Activation of the 9E3/cCAF Chemokine by Phorbol Esters Occurs via Multiple Signal Transduction Pathways That Converge to MEK1/ERK2 and Activate the Elk1 Transcription Factor*

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Using primary fibroblasts in culture, we have investigated the signal transduction mechanisms by which phorbol esters, a class of tumor promoters, activate the 9E3 gene and its chemokine product the chicken chemotactic and angiogenic factor. This gene is highly stimulated by phorbol 12,13-dibutyrate (PDBu) via three pathways: (i) a small contribution through protein kinase C (the commonly recognized pathway for these tumor promoters), (ii) a contribution involving tyrosine kinases, and (iii) a larger contribution via pathways that can be interrupted by dexamethasone. All three of these pathways converge into the mitogen-activated protein kinases, MEK1/ERK2. Using a luciferase reporter system, we show that although both the AP-1 and PDRIIkB (a NFκB-like factor in chickens) response elements are capable of activation in these normal cells, regions of the 9E3 promoter containing them are unresponsive to PDBu stimulation. In contrast, we show for the first time that activation by PDBu occurs through a segment of the promoter containing Elk1 response elements; deletion and mutation of these elements abrogates 9E3/chicken chemotactic and angiogenic factor expression. Electrophoretic mobility shift assays and functional studies using PathDetect systems show that stimulation of the cells by phorbol esters leads to activation of the Elk1 transcription factor, which binds to its element in the 9E3 promoter.

It has been known for some time that chemokines play important roles in leukocyte chemotraction and inflammation (1). More recently, however, it has become increasingly clear that these small cytokines are also involved in wound healing (2), deterrence of retroviral infections (3), and tumorigenesis (4, 5). In the latter case, chemokines can potentially act at several steps in the development of tumors, and their action is dependent not only on the stimulant but also on the environment.

Tumors develop as a result of multiple insults and chemokines could play important roles in these events because a number of them are stimulated by phorbol esters, injury, and oncogenes (2), all of which have been shown to be involved in tumor promotion. Agents that promote tumor development are called tumor promoters; they are not themselves carcinogenic but they promote the development of tumors in areas of the body that have been exposed to a carcinogen (6, 7). There is extensive literature that demonstrates that phorbol esters are very effective tumor promoters (8, 9). Wounding is also a tumor promoter because it can cause cancer to develop at the edges of wounds inflicted in areas that have been exposed previously to a carcinogen (10–15). The v-src oncogene, which is the transforming protein of the Rous sarcoma virus (a retrovirus that causes tumors in chickens), also has been shown to be a tumor promoter (16). The 9E3 gene and its product, the chicken chemotactic and angiogenic factor (cCAF),1 are stimulated to high levels by the v-src oncogene and also by phorbol esters and wounding/inflammation (17–21). Therefore, this chemokine can potentially be a mediator of the tumor-promoting action of these agents. In the case of v-src, during the development of Rous sarcoma virus-induced tumors the expression of the 9E3/cCAF occurs only in the cells of the tissues surrounding the tumor (19, 21). At later stages of tumor growth, when 9E3/cCAF are expressed abundantly, numerous new blood vessels develop in the area (2, 19, 21). Because cCAF is angiogenic (22, 23) and angiogenesis is very important for tumor growth, it is possible that this chemokine is a mediator of the tumor-promoting actions of the v-src oncogene. In the case of wounding, we have shown that after injury the 9E3 gene is highly expressed shortly after injury and in the granulation (repair) tissue during wound healing (19, 21). The persistent expression of 9E3/cCAF during the healing process coupled with its angiogenic properties in tissues that have been exposed to a carcinogen could mediate the tumor promotion stimulated by wounding. The stimulation of 9E3/cCAF to high levels by phorbol esters, again coupled to its angiogenic properties, can potentially mediate the tumor-promoting actions of these molecules.

Until recently, it was believed that gene activation by phorbol esters always involves PKCs (24). PKCs are a family of serine and threonine kinases that contain structural motifs with a high degree of sequence homology. Most PKC isoenzymes have a conserved cysteine-rich (C1) domain at the N terminus of the regulatory domain (25). The C1 domain is involved in binding to diacylglycerol or its potent functional analogues, the phorbol esters, resulting in the translocation of the enzyme to the plasma membrane. The binding also causes a conformational change in PKC that removes the pseudo substrate domain from the active site, allowing substrate binding and catalysis (26). However, several receptors of phorbol esters that are not PKCs now have been identified (25). Examples are

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1 The abbreviations used are: cCAF, chicken chemotactic and angiogenic factor; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; CEF, chicken embryo fibroblasts; qc, quiescent confluent; PFR, polymerase chain reaction; bp, base pairs; EMSA, electrophoretic mobility shift assay; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; dbd, DNA binding domain; AD, activation domain; LPS, lipopolysaccharide, Luc, luciferase; AP1, activating protein 1.
n-chimaerin (27), Unc-13 (28), Vav (29), cathepsin-L (30), and protein kinase D (31). Thus it is clear that not all of the cellular effects of phorbol esters are mediated via PKC. As a consequence, it is important to delineate the signal transduction pathways by which chemokines are turned on by these agents so that crucial steps in the activation process can be identified and potentially used as targets for regulation of these genes. We have investigated the pathway of activation of 9E3/cCAF by phorbol esters such as phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate (PDBu) in primary fibroblasts, the cells that most highly express 9E3/cCAF in vivo. The work presented here shows that activation of this chemokine by phorbol esters involves multiple signaling pathways that culminate in phosphorylation and activation of the MAP kinase ERK2 following by activation of the transcription factor Elk1, leading to 9E3/cCAF expression.

