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Induction of Carcinoma Cell Migration on Vitronectin by NF-κB–dependent Gene Expression

Mayra Yebra,* Edward J. Filardo,* Evelyn M. Bayna,* Ei Kawahara,† Jürgen C. Becker,* and David A. Cheresh*‡

*Departments of Immunology and Vascular Cell Biology, The Scripps Research Institute, La Jolla, California 92037; and †Department of Pathology, Kanazawa University, School of Medicine, 13-I Takara-machi, Kanazawa Ishikawa 920, Japan

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Integrin αvβ5 promotes FG carcinoma cell adhesion to vitronectin yet requires protein kinase C (PKC) activation for migration on this ligand. Here we report that this PKC-dependent cell motility event requires NF-κB–dependent transcription. Specifically, a component within nuclear extracts prepared from PKC-stimulated FG cells exhibited a significant increase in binding activity to a synthetic oligonucleotide containing a consensus κB sequence. These nuclear DNA-binding complexes were shown to be comprised of p65 and p50 NF-κB/rel family members and appeared functionally active because they promoted transcription of a reporter construct containing a κB site. The NF-κB activation event was directly linked to the αvβ5 motility response because the NF-κB–binding oligonucleotide, when introduced into FG cells, inhibited cell migration on vitronectin but not on collagen and had no effect on cell adhesion to either ligand. These results suggest that the detected DNA-binding complexes interact with κB transcriptional elements to regulate gene expression required for αvβ5-dependent cell motility on vitronectin.

INTRODUCTION

Regulation of cell migration is a critical factor during tissue remodeling associated with angiogenesis, embryogenesis, inflammation, and wound healing (Albelda and Buck, 1990). Upon neoplastic transformation, cells acquire a migratory phenotype that facilitates their ability to metastasize. Integrins are cell surface receptors that mediate cell-matrix interactions critical for cell motility (Ruoslahti, 1991; Hynes, 1992; Leavesley *et al.*, 1992, 1993; Klemke *et al.*, 1994). Although integrin-mediated cell adhesion is necessary for migration, it is not always sufficient. For example, recent studies have shown that integrin-dependent cell motility, but not attachment, requires the presence of exogenous growth factors (Chen *et al.*, 1993; Mathay *et al.*, 1993; Klemke *et al.*, 1994), suggesting that cell motility involves activation events not required for adhesion.

‡ Corresponding author.

FG pancreatic carcinoma cells attach to vitronectin utilizing integrin αvβ5, yet are unable to migrate on this ligand even though they attach and migrate on collagen in an α2β1 integrin-dependent manner (Leavesley *et al.*, 1992). These findings indicate that FG cell migration on collagen is constitutive whereas αvβ5-dependent migration on vitronectin requires agonist activation. However, we recently showed that FG cells can be induced to migrate on vitronectin in the presence of soluble growth factors, such as epidermal growth factor and insulin-like growth factor, which act by stimulating a PKC-dependent signaling pathway (Klemke *et al.*, 1994).

PKC is known to activate the adhesive properties of some cells by enhancing the affinity of their cell surface integrins (Shattil and Brass, 1987; Hibbs *et al.*, 1991; Valmu *et al.*, 1991; Wilkins *et al.*, 1991; Martin-Thouvenin *et al.*, 1992). In addition, PKC potentiates signaling events leading to integrin-dependent cell spreading and motility (Vuori and Ruoslahti, 1993; Klemke *et al.*, 1994). Although PKC can directly acti-

vate signaling pathways, it can also regulate gene expression by activating transcription factors such as NF-κB (Dominguez *et al.*, 1993; Diaz-Meco *et al.*, 1993, 1994; Hirano *et al.*, 1995). In this report, we provide evidence that PKC-stimulated carcinoma cell motility on vitronectin is due to a late activation signal leading to the induction of NF-κB–mediated transcription. These findings demonstrate a novel PKC-dependent pathway leading to the modulation of integrin-mediated cell function. In addition, we present the first evidence that transcriptional activation by members of the NF-κB family can regulate integrin-dependent cell motility.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased from Sigma Chemical, St. Louis, MO, unless otherwise specified.

Cell Culture

FG human pancreatic carcinoma cells were cultured in RPMI-1640 containing 10% fetal bovine serum as previously described (Leavesley *et al.*, 1992).

Antibodies

Affinity-purified rabbit polyclonal antibodies specific for p65 (amino acids 3–19), c-rel (amino acids 152–176), relB (amino acids 540–558), and p52 (amino acids 298–324) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and have been previously described (Kunsch and Rosen, 1993; Lewis *et al.*, 1994; Oeth *et al.*, 1994). A rabbit monospecific polyclonal antibody directed to p50 was kindly provided by Paul Oeth and Nigel Mackman, Scripps Research Institute, La Jolla, California.

Adhesive Ligands

Vitronectin was purified as previously described (Yatohgo *et al.*, 1988). Collagen type 1 was obtained from Collaborative Research (New Bedford, MA) or Upstate Biotechnology (Lake Placid, NY).

