

## Integrin ligation and PKC activation are required for migration of colon carcinoma cells

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### SUMMARY

The activation of protein kinases C (PKCs) is an essential step in integrin-dependent cell adhesion and spreading. In this report we examined the effect of the phorbol ester PMA, a PKC activator, on adhesion, spreading and migration of a colon carcinoma cell line, HT29-D4. Treatment with PMA increased the rate of cell spreading and induced the migration of these cells towards purified matrix proteins in haptotaxis assays on Boyden chambers. PMA-induced effects were the result of PKCs activation, as shown by using the inactive isomer 4 $\alpha$ -PMA and PKCs inhibitors. The involvement of integrins in the phorbol ester-induced cell migration was demonstrated both by the absence of migration of cells plated on membranes coated with poly-L-lysine and by the use of function blocking antibodies. Thus, interactions between  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1,  $\alpha$ 6 $\beta$ 4,  $\alpha$ v $\beta$ 5,  $\alpha$ v $\beta$ 6 integrins and their specific ligands are necessary for the PKC-mediated migration. However, adhesion, immunoprecipitation and immunocytofluorometry

experiments clearly showed that HT29-D4 cell haptotaxis induced by PKC activation is not a consequence of quantitative or qualitative changes in the cell surface integrins. We also demonstrated that PKCs were able to activate the MAP kinase pathway and that the impediment of MAP kinase activation resulted in the loss of cell migration. Moreover, stimulation of the insulin-like growth factor I signalling pathway led to MAP kinase activation and to the induction of cell migration. In addition, the growth factor-induced motility of HT29-D4 cells was affected both by PKC and MAP kinase cascade inhibitors. It thus appears that both integrin ligation and MAP kinase activation by PKCs are required to promote the migration of HT29-D4 cells.

Key words: Haptotaxis, MAP kinase, Insulin-like growth factor I, Spreading, Adhesion, Signaling, Extracellular matrix, Phorbol ester

### INTRODUCTION

Epithelial cells interact with the extracellular matrix (ECM) during a variety of biological processes including embryonic development, tumor metastasis and wound healing. Intestinal epithelial injury occurs frequently, impairing barrier function and vectorial transport of epithelium. Epithelial repair occurs rapidly to maintain these functions and needs epithelial cell migration into the defect. The interaction of cells with ECM is, in part, mediated by integrins which are heterodimeric receptors composed by the non covalent association of one of the 16  $\alpha$  subunits and one of the 8  $\beta$  subunits characterized to date (Hynes, 1992; Varner and Cheresch, 1996). Integrins are known to be involved in the regulation of cell adhesion and cytoskeleton remodelling and so in cell spreading and motility. During the last past years, extending results have provided evidence that integrins are also able to induce numerous intracellular signals (reviewed by Schwartz et al., 1995). However, little is known about how integrin signalling

regulates cell motility. Recent in vitro and in vivo studies, showed that integrins are necessary but not sufficient for cell migration, and that activation of cytokine or growth factor signal transduction pathways are also required. Thus, epidermal growth factor or insulin-like growth factor are required for cell migration on vitronectin via the  $\alpha$ v $\beta$ 5 integrin (Klemke et al., 1994; Brooks et al., 1997). The identification of the molecular components common to the integrin and growth factor pathways will allow a better understanding of cell motility regulation.

Protein kinases C (PKCs) represent a family of key enzymes involved in the processes of signal transduction and growth regulation by hormones, neurotransmitters and growth factors. PKCs also play a crucial role in the regulation of various integrin-dependent cellular functions such as cell adhesion, spreading and motility (for a review see Clark and Brugge, 1995). PKC activators, such as the phorbol ester phorbol 12-myristate 13-acetate (PMA), have been used in a number of studies to demonstrate the relationship between PKC activation

and modulation of integrin-dependent functions. These studies indicate that adhesion, spreading and metastasis are, at least partly, regulated by the action of PKCs. Thus, a strong correlation between basal levels of membrane-bound PKCs and the metastatic potential of tumor cells has been reported (Gopalakrishna and Barsky, 1988). Despite numerous studies, it is not clear how PKCs are integrated into the integrin-dependent signal transduction pathway. Since integrins have been shown to be phosphorylated in several systems, the PKC effect may occur through phosphorylation of integrins (Freed et al., 1989; Parise et al., 1990; Shaw et al., 1990), increase of cell surface integrin expression (Eguchi and Horikoshi, 1996), cytoskeletal remodelling (Burn et al., 1988; Sakamoto et al., 1996), or by activating transcription factors (Hirano et al., 1995; Yebra et al., 1995). PKCs have also been shown to activate the adhesive properties of several cells by increasing the affinity of their cell surface integrins (Valmu et al., 1991; Wilkins et al., 1991; Martin-Thouvenin et al., 1992). In addition, PKCs potentiate signalling events leading to integrin-dependent cell spreading and motility (Vuori and Ruoslahti, 1993; Klemke et al., 1994).