MATERIALS AND METHODS

Reagents—The dosages used for particular experiments are indicated in the text or in the figure captions. Bovine thymonin (Sigma) was reconstituted in water and used at 9 units/ml. Calphostin C (100–200 mM), H-7 di-Cl (100–200 mM), tyrphostin AG1478 (5–500 mM), and PD98059 (10 µM) were all purchased from Calbiochem and reconstituted in Me2SO. Phorbol-12,13-dibutyrate and 4a-phorbol-12,13-dibutyrate (Biomol) were dissolved in Me2SO and used at 5–100 mM. Anti-phosphotyrosine PY20 (Transduction Laboratories) and 4G10 (Upstate Biotechnology Inc.) were used for the phosphotyrosine immunoblots. ECL reagents and secondary antibodies were conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). For each inhibitor or activator, a range of doses was tested to determine the optimal dose for the study in question. The Bradford assay was performed using the DC protein assay kit (Bio-Rad).

Cell Culture—Primary cultures of chicken embryo fibroblasts (CEF) were prepared from 10-day-old chicken embryos as described previously (32). On the fourth day, secondary cultures were prepared by trypsinizing and plating the primary cells in 199 medium containing 0.5% tryptose phosphate broth and 2% donor calf serum at a density of 1.2 × 10^6/35-mm dish. To study the effects of phorbol esters, 12-O-tetradecanoylphorbol-13-acetate, PMA, or PDBu on 9E3 expression and cCAF production, we used quiescent confluent CEF (qC5F) cultures and incubated them in serum-free 199 medium containing the specific treatment for varying times (see “Results” and “Discussion”). We found that the addition of the specific activator cells were incubated in serum-free 199 medium containing the appropriate inhibitor for 30 min; incubation at 37 °C was continued for 1 h more before removing the medium and replacing it with fresh serum-free medium containing again the specific inhibitor under study. At the end of the incubation period the supernatant was collected and processed as described earlier (33). Pretreatment with 4a-phorbol for 24 h reduced the down-regulation by approximately 60% and performed for 18–24 h and then removed and replaced with medium containing the appropriate treatment for 18 h when evaluating cCAF levels. In the cases where the cell extracts were analyzed for activation of the ERKs, the cells were pretreated with the PDBu for 24 h, the inhibitor of MEK1/ERK (PD98059) for 1 h, and then the activator, PDBu, at 5 mM for 5 min. Because qC5F are primary cells rather than a cell line, there are small variations in the basal levels of 9E3/cCAF expression from batch to batch of cells. Therefore, for each experiment and/or treatment of a different batch of cells, we used positive (treated) and negative (untreated) controls.

Western Blotting—Volumes of the cell culture supernatant corresponding to equal amounts of protein in the cell extracts were loaded on 10% or 12% polyacrylamide-glycerol gels and electrophoresed at 16–32 mA for about 3 h. The concentration of the protein was determined using the DC protein assay kit (Bio-Rad). The Bradford assay was performed using the ECL reagents (Amersham Pharmacia Biotech). After electrophoresis, the RNA was transferred to MagnaGraph nylon membranes (MSI Inc.), which were photographed to visualize the qualitative aspects of the mRNA and confirm equal loading and even transfer. The RNA was UV-cross-linked to the membrane for 2 min and baked at 80 °C for 2 h. Prehybridization was performed for 6–9 h, and hybridization with a cDNA probe was carried out for 24 h. (33).

Northern Blot Analysis—CEF were homogenized and total RNA was prepared using triZOL reagent (Life Technologies, Inc.). RNA samples (10 µg/ml) were denatured in a formamide-formaldehyde buffer containing ethidium bromide and separated on formaldehyde-agarose gel. After electrophoresis, the RNA was transferred to MagnaGraph nylon membranes (MSI Inc.), which were photographed to visualize the qualitative aspects of the RNA and confirm equal loading and even transfer. The RNA was UV-cross-linked to the membrane for 2 min and baked at 80 °C for 2 h. Prehybridization was performed for 6–9 h, and hybridization with a cDNA probe was carried out for 24 h. (33).
were stimulated with PDBu or LPS for 3 h prior to lysis. For the inhibitor experiments, the qcCEFs were incubated with inhibitors for 30 min before PDBu stimulation. Cell extracts were prepared with reporter lysis buffer according to the protocol provided by the manufacturer (Promega) and stored in a −70 °C freezer.