Adhesion Assay

Polystyrene, nontissue culture-treated, 48-well cluster plates (Costar, Cambridge, MA) were coated for 2 h at 37°C with 10 μg/ml vitronectin or collagen I in phosphate-buffered saline (PBS), pH 7.4; before use, the wells were blocked with RIA grade 1% heat-denatured bovine serum albumin (BSA). The cells were starved for 24 h, then harvested with trypsin/EDTA (Life Technologies, Gaithersburg, MD) and the trypsin was inactivated with soybean trypsin inhibitor (Type I-S). Cells were washed with serum-free Fibroblast Basic Medium (FBM) containing 0.5% BSA (Clonetics, San Diego, CA; BSA/FBM), incubated for 3 h in the presence of 3, 10, or 30 μM of a κB or a mutated κB oligonucleotide, and then treated for 1 h with 5 ng/ml phorbol myristate acetate (PMA) in BSA/FBM before time zero of the adhesion assay. Cells were added at a concentration of 50,000 cells/well in BSA/FBM and allowed to adhere for 2 h. Nonadherent cells were removed by gentle washing and remaining adherent cells were quantified using a colorimetric cell titer assay (CellTiter 96; Promega, Madison, WI). Each data point was calculated from assays performed in triplicate. Nonspecific adhesion as determined by attachment to BSA-coated wells has been subtracted.

Cell Migration Assay

Cellular migration assays were performed as previously described (Klemke *et al.*, 1994).

Oligonucleotides and Radiolabeling Procedures

Single-stranded oligonucleotides encoding the consensus NF-κB binding site d(5′ CAGA**GGGGACTTTCC**GAGA 3′), a mutated NF-κB binding site d(5′ CAGA**GGGGACTTAGA**GAGA 3′), or a consensus Oct-1 binding site d(5′ TGTCGA**ATGCAAAT**CACTAGA 3′) were annealed with their complementary strands and subsequently end labeled with [32γ]ATP (Amersham, Arlington Heights, IL) to a specific activity greater than 10^7 cpm/μg using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared by a modification of the method of Dignam *et al.* (1983). Two micrograms of nuclear extract were incubated with 1–2 ng (approximately 10,000 cpm) of radiolabeled double-stranded oligonucleotide and incubated for 15–30 min at room temperature in a binding buffer containing 20 mM HEPES, pH 7.9, 0.2% NP40, 50 mM KCl, 1 mM dithiothreitol, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg poly-deoxyinosinic-deoxycytidylic acid. In the case of electrophoretic mobility supershift experiments, DNA binding reactions were followed by a 30-min incubation at 4°C with Igs specific for the various NF-κB members. The specificity of p65 binding was demonstrated by addition of a 60-fold molar excess of either a peptide used to generate the p65 antisera (specific peptide) or a peptide directed against relB (irrelevant peptide). After the binding reaction, the samples were fractionated on 6% nondenaturing polyacrylamide gels in 0.5× TBE, dried, and exposed to x-ray film for autoradiography.

Plasmids

The chloramphenicol acetyltransferase (CAT) reporter plasmids pBL-CAT and p2xκB-CAT, containing the thymidine kinase (TK) promoter alone or the TK promoter together with a tandem repeat of an NF-κB binding site have been previously described (Becker *et al.*, 1995).

Cell Transfection and CAT Assays

FG cells were transiently transfected by electroporation. A total of 10^7 cells were transfected per point with equimolar amounts of test plasmids (5 pmol) with a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) at settings of 300 V and 960 mF, with a 0.4-cm electrode gap. The pSV-bgal (Promega, Madison, WI) reporter plasmid was included in all transfections to control for transfection efficiency. Forty-eight hours after transfection, cells were incubated for 3 h either with media alone or with 30 μM double-stranded κB or mutated κB oligonucleotides described above followed by stimulation with PMA (10 ng/ml) for 2 h. At the end of the incubation period cells were harvested, resuspended in 100 μl 0.25 M Tris-HCl (pH 8.0), and subjected to three rapid freeze/thaw cycles in dry ice/ethanol and in a 37°C water bath, respectively. Cell lysates to be used for CAT assays were incubated for 10 min at 60°C, centrifuged for 3 min at 13,000 × *g*, and the supernatant was transferred to a new tube and normalized for protein concentration. CAT assays were done by incubating cell lysate protein extracts with 0.1 mCi [^{14}C]chloramphenicol (60 mCi/mmol, Amersham, Buckinghamshire, England) in the presence of 10 mM *n*-butyryl-coenzyme A for 3 h at 37°C. To terminate the reaction, 500 μl of ethyl acetate was added. Butyrylated and nonbutyrylated chloramphenicol were separated by thin-layer chromatography and subsequently visualized with a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Phosphodiester Oligonucleotide Treatment of Cells

FG cells were harvested and resuspended at 10^6 cells/ml in BSA/FBM containing 3, 10, or 30 μM of double-stranded oligonucleotide for 3 h at 37°C in a humidified incubator. Cells were then treated with 5 ng/ml PMA for 1 h, washed twice in BSA/FBM, and utilized in cellular adhesion or migration assays as described above.

