetics—The time course of PDGF-activated binding to ele-
complexbindingelementIVappears to be a site partially overlap-
in vivo (Fig. 2). The minimal PDGF-inducibility noted in the NF-
pared in protein binding to element IV. These data also establish a
in vitro mobility shift competition experiments. Only those antibodies specific for Rel A (formerly
 characteristic of binding to element IV addback construct) (Fig. 2)
characterized that PDGF-inducible and constitutive protein binding to element IV is a form of NF-
inducible complexes binding element IV, respectively. Addition of the
protein complexes were observed in mobility shifts using PDGF-activating element IV. In agreement
with this prediction, similar PDGF-inducible and constitutive protein binding to element IV was
in the wild type element IV (Fig. 2). The upper arrow shows the positions of the supershifted and
untreated element IV. The middle arrow shows the positions of the unaltered PDGF-
inducible complexes binding element IV. As would be predicted from these data, PDGF-inducible
complexes binding element IV is a form of NF-
parallel the slow kinetics of PDGF-activated binding to element IV. In wild type element IV addback
construct, the slow kinetics of PDGF-activated binding to element IV closely
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raised against Rel B (data not shown), p50, p52, and Rel, as well as the unrelated transcription factor JunD, were also tested and did not supershift the PDGF-inducible species (Fig. 4, A). Two doses of anti-Rel A and anti-p50 (1 and 3 μg) were used in these experiments. PDGF-inducible binding to element IV was quantitatively supershifted upon addition of 1 μg of anti-Rel A (Fig. 4, A, lane 3). In contrast, PDGF-inducible binding to element IV was somewhat diminished, in the absence of a supershift, upon addition of the larger amount of anti-p50 (Fig. 4, A, lane 6). As a positive control for the p50 antibodies used in these experiments, we demonstrated that these antibodies could supershift recombinant p50 homodimers bound to element IV (data not shown). Given that the p50 antibodies quantitatively supershifted a p50-containing complex in these additional mobility shifts, it is unlikely that the PDGF-inducible complex binding element IV contains the p50 subunit of NF-κB. Last, selective UV cross-linking of the PDGF-inducible complex binding element IV detected only a single 65-kDa protein, consistent with the known mass of Rel A (65 kDa) (Fig. 4, B). Taken together, these data suggest that the NF-κB species comprising PDGF-inducible binding to element IV is a Rel A homodimer. It should be noted that the anti-p50 (Fig. 4, a) did not yield a supershift, upon addition of the larger amount of anti-p50 (Fig. 4, A, lane 6). As a control for the integrity of the cytoplasmic extracts, PDGF-inducible binding to element IV was observed in both nuclear and cytoplasmic extracts (Fig. 5, B, upper arrow).

PDGF Induction of MCP-1 Requires Both NF-κB and p90—Mutational analysis of element IV established a correlation between PDGF-inducible and constitutive (p90) proteins binding element IV, and PDGF induction of MCP-1 (Fig. 2, c). Those data did not, however, resolve whether both species together are required for PDGF induction of MCP-1. To address this question, additional mutants of element IV were generated which bound either p90 or NF-κB, but not both, and their PDGF-inducibility determined in transient transfection experiments. Mutant 2 was described previously (Fig. 2) and contains 3 altered nucleotides immediately 5′ to the element IV κB motif. In mobility shift assays, p90 only minimally bound mutant 2 probe (seen only upon overexposure of the gel), whereas PDGF-inducible NF-κB binding to mutant 2 was undiminished compared with the wild type element IV probe (Fig. 6a). A second mutant, mutant 9, contains 2 altered nucleotides at the 3′ end (positions 9 and 10) of the decameric element IV NF-κB binding site. In mobility shift assays p90 bound equally well to mutant 9 and wild type element IV probes, whereas the PDGF-inducible NF-κB species bound a mutant 9 probe very poorly compared with its binding to a wild type element IV probe (Fig. 6, a).