Luciferase and β-Galactosidase Assay—Cell extracts were assayed using a Luminometer with an automated injection device (Monolight 2010, Analytical Luminescence Laboratory). The reaction substrate and buffer are parts of the luciferase assay protocol (Promega). Aliquoted samples were used for the β-galactosidase assay according to the procedure for the β-galactosidase enzyme assay protocol (Promega). A minimum of triplicates for each experiment was performed, and the data were expressed as mean light units of luminescence/unit of β-galactosidase activity.

PathDetect System—We used a cis-PathDetect system for AP-1 and a trans-PathDetect system for the Elk1. These systems were purchased from Promega, and we followed the protocols described by the company with the exception that we used our modified Ca3PO4 precipitation method for cell transfections that we have optimized in our system for lower stress-induced background, rather than transfections with Lipofectin as recommended in the procedure.

Electrophoretic Mobility Shift Assays (EMSA)—Cells were treated as described earlier and nuclear extracts were prepared as described previously (36). Protein concentration was determined with the DC protein assay kit (Bio-Rad). 10 μg of extracted nuclear protein was used for each binding reaction with 2 ng of [γ-32P]ATP-labeled probe containing the conserved Elk-1 binding element (wild type, ATCCAGGATGGTTTTA-ATACCTGCCAC CT (bold indicates the binding site for the Elk1 transcription factor)) in EMSA buffer (188 mM NaCl, 50 mM HEPES, pH 7.9, 2.5 mM EDTA, pH 8.0, 2.5 mM dithiothreitol, and 20% glycerol). 30 ng of unlabeled probe was used for the competition assay, and 1 μL of anti-Phospho Elk1(Ser-383) antibody (NE Biolab) was added for the supershift assay. Samples were assayed in 6% nondenaturing polyacrylamide gel electrophoresis.

RESULTS

Stimulation of 9E3/cCAF by Phorbol Esters—qcCEFs do not express the 9E3/cCAF or they express it at very low levels. However, upon stimulation with PDBu, the levels of the 9E3 mRNA increase dramatically. The rise in mRNA is first seen at 7 min, and it peaks at 3–6 h and declines thereafter (Fig. 1A). cCAF production after stimulation by PDBu shows that the protein accumulates in the culture supernatant (Fig. 1B). Accumulation is dose-dependent (Fig. 1C) and is specific; the Me2SO used as solvent for PDBu did not significantly stimulate 9E3/cCAF (Fig. 1, D and E).

It has been considered for a long time that phorbol esters exert their effects on cells primarily through the activation of PKC isoforms that contain the cysteine-rich (C1) domain to which phorbol esters bind (37). Therefore, we investigated the possibility that stimulation of 9E3/cCAF by phorbol esters occurs via activation of these classical PKCs. We treated cells with PDBu for 24 h to down-regulate total PKC activity by causing proteolytic degradation of these kinases (38–40) followed by restimulation with PDBu. The result was only moderately reduced expression compared with that stimulated by PDBu without pretreatment (Fig. 2A). These observations suggest that activation of PKC represents only part of the stimulation of the 9E3/cCAF by phorbol esters. These results were confirmed by treatment of cells with calphostin C, a specific inhibitor of PKC that competes with phorbol esters for the binding of the C1 domain in the regulatory region of PKC, and we found that 9E3 expression and production of cCAF were minimally blocked by this inhibitor (Fig. 2, B and C). Similar results were obtained when the cells were treated with H7 dihydrochloride, a broad spectrum inhibitor of Ser/Thr kinases that also inhibits PKC (Fig. 2, B and C). None of these inhibitors by themselves cause stimulation of 9E3/cCAF (33). In addition, we also found that the 4α-isomer of PDBu (4α-PDBu), which does not activate PKC (41), stimulates 9E3 expression and cCAF production almost as efficiently as PDBu (Fig. 3, A and B). This stimulation, much like that by PDBu, is time-(Fig.
and dose-dependent, albeit not as efficient as the stimulation by PDBu. These results taken together strongly suggest that 9E3/cCAF stimulation by phorbol esters occurs primarily via PKC-independent pathway(s).

To investigate the possibility that the pathway of activation of 9E3/cCAF by phorbol esters involves tyrosine kinase activation, as it does for stimulation by thrombin (33), we used inhibitors of tyrosine kinases. Herbimycin, an inhibitor of the c-src family of tyrosine kinases, had no inhibitory effect on the PDBu stimulation of 9E3/cCAF (not shown). Similarly, tyrphostin, a selective inhibitor for the epidermal growth factor receptor tyrosine kinase, had essentially no effect on PDBu stimulation of this gene (Fig. 4A), whereas the broad spectrum tyrosine kinase inhibitor genistein produced a distinct decrease in the stimulation of 9E3/cCAF. The same results were observed for cCAF protein levels (not shown). The inhibition of cCAF production when the cells were treated with calphostin C (inhibitor of PKC) and genistein (inhibitor of tyrosine kinases) was additive, but significant cCAF stimulation by PDBu remained (Fig. 4, B and C), suggesting yet other pathways of stimulation for this gene by PDBu.