RESULTS

Vitronectin-mediated Cell Motility Requires a Late Activation Event Induced by PKC

FG carcinoma cells readily adhere to vitronectin using integrin αvβ5, however, they depend on PKC activation to migrate on this ligand (Klemke *et al.*, 1994). This requirement for PKC is a particular attribute of integrin αvβ5, because these same cells readily migrate on collagen using integrin α2β1 without exogenous activation of PKC (Leavesley *et al.*, 1992). It is well documented that certain integrin-mediated adhesive interactions can be activated by agonist stimulation (Shaw *et al.*, 1990; Santala and Heino, 1991; Wilkins *et al.*, 1991; Martin-Thouvenin *et al.*, 1992). In fact, PKC is known to activate multiple signaling pathways resulting in both immediate as well as delayed cellular responses (Davis *et al.*, 1993; Juliano and Haskill, 1993; Shaw *et al.*, 1993; Vuori and Ruoslahti, 1993; Bott *et al.*, 1994). Therefore, we examined the kinetics of PKC induction of FG motility on vitronectin. FG cells were stimulated with phorbol ester for 1 h, washed, and then allowed to migrate on vitronectin for various time intervals. Alternatively, after PMA treatment, FG cells were cultured for 24 h in the absence of serum and then allowed to migrate. As depicted in Figure 1, FG cell migration was delayed for 6 h after PKC stimulation. In contrast, cells pretreated 24 h earlier exhibited rapid vitronectin-directed migration that was measurable within 2 h (Figure 1). These rapid kinetics are comparable to those observed when FG cells were allowed to migrate on collagen without previous PKC activation (our unpublished observations). These findings indicate that αvβ5-dependent cell migration on vitronectin is the consequence of a late activation event mediated by PKC.

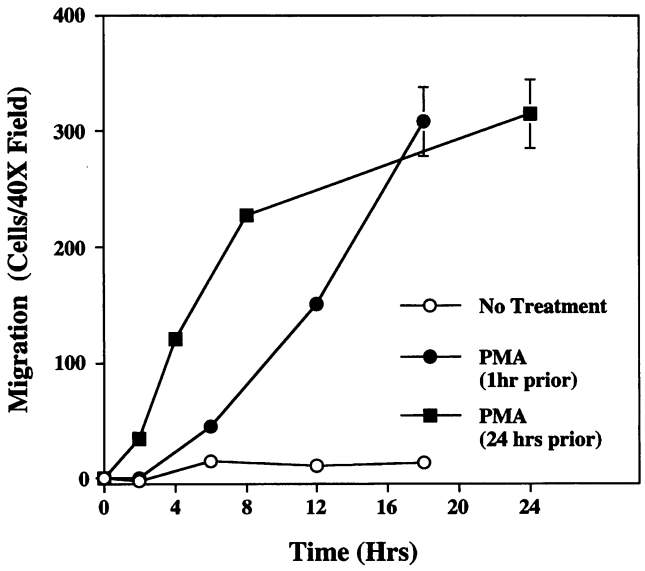

Figure 1. Kinetics of phorbol ester-stimulated FG cell migration on vitronectin. FG cells were treated with 5 ng/ml PMA for 1 h, washed, and allowed to migrate toward vitronectin, for various times in modified Boyden chambers. Alternatively, cells were cultured for 24 h after a 1-h PMA treatment and then allowed to migrate toward vitronectin. After cell migration, cells were stained with crystal violet and enumerated by counting cells in a 40× field. Each data point represents the mean ± SE of four replicate migration chambers.

Migration of FG Cells Requires PKC-induced Gene Expression

PKC not only activates early signaling events but is known to regulate gene transcription (Gross *et al.*, 1993; Bott *et al.*, 1994). Therefore, we tested the possibility that the delayed migration response of FG cells on vitronectin is due to PKC-mediated de novo gene expression. FG cells were stimulated with PMA in the presence or absence of actinomycin D and then allowed to migrate on vitronectin or collagen. As illustrated in Figure 2A, PKC-dependent migration was inhibited with actinomycin D in a dose-dependent manner. In contrast, constitutive collagen migration was independent of de novo gene expression and thus was not inhibited with actinomycin D. Identical results were obtained when cells were stimulated with another PKC-activating phorbol ester, phorbol dibutyrate (our unpublished results). In addition, cycloheximide inhibited cell migration induced by PKC activation (Figure 2B), indicating that de novo protein synthesis of a gene product(s) is required for PKC-induced motility on vitronectin. It is important to note that neither cycloheximide nor actinomycin D inhibited FG cell attachment to vitronectin or collagen (our unpublished results). These findings demonstrate that αvβ5-dependent adhesion of FG cells to vitronectin leads to a motility response only after activation of a PKC-mediated transcriptional event and de novo protein synthesis. These results account for the late acting effects of PKC on αvβ5-dependent vitronectin cell motility (Figure 1). Importantly, this PKC-induced motility event is not due to an increase in αvβ5 levels on the cell surface because no changes in expression of this integrin were detected either by immunoprecipitation or flow cytometric analysis using a monoclonal antibody specific for αvβ5 (our unpublished observations).