In this report we show that activation of PKCs resulted in the migration of colonic carcinoma cells towards various ECM proteins. We demonstrate that cell motility was dependent on MAP activation and that stimulation of the insulin-like growth factor (IGF)-I signalling pathway, which lead to MAP kinase activation, also promoted cell migration. This growth factor-induced motility was affected both by PKC and MAP kinase cascade inhibitors. Thus, activation of MAP kinase by PKCs seems to be a prerequisite in the pathway leading to the integrin-dependent migration of HT29-D4 cells.

## MATERIALS AND METHODS

### Reagents

Fetal bovine serum was from Sera-Lab (Crawley Down, UK), Dulbecco's modified Eagle's medium (DME medium) was from Gibco (Cergy-Pontoise, France) and PBS was from Oxoid (Basingstoke, UK). Calphostin C, human fibronectin and type IV collagen were purchased from Sigma (La Verpillère, France). Bisindolylmaleimide GF109203X was from Boehringer-Mannheim (Meylan, France). 2'-amino-3'-methoxyflavone (PD98059) was from Alexis Biochemicals (San Diego, CA). PMA and 4 $\alpha$ -phorbol 12-myristate 13-acetate (4 $\alpha$ -PMA) were obtained from ICN Pharmaceuticals (Orsay, France). Sulfo succinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) was from Pierce (Rochford, IL). ECL detection kit and streptavidin-horseradish peroxidase (HRP) conjugate were from Amersham (Les Ulis, France). Des(1-3)IGF-I was purchased from GroPep (Adelaide, Australia). Laminin-1 and vitronectin were prepared according to the method of Timpl et al. (1979) and Yatogho et al. (1988), respectively.

Monoclonal antibody (mAb) P4C10 against  $\beta$ 1 subunit and mouse mAb PIB5 against  $\alpha$ 3 subunit were from Gibco-Telios. Rat mAb 69.6.5 against  $\alpha$ v integrin was obtained as previously described (Lehmann et al., 1994). Rat mAb GoH3 against  $\alpha$ 6 subunit, mouse mAb Gi9 against  $\alpha$ 2 subunit, mouse mAb SAM1 against  $\alpha$ 5 subunit, mouse mAb K20 against  $\beta$ 1 subunit and mouse mAb SZ21 against  $\beta$ 3 subunit were from Immunotech (Marseille, France). Anti-active MAPK polyclonal antibody was obtained from Promega (Madison, WI). Donkey anti-rabbit immunoglobulins-HRP conjugate was from Amersham.

### Cell adhesion assays

Adhesion substrata were prepared by coating flat-bottom 96-well microtiter plates (Nunc) overnight at 4°C with 50  $\mu$ l of vitronectin, collagen, laminin or fibronectin at the indicated concentrations. Coated wells were blocked with 1% BSA in PBS for 1 hour and then washed with PBS. The human colon adenocarcinoma cell line HT29-D4 was routinely cultured as described (Fantini et al., 1986). Cells were obtained in single cell suspension by treatment of subconfluent cell monolayers with 0.53 mM EDTA in PBS. After centrifugation, cells were washed twice with Dulbecco modified Eagle's medium (DMEM) containing 0.2% BSA (adhesion buffer) and resuspended in the same medium in the presence or in the absence of PMA. Cells (50,000 cells/50  $\mu$ l) were added to each well and allowed to adhere to the substrata for 2 hours at 37°C in a cell culture incubator. Unattached cells were removed by gently washing three times with adhesion buffer and residual attached cells were fixed by 1% glutaraldehyde. After staining by 0.1% Crystal Violet, cells were lysed with 1% SDS and the optical density was measured at 600 nm by a microplate reader (model  $\Sigma$ 960 from Metertech).

### Cell spreading assays

Spreading substrata, as well as cell preparation, were realized as described for adhesion assays, except that tests were performed in 24-well plates. Cells (25,000 cells/0.5 ml) were seeded in substratum-coated wells and allowed to spread for 3 hours at 37°C. Images acquisition and cell area quantification were performed using the Vision Explorer-VA application from Graftek (Mirmande, France).