It has been known for some time that expression of chemokine genes triggered by a variety of stimuli is inhibited by glucocorticoids (42–45). In most cases tested, these anti-inflammatory agents activate the glucocorticoid receptor that, in turn, interacts with and inactivates transcription factors that are important in chemokine gene expression (46). Dexamethasone is an example of an anti-inflammatory glucocorticoid that inhibits chemokine gene activation (44). Because 9E3/cCAF is stimulated by phorbol esters and plays an important role in the inflammatory response (22), we treated qcCEFs with PDBu in the presence of dexamethasone to determine whether this anti-inflammatory agent inhibited 9E3/cCAF stimulated by this phorbol ester. Dexamethasone significantly decreased cCAF production stimulated by PDBu but did not eliminate it (Fig. 5A). Treatment of qcCEFs with PDBu in the presence of dexamethasone, calphostin C, and genistein simultaneously, however, reduced cCAF production to that of the control (Fig. 5B). These inhibitors were all used at doses chosen for optimal activity (see “Materials and Methods”) without adversely affecting the cells as judged by their morphology, appearance, adhesion to the culture dish, and percent of cell death, even when applied simultaneously (see fig. 5 legend for doses applied). This is further illustrated by the fact that the combination of inhibitors did not significantly affect the basal level of cCAF produced by the treated cells, which is comparable to the levels of the control (Fig. 5B).
Signaling Events Leading to 9E3/cCAF Expression Involve ERK2 Activation—As shown above, phorbol esters stimulate the expression of 9E3/cCAF via multiple pathways; a smaller contribution was made by PKC and tyrosine kinases and a more significant contribution from pathway(s) inhibited by dexamethasone. The MAP kinase ERK2 is a convergence point for many different signaling pathways (47–49). Hence, we investigated if this kinase becomes phosphorylated after stimulation by PDBu and if activation of this enzyme is responsible for stimulation of 9E3/cCAF via any of these pathways.

Immunoblot analysis using anti-phosphotyrosine antibodies showed that a protein with molecular mass of 42 kDa was differentially phosphorylated on tyrosines (Fig. 6A) with the maximum phosphorylation occurring at 2 min after stimulation and declining thereafter. We stripped and reprobed the anti-phosphotyrosine blots with anti-ERK2 antibodies and determined that the 42-kDa protein co-migrated with ERK2 (Fig. 6B). To determine if ERK2 phosphorylation/activation is involved in the signaling pathway(s) inhibited by dexamethasone, we treated cells in the presence or absence of this glucocorticoid and found that this inhibitor partially eliminated ERK2 phosphorylation on tyrosines (Fig. 7, A and B), suggesting that the decrease in cCAF production upon treatment with dexamethasone (Fig. 5B) is linked to ERK2 activation. A specific inhibitor of ERK2 is not available; however, MEK1 (mitogen-activated protein kinase kinase), which can be selectively inhibited by PD98059 (50–54), is directly upstream from ERK2 in the MAP kinase signaling cascade and is the specific kinase responsible for both phosphorylation and activation of ERK2 (55, 56). Therefore, to determine if ERK2 is a convergence point for the various signaling pathways stimulated by PDBu, which lead to expression of this chemokine, we treated cells with PD98059. This inhibitor was highly effective in blocking PDBu-stimulated phosphorylation of ERK2 (Fig. 7C) and eliminated cCAF production when the cells were treated with PDBu in the presence of PD98059 (Fig. 7E). These results, taken together, strongly suggest that the multiple pathways activated by PDBu, which lead to 9E3/cCAF expression, converge in ERK2. In addition, dexamethasone as well as PD98059 eliminated cCAF production stimulated by 4α-PDBu (Fig. 7F).

Analysis of the 9E3/cCAF Promoter in Response to PDBu—Further information on the pathways involved in the stimulation of 9E3/cCAF by PDBu can be elicited by determining the active elements of the gene promoter (Fig. 8A). Therefore, we used a promoter region of the 9E3 gene (∼683 to +32 bp), which contains DNA binding elements that can potentially be...
activated by the pathways identified above, and cloned it into the pGL3 luciferase reporter system (Promega). This region of the promoter (p683) contains the consensus binding sequences for the transcription factors Elk1, AP-1, and PDRII (the chicken equivalent of NFκB) (57–59) and the CAAT box for C/EBP. We found that when this construct was transfected into our primary normal fibroblasts, expression of luciferase was highly stimulated by PDBu (Fig. 8A). To determine the crucial areas of the promoter for activation, we subcloned fragments of the larger promoter region into the reporter system. The intermediate length fragments, 2470 to 132 bp and 2218 to 132 bp, eliminate the two putative Elk1 response elements, and the smallest fragment, 266 to 132 bp, contains only the TATA box. The −470 to +32 bp piece showed a small amount of stimulation (twice as much as the control) (Fig. 8A, II), but the other two constructs showed virtually no stimulation upon PDBu treatment (Fig. 8A, III and IV), although the PDRIIκB and the AP-1 binding elements are still present in the −218 to +32 bp construct. Me2SO, which was used to dissolve the PDBu and the inhibitors, showed a slight increase of activation of the reporter system over the control (Fig. 8). These results correlate very well with those obtained with northern blot and immunoblot analysis. In addition, we found that the stimulation by PDBu was completely inhibited by PD98059 in a dose-dependent manner (Fig. 8B) and that a dose dependence of inhibition was also observed with dexamethasone (Fig. 8C) but
total inhibition was not obtained; the highest dose still left twice as much activation as the control.