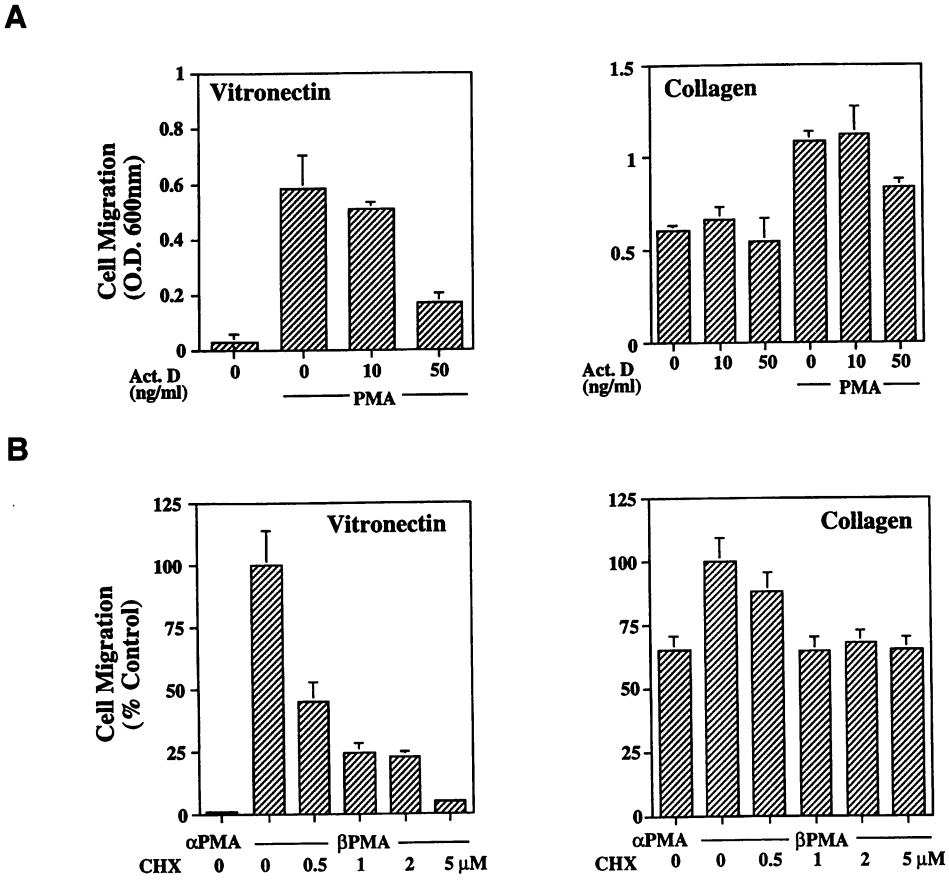

Figure 2. Effect of actinomycin D or cycloheximide on phorbol ester-stimulated FG cell migration toward vitronectin or collagen. (A) FG cells were incubated for 2 h in the presence of 0–50 ng/ml actinomycin D, and then treated with PMA (5 ng/ml) for 1 h. Cells were then washed and allowed to migrate toward vitronectin or collagen for 20 h. Migrant cells were stained with crystal violet and quantified by measuring OD 600 nm values of the eluted dye. Each bar represents the mean ± SE of quadruplicate samples. (B) FG cells were treated with 5 ng/ml αPMA (inactive isomer) or βPMA (active isomer) for 1 h, washed, and allowed to migrate for 20 h toward vitronectin or collagen in the presence or absence of various concentrations of cycloheximide. Cell migration was enumerated by dye uptake and expressed as the percent of control migration (in the absence of cycloheximide), which was designated at 100%. Each bar represents the mean ± of SE of quadruplicates.

Activation of PKC in FG Cells Leads to NF-κB DNA Binding Activity

Several studies have shown that isoforms of PKC can activate NF-κB–mediated transcriptional events (Dominguez *et al.*, 1993; Diaz-Meco *et al.*, 1993, 1994; Hirano *et al.*, 1995). In addition, recent reports have indicated that NF-κB–mediated transcriptional events are associated with the expression of genes involved in diverse cellular adhesive functions (Eck *et al.*, 1993; Narayanan *et al.*, 1993; Shu *et al.*, 1993; Sokoloski *et al.*, 1993). To examine the effects of PKC activation on NF-κB binding activity in FG cells, an electrophoretic mobility shift assay was performed using nuclear extracts prepared from untreated or PMA-treated FG cells. As shown in Figure 3, a component within nuclear extracts from PMA-treated cells bound to a radiolabeled synthetic oligonucleotide containing a consensus NF-κB binding sequence (Figure 3A), but exhibited no measurable binding to an oligonucleotide encoding a mutated NF-κB binding site (Figure 3B). The specificity of this interaction was demonstrated because a 100-fold molar excess of the unlabeled consensus κB oligonucleotide effectively competed for binding while the control oligonucleotide containing the mutated NF-κB binding site failed to do so (Figure 3). These results demonstrate that FG cells undergo a significant increase in specific NF-κB binding activity in response to PKC stimulation.

To further investigate whether the observed NF-κB binding occurs as a consequence of PKC activation, FG cells were pretreated with the PKC inhibitor, calphostin C, and then nuclear extracts of these cells were tested for NF-κB binding activity. As shown in Figure 4A, the increase in NF-κB binding that occurs in response to PMA stimulation could be blocked by pretreatment of these cells with calphostin C, whereas calphostin C had no inhibitory effect on the binding of the constitutive transcription factor oct-1 (Figure 4B). It is important to note that the concentration of calphostin C used (40 nM) to block NF-κB activity was previously shown to selectively block the PMA-induced FG cell migration on vitronectin (Klemke *et al.*, 1994). These results suggest a pathway linking PKC stimulation to the activation of NF-κB binding activity in FG carcinoma cells. More importantly, these data link NF-κB activation with FG cell motility on vitronectin.

