

# The Platelet-derived Growth Factor Controls *c-myc* Expression through a JNK- and AP-1-dependent Signaling Pathway\*

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**Pro-inflammatory cytokines, environmental stresses, as well as receptor tyrosine kinases regulate the activity of JNK. In turn, JNK phosphorylates Jun members of the AP-1 family of transcription factors, thereby controlling processes as different as cell growth, differentiation, and apoptosis. Still, very few targets of the JNK-Jun pathway have been identified. Here we show that JNK is required for the induction of *c-myc* expression by PDGF. Furthermore, we identify a phylogenetically conserved AP-1-responsive element in the promoter of the *c-myc* proto-oncogene that recruits *in vivo* the c-Jun and JunD AP-1 family members and controls the PDGF-dependent transactivation of the *c-myc* promoter. These findings suggest the existence of a novel biochemical route linking tyrosine kinase receptors, such as those for PDGF, and *c-myc* expression through JNK activation of AP-1 transcription factors. They also provide a novel potential mechanism by which both JNK and Jun proteins may exert either their proliferative or apoptotic potential by stimulating the expression of the *c-myc* proto-oncogene.**

A wide range of growth factors, cytokines, and mitogens is able to induce the expression of the *c-myc* proto-oncogene (1, 2). In turn, *c-myc* is necessary for cellular proliferation induced by different oncogenic tyrosine kinases (3). In normal cells as well as in tumors, the ability of *c-myc* to control cellular proliferation has been mostly correlated to changes in its mRNA levels through transcriptional and post-transcriptional mechanisms. In fact, most of the oncogenic alterations that target *c-myc* result in the increase of its messenger RNA and, in turn, of its protein (4). Indeed, overexpression or gene amplification and translocations of *c-myc* are frequent causes of numerous solid and blood human tumors (5). In line with its ability to promote cell cycle progression, in quiescent fibroblasts *c-myc* expression is virtually undetectable. However, upon stimulation with growth factors such as the platelet-derived growth factor (PDGF),<sup>1</sup> its mRNA and then protein levels are rapidly induced

until cells progress through the G<sub>1</sub>/S boundary of the cell cycle (6, 7). Still, the mechanism by which growth factors promote the expression of *c-myc* is poorly understood. In this regard, we have recently described a Rac-dependent signaling pathway initiated by PDGF, controlling the expression of the *c-myc* proto-oncogene (6). Nonetheless, the mechanism by which Rac mediates PDGF stimulation of the *c-myc* promoter has remained elusive.

In this study, we show that JNK is required for PDGF induction of *c-myc* expression. Furthermore, we identify a phylogenetically conserved AP-1-responsive element in the human, mouse, and even *Drosophila c-myc* promoter. Such element binds *in vivo* to members of the Jun family of transcription factors, c-Jun and JunD, as indicated by chromatin immunoprecipitation analysis. Finally, we show that through this element PDGF is able to control the activity of the *c-myc* promoter in an AP-1-dependent fashion, implying the existence of a novel signaling pathway linking the PDGF receptor through JNK and Jun proteins to nuclear events culminating in the expression of the *c-myc* proto-oncogene.

## EXPERIMENTAL PROCEDURES

**Reagents**—Human recombinant PDGF (Intergen) was used at a final concentration of 12.5 ng ml<sup>-1</sup>. The selective JNK inhibitor SP600125 (Biomol) was added to the cells 30 min before stimulation at the indicated concentrations. pcDNAIII/GS-Myc-V5 was purchased from Invitrogen. Myelin basic protein was purchased from Sigma. Expression vectors for Rac12V and the corresponding effector domain mutants Rac12V/33N, Rac12V/37L, and Rac12V/40H were kindly provided by C. J. Der (8). The bacterial expression vector pGEX-4T3 glutathione S-transferase-ATF2 and expression vectors for MEKK1 and MLK3 were described previously (9, 10). pcDNAIII-Sis was generated by cloning the *sis* (PDGF) oncogene in the EcoRI and NotI restriction sites. The *pmcAP-1* luciferase reporter vector was obtained by cloning two mouse AP-1 elements in the pGL3 reporter vector (Promega). PCR amplifications of the c-Fos and c-Jun cDNAs were cloned in the pCEFL AU5 and pCEFL AU1 expression vectors, respectively. The JunDBD-Sin3-binding domain of Mad (SID) expression vector was prepared cloning in pCEFL HA the DNA-binding domain of c-Jun and the Sin3-binding domain of Mad. The Gal4-driven luciferase reporter plasmid pGal4-Luc was constructed by inserting six copies of a Gal4-responsive element and a TATA oligonucleotide to replace the simian virus 40 minimal promoter in the pGL3 vector (Promega). The Gal4-VP16 expression

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<sup>1</sup> The abbreviations used are: PDGF, platelet-derived growth factor;

HDACs, histone deacetylases; HA, hemagglutinin; SID, Sin3-binding domain; JNK, c-Jun N-terminal kinase; AP-1, activator protein 1; MAPK, mitogen-activated protein kinase; MEKK, MAPK/extracellular signal-regulated kinase kinase; Erk, extracellular signal-regulated kinase; ChIP, chromatin immunoprecipitation assay; EMSA, electrophoretic mobility shift assay; Stat-3, signal transducer and activator of transcription-3; DBD, DNA-binding domain; Luc, luciferase; ATF, activating transcription factor; MLK, mixed lineage kinase.

vector was prepared cloning the transactivation domain of the VP16 transcription factor in-frame with the DNA-binding domain of Gal4 into the pCDNA III vector. Specific maps and restriction sites will be made available upon request.