The results presented above lead us to conclude that the region of the promoter between 2683 and 2470 bp contains the dominant elements important for activation of 9E3/cCAF by PDBu. This region contains two elements that are highly conserved for binding of the Elk1 transcription factor with one starting at 2534 bp and the other at 2493 bp. Elk1 is an important substrate for ERK2 that we have shown here is critical for expression of 9E3/cCAF after stimulation of the cells by PDBu. Therefore, we deleted the Elk1 starting at 2534 bp and mutated the one starting at 2493 bp to obtain a construct containing -493 to +32 bp (pmElk1) and performed transfection studies similar to those described above. The activity of the two promoters containing the two Elk1 elements (-683 to +32 bp and -542 to +32 bp) was not significantly different. The promoter containing only the first Elk1 element (-485 to +32 bp) showed a significant decrease in activity, whereas the mutated promoter (pmElk1) showed a very low level of activity that was similar to that obtained with the -470 to +32 bp construct (Fig. 9A). These results suggest that the two Elk-1 elements are the major regulatory elements for cCAF expression when cells are stimulated by phorbol esters.

To complement the mutagenesis studies, we tested whether the Elk1 transcription factor is activated in response to PDBu. For these experiments, we used a PathDetect reporter system
developed by Stratagene and applied it to our system (Fig. 9B). Cells were cotransfected with the pFR-Luc construct, which contains the Gal4 DNA binding sequence upstream of the luciferase gene, and with the pFA-Gal4dbd-Elk1AD construct (pFA-dbd-Elk1), which is an expression vector for a fusion protein containing Gal4dbd and the Elk1 activation domain (Elk1AD). The Gal4dbd part of this fusion protein binds to the Gal4 element on pFR-Luc, and the Elk1AD portion of the fusion protein is capable of activating the luciferase gene. As a negative control we used an expression vector for the Gal4 DNA binding domain alone (pFC-dbd), and as a positive control we used an expression vector for MEK1 (pFC-MEK1). Our results show that when pFC-Gal4dbd was cotransfected with the reporter construct (pFR-Luc) and subsequently treated with PDBu, there was a very small activation of the luciferase gene, which was not significantly different from the control (Fig. 9B, b); the same was observed if cotransfection was performed with pFA-
Gal4dbd-Elk1AD in the absence of stimulation by PDBu (Fig. 9B, c). However, when this phorbol ester was used to stimulate the cells cotransfected with the pFA-Gal4dbd-Elk1AD, we observed a 4-fold increase in activation (Fig. 9B, d), which was eliminated by PD98059 (Fig. 9B, e), the inhibitor for MEK1. Cotransfection of a MEK1 expression vector with the reporter construct and the expression vector for the fusion protein Gal4dbd-Elk1AD but in the absence of PDBu stimulation caused significant activation of the reporter gene (Fig. 9B, f). Therefore, overexpression of MEK1 activated its substrate ERK2 followed by activation of the Elk1 transcription factor. The results show that PDBu can stimulate activation of the Elk1 transcription factor leading to gene expression.

To determine if Elk1 activated by PDBu binds specifically to its element in these primary fibroblasts, we performed EMSA and supershifts using antibodies specific for activated Elk1. Our normal primary cells express very low levels of the Elk1 transcription factor making it difficult to obtain strong shift and supershift bands. Therefore, we overexpressed Elk1 using pCMV-Elk1 (60), treated the cells with PDBu, and incubated the nuclear extracts with a radiolabeled 27-mer oligonucleotide containing a sequence identical to that of the Elk1 elements present in our promoter. As observed by others (60, 61), EMSA showed multiple shifted bands (Fig. 9C, arrowheads). These shifted bands were competed out with cold oligonucleotide (Fig. 9C, lane 2) and disappeared when incubated with the mutated radiolabeled oligonucleotide (Fig. 9C, lane 3). Incubation with a specific antibody to activated Elk1, which is phosphorylated on serine 383 (60), resulted in a supershift of two of the Elk1 bands to a common band (Fig. 9C, lane 4, double arrowhead). Because Elk1 can bind to its element even when it is not activated (61) to verify that the supershifted band represents activated Elk1, we treated the same batch of primary cells with PDBu in the presence of the inhibitor PD98059. As described above, PD98059 is a specific inhibitor of MEK1 that directly activates ERK2, which phosphorlates/activates Elk1. Therefore, the Elk1 transcription factor bound to the oligonucleotide should not be phosphorylated on Ser-383 and should not be recognized by the antibody specific to this Ser. When the nuclear extracts from these cells were incubated with the antibody against activated Elk1, there was no supershift (Fig. 9C, lane 5). Thus, EMSA confirms that Elk-1 binds specifically to its element in the 9E3 gene promoter and that this transcription factor is activated by PDBu. The results of transcription activation taken together with those on signal transduction show that PDBu stimulates MEK1/ERK2, which results in the activation of the Elk1 transcription factor and stimulation of 9E3/cCAF.