### Cell migration assays

Migration assays were performed using modified Boyden chambers (NeuroProbe Inc., Bethesda, MD) with 8- $\mu$ m pore polycarbonate Nucleopore membranes (Costar, Cambridge, MA). The undersurface of the membrane was precoated with purified matrix proteins in DMEM containing 0.1% BSA for 1 hour at 37°C. The lower reservoir was filled with the appropriate protein and the membrane was placed in the chamber. Migration towards Matrigel (50-fold dilution) was performed without precoating the filter. Cells were prepared as for adhesion assays, treated or not with PMA prior to being added into the chamber at low density (100,000 cells/cm<sup>2</sup>) to prevent cell-cell contacts and then incubated for 5 hours at 37°C. When calphostin C was used, incubations were carried out under fluorescent light to activate calphostin C (Bruns et al., 1991). Following incubation, non-migratory cells on the upper surface of the filter were wiped with a cotton swab. Migrated cells on the lower surface of the filter were fixed and stained with 0.125% Coomassie Blue in a methanol:acetic acid:water mixture (45:10:45). Haptotaxis was determined by counting cells in ten microscopic fields per well. Migration results are expressed as the average number of cells per microscopic field.  $\times$ 320.

### Flow cytometry analysis

Cell surface expression of integrin subunits on untreated or PMA-treated HT29-D4 cells was determined by flow cytometry. Subconfluent cells were harvested and resuspended in DMEM containing 20% fetal calf serum and 1% BSA. The single cell suspension (10<sup>6</sup> cells/ml) was incubated for 90 minutes at 4°C in the presence of anti-integrin mAbs (10  $\mu$ g/ml each). Cells were rinsed once with ice-cold DMEM containing 0.1% BSA and then incubated for 30 minutes at 4°C with the appropriate secondary FITC-conjugated antibody. After washing, cells were fixed with 1% paraformaldehyde and cell-bound fluorescence was quantified using a Becton-Dickinson FACScan flow cytometer. To obtain a correct quantification, all samples were prepared with the same cell suspension.

### Cell surface labeling and immunoprecipitation

Subconfluent cells were washed twice with PBS and incubated with 1 mg/ml NHS-LC-biotin in PBS for 20 minutes at 4°C under agitation.

Cells were then washed with PBS and solubilization was performed by a 30-minute incubation at 4°C with 50 mM Tris-HCl, pH 8, 200 mM NaCl and 1% Triton X-100 (RIPA buffer) containing 0.5% BSA and a mixture of proteinase inhibitors (1 mM PMSF, 500 units/ml aprotinin, 1 µg/ml leupeptin, 1 µM pepstatin, 1 mM iodoacetamide and 1 mM ortho-phenantroline). Clarified cell lysates were then incubated with antibodies overnight at 4°C. After adding Protein G-agarose for 1 hour, the suspension was centrifuged and pellets were washed four times with RIPA buffer containing 500 mM NaCl and once with PBS. Immunoprecipitated proteins were solubilized in Laemmli sample buffer, heated at 100°C for 5 minutes and submitted to SDS-PAGE under reducing or nonreducing conditions. The gel was then blotted onto a nitrocellulose sheet and revealed, after streptavidin-HRP incubation, by the light-based ECL system.

### Detection of activated MAPK

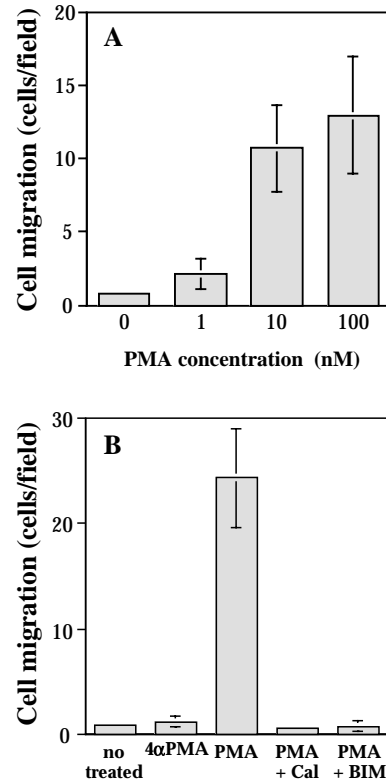
Confluent cells were washed twice with PBS, scraped into boiling 63 mM Tris-HCl buffer, pH 6.8, containing 10% glycerol, 4% SDS, 1 mM orthovanadate and the mixture of proteinase inhibitors. After sonication to fragment DNA, lysates were clarified by centrifugation at 15,000 g for 15 minutes. Extracted proteins (75 µg) were separated by 10% acrylamide SDS-PAGE and electrotransferred onto a nitrocellulose sheet. After saturation overnight at 4°C with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) containing 1% BSA, the nitrocellulose sheet was incubated with anti-active MAPK polyclonal antibodies for 2 hours at room temperature in TBS containing 0.05% Tween-20 and 0.1% BSA. Bound antibodies were detected by HRP-conjugated antibodies to rabbit immunoglobulins and revealed with the light-based ECL system.

