Induction of NF- κ B-Like Activity by Platelet-Derived Growth Factor in Mouse Fibroblasts

Nancy E. Olashaw,* Timothy F. Kowalik,† Eng-Shang Huang,†‡ and W.J. Pledger*

*Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232; and †Lineberger Comprehensive Cancer Center and ‡Departments of Microbiology and Immunology and Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7295

Submitted October 10, 1991; Accepted August 3, 1992

Nuclear factor kappa B (NF- κ B) modulates the expression of numerous genes via interaction with a specific DNA sequence termed the κ B site. Its activity is modulated by a cytosolic inhibitor protein termed I κ B, and its activation occurs in response to a variety of agents in a variety of cell types, most notably B and T lymphocytes. Data presented here show that an activity (designated complex I) that binds specifically to the κ B site is induced in density-arrested Balb/c-3T3 mouse fibroblasts by platelet-derived growth factor (PDGF), a potent mitogen for these cells. Increased levels of complex I, as evaluated by electrophoretic mobility shift assays of nuclear extracts, were observed in cells treated for 1–4 h (but not 15 min) with the BB isoform of PDGF. 12-O-tetradecanoylphorbol 13-acetate (TPA) and the AA isoform of PDGF also stimulated this response and both isoforms, but not TPA, were effective in cells depleted of protein kinase C. Complex I most likely is authentic NF- κ B, a p50-p65 heterodimer, or a closely related factor because it exhibited properties characteristic of those previously described for NF- κ B including inducibility by deoxycholate and cycloheximide and sensitivity to I κ B. A second κ B binding activity (complex II), which apparently contained p50 homodimers, displayed limited induction by PDGF, whereas a third complex (complex III) migrated faster than but behaved similarly to complex I. These studies suggest that NF- κ B or an NF- κ B-like factor may participate in the expression of PDGF-inducible genes.

INTRODUCTION

Hormone-mediated biological processes such as cell proliferation and differentiation require the expression of specific genes, a process effected by the binding of trans-acting protein factors to regulatory gene elements. The activities of many transcription factors are hormonally controlled; one such hormone-responsive factor is nuclear factor kappa B (NF- κ B),¹ an activity first identified in B cells by its capacity to complex to a 10-bp sequence present in the κ light chain enhancer (the κ B site; Sen and Baltimore, 1986a,b). Activation of NF- κ B

is accomplished by numerous agents including lipopolysaccharide, phytohemagglutinin, and 12-O-tetradecanoylphorbol 13-acetate (TPA; Sen and Baltimore, 1986b) and entails the release of NF- κ B from a cytosolic inhibitor protein termed I κ B and the subsequent translocation of NF- κ B to the nucleus (Baeuerle and Baltimore, 1988a). Data from in vitro studies suggest that dissociation of NF- κ B from I κ B involves phosphorylation of I κ B as I κ B phosphorylated by purified kinases (e.g., protein kinase C) does not bind NF- κ B (Ghosh and Baltimore, 1990). It is noted that NF- κ B consists of two subunits—p50 and p65—both of which share sequence similarity in their DNA binding domains with the products of the *rel* oncogene family (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991) and that NF- κ B interacts with I κ B (both the α and β forms) via

¹ Abbreviations used: EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; GST, glutathione-S-transferase; IGF-1, insulin-like growth factor 1; NF- κ B, nuclear factor kappa B; PDGF, platelet-derived growth factor; TGF β ₁, transforming growth factor β ₁; TPA, 12-O-tetradecanoylphorbol 13-acetate.

p65 (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Zabel and Baeuerle, 1990).

NF- κ B participates in the expression of a variety of genes in a variety of cell types; many of these genes encode cytokines, suggestive of a role of NF- κ B in wound healing and inflammation (reviewed by Baeuerle, 1991). Additionally, at least in some systems, this factor may be involved in growth control. T cell proliferation, for example, is dependent on the expression of two NF- κ B-modulated genes, interleukin-2 (Hoyos *et al.*, 1989) and the interleukin-2 receptor α subunit (Ballard *et al.*, 1988; Leung and Nabel, 1988) and is deregulated by the human leukemia virus HTLV-1 via a process involving activation of NF- κ B by a virally encoded protein (Ballard *et al.*, 1988; Leung and Nabel, 1988).

Although NF- κ B has been extensively studied in lymphocytes, its activity and function in fibroblasts have been incompletely addressed. Platelet-derived growth factor (PDGF) is a potent mitogen for fibroblasts that like T cell mitogens (e.g., phytohemagglutinin and TPA) promotes proliferation by initiating the cell cycle traverse of G₀-arrested cells (Pledger *et al.*, 1977; Stern and Smith, 1986). Biologically active PDGF is a disulfide-linked dimer of two A chains (PDGF-AA), two B chains (PDGF-BB, the product of the *c-sis* protooncogene), or one A and one B chain and interacts with target cells via specific membrane-bound receptors possessing an intrinsic ligand-activated tyrosine kinase activity (reviewed by Olashaw *et al.*, 1991). Binding of PDGF to its receptors elicits the formation of a variety of second messengers including inositol 1,4,5-trisphosphate and diacylglycerol, which mobilize intracellular calcium and activate protein kinase C, respectively (Olashaw *et al.*, 1991). Of the numerous genes transcriptionally induced by PDGF at least three— β -interferon (Kohase *et al.*, 1987; Lenardo *et al.*, 1989), melanoma growth stimulatory activity/*gro* (Bordoni *et al.*, 1989; Anisowicz *et al.*, 1991) and *c-myc* (Kelly *et al.*, 1983; Duyao *et al.*, 1990)—contain κ B sites.

As PDGF both stimulates protein kinase C, a potential mediator of NF- κ B activation, and induces the expression of genes containing κ B sites, we assessed its capacity to activate NF- κ B in Balb/c-3T3 cells, a nontransformed mouse fibroblast line. Our data show that PDGF induces an activity (referred to as complex I) that specifically and in an I κ B-sensitive manner binds the κ B site in electrophoretic mobility shift assays. These data identify NF- κ B or a closely related complex as a putative and previously unrecognized mediator of PDGF-induced gene expression.