Because it has been previously shown that phorbol esters activate the NFκB and AP-1 transcription factors (62–64) although it is not the case in our transfection experiment (Figs. 8A and 9A), we tested the possibility that the PDR1x1B and the AP-1 binding elements were somehow inactivated by alterations of our constructs during the PCR reaction. Sequence analysis showed that they contain no mutations. Further testing of the PDR1x1B was obtained by treating the cells transfected with the −218 to +32 bp construct with LPS, which is known to activate the CINC chemokine gene through NFκB (45). Treatment of this region of the 9E3 gene promoter with LPS activated the reporter system (Fig. 10A), showing that PDR1x1B is functional in the 9E3/cCAF promoter but does not respond to PDBu. Testing of the functionality of the AP-1 binding element was performed by cotransfecting the fibroblasts with the p683 construct and the expression vectors containing the c-Fos and c-Jun cDNAs. The latter vectors, when expressed, should form an active AP-1 transcription factor complex (60–65). To determine if the expressed AP-1 transcription factor was functional in our normal fibroblasts, the cis-PathDetect system (Stratagene) for the AP-1 binding element was used. This system contains seven AP-1 elements in series in a Cis-reporter backbone containing the luciferase reporter gene (pAP-1-Luc). The Cis-reporter by itself caused virtually no activation of the luciferase gene (Fig. 10B, a), whereas in the presence of PDBu (PdiBt) stimulation alone (Fig. 10B, b), c-Fos + c-Jun (Fig. 10B, c), or c-Fos + c-Jun + PdiBt (Fig. 10B, d) there was a 3–4-fold increase in activation. MEK kinase was used as a positive control to verify that the Cis-reporter system working correctly (Fig. 10B, e). Thus, the AP-1 transcription factor complex made from the expression vectors is functional in our cells. However, when cells were cotransfected with p683 and the expression vectors for the AP-1 complex, we found that c-Fos and c-Jun individually or in combination do not stimulate expression of the reporter gene above that of the control (Fig. 10C, c, e, and g). When these cells were simultaneously stimulated with PDBu, there was an increase in the expression of the reporter to slightly lower levels (Fig. 10C, d, f, and h) than those of the positive control (cells transfected with the p683 alone and treated with PDBu) (Fig. 10C, b). These results show that overexpression of a functional c-Fos/cJun complex (that can bind to AP-1 response elements and activate transcription) does not activate the reporter gene in the context of our promoter. To further establish the lack of involvement of AP-1 in 9E3/cCAF expression, we used site-directed mutagenesis to mutate the AP-1 binding element in the context of the p683 promoter (pμAP-1). In comparison to the native p683, the p683 with the mutated AP-1 element showed a small decrease in luciferase activity upon stimulation with PDBu, demonstrating that AP-1 in not significantly involved in the activation of 9E3/cCAF by this tumor promoter.

**DISCUSSION**

Although it is clear that chemokines play important roles in tumorigenesis, very little is known about the way tumor-promoting agents activate chemokine genes. We have used the 9E3 gene and its product cCAF as a model to study the activation of chemokines in primary fibroblasts by one class of tumor promoters, the phorbol esters. We show the following: (i) 9E3/cCAF stimulation by PDBu is only moderately inhibited by down-regulation of C1 domain-containing PKCs or by inhibition of this enzyme with the specific inhibitor calphostin C and that stimulation can also be achieved by 4α-PDBu, the isomer of PDBu that does not activate PKC; (ii) general tyrosine kinase inhibitors, although more effective than PKC inhibitors, also did not completely abolish the activation of 9E3/cCAF by PDBu; (iii) dexamethasone is much more effective than PKC or the tyrosine kinase inhibitor in inhibiting the activation of this chemokine by PDBu; (iv) complete inhibition of 9E3/cCAF was not accomplished until the three types of inhibitors were applied simultaneously; (v) inhibition of MEK1/ERK2 by PD98059 also completely eliminated 9E3/cCAF expression; (vi) Elk1 binding elements and the Elk1 transcription factor are crucial for activation of this chemokine gene by PDBu, whereas the AP-1 and the PDR1x1B binding elements, although potentially fully functional, are not responsive to the activation of the 9E3/cCAF by PDBu.