## RESULTS

### PMA induces migration of HT29-D4 cells via PKC activation

To study HT29-D4 cell motility, we used haptotaxis assays towards attractive proteins in modified Boyden chambers. Cell motility was quantified by counting the number of cells that migrated to the underside of the membrane. We first used Matrigel, a complex material obtained from EHS (Engelbreth-Holm-Swarm) tumor cells, as a matrix. Matrigel contains not only ECM proteins (laminin-1, type IV collagen, heparan sulfate proteoglycans and entactin), but also some other components produced by tumor cells, such as growth factors (EGF, TGF-β), collagenase and plasminogen activator. Although HT29-D4 cells readily attached to Matrigel (not shown), they failed to migrate towards this complex ECM (Fig. 1A), even after a long period of exposure of more than 24 hours (data not shown). When we added PMA during the migration assays, we measured a significant migration of HT29-D4 cells after 5 hours in a dose-dependent manner (Fig. 1A). The fraction of motile cells after 5 hours (above 5% of the input) doubled when cells were allowed to traverse the membrane for 24 hours (data not shown). However, to give good interpretations of our results, we should take into account that after such a long period cells started to grow and that, more importantly, PMA could induce a down-regulation of PKCs (Hug and Sarre, 1993). Thus, subsequent experiments were limited to 5 hours and we used the optimal PMA concentration (10 nM).

To determine whether the effect of PMA on cell migration occurs as a consequence of PKC activation, we used two



**Fig. 1.** Activation of PKC induces HT29-D4 cell migration.

(A) Migration was performed as described in Materials and Methods using filters coated with Matrigel diluted 50-fold in DME medium containing 0.1% BSA. Cells were treated or not with various concentrations of PMA prior to be added into the chamber. After 5 hours at 37°C, cells that migrated into the lower surface of the filter were fixed, stained and quantitated. (B) Migration was performed as described above in the absence or in the presence of 10 nM 4α-PMA or PMA alone or in combination with 1 µM calphostin C (Cal) or 5 µM bisindolylmaleimide (BIM). All results are expressed as the average number of cells (±s.d.) per microscopic field (magnification, ×320) from 3 independent experiments performed in triplicate.

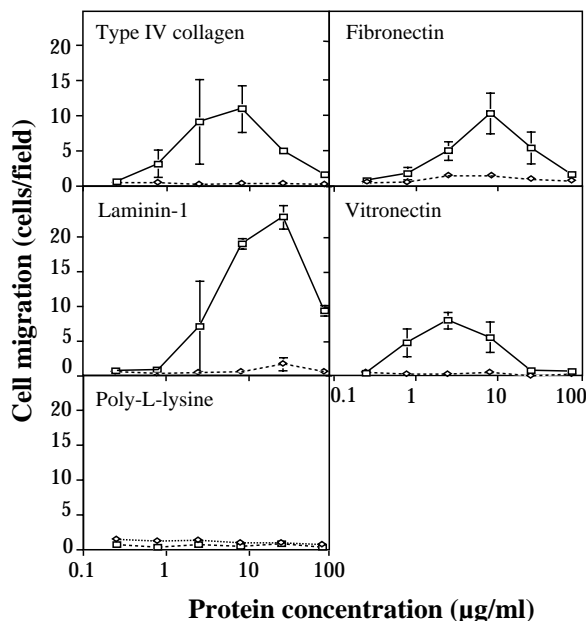
approaches. First, PMA was replaced by its inactive isomer 4α-PMA. Second, we determined whether the PMA effect could be blocked by two PKC inhibitors with different action sites on the kinase: calphostin C and bisindolylmaleimide GF109203X. As illustrated in Fig. 1B, 4α-PMA was unable to promote the migration of HT29-D4 cells. Moreover, both PKC inhibitors prevented PMA-induced migration. These results indicate that PKC activation is required for motility of HT29-D4 cells towards Matrigel.