MATERIALS AND METHODS

Cell Culture

Balb/c-3T3 and Hela cells were grown in Dulbecco-Vogt modified Eagle's medium supplemented with 10% calf serum, 4 mM L-gluta-

mine, 50 units/ml penicillin, and 50 μ g/ml streptomycin in humidified 5% CO₂-95% air. Confluent cultures were used in all experiments. WEHI 231 cells were grown in suspension and were provided by Dr. A. Lawton (Vanderbilt University).

Preparation of Cytosolic and Nuclear Extracts

Cytosolic and nuclear extracts were prepared essentially as described by Dignam (1983); two or three 100-mm plates of Balb/c-3T3 cells or one of Hela cells or $\sim 5 \times 10^6$ WEHI cells were used for each experimental treatment. Cells were rinsed with phosphate buffered saline and scraped from the plates (Balb/c-3T3, Hela) or pelleted (WEHI), and were lysed in Buffer A (Dignam, 1983) containing 1% Nonidet P40. The supernatant (cytosol) was collected, and the nuclear pellet was rinsed and incubated for 2 $\times$ 15 min in a small volume of Buffer C (Dignam, 1983) containing 0.45 M NaCl. After removal of nuclei by centrifugation, the supernatant (nuclear extract) was diluted to give concentrations of the buffer ingredients as specified for Buffer D (Dignam, 1983) with the exception that final KCl concentration was 50 mM. Cytosolic extracts were also adjusted to Buffer D (Dignam, 1983). All procedures were done at 4°C.

Electrophoretic Mobility Shift Assays (EMSA)

Cytosolic or nuclear extracts were preincubated for 15 min at 4°C in a buffer containing 10 mM Tris (hydroxymethyl) aminomethane (Tris) (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1.8 μ g salmon sperm DNA, 0.2% NP40, and 20 μ g bovine serum albumin. Where indicated, deoxycholate, glutathione-S-transferase (GST), GST/I κ B or cold oligonucleotide was present during the preincubation. Double-stranded end-labeled oligonucleotide (0.25–0.5 ng/reaction) was added and the mixture incubated for 20 min at room temperature; final sample volume was 25 μ l. In some experiments, preimmune serum or p50 antiserum was added at this time and the samples incubated for 3 hr at 4°C. Samples were electrophoresed on 6% polyacrylamide gels containing 25 mM Tris, 22 mM borate, and 0.25 mM EDTA. After electrophoresis, the gels were dried and exposed to film overnight. Synthetic oligonucleotides were end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase before annealing. The sequence of the wild-type NF- κ B probe was 5'-TCGACAGAGGGGACTTCCGAGAGGC-3' and that of the mutant oligonucleotide was 5'-TCGACAGAAATTCCTTCCGAGAGGC-3' (Lenardo *et al.*, 1989).

RESULTS

Stimulation of κ B Binding Activity by PDGF in Balb/c-3T3 Cells

To determine whether PDGF stimulates κ B binding activity in Balb/c-3T3 cells, density-arrested cultures were treated for various times with PDGF-BB or, for comparative purposes, TPA. Activation of NF- κ B by TPA has been observed in numerous systems (e.g., Hela [Sen and Baltimore, 1986b]) and, like PDGF, TPA is mitogenic for Balb/c-3T3 cells (Frantz *et al.*, 1979). After exposure of cells to PDGF-BB or TPA, nuclear extracts were prepared and electrophoretic mobility shift assays (EMSA) performed with a radiolabeled oligonucleotide containing the κ B site of the κ light chain gene (Lenardo *et al.*, 1989). As a positive control, nuclear extracts of TPA-treated Hela cells were also assayed.

As shown in Figure 1, A and B, exposure of Balb/c-3T3 cells to PDGF-BB or TPA for 1–4 h stimulated the formation of a DNA/protein complex (designated com-

plex I) that migrated in the gel at the same position as a complex present in TPA-treated but not untreated Hela cells. This band shift presumably represents specific binding to the κ B site because it was abolished by excess unlabeled wild-type oligonucleotide but was unaffected by excess oligonucleotide containing a mutation in the κ B site that precludes NF- κ B binding (Figure 1C; Lenardo *et al.*, 1989). The kinetics of complex I formation by PDGF-BB and TPA were similar and co-treatment of cells for 4 h with both agents did not increase binding activity above that induced by either agent alone (Figure 1A). It is noted that although protein kinase C is activated within minutes of addition of PDGF-BB and TPA to cells, as is the PDGF receptor tyrosine kinase in response to PDGF-BB (Rodriguez-Pena and Rozengurt, 1985; Kazlauskas *et al.*, 1988), κ B binding activity at 15 min after stimulation was not detectably increased above basal level. The reason for this delay is unclear.

Two additional κ B binding complexes (designated II and III) were also seen in Balb/c-3T3 cells (Figure 1). Although present to a lesser extent than complex I, complex III displayed similar kinetics of induction as did complex I and was effectively competed by excess wild-type but not mutant oligonucleotide. Complex II, which (for the most part) exhibited higher basal levels than complexes I and III, was only minimally elevated by PDGF-BB and TPA and only partially competed. This incomplete competition may reflect the presence of both a specific and a nonspecific DNA binding activity in this band shift.