**Cell Culture and Transfections**—NIH 3T3 fibroblasts were maintained at 37 °C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) bovine calf serum (BioWhittaker), 2 mM L-glutamine, and penicillin-streptomycin (Invitrogen). NIH 3T3 cells were transfected by the LipofectAMINE Plus reagent or LipofectAMINE reagent (Invitrogen) in accordance with the manufacturer's instructions.

**Northern Blot Analysis**—After 24 h of starvation, NIH 3T3 cells were washed with cold phosphate-buffered saline and total RNA was extracted by homogenization with TRIzol (Invitrogen) in accordance with manufacturer's specifications. Total RNA (10 µg) was fractionated in 2% formaldehyde-agarose gels, transferred to Hybond-XL nylon membranes (Amersham Biosciences), and hybridized with <sup>32</sup>P-labeled DNA probes prepared with the Prime-a-Gene labeling system (Promega). As a probe, we used a 450-bp PstI DNA fragment from the human *c-myc* gene (pcDNAIII/GS-Myc-V5). The RNA membranes were pre-hybridized for >2 h in hybridization solution (ExpressHyb, Clontech) at 70 °C. The <sup>32</sup>P-labeled probe was added to the blots and hybridized for another 16 h at 60 °C. The blots were washed twice for 30 min each in 2× SSC, 0.1% SDS at room temperature and then washed twice for 30 min each in 0.2× SSC, 0.1% SDS at 60 °C. Accuracy of RNA loading and transfer was confirmed by fluorescence under ultraviolet light after staining with ethidium bromide.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assays were performed using the chromatin immunoprecipitation assay kit (Upstate Biotechnology), in accordance with the manufacturer's instructions. Chromatin from NIH 3T3 cells has been fixed by directly adding formaldehyde (1% final) to the cell culture medium. Nuclear extracts have been isolated from the cells and then sonicated to obtain mechanical sharing of the fixed chromatin. Transcription factor-bound chromatin has been immunoprecipitated with specific antibodies, cross-linking has been reversed, and the isolated genomic DNA has been amplified by PCR using specific primers encompassing the murine *c-myc* promoter: forward AP66 (5'-ATACCTGTGACTATTCATT-3') and reverse AP67 (5'-GATGCTTCCTTGCCTAAGAC-3'). The PCR products were separated on a 2% agarose gel. Primers used as a control for the ChIP analysis amplify an unrelated DNA sequence located on murine chromosome 5: forward DE1 (5'-AGGACCCATCCAGATGGAGT-3') and reverse DE2 (5'-CAATGCTCCAATCCTGTCA-3').

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts were obtained from NIH 3T3 cells plated in 10-cm plates and grown to 70% confluency, starved overnight, and then stimulated with PDGF when needed. Cells were washed in cold phosphate-buffered saline and lysed in 400 µl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After 15 min on ice, 25 µl of 10% Nonidet P-40 was added and vigorously vortexed for 10 s. Homogenates were centrifuged for 30 s. Nuclear pellets were resuspended in 50 µl of ice-cold hypotonic buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and rocked at 4 °C for 15 min. Homogenates were centrifuged for 5 min, and the supernatants (nuclear extracts) were aliquoted and stored at -70 °C. After determining protein concentrations using Bio-Rad protein assay (Bio-Rad), 2 µg of proteins were incubated at room temperature with 1 µg of poly(dI-dC) and 0.1 µg of salmon sperm DNA in 20 µl of binding buffer (12 mM HEPES, pH 7.8, 60 mM KCl, 2 mM MgCl<sub>2</sub>, 0.12 mM EDTA, 0.3 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 12% glycerol) for 15 min. Complementary synthetic oligonucleotides containing the AP-1-responsive element plus adjacent sequences from the mouse *c-myc* promoter (AP1F, 5'-ATACCTGTGACTATTCATT-3', and AP1R, 5'-AAATGAATGAGTCACAGGTAT-3') were obtained from MWG Biotech and labeled with [<sup>32</sup>P]ATP using T4 polynucleotide kinase (Invitrogen). Labeled oligonucleotides were purified using G25 columns (Amersham Biosciences) and used as probes (20,000 cpm/reaction) and added to the reactions for an additional 15 min. Complexes were analyzed on non-denaturing (4.5%) polyacrylamide gels in TGE buffer (40 mM Tris, 270 mM glycine, 2 mM EDTA, pH 8.0) and run at 13 V/cm at 4 °C. For supershift assays, 1 µg of the indicated antisera was added to the binding reaction.

**Kinase Assays**—Methods to evaluate the phosphorylating activity of MAPK and JNK by *in vitro* kinase assays have been described previously (11).

**Western Blot Analysis and Antibodies**—Methods to evaluate the phosphorylating activity of MAPK and JNK by *in vitro* kinase assay

have been described previously (11). Lysates of total cellular proteins were analyzed by protein immunoblotting after SDS-PAGE with specific rabbit antisera or mouse monoclonal antibodies. Immunocomplexes were detected by ECL Plus (Amersham Biosciences) by using goat antiserum against rabbit or mouse IgG coupled to horseradish peroxidase (Amersham Biosciences). EMSA, Western blots, immunoprecipitations, and CHIP analysis were performed using rabbit polyclonal antibodies against JNK1 (C-17), Erk2 (C-14), Rac1 (C-14), c-Jun (H-79), JunD (329), JunB (N-17), and ATF2 (C-19) (Santa Cruz Biotechnology); phospho-c-Jun (Ser-63) and phospho-c-Jun (Ser-73) (Cell Signaling Technology); mouse monoclonal antibodies against HA and AU5 epitopes (Berkley Antibody Company); and JNK1 (BD Biosciences).