### PKC-induced migration is mediated by specific integrin-ECM protein interactions

In order to investigate which components of Matrigel were involved in PKC-dependent haptotaxis, we next studied the effect of PMA treatment on HT29-D4 cell migration towards purified ECM proteins. We tested two major components of Matrigel, (laminin-1 and type IV collagen), as well as vitronectin and fibronectin in concentrations ranging from 0.25 to 80 µg/ml. As observed with Matrigel, although HT29-D4 cells readily adhered to the four matrix proteins tested (see results below), they were unable to spontaneously migrate

towards any of these proteins (Fig. 2). However, PMA treatment allowed cells to cross the membranes coated with all the four matrix proteins. The maximal migration was observed in a narrow range of protein concentration (2.5 to 25  $\mu\text{g/ml}$ ), depending on the ECM protein. Lower or higher concentrations were unable to allow cell migration. Only laminin, at concentrations between 10 and 30  $\mu\text{g/ml}$ , was able to consistently support cell migration as efficiently as Matrigel. Importantly, with or without PMA in the culture medium, cells remained unable to reach the underside of a membrane coated with an integrin-independent substratum such as poly-L-lysine (Fig. 2). Moreover, migration of PMA-treated cells on purified ECM proteins was PKC-dependent as shown by the fact that 4 $\alpha$ -PMA remained inactive and that both calphostin C and bisindolylmaleimide GF109203X inhibited cell migration (data not shown). Taken together, these observations indicate that ECM proteins-cell interactions are necessary to support the PKC-dependent HT29-D4 haptotaxis, and that various ECM proteins are able to do so.

Integrins are the main receptors for ECM proteins. HT29-D4 express the  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha v\beta 5$  and  $\alpha v\beta 6$  integrins (Lehmann et al., 1994, 1996, and unpublished results). To investigate whether these integrins were involved in HT29-D4 cell migration and which integrin chains mediated the PKC-dependent cell migration on purified ECM proteins, we performed migration assays in the presence of specific function-blocking antibodies. As shown in Fig. 3, antibodies against the  $\alpha 2$ ,  $\alpha 3$  and  $\beta 1$  subunits inhibited cell migration towards type IV collagen. On the contrary, mAbs against the  $\alpha 6$  or  $\alpha v$  subunits failed to affect the PKC-dependent motility to type IV collagen. Similar experiments clearly showed that



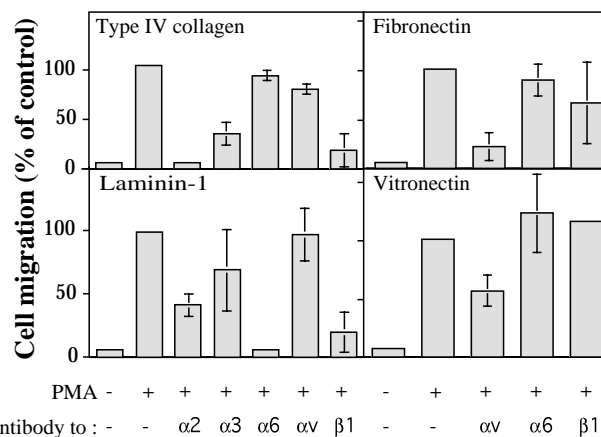
**Fig. 2.** HT29-D4 cell migration depends on purified ECM protein concentration. Migration assays were performed using filters coated with various concentrations of purified ECM proteins. Cells were treated ( $\square$ ) or not ( $\circ$ ) with 10 nM PMA prior to be added into the chamber. Results are from at least 3 independent experiments performed in triplicate.

only the mAb 69-6-5, blocking the  $\alpha v$  subunit, was efficient to specifically prevent cell motility towards vitronectin or fibronectin, suggesting that  $\alpha v$ -containing integrins are involved in this process. Haptotaxis towards laminin-1 was specifically blocked by mAbs against  $\alpha 2$ ,  $\alpha 6$  or  $\beta 1$ , while mAbs against  $\alpha 3$  and  $\alpha v$  had little effect, if not at all.

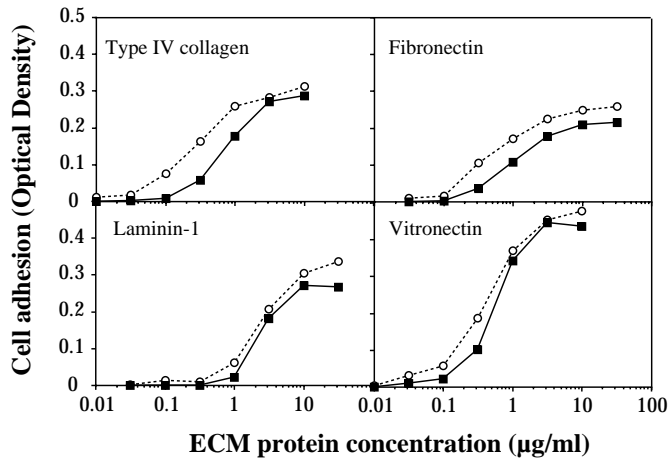
### PMA promotes cell spreading, but has no effect on cell adhesion