Specificity of κ B Binding Activity in Balb/c-3T3 Cells

To assess whether stimulation of κ B binding activity was restricted to specific factors or was a generalized response to growth factor treatment, we exposed Balb/c-3T3 cells to epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and transforming growth factor β_1 (TGF β_1), as well as both the BB and AA iso-

Figure 1. Stimulation of κ B binding activity by PDGF-BB and TPA. (A) Density-arrested Balb/c-3T3 cells were prestarved overnight in medium containing 0.5% calf serum. Prestarved cells then received either no addition (lane 1), PDGF-BB (final concentration 20 ng/ml; lanes 2–5), TPA (final concentration 200 nM; lanes 6–9), or PDGF-BB and TPA (both; lane 10) for the indicated times. Nuclear extracts were prepared and 5 μ g analyzed for κ B-binding activity by EMSA. (B) Nuclear extracts of Balb/c-3T3 (10 μ g) and Hela (3 μ g) cells were assayed for κ B binding activity. Hela cells were treated with (lane 2) or without (lane 1) 200 nM TPA for 1 h. Balb/c-3T3 cells were treated with 20 ng/ml PDGF-BB (lane 4) or 200 nM TPA (lane 5) for 2 h or left untreated (lane 3). (C) Nuclear extracts (2 μ g) prepared from untreated Balb/c-3T3 cells (lane 1) or cells treated with 20 ng/ml PDGF-BB for 2 h (lanes 2–10) were assayed for κ B binding activity in the absence (lanes 1 and 2) or presence of excess wild-type (lanes 7–10) or mutant (lanes 3–6) oligonucleotide. Complexes I, II, and III are indicated.

Figure 2. Specificity of PDGF-BB induction of κ B-binding activity. Density-arrested Balb/c-3T3 cells were refed with medium containing 0.25% platelet-poor plasma (vehicle; lane 1) and either 100 ng/ml PDGF-BB (lane 2), 100 ng/ml PDGF-AA (lane 3), 50 ng/ml EGF (lane 4), 10 ng/ml TGF β (lane 5), or 50 ng/ml IGF-1 (lane 6). Cells were harvested 4 h after refeeding. Nuclear extracts (10 μ g) were analyzed for κ B binding activity by EMSA. Upper arrow denotes complex I, lower arrow denotes complex III.

forms of PDGF, for 4 h. Nuclear extracts were prepared and EMSA was performed. Whereas TPA and PDGF act to initiate the cell-cycle traverse of G₀-arrested Balb/c-3T3 cells (Pledger *et al.*, 1977; Frantz *et al.*, 1979), EGF and IGF-1 are required for traverse of early and late G₁, respectively (Leof *et al.*, 1982, 1983); TGF β , while mitogenic for some fibroblast lines (Leof *et al.*, 1986), has no effect on the proliferation of Balb/c-3T3 cells (Pledger, unpublished data). The data in Figure 2 show that EGF and PDGF-AA, like PDGF-BB, stimulated κ B binding (complexes I and III) whereas IGF-1 and TGF β , at least during a 4-h treatment, did not appreciably induce this response; thus, induction of κ B-binding activity in Balb/c-3T3 cells may be limited, at least in part, to factors functioning early in G₀/G₁.

Effect of TPA Pretreatment on PDGF-Stimulable κ B Binding Activity

As noted above, TPA and PDGF-BB, when added in combination to cells, stimulated κ B binding activity to an extent comparable to that produced by either agent alone (Figure 1A). This lack of additivity suggests that these agents induce this response by a similar mechanism, a premise supported by the similar time courses shown in Figure 1A. As the actions of TPA are mediated by protein kinase C (Castagna *et al.*, 1982), an enzyme previously shown to inactivate I κ B in vitro (Ghosh and Baltimore, 1990), we asked whether this kinase was required for the induction of κ B binding activity by PDGF. In these experiments, cells were pretreated for 48 h with 400 nM TPA before addition of PDGF-BB, PDGF-AA, or TPA. Prolonged exposure of cells to TPA has been shown previously to abrogate protein kinase C activity (Rodriguez-Pena and Rozengurt, 1984), a phenomenon verified in our system by immunoblotting of the protein kinase C isozymes (all isozymes detectable in untreated Balb/c-3T3 cells [α , β , δ , ϵ] were not observed in TPA-pretreated cells; Coats, Pledger, and Mochly-Rosen,

unpublished data). As shown in Figure 3, TPA induced κ B binding activity (complexes I and III) in nonpretreated cells but, consistent with the loss of protein kinase C activity, was ineffective when readded to TPA-pretreated cells. In contrast, PDGF-BB and PDGF-AA stimulated this response as efficiently in pretreated cells as in control cells. Thus, the data show that the PDGF isoforms, unlike TPA, retain the capacity to induce κ B binding in protein kinase C-depleted cells and suggest that agents other than or in addition to protein kinase C mediate this action of PDGF.

Involvement of I κ B in the Activation of PDGF-Inducible κ B Binding Activity

Although the first identified, NF- κ B is not the only factor capable of binding the κ B site (Baeuerle, 1991). Thus whether any of the activities in our system represent authentic NF- κ B is not clear. Factors binding the κ B site include those whose activity is modulated by I κ B (e.g., NF- κ B and c-Rel, the product of the *rel* protooncogene) and those whose activity is unaffected by this inhibitor (e.g., KBF1, a p50 homodimer). With regard to this distinction, the data presented in Figure 4 support an involvement of I κ B in the regulation of two of the κ B binding activities (complexes I and III) in Balb/c-3T3 cells. As shown in Figure 4A, exposure of cytosol from quiescent unstimulated Balb/c-3T3 cells to concentrations of deoxycholate up to 0.5% increased progressively the formation of complex I (or of a complex with similar mobility as complex I). Previous studies have shown

Figure 3. Effect of protein kinase C depletion on PDGF induction of κ B binding activity. Density-arrested Balb/c-3T3 cells were incubated in medium containing either 400 nM TPA (lanes 5–8) or 2% platelet-poor plasma and vehicle (lanes 1–4) for 48 h. Cells were either left untouched (lanes 1 and 5) or were refed with medium containing 2% platelet-poor plasma and either 400 nM TPA (lanes 2 and 6), 10 ng/ml PDGF-BB (lanes 3 and 7), or 10 ng/ml PDGF-AA (lanes 4 and 8). Cells were harvested 4 h after refeeding. Nuclear extracts (8.5 μ g) were examined for κ B binding activity by EMSA. Upper arrow denotes complex I, lower arrow denotes complex III.