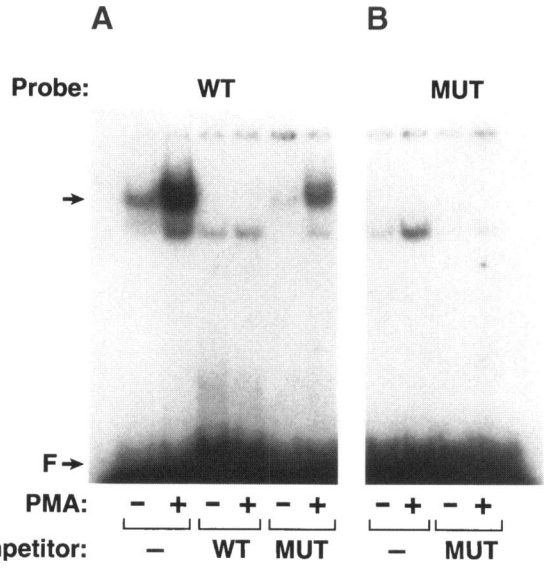

Figure 3. PMA-induced NF-κB binding activity in FG carcinoma cells. Electrophoretic mobility shift assay utilizing 2 μg of nuclear extract prepared from untreated (−) or PMA-treated (+) FG carcinoma cells and 1 ng of radiolabeled double-stranded oligonucleotide containing either κB (WT) CAGA**GGGGACTTAGA**GAGA (A) or mutant κB (MUT) CAGA**GGGGACTTTCC**GAGA (B) consensus sequences. DNA binding reactions were conducted at room temperature for 20 min in the presence or absence of a 100-fold molar excess of unlabeled competitor. After this incubation, bound nucleotide was fractionated from unbound nucleotide on 6% nondenaturing polyacrylamide gels and exposed to x-ray film for autoradiography. The slower-migrating band (arrow) represents the NF-κB complex. The band immediately below the NF-κB complex observed with both probes is nonspecific. Free oligonucleotide (F) is evident at the bottom of the gel.

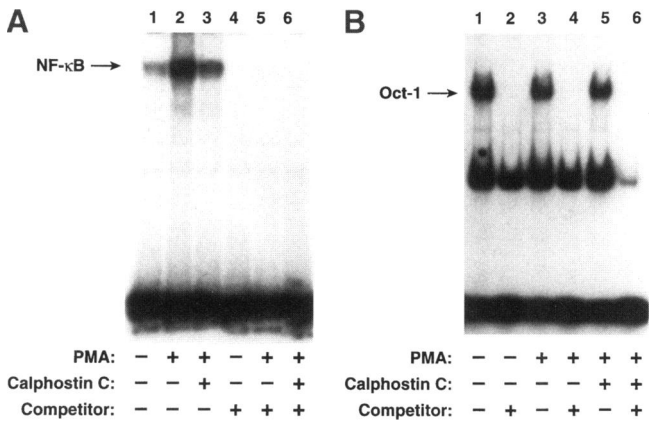

Figure 4. Calphostin C blocks PMA-stimulated NF-κB binding. (A) Electrophoretic mobility shift assay utilizing 1 ng of radiolabeled κB oligonucleotide (as in Figure 3 legend) and 2 μg of nuclear extract prepared from FG cells, unstimulated (lanes 1 and 4), PMA-stimulated (lanes 2 and 5), or PMA stimulated and calphostin C pretreated (lanes 3 and 6) in the absence (lanes 1–3) or presence of 100-fold molar excess of cold competitor (lanes 4–6). (B) Nuclear extracts used in panel A were analyzed by electrophoretic mobility shift assay for binding 1 ng of radiolabeled oct-1 oligonucleotide as follows: unstimulated (lanes 1 and 2), PMA stimulated (lanes 3 and 4), or PMA stimulated and calphostin C pretreated (lanes 5 and 6) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 100-fold molar excess cold competitor.

PKC Activation in FG Cells Promotes Formation of NF-κB Transcription Complexes Containing p65 and p50

NF-κB represents a family of five distinct subunits that form hetero- and homodimers that bind to and transcribe DNA (Grilli *et al.*, 1993; Liou and Baltimore, 1993). The major form of NF-κB is a heterodimer composed of p65 and p50 subunits present in an inactive form in the cytoplasm complexed to inhibitors, termed IκBs, the prototype being IκBα (Baeuerle and Baltimore, 1989; Rice and Ernst, 1993). Activation of cells with a variety of stimuli results in phosphorylation and proteolytic degradation of IκBα leading to the translocation of NF-κB to the nucleus where it binds DNA (Liou and Baltimore, 1993; DiDonato *et al.*, 1995; Lin *et al.*, 1995). To identify which NF-κB family members were capable of binding a κB regulatory element in response to PKC activation of FG carcinoma cells, supershift gel assays were performed using antibodies specific for each of the NF-κB subunits. As demonstrated in Figure 5, antibodies directed against either p65 or p50 induced a supershift, whereas antibodies to c-rel, rel B, and p52 did not. The specificity of anti-p65 in this assay was established because the immunizing peptide used to generate this antibody completely blocked the supershifted band (Figure 5). The specificity of anti-p50 has been previously established (Oeth *et al.*, 1994). We further tested five different antibodies directed to c-rel in gel supershift assays, yet failed to detect measurable c-rel binding to the consensus κB site (our unpublished observations). These findings suggest that PKC activation of FG cells promotes an NF-κB binding activity consisting of p65 and p50.