Cell migration requires adhesive interactions between cell surface adhesion receptors and the substratum at the leading edge and the release of adhesion at the trailing edge of the cell (Lauffenburger and Horwitz, 1996). In several studies, PMA has been shown to modulate cell adhesiveness (Wilkins et al., 1991; Vuori and Ruoslahti, 1993; van Leeuwen et al., 1994; Eguchi and Horikoshi, 1996). We thus examined whether PKC stimulation could activate HT29-D4 cell integrins and so increase cell adhesion to ECM proteins. Cells were treated or not with 10 nM PMA and then allowed to attach to increasing amounts of purified ECM proteins. As already reported (Lehmann et al., 1994), untreated HT29-D4 cells attached to the four matrices with similar efficiency (Fig. 4). When cells were treated with 10 nM PMA, we observed a very slight increase of adhesion for small concentrations of type IV collagen and fibronectin, while no effect could be observed on laminin-1 or vitronectin.

Cell spreading is another critical event required for cell migration. It reflects the capacity of cells to change shape after interaction with the ECM. To determine whether PMA modulates spreading, we plated HT29-D4 cells on ECM protein substratum in the absence or in the presence of 10 nM PMA. After a 3-hour incubation at 37°C, untreated cells began to spontaneously spread on type IV collagen, laminin-1 and vitronectin (Fig. 5A). Treatment of cells by PMA resulted in a



**Fig. 3.** Inhibition of PMA-induced migration by anti-integrin antibodies. Migration test was performed as described in Materials and Methods using filters coated with the appropriate matrix protein at 2.5  $\mu\text{g/ml}$  (vitronectin) or 8  $\mu\text{g/ml}$  (type IV collagen, laminin-1, fibronectin). Cells were treated with 10 nM PMA and incubated in the presence or not of blocking antibodies. Antibodies were used at 10  $\mu\text{g/ml}$  excepted for anti- $\beta 1$  mAb, used at a 1/1,000 dilution. Data shown are means ( $\pm$ s.d.) from at least 3 experiments performed in triplicate and are expressed as a percentage of migration in the absence of antibody.



**Fig. 4.** Effect of PMA treatment on HT29-D4 cell adhesion. 96-well microtiter plates were coated with matrix proteins at the indicated concentrations. Cells in suspension were treated (○) or not (■) with 10 nM PMA and adhesion assays were performed as described in Materials and Methods. Data shown are means from 2 experiments performed in triplicate.

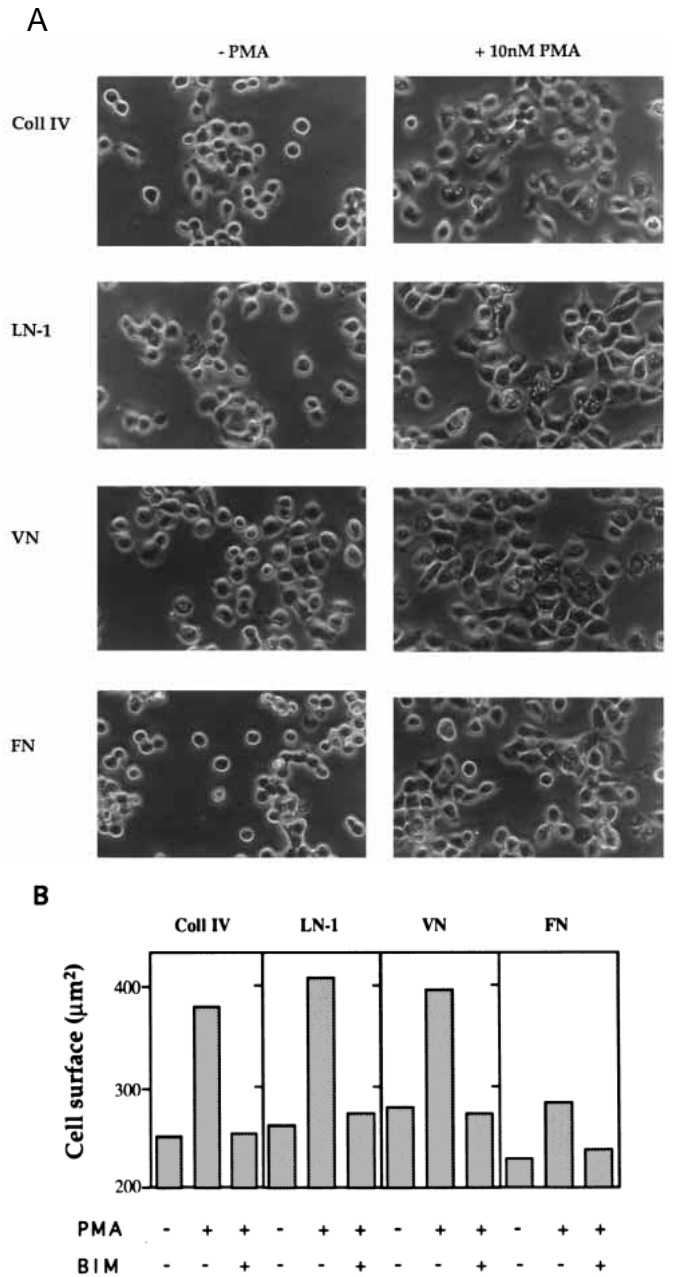
marked increase in cell spreading on all these proteins. More interestingly, cells incubated in the absence of PMA did not spread on fibronectin after 3 hours (Fig. 5A) or even after 24 hours of incubation (data not shown). However, when PMA was added, cells were then able to spread rapidly on fibronectin after only 3 hours (Fig. 5A). Inhibition of the enzymatic activity of PKC by the selective inhibitor bisindolylmaleimide GF109203X prevented the spreading of HT29-D4 cells induced by PMA treatment on all four ECM proteins (Fig. 5B). Moreover, when PMA was replaced by its inactive isomer, 4 $\alpha$ -PMA, there was no increase in cell spreading, as compared to the untreated cells (data not shown). Thus, the activation of PKCs by PMA results in changes in the cell shape leading to an increase in cell spreading, as well as cell motility capacities.