Figure 4. Deoxycholate and cycloheximide induction of κ B binding activity. (A) Cytosolic extracts were prepared from density-arrested prestarved Balb/c-3T3 cells as described in MATERIALS AND METHODS. Before addition of the labeled κ B probe cytosol (13 μ g) was preincubated for 15 min at 4°C in binding buffer supplemented with 0–0.8% deoxycholate (lanes 3–10). Final Non-idet P40 concentration in the reaction mixture was 0.2%. Nuclear extracts were prepared from untreated (lane 1) or PDGF-BB-treated (20 ng/ml, 2 h; lane 2) Balb/c-3T3 cells; 5.7 μ g were used in the binding assay. (B) Density-arrested prestarved Balb/c-3T3 cells received either no addition (v; lane 1) or 10 μ g/ml cycloheximide (lanes 2–6) for the indicated times. Nuclear extracts (7.8 μ g) were assayed for κ B-binding activity by EMSA. Complexes I, II, and III are indicated.

that deoxycholate activates NF- κ B by disrupting the NF- κ B/I κ B complex (Baeuerle and Baltimore, 1988b); activation of c-Rel κ B binding activity by this detergent has also been observed (Kerr *et al.*, 1991). The deoxycholate-inducible κ B binding activity was ablated by wild-type but not mutant oligonucleotide and, in accord with previous findings of Baeuerle and Baltimore (1988b), was inhibited by concentrations of deoxycholate >0.5% (Figure 4A). Complex II was also present in Balb/c-3T3 cytosol but was not increased by deoxycholate, whereas complex III was not detected.

Cycloheximide, which has been shown to activate NF- κ B in a variety of cell types presumably by preventing resynthesis of I κ B which when dissociated from NF- κ B is unstable (Sen and Baltimore, 1986b; Baeuerle and Baltimore, 1988a), also stimulated κ B binding in Balb/c-3T3 cells (Figure 4B). Increased formation of complexes I and III, but not complex II, was evident and progressive in cells treated for 1–6 h with this protein synthesis inhibitor.

To directly assess the capacity of I κ B to interact with the κ B binding activities in our system, nuclear extracts of Balb/c-3T3 cells were incubated with a glutathione-S-transferase/I κ B (GST/I κ B) fusion protein. This protein was prepared using the I κ B cDNA (termed MAD-3) cloned by Haskill *et al.* (1991) from adherent human monocytes (Kowalik and Huang, unpublished data), and it is noted that inhibition of NF- κ B/DNA binding by *in vitro* translated MAD-3 has been demonstrated previously and apparently occurs via interaction of the MAD-3 protein with the p65 subunit of NF- κ B (Haskill *et al.*,

1991). Regardless of whether nuclear extracts were prepared from PDGF-BB-treated, TPA-treated or untreated cells, inclusion of GST/I κ B in the binding reaction virtually abolished the band shifts corresponding to complexes I and III, whereas GST alone had no effect (Figure 5). This finding indicates that these complexes contain a component (e.g., p65 or c-Rel) that binds I κ B in a manner that precludes interaction with the κ B site. In contrast, the binding of complex II to this site was not affected by the I κ B fusion protein.

Recognition of Complex II by an Antiserum to p50

The κ B-binding activities in Balb/c-3T3 cells were further characterized by use of an antipeptide antiserum to mouse p50 (Ghosh *et al.*, 1990). This antiserum does not recognize c-Rel and has a preferential affinity for p50 homodimers as compared with p50-p65 heterodimers (Sen, personal communication). In our experiments, this antiserum was added to nuclear extracts after the binding reaction; the presence of p50 in the extracts is indicated by the appearance of a low mobility band shift (or supershift) representing the antibody/antigen/oligonucleotide complex, and the concomitant loss of the corresponding higher mobility band. Nuclear extracts of Balb/c-3T3 cells and, as a positive control, untreated WEHI 231 mouse B cells were assayed; previous studies have shown that WEHI cells constitutively express NF- κ B (Sen and Baltimore, 1986a).

As shown in Figure 6, WEHI nuclear extracts contained two κ B binding activities: 1) a faster migrating

Figure 5. Inhibition of complex I and III κ B binding activity by $I\kappa B$ fusion protein. Density-arrested Balb/c-3T3 cells were prestarved overnight in medium containing 0.5% calf serum. Prestarved cells were then received either no addition (lanes 1, 4, and 7), PDGF-BB (final concentration 20 ng/ml; lanes 2, 5, and 8) or TPA (final concentration 200 nM; lanes 3, 6, and 9) for 2 h. Nuclear extracts (6 μ g) were preincubated in binding buffer alone (lanes 1–3) or containing GST (lanes 4–6) or GST/ $I\kappa B$ (200 ng/rxn; lanes 7–9) for 15 min before addition of labeled κ B probe. Complexes I, II, and III are indicated.

form that was unaffected by GST/ $I\kappa B$ but supershifted by p50 antiserum 2) a slower migrating form that was abolished by GST/ $I\kappa B$ but unaffected by p50 antiserum. These findings are consistent with the presence of p50 homodimers, which do not bind MAD-3/ $I\kappa B$ (Haskill *et al.*, 1991), in the faster migrating complex and of p50-p65 heterodimers in the slower migrating complex. The faster migrating complex in WEHI extracts comigrated with complex II in Balb/c-3T3 extracts, whereas the slower migrating complex ran slightly faster than complex I.

Addition of p50 antiserum to nuclear extracts of PDGF-treated Balb/c-3T3 cells supershifted (at least in part) complex II; this supershifted band comigrated with the supershift seen in antiserum-treated WEHI extracts. Preimmune serum did not produce a supershift in either cell line and complexes I and III in Balb/c-3T3 cells were unaffected by the p50 antiserum. These findings indicate that complex II contains authentic p50; whether p50 is also present in complexes I and III in combination with $I\kappa B$ -sensitive factors is not clear but not unlikely.