Functional Activity of NF-κB Transcription Complexes in PMA-activated FG Cells

Introduction of a tandem repeat of an NF-κB binding site upstream of the TK promoter of pBL-CAT enabled us to test the functional relevance of the detected NF-κB transcription complexes. After transfection into FG cells, the CAT expression from this construct in resting cells was compared with the expression 2 h after stimulation with PMA (Figure 6). In resting cells, only a low level of CAT expression from this construct was evident; stimulation with PMA led to a fivefold increase in CAT expression. To confirm the specificity of the observed induction of CAT activity, before PMA stimulation we incubated FG cells for 3 h with an excess of the double-stranded oligonucleotide previously used for detection of NF-κB binding activity.

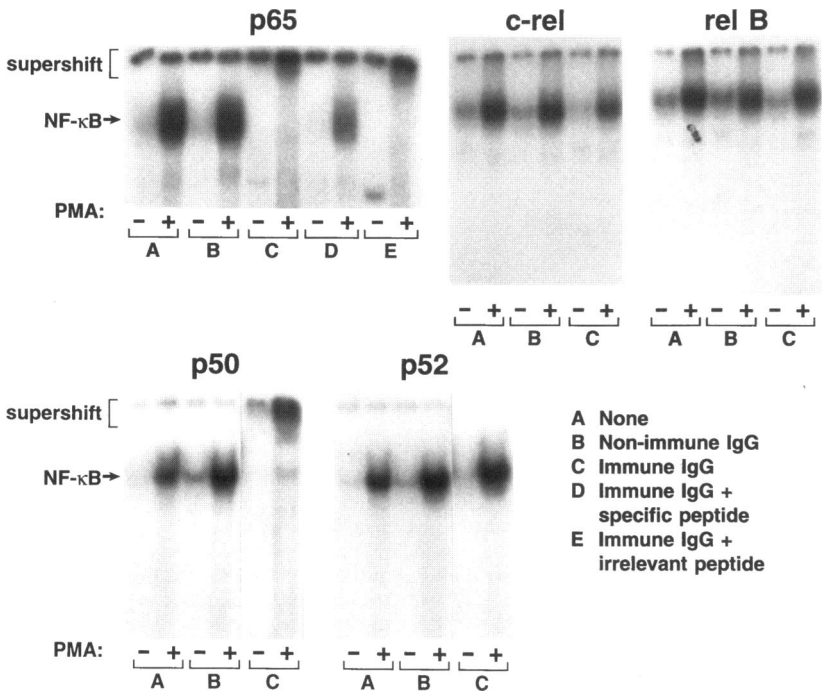

Figure 5. NF-κB binding activity is mediated by p65 and p50. Nuclear extracts from untreated (−) or PMA-treated (+) FG carcinoma cells were incubated for 20 min at room temperature with 1 ng of a radiolabeled NF-κB consensus oligonucleotide. DNA binding reactions were followed by the addition of nonimmune rabbit IgG or Igs that specifically recognize p65, c-rel, relB, p50, and p52, and immunizing or irrelevant competing peptide (D and E) as described in MATERIALS AND METHODS. The slower-migrating supershifted bands are indicated by the bracket.

This treatment virtually abolished the PMA-induced increase of CAT expression from p2xκBCAT (Figure 6). In contrast, the control mutant κB oligonucleotide did not interfere with the induction of CAT expression from the reporter construct after PMA stimulation. These findings demonstrate that the κB oligonucleotide used in the DNA-binding assays directly impacts NF-κB–mediated gene transcription in vivo.

FG Cell Motility Depends on PKC Induction of NF-κB

To determine whether the vitronectin migration of FG cells was dependent on PKC activation of NF-κB, FG cells were incubated with a double-stranded oligonucleotide containing the same NF-κB consensus binding site used in the DNA binding studies (above) before PMA treatment. This approach has been shown to cause significant cellular uptake of oligonucleotides (Eck *et al.*, 1993). As shown in Figure 7, the NF-κB–specific oligonucleotide blocked PKC-mediated vitronectin cell migration in a dose-dependent manner, yet it had no effect on the α2β1-dependent collagen migration of these cells. In contrast, the control oligonucleotide containing the mutant κB site had no effect on FG cell migration. Moreover, neither oligonucleotide affected FG cell adhesion to vitronectin or collagen (Figure 7). These findings demonstrate that activation of NF-κB by PKC is involved in αvβ5-dependent cell migration but not adhesion on vitronectin. Moreover, FG cell migration on collagen is constitutive and thus is independent of the ability of PKC to activate an NF-κB–mediated transcriptional event.