Mutant 2 and 9 addback constructs exhibited considerably decreased PDGF inductions at both 1.5 and 3 h of stimulation compared with the wild type element IV addback construct in transient transfections (62 and 78% decreases at 3 h, respectively, the time of maximum induction of the wild type element IV addback construct) (Fig. 6, b). Hence, two element IV mutants which bind either the PDGF-inducible NF-κB (Rel A) species or p90, but not both, each confer significantly decreased PDGF-inducibility on a non-inducible MCP-1 reporter construct when added back in single copy. These data suggest that both NF-κB and p90 together are required for optimum PDGF induction of MCP-1 via element IV.

p90 is a Serine/Threonine Phosphoprotein—Pretreatment of nuclear extracts from quiescent or PDGF-stimulated fibroblasts with CIP decreased the migration of the p90 complex in native gels, but did not significantly decrease p90 binding to element IV (Fig. 7, A, lower arrow). In contrast, migration of the PDGF-inducible complex binding element IV was not significantly altered by CIP treatment (Fig. 7, A, upper arrow). As an additional control, no difference in migration of the p90 complex was noted after pretreatment of nuclear extracts with CIP storage buffer alone (Fig. 7, A). Furthermore, no change in migration of free element IV probe was noted after pretreatment of nuclear extracts with CIP or storage buffer alone (data not shown). Changes in p90 migration after CIP treatment are consistent with a loss of phosphate(s), thereby decreasing the mobility of p90 in native gels through loss of negative charges.

To determine the identity of the phosphorylation site(s) on p90, nuclear extracts prepared from quiescent or PDGF-treated fibroblasts were used in mobility "supershift" assays in constitutive complexes and PDGF-induced complexes. In both nuclear and cytoplasmic extracts prepared from quiescent fibroblasts (data not shown), selective UV cross-linking of the constitutive complex binding element IV was excised from the nondenaturing gel and resolved by electrophoresis in an 8% SDS-polyacrylamide gel. Molecular mass standards (kDa) are shown at the left. Similar results are observed with 500 μl UV irradiation (data not shown). No cross-linking was observed in the absence of UV irradiation (data not shown). B, a radiolabeled double-stranded oligonucleotide probe corresponding to element IV was used in mobility-shift assays with 15 μg of nuclear (N) or cytoplasmic (C) extracts prepared from quiescent fibroblasts (−) or fibroblasts treated with the B:B isofrom of PDGF (30 ng/ml) for 1.75 h (+). Upper and lower arrows show the positions of the PDGF-inducible and constitutive element IV binding complexes, respectively. Free probe is not shown in this experiment. No complexes were observed with probe alone in the absence of extract (data not shown).
FIG. 6. PDGF induction of MCP-1 requires both NF-κB and p90. A, radiolabeled double-stranded oligonucleotide probes, corresponding to wild type element IV (wt) and element IV mutants 2 or 9 (mut 2 and mut 9, respectively), were used in mobility shift assays with 15 μg of nuclear extracts prepared from quiescent fibroblasts (–) or fibroblasts treated with the B:B isofrom of PDGF (30 ng/ml) for 1.75 h (+). Sequences of the oligonucleotides are listed in “Experimental Procedures.” The upper and lower arrows show the positions of the PDGF-inducible and constitutive (p90) element IV binding complexes, respectively. Free probe is not shown in this experiment. No complexes were observed with probe alone in the absence of extract (data not shown). B, A, shown in schematic form (construct T) is the structure of the 5′ end of a non-inducible tagged MCP-1 reporter gene (pGMJE-1) which includes 466 bp of 5′-flanking sequences and 1.9 kb of 3′-untranslated sequences. The additional constructs are derived from construct T by addition of the shown double-stranded oligonucleotides corresponding to wild type element IV (wt) or element IV mutants 2 and 9 (mut 2 and mut 9, respectively). The rightward-facing bold arrow above an element denotes readdition in the in vivo orientation. B, RNase protection assays on 30 μg of total cellular RNA from fibroblasts transfected with 35 μg of the shown constructs, allowed to become quiescent, and then not exposed (–) or exposed to the B:B isofrom of PDGF (30 ng/ml) for 1.5 or 3 h. The upper and lower arrows highlight the 305- and 241-nucleotide protected fragments corresponding to expression of transfected MCP-1 constructs and endogenous MCP-1, respectively. Phosphorimagger images were designed to optimally demonstrate differences in expression of the transfected constructs. C, RNase protection assays on 15 μg of total cellular RNA from the transfections shown in B with an α-globin riboprobe.