**Reporter Gene Assays**—For each well, cells were transfected by the LipofectAMINE reagent with different expression plasmids together with 50 ng of the indicated reporter plasmid and 10 ng of pRL-null (a plasmid expressing the enzyme *Renilla* luciferase from *Renilla reniformis*) as an internal control. In all of the cases, the total amount of plasmid DNA was adjusted with empty vector. After 16–20 h from transfection, firefly and *Renilla* luciferase activities present in cellular lysates from serum-starved cells were assayed using the Dual-luciferase reporter system (Promega) and light emission was quantified using the Lumat LB9507 luminometer (EG&G Berthold) as specified by the manufacturer.

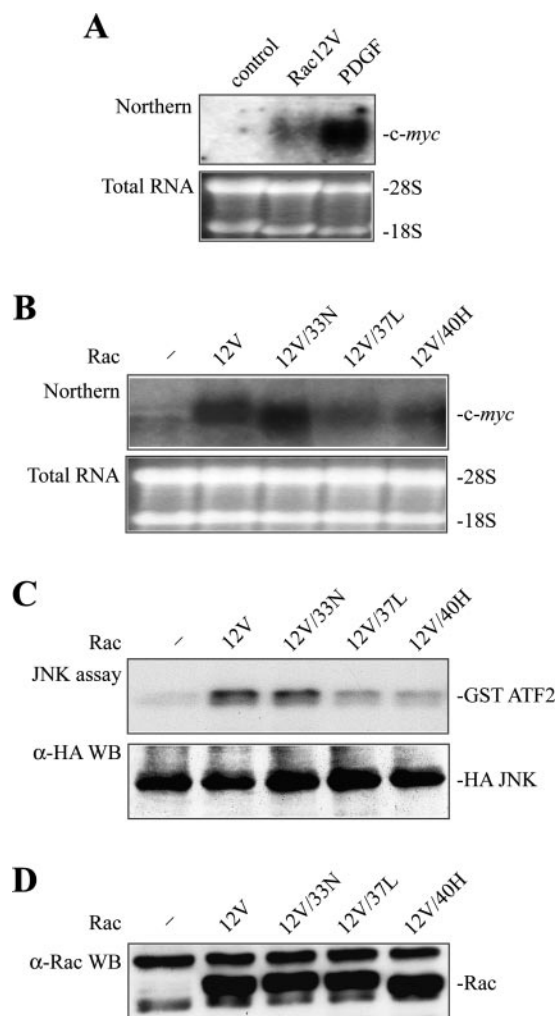
## RESULTS AND DISCUSSION

**Rac Effector Domain Mutants Differentially Impair Endogenous *c-myc* Expression**—To investigate the signaling pathways activated by Rac impinging on the regulation of *c-myc* expression, we used specific constitutively active Rac effector domain mutants that are differentially impaired in their downstream signaling activities (8, 12). Therefore, we compared the ability to stimulate *c-myc* expression of a constitutively active Rac12V mutant with that of Rac alleles harboring additional mutations in their effector domain (Rac12V/33N, Rac12V/37L, and Rac12V/40H). We first explored, by Northern blot analysis, the ability of Rac12V to induce *c-myc* expression using PDGF as a positive control. As expected, the activated Rac12V mutant significantly induced *c-myc* expression as evidenced by an increase in the level of *c-myc* mRNA (Fig. 1A). We next investigated the effect of the double mutants. As shown in Fig. 1B, both the Rac12V/37L- and Rac12V/40H-mutated proteins were ineffective in stimulating the expression of *c-myc*, whereas the Rac12V/33N protein was fully competent to induce the transcription of the *c-myc* proto-oncogene.

Recent work has established an impairment of JNK activation as a consequence of the transfection of the Rac12V/37L and Rac12V/40H effector domain mutants (8). In line with these studies, the data in Fig. 1C show that both Rac12V/37L and Rac12V/40H not only were unable to stimulate *c-myc* expression but they were also incapable of stimulating the activity of JNK, thereby suggesting the involvement of this kinase in Rac-induced *c-myc* expression. Conversely, the Rac12V/33N-mutated protein activated JNK at a level similar to the positive control, Rac12V (Fig. 1C). All of the Rac mutants were expressed at comparable levels (Fig. 1D). Together, these results strongly suggest that the JNK pathway is involved in the regulation of *c-myc* expression.