DISCUSSION

Our studies show that PDGF induces three specific κ B-binding activities in density-arrested Balb/c-3T3 cells (Figure 1). These complexes have been termed complex I, II, and III in order of decreasing mobility in native gels. Similar complexes have been observed in serum-

treated Balb/c-3T3 cells (Baldwin *et al.*, 1991). As evaluated by EMSA of nuclear extracts with a radiolabeled oligonucleotide probe containing the κ B site, complex I was the most dramatically increased by PDGF, whereas complex II showed only limited induction. On the basis of the data presented and as discussed below, we suggest that complex I is NF- κ B (i.e., a p50-p65 heterodimer) or a closely related complex, whereas complex II is, at least in part, KBF1 (p50 homodimer). With regard to complex III, its similar pattern of induction as but greater mobility than complex I and its low abundance suggest that this complex is a proteolytic product of complex I. The capacity of a 42-kd fragment of p50 to dimerize with p65 and bind the κ B site has been demonstrated previously (Baeuerle and Baltimore, 1989).

The salient feature of NF- κ B is modulation of its activity by the cytosolic protein $I\kappa B$. Two forms of $I\kappa B$, termed $I\kappa B$ - α and $I\kappa B$ - β , have been identified (Zabel and Baeuerle, 1990) and both, although structurally and immunologically distinct (Kerr *et al.*, 1991), interact with p65 and not p50 (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Zabel and Baeuerle, 1990). As noted above, activation of NF- κ B requires its release from $I\kappa B$ (Baeuerle and Baltimore, 1988a); a method of activation involving modification but not dissociation of $I\kappa B$ has also been proposed (Kerr *et al.*, 1991). Both complex I and complex III contained a component sensitive to $I\kappa B$.

Figure 6. Recognition of complex II by p50 antiserum. Nuclear extracts were prepared from untreated WEHI 231 cells (lanes 5–8) and density-arrested prestarved Balb/c-3T3 cells either untreated (lane 1) or treated with 20 ng/ml PDGF-BB for 2 h (lanes 2–4). Balb/c-3T3 extracts (2 μ g) and WEHI extracts (2 μ g) were preincubated in binding buffer alone (lanes 1–5, 7, and 8) or containing GST/ $I\kappa B$ (lane 6) for 15 min before addition of labeled probe. Extracts then received either no addition (lanes 1, 2, 5, and 6), 1- μ l preimmune serum (pi; lanes 3 and 7) or 1- μ l p50 antiserum (p50; lanes 4 and 8) and were incubated on ice for 3 h. The Balb/c-3T3 bands were overexposed to allow detection of the supershift. Complexes I, II, and III in Balb/c-3T3 cells are indicated. Large arrow denotes supershift.

As shown in Figure 5, the binding of these complexes to the κ B site was ablated by a GST/I κ B fusion protein prepared from the cDNA encoding the I κ B protein cloned from human monocytes (MAD-3; Haskill *et al.*, 1991). Additionally, agents known to preclude NF- κ B/I κ B interaction induced the formation of complex I (deoxycholate and cycloheximide) and complex III (cycloheximide; Figure 4).

Both p50 and p65 are part of a rapidly growing family of Rel-related κ B-binding proteins that includes v-Rel, c-Rel, RelB, p50B, and the Drosophila protein dorsal (Ballard, *et al.*, 1990; Kabrun *et al.*, 1990; Inoue *et al.*, 1991; Ip *et al.*, 1991; Ryseck *et al.*, 1992; Bours *et al.*, 1992) and previous studies (Bours *et al.*, 1992; Ryseck *et al.*, 1992) have shown that many of these proteins dimerize with each other, at least in vitro, to produce a variety of transcriptionally active DNA binding complexes. As MAD-3 (the I κ B used in our study) is related to I κ B- β (Davis *et al.*, 1991; Inoue *et al.*, 1992), and as I κ B- β has been shown to interact with c-Rel (Kerr *et al.*, 1991), it is possible that the I κ B-sensitive component of complexes I and III is c-Rel and not p65. Bull *et al.* (1989), however, have shown that c-rel mRNA is barely detectable in quiescent NIH-3T3 cells. Although both c-rel message and c-Rel are induced rapidly by TPA in NIH-3T3 cells, our data show that cycloheximide increases the formation of complexes I and III in Balb/c-3T3 cells (Figure 4B) and thus suggest that these complexes do not contain c-Rel as its synthesis would be precluded by cycloheximide.

Although the presence of p65 in complexes I and III remains unproven, data from immunological studies indicate that complex II contains p50 (Figure 6). The presence of p50 homodimers in complex II is consistent with the lack of effect of 1) GST/I κ B on its κ B binding activity (Figure 5) 2) deoxycholate and cycloheximide on its induction (Figure 4). The existence of KBF1 (p50 homodimer) in various cell types and its capacity to bind specific κ B motifs and modulate transcription have been demonstrated previously (Baldwin and Sharp, 1987; Israël *et al.*, 1987; Fujita *et al.*, 1992). As noted above, the p50 antiserum used in our experiments preferentially recognizes homodimerized as compared with heterodimerized p50. Thus, the lack of effect of this antiserum on complexes I and III does not exclude the existence p50 in these complexes. As shown in Figure 6, this antiserum only supershifted an I κ B-insensitive κ B binding activity (presumably KBF1) in WEHI cells, a B cell line known to possess p50-p65 heterodimers (presumably, at least in part, the I κ B-sensitive band shift in Figure 6; Sen and Baltimore, 1986a; Inoue *et al.*, 1991). As p50 is present in our cells, it is likely that it interacts with proteins other than itself to form additional κ B-binding complexes (e.g., complex I and complex III).