Our studies show that all three of these signaling pathways, C1 domain PKCs, tyrosine kinases, and those mediated by dexamethasone, converge to MEK1/ERK2. The activation of ERK2 via multiple signaling pathways is consistent with the recent demonstration that full activation of ERKs, in particular in fibroblasts, may require the cooperation of various signaling pathways (66, 67). Furthermore, ERK1 and ERK2 are central transducers of extracellular signals from hormones, growth
factors, and cytokines (55, 63) and are known to be points of convergence of signals emanating from tyrosine kinase-coupled receptors (68) and G-protein-coupled receptors (69). The results presented here further these studies and show that ERK2 can also be a point of convergence of several signaling pathways generated independently of cell surface receptors.

The inhibition of PDBu stimulation of 9E3/cCAF by dexamethasone is intriguing, because this glucocorticoid is primarily known for its inhibition of the NFkB transcription factor, and yet our results show that PDRIIxB DNA binding element, p66 contains only the TATA box therefore, stimulation was insignificant. B, testing the functionality of the c-Fos and c-Jun expression systems in normal fibroblasts. For these studies a promoter containing 7 AP-1 binding elements in a series in front of the luciferase reporter gene (cis-PathDetect system from Stratagene) was used. Overexpression of c-Fos and c-Jun in the absence (c) or presence (d) of PDBu (Pdbt), caused a 4–5-fold increase in transcription of the reporter system. The functionality of the cis-PathDetect system was shown by cotransfection of the cells with an expression vector for MEK kinase, which functions as the positive control for the system (e). C, the activity of the AP-1 binding element in the 9E3 promoter was studied by transfection of the cells with p683 and the expression vectors for c-Fos, c-Jun, or both. c-Fos and c-Jun individually or in combination do not stimulate expression of the reporter gene above that of the control (c). When these cells were simultaneously stimulated by PDBu there was an increase in expression of the reporter to slightly lower levels (d, f, and h) than those of the positive control (b). D, to further determine the lack of involvement of AP-1 in 9E3/cCAF expression, the AP-1 binding element was mutated within the context of the p683 promoter (pmAP-1) by site-directed mutagenesis using Quickchange (Stratagene), and the mutation was verified by restriction enzyme digestion and sequencing. Our results show only an insignificant reduction in activation of the reporter system when the AP-1 binding element was mutated, demonstrating that in primary normal fibroblasts activation of 9E3/cCAF by PDBu does not involve AP-1. Each experiment was performed at least twice with two different batches of cells. The bars represent S.E. of three samples/condition.

FIG. 10. Activity of the PDRIIxB (NFkB-like) and the AP-1 binding elements in response to PDBu stimulation. A, stimulation of the pGL3/9E3/p218 and pGL3/9E3/p66 by LPS. LPS stimulated transcription from the p218 promoter because this promoter contains the PDRIIxB DNA binding element. p66 contains only the TATA box therefore, stimulation was insignificant. B, testing the functionality of the c-Fos and c-Jun expression systems in normal fibroblasts. For these studies a promoter containing 7 AP-1 binding elements in a series in front of the luciferase reporter gene (cis-PathDetect system from Stratagene) was used. Overexpression of c-Fos and c-Jun in the absence (c) or presence (d) of PDBu (Pdbt), caused a 4–5-fold increase in transcription of the reporter system. The functionality of the cis-PathDetect system was shown by cotransfection of the cells with an expression vector for MEK kinase, which functions as the positive control for the system (e). C, the activity of the AP-1 binding element in the 9E3 promoter was studied by transfection of the cells with p683 and the expression vectors for c-Fos, c-Jun, or both. c-Fos and c-Jun individually or in combination do not stimulate expression of the reporter gene above that of the control (c). When these cells were simultaneously stimulated by PDBu there was an increase in expression of the reporter to slightly lower levels (d, f, and h) than those of the positive control (b). D, to further determine the lack of involvement of AP-1 in 9E3/cCAF expression, the AP-1 binding element was mutated within the context of the p683 promoter (pmAP-1) by site-directed mutagenesis using Quickchange (Stratagene), and the mutation was verified by restriction enzyme digestion and sequencing. Our results show only an insignificant reduction in activation of the reporter system when the AP-1 binding element was mutated, demonstrating that in primary normal fibroblasts activation of 9E3/cCAF by PDBu does not involve AP-1. Each experiment was performed at least twice with two different batches of cells. The bars represent S.E. of three samples/condition.

Phorbol Esters Activate 9E3/cCAF via Multiple Signal Pathways

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elements and activate transcription does not activate the reporter gene in the context of the 9E3 promoter AP-1 binding element. Furthermore, mutation of the AP-1 binding element within the context of the 9E3/cCAF promoter did not result in significant reduction of activity of the reporter system. These results taken together demonstrate that AP-1 is not importantly involved in the stimulation of 9E3/cCAF by PDBu. Therefore, it is possible that the AP-1 binding element is inaccessible for activation of this chemokine gene in normal fibroblasts. Interestingly, we have identified a consensus binding sequence for the Oct-1 repressor near the AP-1 binding element. Oct-1 is a member of the POU domain family and has been shown to repress the expression of several genes (72–75). Therefore, it is possible that this repressor in normal cells inhibits the ability of the activated c-Fos and c-Jun complex to bind to its element and/or to activate gene expression, whereas in transformed cells this repression is lifted. Work is in progress to analyze the role of this repressor in the tightly regulated expression of 9E3/cCAF in normal cells to compare with the constitutive expression observed in transformed fibroblasts.