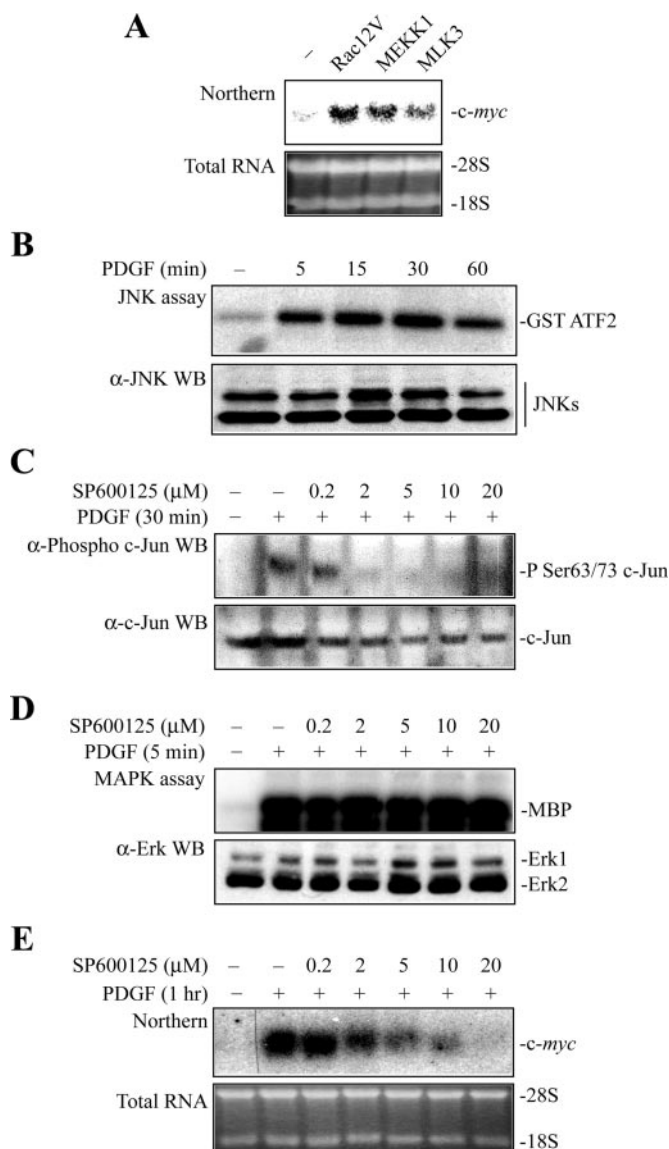
**JNK Activity Is Necessary for PDGF Induction of *c-myc* Expression**—The possibility that JNK participates in the regulation of *c-myc* expression prompted us to test whether the constitutive activation of this signal transduction pathway could stimulate the expression of the endogenous *c-myc* proto-oncogene. Therefore, we transfected NIH 3T3 cells with vectors expressing two upstream activators of the JNK cascade, the MEKK1 and MLK3 MAPK kinase kinases (10, 13). As shown in Fig. 2A, both proteins induced the transcription of the endogenous *c-myc* gene at levels comparable to the positive control Rac12V, indicating that JNK activation is sufficient to trigger the expression of *c-myc*.

Although JNKs have been isolated and characterized as



**FIG. 1. Effects of Rac effector domain mutants on the expression of the endogenous *c-myc* gene.** *A*, analysis of *c-myc* mRNA expression in NIH 3T3 cells stimulated 1 h with PDGF or transiently expressing the activated form of Rac1, Rac12V (2  $\mu$ g). *B*, analysis of *c-myc* mRNA in NIH 3T3 cells transiently transfected with constitutively active Rac effector domain mutants (2  $\mu$ g) as indicated. *C*, stimulation of HA-JNK (1  $\mu$ g) activity in transiently transfected NIH 3T3 cells expressing specific constitutively active Rac effector domain mutants (2  $\mu$ g). *D*, analysis of the expression of the constitutively active Rac effector domain mutants (2  $\mu$ g). –, no treatment; WB, Western blot.

stress-activated kinases based on their strong response to environmental stresses and inflammation stimuli, different growth factors are also able to stimulate their activity (14). Moreover, they have recently been involved in mediating the proliferative effects of some oncogenes, including the product of the *bcr-abl* oncogene (15). Based on our data, we next explored the participation of JNK in the regulation of *c-myc* expression induced by PDGF. We began exploring the ability of this mitogen to activate JNK. As shown in Fig. 2*B*, exposure of NIH 3T3 fibroblasts to PDGF induced the activation of JNK, which peaked 30 min after stimulation. As an approach to examine the involvement of JNK in PDGF-induced *c-myc* expression, we took advantage of the availability of a synthetic compound, SP600125, a reversible ATP-competitive inhibitor that blocks JNK without significantly affecting other related kinases (16). We first confirmed the ability of the drug to inhibit JNK-dependent pathways in our experimental model. Indeed, SP600125 abolished PDGF-induced phosphorylation of the endogenous *c-Jun* protein in a dose-dependent manner as scored by Western blot analysis using a mixture of anti-phospho-Ser-63 and phospho-Ser-73 *c-Jun* antibodies (Fig. 2*C*). Con-



**FIG. 2. A role for JNK in PDGF induction of *c-myc* expression.** *A*, analysis of *c-myc* mRNA expression in NIH 3T3 cells transfected with expression vectors for Rac12V, MEKK1, and MLK3 (2  $\mu$ g each). *B*, stimulation of endogenous JNK activity in NIH 3T3 cells treated with PDGF for the indicated durations. *C*, analysis of serine 63 and serine 73 phosphorylation of the endogenous *c-Jun* in NIH 3T3 cells pretreated with increasing concentrations of the specific JNK inhibitor, SP600125, and then stimulated for 30 min with PDGF. *D*, analysis of MAPK activity in NIH 3T3 cells pretreated with increasing concentrations of specific JNK inhibitor, SP600125, and then stimulated for 5 min with PDGF. *E*, analysis of *c-myc* mRNA in NIH 3T3 cells pretreated with increasing concentrations of the specific JNK inhibitor, SP600125, and then stimulated for 1 h with PDGF. –, no treatment; WB, Western blot.