Regardless of the identity of the κ B-binding activities in our system, our data show that two of these activities

(complexes I and III) are effectively induced by PDGF in density-arrested Balb/c-3T3 cells. Data examining the growth-factor specificity of this response (Figure 2) suggest that it may be limited to factors functioning in early G₀/G₁. Similarly, a role of NF- κ B-like activity in the G₀/G₁ transition of serum-stimulated Balb/c-3T3 cells has been proposed (Baldwin *et al.*, 1991). Our data also demonstrate that both the AA and BB isoforms of PDGF enhance κ B-binding activity in quiescent Balb/c-3T3 cells (Figures 2 and 3). Previous studies (Hart *et al.*, 1988; Heldin *et al.*, 1988) have shown that whereas PDGF-BB interacts with both α and β PDGF receptors, PDGF-AA recognizes α receptors only; thus, the ability of PDGF-AA to stimulate κ B binding activity indicates that α receptors are capable of generating this response.

As noted above, activation of NF- κ B requires its release from I κ B, a process most likely accomplished by phosphorylation of I κ B. Kinases potentially responsible for I κ B phosphorylation in vivo include protein kinase C and cyclic AMP-dependent protein kinase, both of which have been shown to activate NF- κ B in in vitro assays and to phosphorylate one or both forms of I κ B (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990; Kerr *et al.*, 1991). Although PDGF-BB and PDGF-AA activate protein kinase C (Kazlauskas *et al.*, 1988), down regulation of protein kinase C activity by chronic exposure of cells to TPA did not affect subsequent induction of κ B binding activity by either isoform (Figure 3). Similarly, as reported by others (Meichle *et al.*, 1990; Bomszyk *et al.*, 1991), ablation of protein kinase C activity by either TPA pretreatment or specific inhibitors did not preclude NF- κ B activation in B cells by interleukin-1 or lipopolysaccharide or in T cells by tumor necrosis factor. In these studies as well as ours, TPA stimulated κ B binding in the presence but not the absence of protein kinase C activity; thus both protein kinase C-independent and -dependent pathways may participate in this process. It is possible of course that residual protein kinase C activity or TPA-insensitive forms of this kinase account for the PDGF induction of κ B-binding activity in TPA-pretreated cells.

PDGF stimulation of κ B binding most likely is also independent of cyclic AMP-dependent protein kinase because 1) PDGF does not appreciably increase cellular cyclic AMP content in Balb/c-3T3 cells (Wharton *et al.*, 1982) 2) cyclic AMP agonists only marginally stimulated κ B binding activity (Olashaw, unpublished data). Direct phosphorylation of I κ B by the PDGF receptor tyrosine kinase remains a possibility because some forms of I κ B have been shown to possess a consensus site for tyrosine kinase phosphorylation (Davis *et al.*, 1991; Haskill *et al.*, 1991). Our preliminary data show that vanadate, an inhibitor of tyrosine phosphatases, increases κ B binding activity (complexes I and III) in Balb/c-3T3 cells; whether this reflects tyrosine phosphorylation of I κ B, PDGF receptors, or other substrates is not known. Al-

ternative to I κ B phosphorylation, a mechanism of NF- κ B activation by oxidation-reduction has also been proposed (Staal *et al.*, 1990; Toledano and Leonard, 1991).

PDGF has been shown to modulate the expression of a wide variety of genes and, therefore, presumably regulates the activity of a numerous transcription factors. Studies thus far have identified *c-fos* and *c-jun* as PDGF-inducible trans-acting factors and have shown that PDGF controls the activity of these factors at the level of transcription (reviewed by Ransone and Verma, 1990). The data presented here potentially add another trans-acting factor—NF- κ B (or an NF- κ B-like factor)—to this list. Obviously further studies are required to determine whether the stimulation of NF- κ B-like activity by PDGF is accompanied by transactivation. Identification of specific genes induced by PDGF via the κ B site will aid in assessing the role of NF- κ B-like factors in growth control.

ACKNOWLEDGMENTS

The authors thank John Neff for technical assistance, Dr. Larry Kerr for helpful comments and suggestions, and Dr. Ranjan Sen for his generous gift of p50 antiserum. This work was supported by NIH grants CA42713 (W.J.P.), CA43720 (N.E.O.), and CA21773 (E.-S. Huang). T.F.K. was supported by Damon Runyon-Walter Winchell Cancer Research Fund Fellowship DRF-1097.