FIG. 7. p90 is a serine-threonine phosphoprotein. A, a radiolabeled double-stranded oligonucleotide probe corresponding to element IV was used in mobility shift assays with 15 μg of nuclear extracts prepared from quiescent fibroblasts (–) or fibroblasts treated with the B:B isofrom of PDGF (30 ng/ml) for 1.75 h (+). CIP pretreatment was with 2 units/μl for 30 min at 30°C. Buffer denotes addition of the CIP storage buffer alone for a 30-min incubation at 30°C. Upper and lower arrows show the positions of the PDGF-inducible and constitutive element IV binding complexes, respectively. Free probe is not shown in this experiment. No complexes were observed with probe alone in the absence of extract (data not shown). B, A, radiolabeled double-stranded oligonucleotide probe corresponding to element IV mutant 9 was used in mobility shift assays with 15 μg of nuclear extracts prepared from quiescent fibroblasts (–) or fibroblasts treated with the B:B isofrom of PDGF (30 ng/ml) for 1.75 h (+). S, monoclonal anti-phosphoserine antibodies (Ab) added to supershifts (25 and 50 μg in lanes 2 and 3 or 8 and 9, respectively). T, monoclonal anti-phosphothreonine antibodies added to supershifts (6 and 13 μg in lanes 4 and 5 or 10 and 11, respectively). Y, monoclonal anti-phosphotyrosine antibodies added to supershifts (33 μg in lanes 6 and 12). Only the constitutive (p90) element IV binding complexes is shown. Free probe is not shown in this experiment. No complexes were observed with probe alone in the absence of extract (data not shown).

DISCUSSION

Three Discrete PDGF-regulated Genomic Elements Mediate Induction of the MCP-1 Gene—The mechanisms underlying PDGF induction of a growing class of slow immediate-early genes, induced with slower kinetics than the well studied c-fos gene, remain incompletely resolved. In this study we show that four distinct elements, contained within a 240-bp PDGF-regulated enhancer present 2.3 kb upstream of the MCP-1 transcription start site, are capable of restoring PDGF-inducibility to different degrees in transient transfections (Fig. 1). Interestingly, the sum of the inductions obtained with the individual elements is similar to the PDGF induction obtained with the complete 240-bp enhancer (Fig. 1), suggesting that synergistic interactions between the four elements are not occurring to a significant extent in these transfections. We have shown previously that the proteins binding MCP-1 elements II, III, and IV are unrelated in mobility shift competitions (elements I and IV contain similar sequences and bind related PDGF-activated proteins) (42), suggesting that PDGF induction of MCP-1 in fibroblasts occurs via at least three different PDGF-activated signaling pathways.

Whether the three PDGF-activated signal transduction pathways originate from different signal-generating regions of the PDGF receptor, or represent bifurcations from a common cytoplasmic or membrane signaling protein, remains to be determined. Given the marked sequence dissimilarity of elements II, III, and IV, and the apparent unrelatedness in mobility shifts of the proteins binding these elements, a less likely scenario would include a common signaling intermediate act-
ing directly on all the DNA-binding proteins involved. Recent evidence suggests that src kinase is required for PDGF induction of c-myc, another member of the slow kinetics subset of immediate-early genes (51). Given the conserved nature of control elements among members of the fast kinetics subset of immediate-early genes (17), it is not unlikely that a src kinase is an intermediate in at least one of the pathways involved in PDGF induction of MCP-1.

A PDGF-inducible NF-κB Species and a Constitutively Present Phosphoprotein (p90) Bind Element IV—Mobility shifts using nuclear extracts prepared from quiescent and PDGF-treated fibroblasts demonstrated two distinct protein complexes binding element IV. One complex is PDGF-inducible (Fig. 2a, upper arrow). Evidence presented herein suggests the PDGF-inducible complex binding to element IV is a Rel A homodimeric species of NF-κB (Figs. 2 and 4). Of note, the κB motif present within element IV (GGGAGTTTCC) has been shown previously to be an ideal binding sequence for Rel A homodimers (50).

We have also provided evidence that a second, constitutively binding protein complex contains a 90-kDa nuclear-restricted, serine/threonine phosphoprotein which we have termed p90 (Figs. 5 and 7). Mutational analysis of element IV, most informatively element IV mutants 2 and 9, demonstrated the binding site for p90 partially overlaps the κB site within element IV (Fig. 6). From these results the maximal sequence required for p90 binding can be inferred to be an 11-mer, AATGGGAATTT. Sequence comparisons reveal no known transcription factor-binding sequences matching this predicted 11-bp p90-binding site. These findings suggest p90 is a novel DNA-binding phosphoprotein.