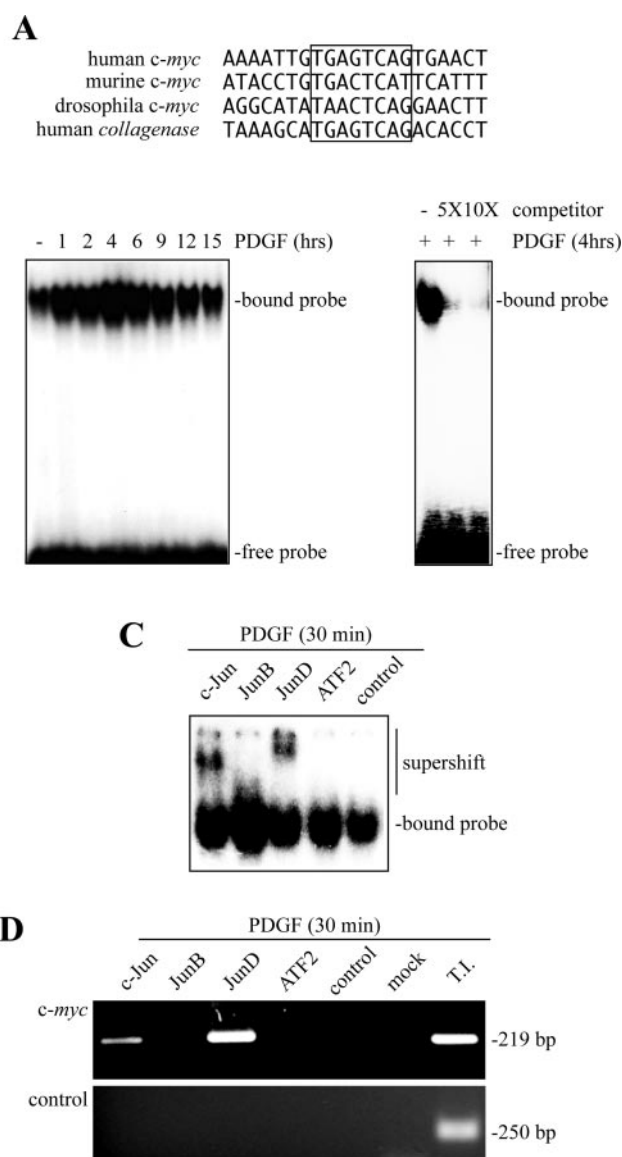
versely, identical concentrations of the drug had no effect on PDGF-induced Erk1/2 activation (Fig. 2*D*) and on Erk-dependent *c-Fos* phosphorylation (data not shown), indicating the specificity of the SP600125 for the JNK pathway as compared with other highly related MAPK-signaling pathways. To test the involvement of JNK in PDGF-induced *c-myc* expression, we performed Northern blot analysis on NIH 3T3 cells pretreated with increasing concentrations of the JNK inhibitor and then stimulated with PDGF for 1 h. As a result, the drug strongly inhibited PDGF-induced *c-myc* expression, even at the lowest concentration of the drug (Fig. 2*E*). Remarkably, the kinetic of inhibition of *c-myc* expression was coincident with the results obtained for the inhibition of PDGF-induced JNK activation by SP600125 (Fig. 2*C*). Thus, the emerging picture from these



data is that activation of the JNK pathway may regulate *c-myc* expression and, in turn, PDGF exploits JNK as a key molecule to promote *c-myc* expression, possibly through phosphorylation and activation of nuclear transcription factors.

**A Typical AP-1-responsive Element in the *c-myc* Promoter**—Two principal promoters, P1 and P2, drive the transcription of the human *c-myc* gene (17). Despite the extraordinary complexity in the regulation of *c-myc* expression, the rate of transcription from these two promoters is mainly governed by composite negative and positive regulatory elements comprised within a 2.3-kb domain located upstream of the promoters (18). Among these elements, E2F, Stat-3, NF- $\kappa$ B, and F cell receptor 4-binding sites have been identified (19–22). In search for additional responsive elements that could mediate the JNK-dependent regulation of the *c-myc* gene, we performed computer-assisted analysis of its promoter region by the TRANSFAC data base (23). Surprisingly, we could identify 1.3 kb upstream the human *c-myc* transcription start site, a TGAGTCA motif perfectly matching the canonical AP-1-responsive element (Fig. 3A) (24). Interestingly, a similar analysis found conserved responsive elements also in the promoters of murine and even *Drosophila c-myc* genes (Fig. 3A). The sequences of the respective responsive elements were highly related to each other (Fig. 3A, boxed nucleotides) as opposed to their immediate flanking regions, suggesting that a strong selective pressure was exerted to maintain these sites intact during evolution.

Several short sequences similar to known response elements are frequently found in promoter regions of a variety of genes. However, the arrangement of these sites in relation to neighboring sequences often determines the functionality of the predicted binding site. Thus, we first studied the ability of an oligonucleotide containing the murine *c-myc* AP-1-responsive element plus adjacent sequences to form DNA/protein complexes by means of EMSA. As shown in Fig. 3B, left panel, proteins from NIH 3T3 nuclear extracts recognized and strongly bound the *c-myc* AP-1-responsive element as evidenced by the presence of a shifted complex that was more prominent 4 h after PDGF addition as a consequence of the accumulation of AP-1 proteins in the stimulated NIH 3T3 cells (25). The binding was specific, because it was efficiently competed by adding an excess of unlabeled *c-myc* AP-1 oligonucleotide (Fig. 3B, right panel). To further investigate the nature of the transcription factors bound to the described *c-myc* AP-1 element, we next performed supershift experiments by incubating the binding reactions in the presence of specific antibodies against Jun family members. These proteins have been in fact described as substrates of the JNK signaling pathway, and they could therefore possibly mediate the effect of this kinase on the *c-myc* promoter. As shown in Fig. 3C, both *c-Jun* and JunD antibodies strongly decreased the electrophoretic mobility of the complexes derived from NIH 3T3 cells stimulated 30 min with PDGF, whereas the JunB antibody had a much lower effect. As an additional control, no ATF2 was detected in the complexes (Fig. 3C), in line with the fact that Jun:ATF2 heterodimers bind more efficiently atypical 8-bp TGACGTCA sites (26). Conversely, among Fos proteins, only Fra2 was detected as part of the complexes (data not shown). On the basis of the binding observed *in vitro*, we next examined by ChIP analysis whether members of the Jun family could actually bind *in vivo* the endogenous *c-myc* promoter. In NIH 3T3 cells, ChIP assays clearly demonstrated the binding of both *c-Jun* and JunD to the endogenous *c-myc* promoter 30 min after PDGF addition (Fig. 3D), coincidentally with the time point at which PDGF induces maximal JNK stimulation (see Fig. 2B). Conversely, we did not observe any *in vivo* binding of JunB to the promoter (Fig. 3D). As expected (see above), we could not detect ATF2 bound to the