In addition to its role in the inhibition of NFkB and AP-1, dexamethasone also inhibits the phosphorylation of the Raf1 kinase (76, 77). Raf1 is known to act upstream from MEK1 and ERK2 in the MAP kinase cascade (77, 78) and, therefore, can be importantly involved in ERK2 activation. Our ongoing work in deciphering the signal transduction pathways of stimulation of 9E3/cCAF has shown that Raf1 activation is required for 9E3/cCAF expression stimulated by phorbol esters, suggesting that dexamethasone acts on Raf1 to inhibit MEK1/ERK2 activity and 9E3/cCAF expression.

ERK2 is known to activate gene expression via a variety of transcription factors including Elk1 (79–81). Our data show that 80–90% of the activation of 9E3/cCAF by PDBu occurs in the −543 to −470 bp region of the 9E3 promoter (Figs. 8A and 9A), which contains two Elk1 responsive elements; deletion and mutation of the two Elk1 elements present in this region of the promoter eliminated this effect. Using a Gal4-Elk1 fusion protein, we also show that PDBu stimulation leads to the activation of the Elk1 transcription factor and subsequent activation of the reporter system (Fig. 9B). EMSA with nuclear extracts of cells overexpressing Elk1 shows that in vitro the oligonucleotide sequence representing the Elk1 binding elements in the promoter of 9E3/cCAF was specifically bound by nuclear protein(s). The shift band can be retarded by an antibody specific to Ser-383 phosphorylated/activated Elk1, demonstrating that after treatment with PDBu the factor that binds to the elements is activated Elk1 (Fig. 9C). The interesting supershift of two bands to the same heavier band by the antibody to activated Elk1, demonstrating that after treatment with PDBu the factor that binds to the elements is activated Elk1 (Fig. 9C).

Using transcription activation reporter systems, site-directed mutagenesis, heterologous expression systems, and EMSA, we have shown that the major transcriptional activation pathway for 9E3/cCAF expression upon stimulation by PDBu occurs via ERK2/Elk1.

The remaining 10–20% of activation of 9E3/cCAF by PDBu occurs almost exclusively in the −470 to −218 bp section of the promoter (Figs. 8A and 9A). This region does not contain a consensus sequence for the Elk1 element, but it does contain a closely related Ets sequence, which could be responsible for this smaller activation. It has been shown that several Ets domain binding factors are targets for the ERK proteins (80), making it possible that ERK2 activates one of the Ets-binding proteins, which in turn could be responsible for the small level of activation we observed with this shorter promoter.

When taken together, our results lead to a potential model for activation of 9E3/cCAF by phorbol esters in primary normal fibroblasts. Complete inhibition of PDBu stimulation of 9E3/cCAF by PD98059 (downstream inhibitor of the MAP kinase cascade) indicates that all three signaling pathways go through MEK1/ERK2, whereas dexamethasone (upstream inhibitor of the MAP kinase cascade) inhibits only about 80% of the stimulation, suggesting that one of the less active pathways (PKC or tyrosine kinase activated) is not inhibited by dexamethasone. Thus, the major pathway by which PDBu stimulates 9E3/cCAF and one of the two minor pathways must merge together before the point of dexamethasone inhibition, and the third pathway involved must merge in the cascade after the point of dexamethasone inhibition. Dexamethasone is known to interfere with Raf1 (76, 77), which is the kinase that acts upon MEK1 in the MAP kinase signaling cascade, hence the minor pathway independent of dexamethasone inhibition could merge either at the point of MEK1 or immediately before it. Our observation that both dexamethasone and PD98059 completely inhibit 9E3/cCAF stimulation by 4α-PDBu (which does not activate PKC), whereas dexamethasone only partially inhibits stimulation by PDBu, suggests that the dexamethasone-independent pathway is the pathway involving PKC. Alternatively, PKC can stimulate Raf1 activation in a way that cannot be inhibited by dexamethasone.

In summary, we show for the first time that phorbol esters can activate a chemokine gene via multiple pathways that involve simultaneously a dexamethasone-sensitive pathway and to a much lesser extent tyrosine kinases and C1 domain-containing PKCs. These pathways all culminate in ERK2 activation and in dominant stimulation of a major regulatory region of the gene that contains two Elk1 consensus binding sites. Furthermore, our functional studies show that stimulation of this gene by phorbol esters leads to activation of the Elk1 and that this transcription factor binds to its elements in the 9E3/cCAF promoter. We conclude that the predominant signaling pathway for the activation of 9E3/cCAF by PDBu goes through the MAP kinase cascade enzymes MEK1/ERK2 and involves activation of the transcription factor Elk1. The existence of multiple pathways to activate chemokine genes is important because these are immediate early response genes that are expressed in response to stresses such as tumors and injuries. Agonists that use several pathways are more versatile in turning on genes potentially allowing for a more reliable response.

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