**PMA does not alter integrin expression at the HT29-D4 cell surface**

To determine whether PMA-induced effects were the result of quantitative changes in the number of cell surface receptors, cells were treated in the presence or in the absence of PMA and examined for cell surface integrins. We first quantitatively compared cell surface expression of the different integrins by indirect immunofluorescence and flow cytometry analysis. Exposure of cells to PMA for 5 hours (Fig. 6) or 18 hours (not shown) did not influence the expression level of the  $\beta$ 1,  $\beta$ 4,  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 6 or  $\alpha$ v integrins, and did not induce the expression of integrins such as  $\beta$ 3 and  $\alpha$ 5 which are not displayed on untreated cells. Moreover, after cell-surface biotinylation, the electrophoretic profile of immunoprecipitated integrins was unchanged by PMA treatment (Fig. 7). Thus, clearly, the HT29-D4 cell haptotaxis induced by PKC activation is not a consequence of a quantitative or qualitative changes in the integrins expression at the cell surface.

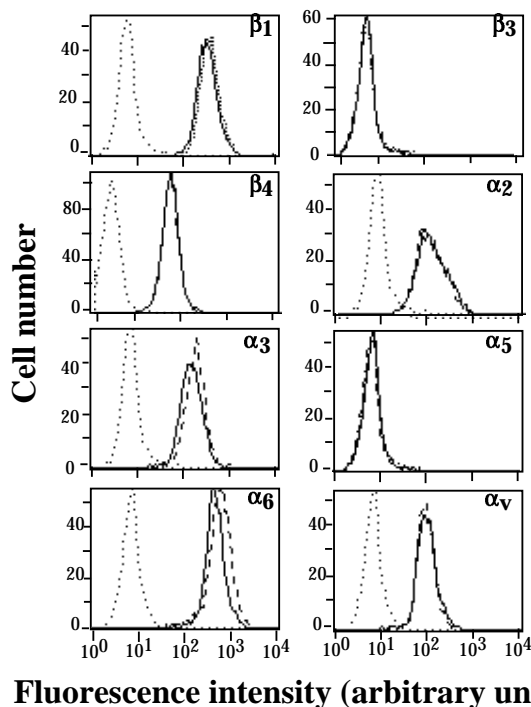
**MAPK is involved in PKC-induced cell migration**

Activation of the mitogen-activated protein kinase (MAP kinase) pathway leads to the regulation of gene transcriptional



**Fig. 5.** Effect of PMA treatment on HT29-D4 cell spreading. 24-well plates were coated with ECM proteins at 25 µg/ml. Cells were seeded in the presence or in the absence of 10 nM PMA and allowed to spread for 3 hours at 37°C. (A) Pictures shown are representative of 4 experiments. Bar, 20 µm. (B) Changes in cell surface in response to PMA alone or in combination with 5 µM bisindolylmaleimide (BIM) were determined by computer image analysis and expressed as the average cell area.