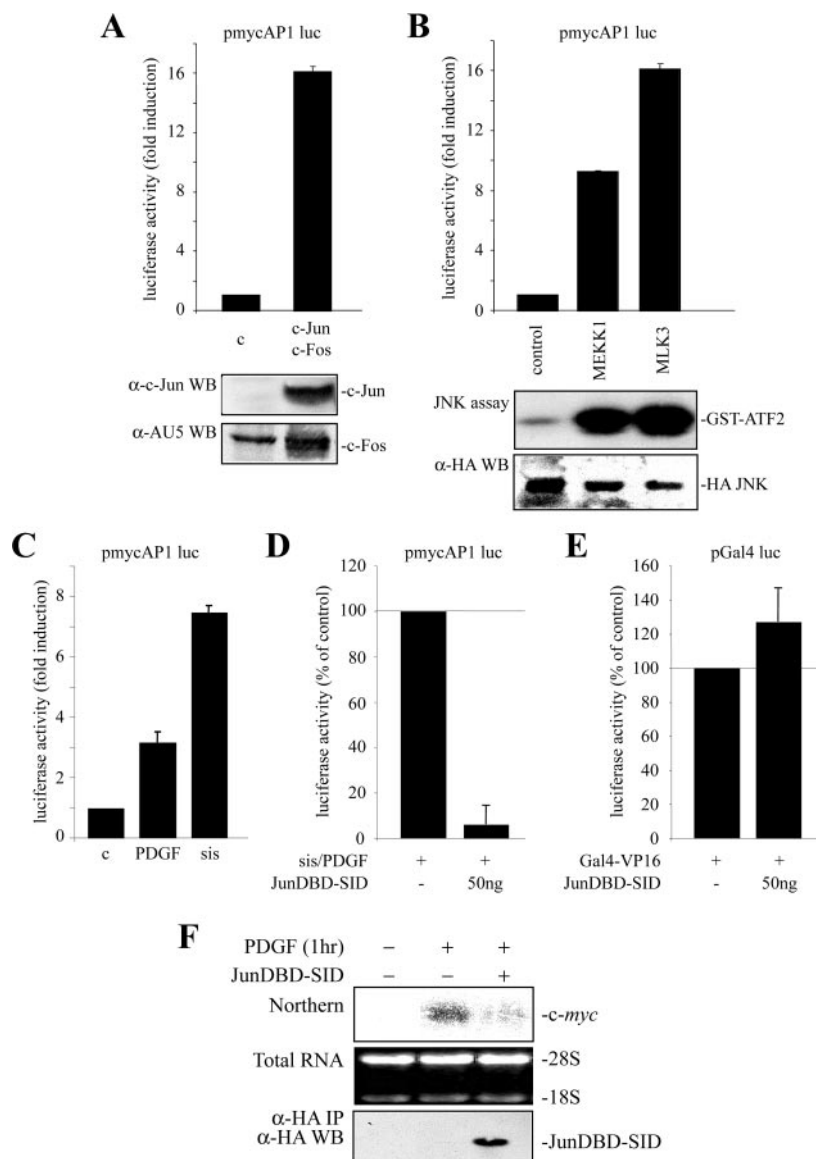


**FIG. 3. A typical AP-1 element in the *c-myc* promoter, recognized by the *c-Jun* and *JunD* AP-1 family members.** A, sequences of predicted AP-1-responsive elements from human, mouse, and *Drosophila c-myc* promoters obtained by the TRANSFAC data base (23). The well established AP-1 element of the human *collagenase* gene is used as a comparison. B, left panel, binding of nuclear proteins to the AP-1-responsive element from the mouse *c-myc* promoter determined by EMSA upon PDGF stimulation of NIH 3T3 cells. Right panel, control of the specificity of the EMSA analysis on the AP-1 element of the mouse *c-myc* promoter using the unlabeled oligonucleotides as competitors in a concentration of 5- and 10-fold-molar excess versus the probe. C, supershift analysis of nuclear factors bound to the AP-1 element of the mouse *c-myc* promoter. D, ChIP analysis of the murine *c-myc* promoter from NIH 3T3 cells treated 30 min with PDGF. Immunoprecipitates from each sample were analyzed by PCR using primers for the mouse *c-myc* promoter as specified under "Experimental Procedures." As a control, a sample representing linear amplification of the total input chromatin was included in the PCR (T.I.). Additional controls included an immunoprecipitation lacking specific antibody (control) and amplification of a sample without chromatin (mock). In the lower panel, samples from the same immunoprecipitates were analyzed by PCR using primers recognizing an unrelated DNA sequence present on murine chromosome 5. -, no treatment.