DISCUSSION

Cell motility is a highly regulated process that occurs during embryonic development, inflammation, wound healing, and cancer (Albelda and Buck, 1990). Although little is known regarding the molecular regulation of cell movement, it is clear that integrin-mediated adhesion is involved in this process. However, it is also apparent that such adhesion events are not always sufficient for the expression of a migratory phenotype. For example, we have previously shown that FG pancreatic carcinoma cells attach to vitronectin via integrin αvβ5, yet fail to migrate on this ligand even though these cells express α2β1, enabling them to attach and constitutively migrate on a collagen substrate (Leavesley *et al.*, 1992). Moreover, transfection of FG cells with a cDNA encoding the integrin β3 subunit promoted expression of αvβ3 and enabled them to migrate on vitronectin without previous activation (Leavesley *et al.*, 1992). These findings demonstrate that different integrins on the cell surface, which recognize the same ligand, differ with respect to their ability to promote cell migration and that αvβ5 promotes adhesion yet is unable to engage the motility machinery. In this report we demonstrate that the specific activation of αvβ5-dependent motility in FG cells depends on a transcriptional event mediated by PKC.

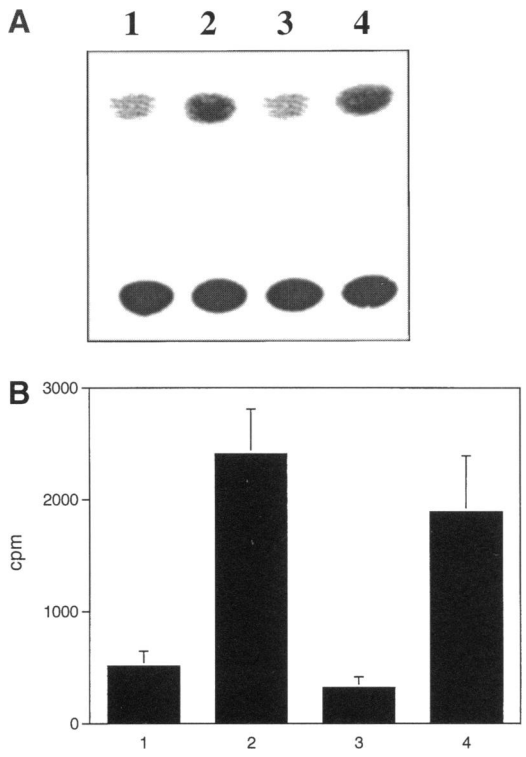

Figure 6. Functional activity of NF-κB transcription complexes. Cellular extracts of FG cells transfected with p2xκB-CAT were obtained after no stimulation (1), PMA treatment alone (2), or PMA treatment in the presence of 30 μM oligonucleotides containing either the consensus κB recognition sequence (3) or a mutated κB recognition sequence (4), were assayed for CAT activity. Results were displayed as autoradiographs of a thin-layer chromatography (A) or as a quantification of the amount of dibutyrylated [^{14}C]chloramphenicol (B).

Recent reports have shown that integrins can respond to intracellular signals enabling them to undergo conformational changes resulting in enhanced ligand binding (Shaw *et al.*, 1990, 1993; Hibbs *et al.*, 1991; Valmu *et al.*; 1991; Wilkins *et al.*, 1991). For example, cellular activation induces immediate integrin function as measured by platelet aggregation (Shattil and Brass, 1987; Ginsberg *et al.*, 1992) or leukocyte adhesion to endothelium (Lo *et al.*, 1989). In each of these cases, agonists, such as cytokines or phorbol esters, were used to activate the integrin-dependent cellular response. Phorbol esters are known to activate the serine/threonine kinase PKC, which in turn, impacts a wide range of biological properties. This is likely due to the fact that PKC not only directly activates signaling molecules, but in addition, is capable of inducing gene transcription (Ghosh and Baltimore, 1990; Diaz-Meco *et al.*, 1993; Gross *et al.*, 1993; Bott *et al.*, 1994; Hirano *et al.*, 1995).

Transcriptional events have been linked to cell movement in vivo. For example, both the C/EBP and the ETS transcription factor families have been shown to play a role in cell migration events during *Drosophila* development (Montell *et al.*, 1992; Klambt, 1993). In addition, the ETS family has been implicated in endothelial cell migration and neovascularization during mouse development (Grevin *et al.*, 1993). These findings reveal that during various biological processes in vivo, cell migration is only achieved after induction of specific transcriptional events. Thus, it is conceivable that such transcriptional events are related to those involved in FG cell migration on vitronectin.

We present several lines of evidence that the PKC-dependent transcriptional event involved in FG cell motility is mediated by NF-κB family members, and in particular p65 and p50. First, PKC activation leads to enhanced binding of a component within FG cell nuclear extracts to a consensus κB element whereas these extracts exhibit no binding activity to an oligonucleotide containing a mutated κB site. Second, pretreatment of cells with the specific PKC inhibitor calphostin C, which blocks αvβ5-dependent FG cell motility on vitronectin (Klemke *et al.*, 1994), leads to a dramatic reduction of this NF-κB–dependent molecular interaction. Furthermore, antibodies specific for p65 and p50 promote a gel supershift when incubated with FG cell nuclear extracts in the presence of a κB oligonucleotide indicating a potential nuclear complex containing p65 and p50. The functional relevance of this binding activity is demonstrated by transfection of FG cells with CAT reporter constructs containing a tandem repeat of a consensus κB binding site followed by PMA-induced CAT transcription from these constructs, an event that is inhibited by the oligonucleotide used for detection of the NF-κB binding activity. Finally, this same oligonucleotide abolishes PKC-induced FG cell motility on vitronectin while having no effect on the constitutive, PKC-independent motility on collagen or on adhesion to either ligand.