REFERENCES

- Anisowicz, A., Messineo, M., Lee, S.W., and Sager, R. (1991). An NF- κ B-like transcription factor mediates IL-1/TNF- α induction of *gro* in human fibroblasts. *J. Immunol.* 147, 520–527.
- Baeuerle, P. (1991). The inducible transcription activator NF- κ B: regulation by distinct subunits. *Biochem. Biophys. Res. Commun.* 1072, 63–80.
- Baeuerle, P., and Baltimore, D. (1988a). I κ B: a specific inhibitor of the NF- κ B transcription factor. *Science* 242, 540–546.
- Baeuerle, P., and Baltimore, D. (1988b). Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF- κ B transcription factor. *Cell* 53, 211–217.
- Baeuerle, P., and Baltimore, D. (1989). A 65-kD subunit of active NF- κ B is required for inhibition of NF- κ B by I κ B. *Genes Dev.* 3, 1689–1698.
- Baldwin, A.S., Azizkhan, J.C., Jensen, D.E., Beg, A.A., and Coodly, L.R. (1991). Induction of NF- κ B DNA-binding activity during the G₀-to-G₁ transition in mouse fibroblasts. *Mol. Cell. Biol.* 11, 4943–4951.
- Baldwin, A.S., and Sharp, P.A. (1987). Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse H-2K^b class I major histocompatibility gene. *Mol. Cell. Biol.* 7, 305–313.
- Ballard, D.W., Bohnlein, E., Lowenthal, J.W., Wano, Y., Franza, B.R., and Greene, W.C. (1988). HTLV-1 Tax induces cellular proteins that activate the κ B element in the IL-2 receptor α gene. *Science* 241, 1652–1655.
- Ballard, D.W., Walker, W.H., Doerre, S., Sista, P., Molitor, J.A., Dixon, E.P., Pepper, N.J., Hannink, M., and Greene, W. (1990). The *v-rel* oncogene encodes a κ B enhancer binding protein that inhibits NF- κ B function. *Cell* 63, 803–814.
- Bomsztyk, K., Rooney, J.W., Iwasaki, T., Rachie, N.A., Dower, S.K., and Sibley, C.H. (1991). Evidence that interleukin-1 and phorbol esters activate NF- κ B by different pathways: role of protein kinase C. *Cell Regul.* 2, 329–335.
- Bordoni, R., Thomas, G., and Richmond, A. (1989). Growth factor modulation of melanoma growth stimulatory activity mRNA expression in human malignant melanoma cells correlates with cell growth. *J. Cell. Biochem.* 39, 421–428.
- Bours, V., Burd, P.R., Brown, K., Villalobos, J., Park, S., Ryseck, R.-P., Bravo, R., Kelly, K., and Siebenlist, U. (1992). A novel mitogen-inducible gene product related to p50/p105 NF- κ B participates in transactivation through a κ B site. *Mol. Cell. Biol.* 12, 685–695.
- Bull, P., Hunter, T., and Verma, I.M. (1989). Transcriptional induction of the murine *c-rel* gene with serum and phorbol-12-myristate-13-acetate in fibroblasts. *Mol. Cell. Biol.* 9, 5239–5243.
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257, 7847–7851.
- Davis, N., Ghosh, S., Simmons, D.L., Tempst, P., Liou, H.-C., Baltimore, D., and Bose, H.R. (1991). Rel-associated pp40: an inhibitor of the Rel family of transcription factors. *Science* 253, 1268–1271.
- Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11, 1475–1489.
- Duyao, M.P., Kessler, D.J., Spicer, D.B., and Sonenshein, G.E. (1990). Binding of NF- κ B-like factors to regulatory sequences of the *c-myc* gene. *Curr. Top. Microbiol. Immunol.* 166, 211–218.
- Frantz, C.N., Stiles, C.D., and Scher, C.D. (1979). The tumor promoter 12-O-tetradecanoylphorbol-13-acetate enhances the proliferative response of BALB/c-3T3 cells to hormonal growth factors. *J. Cell. Physiol.* 100, 413–424.
- Fujita, T., Nolan, G.P., Ghosh, S., and Baltimore, D. (1992). Independent modes of transcriptional activation by the p50 and p65 subunits of NF- κ B. *Genes Dev.* 6, 775–787.
- Ghosh, S., and Baltimore, D. (1990). Activation in vitro of NF- κ B by phosphorylation of its inhibitor I κ B. *Nature* 344, 678–682.
- Ghosh, S., Gifford, A.M., Riviere, L.R., Tempst, P., Nolan, G.P., and Baltimore, D. (1990). Cloning of the p50 DNA binding subunit of NF- κ B: Homology to *rel* and *dorsal*. *Cell* 62, 1019–1029.
- Hart, C.E., Forstrom, J.W., Kelly, J.D., Seifert, R.A., Smith, R.A., Ross, R., Murray, M.J., and Bowen-Pope, D.F. (1988). Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* 240, 1529–1531.
- Haskill, S., Beg, A.A., Tompkins, S.M., Morris, J.S., Yurochko, A.D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A.S. (1991). Characterization of an immediate-early gene induced in adherent monocytes that encodes I κ B-like activity. *Cell* 65, 1281–1289.
- Heldin, C.-H., Backstrom, G., Ostman, A., Hammacher, A., Ronnstrand, L., Rubin, K., Nister, M., and Westermark, B. (1988). Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J.* 7, 1387–1393.
- Hoyos, B., Ballard, D.W., Bohnlein, E., Siekevitz, M., and Greene, W.C. (1989). Kappa B-specific DNA binding proteins: role in the regulation of human interleukin-2 gene expression. *Science* 244, 457–460.
- Inoue, J.-I., Kerr, L.D., Kakizuka, A., and Verma, I.M. (1992). I κ B γ , a 70 kd protein identical to the C-terminal half of p110 NF- κ B: a new member of the I κ B family. *Cell* 68, 1109–1120.