*c-myc* promoter (Fig. 3D), whereas it was able to bind the *c-jun* promoter, which harbors an atypical 8-bp TGACATCA element (data not shown). The same analysis, performed on untreated quiescent NIH 3T3 cells, gave similar results (data not shown), confirming that Jun family members are pre-bound to their

**FIG. 4. PDGF stimulation of *c-myc* expression is controlled by the AP-1-responsive element.**

**A**, stimulation of the *c-myc* AP-1 in NIH 3T3 cells cotransfected with the pmcAP-1-Luc reporter (50 ng) and expression vectors for c-Jun and c-Fos (0.5  $\mu$ g each). Expression of the AU1-c-Jun and AU5-c-Fos proteins was confirmed by anti-c-Jun and anti-AU5 immunoblots, respectively. **B**, stimulation of the *c-myc* AP-1-responsive element in NIH 3T3 cells cotransfected with pmcAP-1-Luc (50 ng) and expression vectors for MEKK1 and MLK3 (0.5  $\mu$ g each). As a control, NIH 3T3 cells co-transfected with an epitope-tagged HA-JNK (0.5  $\mu$ g) and expression vectors for MEKK1 and MLK3 (0.5  $\mu$ g each) were also subjected to JNK *in vitro* kinase assay (lower panels). **C**, stimulation with PDGF of the *c-myc* AP-1-responsive element in NIH 3T3 cells cotransfected with pmcAP-1-Luc (50 ng). In the case of sis-PDGF, the expression vector for the oncogene (0.5  $\mu$ g) was cotransfected with the reporter (50 ng). **D**, analysis of the stimulation of the *c-myc* AP-1 element by the sis-PDGF oncogene in NIH 3T3 cells cotransfected with pmcAP-1-Luc (50 ng) with expression vector for the sis-PDGF oncogene (0.5  $\mu$ g) and with the AP-1 repressor, JunDBD-SID (50 ng), as indicated. **E**, analysis of the stimulation of the Gal4-responsive element by the Gal4-VP16 chimerical transcription factor in NIH 3T3 cells cotransfected with pGal4-Luc (100 ng) with expression vector for the Gal4-VP16 molecule (0.5  $\mu$ g) and with the AP-1 repressor (50 ng), JunDBD-SID, as indicated. **F**, analysis of *c-myc* mRNA expression in NIH 3T3 cells transfected with an expression vector for the JunDBD-SID molecule (2  $\mu$ g) and then stimulated with PDGF for 1 h. Equal loading of RNA was confirmed. Expression of the HA-epitope tagged Jun DBD-SID protein was confirmed by anti-HA immunoblot performed after anti-HA immunoprecipitation of the samples. -, no treatment; c, control; WB, Western blot; IP, immunoprecipitation.



responsive elements (25) and can be rapidly trans-activated by phosphorylation in response to external stimuli (27). As an additional control, no amplification was observed from the same immunoprecipitates when using primers recognizing DNA sequences unrelated to the *c-myc* promoter (Fig. 3D, lower panel). At this point, it is important to notice that although ChIP analysis was not able to detect binding of JunB to the *c-myc* promoter, EMSA experiments showed a small amount of this protein bound to the *c-myc* AP-1-containing EMSA probes. We have attributed this apparent difference to the *in vitro* nature of the EMSA and its limitations to precisely recapitulate the binding of the transcription factors at the level of the endogenous promoters. At the same time, this situation underscores the importance of the results from the ChIP assay, showing *in vivo* binding of c-Jun and JunD to the promoter. Altogether, these results indicate that proteins of the AP-1 family, specifically c-Jun and JunD, are able to recognize and bind *in vivo* the AP-1 element present in the *c-myc* promoter, thus suggesting this element as a potential mediator of JNK-dependent regulation of *c-myc* expression induced by PDGF.

**The AP-1 Element Controls PDGF Stimulation of *c-myc* Expression**—We next investigated whether the *c-myc* AP-1 element was able to mediate PDGF-induced stimulation of *c-myc* expression. The control of histone acetylation is a key step in

the general regulation of cellular transcriptional events (28). In turn, a model has been recently proposed in which the trans-activation potential of c-Jun and possibly that of its related proteins is constitutively repressed by a histone deacetylases (HDACs) containing complex, which physically interacts with c-Jun itself and can be released upon JNK-dependent phosphorylation of the protein (29). Therefore, we reasoned that an artificial molecule specifically targeting HDACs to AP-1 elements could recapitulate HDAC-dependent negative regulation of AP-1-containing promoters but in a dominant repressive fashion (unable to be relieved by upstream stimuli). Therefore, we engineered a molecule in which the DBD of c-Jun has been fused to the SID. The resulting protein (JunDBD-SID) is able to bind Sin3 and, through this, recruit HDACs (30). We expect this repressor to be able to specifically inhibit transcription by targeting through the c-Jun DBD, AP-1 elements that are present in the endogenous promoters. To control the specificity of the repressor, we first engineered a reporter plasmid carrying the luciferase gene expressed under the control of a tandemly repeated AP-1 element from the murine *c-myc* gene (pmcAP-1-Luc). Such a construct behaves as a typical AP-1 reporter, its activity being readily induced by the c-Jun and c-Fos members of the AP-1 family (Fig. 4A) and by upstream stimulators of the JNK pathway, MEKK1 and MLK3 (Fig. 4B)

(10, 13). As expected, the activity of the pmycAP-1-Luc plasmid was also readily induced by PDGF, its effect being much stronger when the corresponding oncogene (*Sis*) was cotransfected with the reporter (Fig. 4C). We hypothesized that the expression of luciferase from this construct should be strongly inhibited by the JunDBD-SID repressor through specific targeting to the *c-myc* AP-1 and recruitment of HDACs. As expected, very low amounts of the JunDBD-SID repressor were sufficient to completely abolish the activity of the pmycAP-1-Luc reporter induced by *sis*, the oncogenic form of the PDGF-proto-oncogene (Fig. 4D), while not affecting the luciferase activity induced by a Gal4-VP16 (Fig. 4E) or a p53 molecule (data not shown) on their respective reporter vectors. Therefore, these data confirmed the effectiveness and specificity of the engineered protein and the dependence of its activity upon the presence of functional AP-1 elements. It is also important to notice that such experiments not only control the specificity of our approach but also contribute to establish that the *c-myc* AP-1 is a fully functional element that can be stimulated by PDGF and the JNK pathway. This finding further supports the hypothesis that JNK plays a key role in the regulation of *c-myc* expression, possibly induced by PDGF activation of its cognate receptors.