NF-κB is an inducible transcription factor originally identified as a heterodimer consisting of a 65-kDa subunit (p65) and a 50-kDa subunit (p50) (Baeuerle and Baltimore, 1989). Both subunits share a 300-amino acid region of N-terminal homology with various members of the Rel transcription factor family (Blank *et al.*, 1992). NF-κB is found in the cell cytosol as an inactive complex with inhibitors, termed IκBs (Baeuerle and Baltimore, 1988; Liou and Baltimore, 1993). Earlier in vitro studies have shown that PKC can phosphorylate IκB, resulting in the failure of the phosphorylated IκB to inhibit NF-κB DNA binding (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). However, recent in vivo observations indicate that IκB phosphorylation is necessary but not sufficient for its proteolytic degradation and that this degradation is required for the release of active NF-κB, which then translocates to the nucleus where it binds its target genes (DiDonato *et al.*, 1995; Lin *et al.*, 1995).

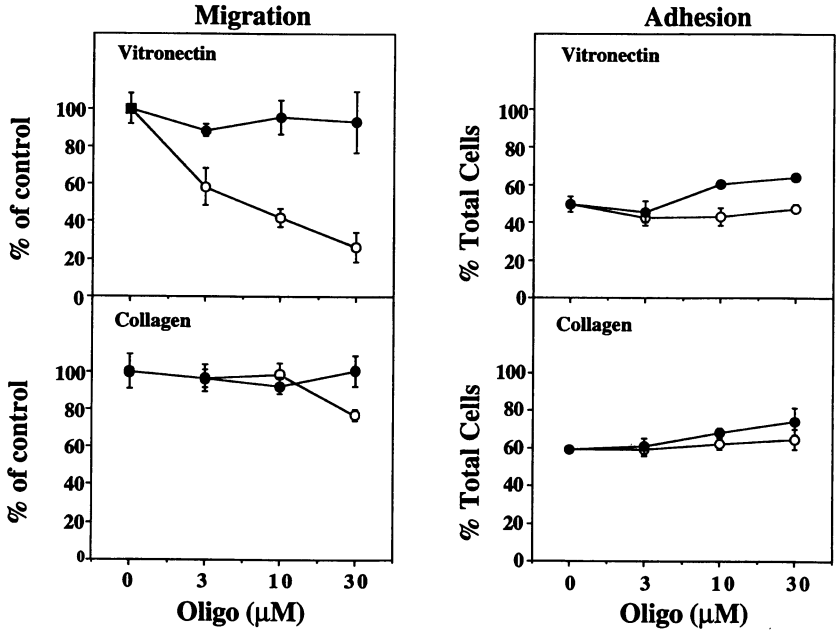

Figure 7. An oligonucleotide containing a κB consensus sequence inhibits migration of PMA stimulated FG cells on vitronectin but not collagen. FG cells were incubated with various concentrations of κB (○) or mutated κB (●) oligonucleotides for 3 h, treated with PMA (5 ng/ml) for 1 h, washed, and allowed to migrate for 20 h toward vitronectin or collagen (left panel) or adhere to immobilized ligands for 2 h (right panel). Cell migration was enumerated by dye uptake as described above. Cell adhesion is presented as percent of total adherent cell population. Cell attachment to BSA-coated wells was subtracted as background adhesion and was typically less than 5%. Each point represents the mean ± SE of triplicates.

It is well documented that several agents such as the active phorbol ester PMA, mitogens, and cytokines induce the nuclear expression and DNA-binding activity of NF-κB family members in various cell types (Grilli *et al.*, 1993; Liou and Baltimore, 1993). In this report we demonstrate enhanced DNA-binding activity of p65 and p50 as well as NF-κB–dependent transcription upon PKC activation in FG cells.

The present study demonstrates a role for an NF-κB–regulated transcriptional event resulting in specific integrin-mediated motility independent of cell adhesion. Our results show that αvβ5, unlike α2β1 or αvβ3, requires PKC activation resulting in NF-κB–dependent transcription to engage the motility machinery. We propose that structural component(s) within the β5 subunit may make αvβ5 particularly responsive to this activation pathway in FG cells. Specifically, α2β1 or αvβ3 expressed in these cells leads to constitutive PKC-independent cell motility (Leavesley *et al.*, 1992). It is possible that regions within the β5 cytoplasmic tail or within the ectodomain of this subunit require an interaction with a protein(s) whose expression depends on PKC-mediated gene transcription. Alternatively, without previous PKC activation, the affinity of αvβ5 for vitronectin may not be compatible with receptor clustering necessary for actin assembly and cell motility.

The data in this report define a novel activation pathway leading to the regulation of integrin-mediated cell motility. Although we have implicated a role for NF-κB in PKC-dependent migration via integrin αvβ5, we do not exclude participation of other transcription factor families. Indeed it has recently been shown that other transcription factors can synergize with NF-κB to potentiate its function in gene transcription. Our findings suggest that transcriptional control of cell motility may ultimately impact our understanding of cell matrix interactions important during wound healing, inflammation, and cancer.

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