NF-κB and p90 Together Are Required for PDGF Induction of MCP-1 via Element IV—Two element IV mutants, mutants 2 and 9, were shown to bind only NF-κB or p90, respectively, in mobility shifts (Fig. 6). MCP-1 addback constructs containing either mutant 2 or mutant 9, however, demonstrate significantly less PDGF-inducible expression than a wild type element IV addback construct in transient transfections (Fig. 6). Since PDGF induction of MCP-1 has been shown previously to be the result of increased transcription (31, 38–40, 52), these data are strongly consistent with NF-κB and p90 together being required for increased transcription of the MCP-1 immediate-early gene in response to PDGF. p90 therefore appears to function as a coactivator with NF-κB in PDGF induction of MCP-1 via element IV. These results would also predict that in vivo both NF-κB and p90 bind element IV simultaneously, forming a large ternary complex, while participating in PDGF induction of MCP-1. Consistent with this prediction are the results of mobility shift experiments using increasing amounts of nuclear extracts prepared from PDGF-treated fibroblasts, which demonstrated an additional complex binding element IV specifically and migrating more slowly than the individual NF-κB or p90 complexes, under conditions of extract excess (data not shown).

Also consistent with a dual requirement for NF-κB and p90 in PDGF induction of MCP-1, are the results of transient transfections using an element I MCP-1 addback construct. Element I contains a κB motif within its sequences (GGGCTTTTCC), and in mobility shift assays binds a PDGF-inducible complex which co-migrates with the PDGF-inducible (NF-κB) complex binding element IV (data not shown). Element I, however, does not bind p90 (data not shown), and the element I containing MCP-1 reporter construct is only minimally PDGF-inducible in transient transfections (Fig. 1).

Our data suggest that the β-chemokine MCP-1 (36), a member of the slow kinetics subset of immediate-early genes, is one of a small group of genes regulated by the Rel A homodimeric form of NF-κB. Another group has recently suggested a role for NF-κB in inducible transcription of MCP-1 by several tumor cell lines (53). This study, however, did not characterize the species of NF-κB mediating transcriptional stimulation, nor was the apparent requirement for a coactivator with NF-κB noted in the study (53). The latter discrepancy is likely due to the use of chloroamphenicol acetyltransferase constructs. Chloroamphenicol acetyltransferase constructs often exhibit high degrees of basal (unstimulated) expression in transient transfections, thereby minimizing the induction observed after a growth factor or cytokine stimulus (potentially obscuring the effects of a required coactivator with NF-κB).

A large body of literature strongly supports the participation of NF-κB, primarily as a p50/p65 (Rel A) heterodimer, in the transcription of many genes (54–57). In contrast, Rel A homodimers have been proposed to play a role in the transcriptional control of a much smaller group of genes including, intracellular adhesion molecule-1 (58), mucosal vascular addressin cell adhesion molecule 1 (59), interleukin 2 (60), and interleukin 8 (61). It is interesting that the genes for the α-chemokine interleukin-8 (36) and β-chemokine MCP-1 both appear to require activation of the Rel A homodimeric form of NF-κB for transcriptional induction in response to growth factors or cytokines. Given the conserved nature of control elements among members of the fast kinetics subset of immediate-early genes, it is quite possible that Rel A homodimers, or other members of the NF-κB/Rel family of transcription factors, also play a role in growth factor induction of other slow kinetics immediate-early genes such as c-myc.

Our results also suggest that p90 functions as a coactivator with NF-κB participating in PDGF induction of MCP-1. Another coactivator with NF-κB, the high mobility group protein, HMG I(Y), has been shown to operate in viral induction of the human interferon-β gene (62). p90 appears to be distinct from HMG I(Y) in several important respects. HMG I(Y) was described as an approximately 13-kDa basic protein which binds to A-T rich regions within the β-interferon κB site, thereby facilitating the binding of NF-κB to its decameric site (62). Furthermore, HMG I(Y) appears to act specifically with the p50-Rel A heterodimeric form of NF-κB (63). In contrast, p90 is considerably larger than HMG I(Y) and binds to a previously unreported sequence which only partially overlaps the MCP-1 element IV κB motif. p90 also appears to interact with the Rel A homodimeric form of NF-κB. Last, binding of p90 and NF-κB to element IV appear to be mutually independent events (Figs. 2a and 6a). The apparent requirement for p90 in PDGF induction of MCP-1 effectively adds an additional level of regulation on the function of the potent transcription factor NF-κB. Requirements for coactivators like p90, therefore, can be a mechanism by which specificity of growth factor-stimulated transcriptional responses may be achieved using powerful and widely present transcription factors such as NF-κB.

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