- Inoue, J.-I., Kerr, L.D., Ransone, L.J., Bengal, E., Hunter, T., and Verma, I.M. (1991). *C-Rel* activates but *v-Rel* suppresses transcription from κ B sites. *Proc. Natl. Acad. Sci. USA* 88, 3715–3719.
- Ip, Y.T., Kraut, R., Levin, M., and Rushlow, C.A. (1991). The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long range repression element in *Drosophila*. *Cell* 64, 439–446.
- Israël, A., Kimura, A., Kieran, M., Yano, O., Kanellopoulos, J., LeBail, O., and Kourilsky, P. (1987). A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and β 2-microglobulin genes. *Proc. Natl. Acad. Sci. USA* 84, 2653–2657.
- Kabrun, N., Hodgson, J.W., Doemer, M., Mak, G., Franza, B.R., and Enrietto, P.J. (1990). Interaction of the *v-rel* protein with an NF- κ B DNA binding site. *Proc. Natl. Acad. Sci. USA* 88, 1783–1787.
- Kazlauskas, A., Bowen-Pope, D., Seifert, R., Hart, C.E., and Cooper, J.A. (1988). Different effects of homo- and heterodimers of platelet-derived growth factor A and B chains on human and mouse fibroblasts. *EMBO J.* 7, 3727–3735.
- Kelly, K., Cochran, B., Stiles, C.D., and Leder, P. (1983). Cell-specific regulation of the *c-myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell* 35, 603–610.
- Kerr, L.D., Inoue, J., Davis, N., Bose, H.R., Baeuerle, P.A., and Verma, I.M. (1991). The *Rel*-associated pp40 protein prevents DNA binding of *Rel* and NF- κ B: Relationship with I κ B β and regulation by phosphorylation. *Genes Dev.* 5, 1464–1476.
- Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M.B., Kourilsky, P., Baeuerle, P.A., and Israel, A. (1990). The DNA binding subunit of NF- κ B is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* 62, 1007–1018.
- Kohase, M., May, L.T., Tamm, I., Vilcek, J., and Sehgal, P.B. (1987). A cytokine network in human diploid fibroblasts: interactions of β -interferons, tumor necrosis factor, platelet-derived growth factor, and interleukin-1. *Mol. Cell. Biol.* 7, 273–280.
- Lenardo, M.J., Fan, C.H., Maniatis, T., and Baltimore, D. (1989). The involvement of NF- κ B in β -interferon gene regulation reveals its role as a widely inducible mediator of signal transduction. *Cell* 57, 287–294.
- Leof, E.B., Proper, J.A., Goustin, A.S., Shipley, G.D., DiCorleto, P.E., and Moses, H.L. (1986). Induction of *c-sis* mRNA and platelet-derived growth factor-like activity by transforming growth factor type- β : a proposed model for indirect mitogenesis involving autocrine activity. *Proc. Natl. Acad. Sci. USA* 83, 2453–2457.
- Leof, E.B., Van Wyk, J.J., O'Keefe, E.J., and Pledger, W.J. (1983). Epidermal growth factor is required only during the traverse of early G₁ in PDGF-stimulated BALB/c-3T3 cells. *Exp. Cell Res.* 147, 202–208.
- Leof, E.B., Wharton, W., Van Wyk, J.J., and Pledger, W.J. (1982). Epidermal growth factor (EGF) and somatomedin C regulate G₁ progression in competent BALB/c-3T3 cells. *Exp. Cell Res.* 141, 107–115.
- Leung, K., and Nabel, G.J. (1988). HTLV-1 transactivator induces interleukin-2 receptor expression through an NF- κ B-like factor. *Nature* 333, 776–778.
- Meichle, A., Schutze, S., Hensel, G., Brunsing, D., and Kronke, M. (1990). Protein kinase C-independent activation of nuclear factor κ B by tumor necrosis factor. *J. Biol. Chem.* 265, 8339–8343.
- Nolan, G.P., Ghosh, S., Liou, H.C., Tempst, P., and Baltimore, D. (1991). DNA binding and I κ B inhibition of the cloned p65 subunit of NF- κ B, a *rel*-related polypeptide. *Cell* 64, 961–969.
- Olashaw, N.E., Olson, J.E., Drozdoff, V., and Pledger, W.J. (1991). Growth factors: their role in the control of cell proliferation. In: *Molecular and Cellular Approaches to the Control of Proliferation and Differentiation*, ed. G.S. Stein and J.B. Lian, New York: Academic Press, 3–27.
- Pledger, W.J., Stiles, C.D., Antoniades, H.N., and Scher, C.D. (1977). Induction of DNA synthesis in BALB/c-3T3 cells by serum components: Reevaluation of the commitment process. *Proc. Natl. Acad. Sci. USA* 74, 4481–4485.
- Ransone, L.J., and Verma, I.M. (1990). Nuclear proto-oncogenes *fos* and *jun*. *Annu. Rev. Cell Biol.* 6, 539–557.
- Rodriguez-Pena, A., and Rozengurt, E. (1984). Disappearance of Ca²⁺-sensitive, phospholipid-dependent kinase activity in phorbol ester-treated 3T3 cells. *Biochem. Biophys. Res. Commun.* 120, 1053–1059.
- Rodriguez-Pena, A., and Rozengurt, E. (1985). Serum, like phorbol esters, rapidly activates protein kinase C in intact quiescent fibroblasts. *EMBO J.* 4, 71–76.
- Ruben, S.M., Dillon, P.J., Schreck, R., Henkel, T., Chen, C.-H., Maher, M., Baeuerle, P.A., and Rosen, C.A. (1991). Isolation of a *rel*-related human cDNA that potentially encodes the 65 kD subunit of NF- κ B. *Science* 251, 1490–1493.
- Ryseck, R.-P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P., and Bravo, R. (1992). RelB, a new Rel family transcription activator that can interact with p50-NF- κ B. *Mol. Cell. Biol.* 12, 674–684.
- Sen, R., and Baltimore, D. (1986a). Multiple nuclear factors interact with immunoglobulin enhancer sequences. *Cell* 46, 705–716.
- Sen, R., and Baltimore, D. (1986b). Inducibility of κ immunoglobulin enhancer-binding protein NF- κ B by a posttranslational mechanism. *Cell* 47, 921–928.
- Shirakawa, F., and Mizel, S. (1989). In vitro activation and nuclear translocation of NF- κ B catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol. Cell. Biol.* 9, 2424–2430.
- Staal, F.J.T., Roederer, M., Herzenberg, L.A., and Herzenberg, L.A. (1990). Intracellular thiols regulate activation of nuclear factor κ B and transcription of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 87, 9943–9947.
- Stern, J.B., and Smith, K.A. (1986). Interleukin-2 induction of T-cell G₁ progression and *c-myc* expression. *Science* 233, 203–206.
- Toledano, M.B., and Leonard, W.J. (1991). Modulation of transcription factor NF- κ B binding activity by oxidation-reduction in vitro. *Proc. Natl. Acad. Sci. USA* 88, 4328–4332.
- Urban, M.B., and Baeuerle, P.A. (1990). The 65-kD subunit of NF- κ B is a receptor for I κ B and a modulator of DNA-binding specificity. *Genes Dev.* 4, 1975–1984.
- Wharton, W., Leof, E.B., Olashaw, N., Earp, H.S., and Pledger, W.J. (1982). Increases in cyclic AMP potentiate competence formation in BALB/c-3T3 cells. *J. Cell. Physiol.* 111, 201–206.
- Zabel, U., and Baeuerle, P.A. (1990). Purified human I κ B can rapidly dissociate the complex of the NF- κ B transcription factor with its cognate DNA. *Cell* 61, 255–265.