Finally, to prove the ability of the AP-1 element to regulate the transcription of the *c-myc* promoter, we analyzed by Northern blot the RNAs produced by PDGF-treated cells expressing the JunDBD-SID protein. Strikingly, the AP-1 repressor clearly inhibited PDGF-induced accumulation of *c-myc* mRNA (Fig. 4F). Altogether, these findings strongly support the idea that the AP-1 sequence identified in the *c-myc* promoter is functional, being able to control the *c-myc* expression induced by PDGF through the recruitment of members of the AP-1 family of transcription factors. In all, these findings also contribute to understand some of the molecular mechanisms by which *c-Jun* acts as a positive regulator of the cell cycle (24, 27) because only very few *c-Jun* targets involved in the control of the cell cycle have been yet identified. In fact, this study adds *c-myc* to the short list of prototypes genes, such as *cyclin D1* and *p53* (31, 32), that are regulated by *c-Jun* and involved in cellular proliferation. Our finding also show that JunD is bound to the *c-myc* promoter and can regulate *c-myc* expression, which may help to explain the role of this protein as mediator of cellular survival (33, 34).

The ability of JNK to mediate a typical proliferative pathway such as the one connecting PDGF to *c-myc* induction contrasts with the pro-apoptotic effects attributed to JNK in response to different environmental stresses (14). Nonetheless, JNK has been also implicated in mediating survival signals in response to integrins (35), tumor necrosis factor- $\alpha$  (34), and cellular oncogenes (15). Interestingly, recent evidence shows that JunD is able to act downstream of JNK as a sensor that transmit survival or apoptotic signals depending on the state of others transcription factors (34). Two non-mutually exclusive mechanisms are usually taken into account to explain the possibility for JNK to mediate both apoptotic and survival signaling. The latter mechanism considers the possibility that cells may interpret a transient JNK activation as a survival signal as opposed to the apoptotic response elicited by a sustained JNK activity (36). The latter mechanism considers the possibility that JNK signals for cellular survival depend on the cooperation with other pathways such as Akt and NF- $\kappa$ B (34). Indeed, both these mechanisms could operate in our PDGF-dependent system. In fact, we have shown a transient activation of JNK in response to PDGF (Fig. 2B) and PDGF itself is able to contemporarily stimulate multiple signaling pathways, including Akt and NF- $\kappa$ B (37). Nonetheless, the possibility remains that specific stimuli, activating JNK in the absence of pro-survival

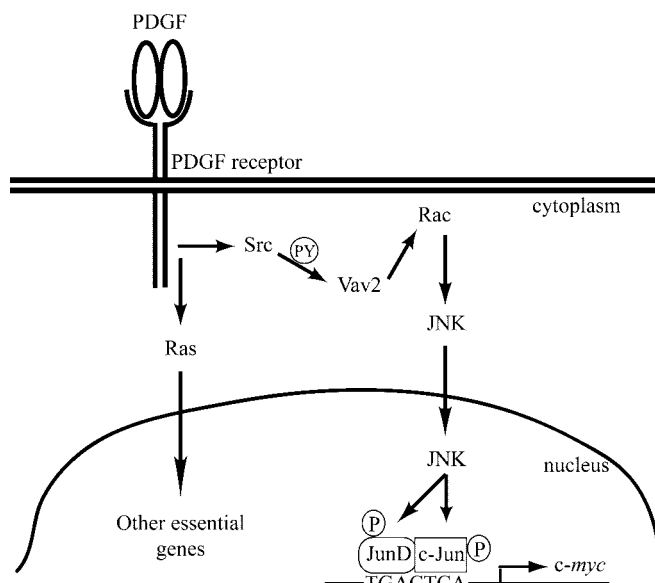


FIG. 5. Schematic representation of the pathway connecting PDGF receptors to the stimulation of *c-myc* expression through the Src-dependent activation of Vav2 and Rac (6) and, in turn, stimulation of JNK activity, impinging on the regulation of the activity of AP-1 transcription factors bound to the promoter of the *c-myc* proto-oncogene.

signals, may induce apoptosis through a *c-myc*-dependent pathway. As *c-myc* itself has been involved in both pro- and anti-apoptotic responses, the mechanism by which regulation of *c-myc* expression by JNK-*c-Jun*/*JunD* relates to these two opposite responses will warrant further investigation.

Our previous results indicated that PDGF induces *c-myc* expression through the Src-dependent activation of the Vav2 exchange factor, acting on the small GTPase Rac (6). By studying the downstream components of the Rac pathway, we now show that JNK and two AP-1 family members, *c-Jun* and *JunD*, are essential components of the signaling cascade that mediate PDGF stimulation of *c-myc* expression (Fig. 5), which significantly establishes a new functional connection between Jun proteins and the *c-myc* proto-oncogene. The proposed pathway also suggests a mechanism by which both JNK and Jun proteins might exert their proliferative or apoptotic potential through the expression of the *c-myc* proto-oncogene. Further work will be required to establish the contribution of the “JNK-*Jun* pathway” to the biological responses of tyrosine kinase receptors such as the PDGF receptors as well as other membrane receptors that use the c-Myc protein to signal cellular proliferation.

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## **The Platelet-derived Growth Factor Controls *c-myc* Expression through a JNK- and AP-1-dependent Signaling Pathway**

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