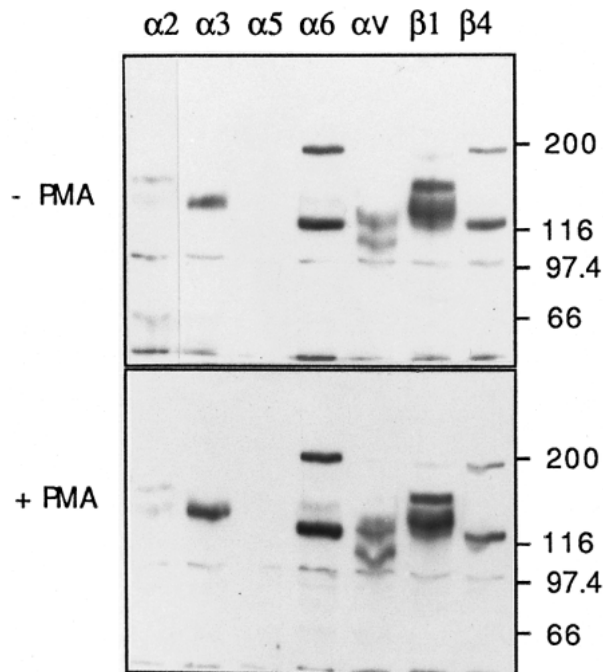
events. Both growth factor receptors and integrins promote signalling events leading to MAP kinase activation and the induction of cell motility. To further investigate the role of mitogen-activated protein (MAP) kinase in PKC-induced cell migration, we used PD98059, described as a potent inhibitor of MAP kinase kinase (MEK) (Dudley et al., 1995). Cells were incubated for 2 hours with PD98059 before performing a cell migration assay in modified Boyden chambers. As illustrated



**Fig. 6.** Quantification of cell surface integrins on PMA-treated HT29-D4 cells. Cells were incubated for 5 hours in the presence (-) or in the absence (—) of 10 nM PMA. Cells were harvested and resuspended in the presence of anti-integrin subunits antibodies. After incubation with the appropriate secondary FITC-conjugated antibody, cell-bound fluorescence was quantified using a Becton-Dickinson FACSscan flow cytometer. Non specific labeling (.....) was determined by incubating cells with the secondary FITC-conjugated antibody alone. Results shown are representative of 2 experiments.

in Fig. 8A, PD98059 reduced cell migration by 50% to 75% depending on the matrix protein tested, indicating that MAP kinase activity is required for PMA-induced HT29-D4 migration. In order to confirm that MAP kinase could be activated by PMA treatment, we used a specific antibody raised against the dually-phosphorylated region within the catalytic core of the active form of the MAP kinase enzymes. Incubation with 10 nM PMA resulted in a rapid activation of ERK2, whereas no activation of ERK1 could be observed (Fig. 8B, lanes 2 to 4).

Insulin-like growth factor I (IGF-I), a multifunctional regulatory peptide that share structural homology with proinsulin, mediates part of its effects by activating the ERK1/2 signal transduction cascade (Parrizas et al., 1997). As shown in Fig. 8B, incubation of HT29-D4 cells with des(1-3)IGF-I, an IGF-I truncated analog, caused a significant increase of ERK2 activation (lane 5). Moreover, several studies have reported evidence for PKCs requirement in IGF-I-induced cell migration (Miyata et al., 1989; Klemke et al., 1994). We therefore sought to determine whether activation of the MAP kinase pathway by IGF-I could lead to HT29-D4 cell migration. As illustrated in Fig. 8C, des(1-3)IGF-I was able to induce migration of HT29-D4 cells in Boyden chamber assays. The des(1-3)IGF-I-induced migration was dramatically inhibited by bisindolylmaleimide GF109203X and PD98059



**Fig. 7.** Cell surface expression of integrin heterodimers. Cells were treated or not for 16 hours with 10 nM PMA and then cell surface proteins were biotinylated. Immunoprecipitation and detection of integrins were performed as described in Materials and Methods.

(Fig. 8C), indicating that the migratory behavior induced by des(1-3)IGF-I requires both PKC and MAP kinase activities.

## DISCUSSION

Motility of cells such as fibroblasts and macrophages has been investigated in detail (for review see Lauffenburger and Horwitz, 1996). However, the mechanisms by which polarized epithelial cells change to the flattened phenotype and become motile is not as extensively characterized. HT29-D4 cells are able to undergo an enterocyte-like differentiation and may thus provide an interesting model for such in vitro studies (Fantini et al., 1986). In this report, we used modified Boyden chambers to determine the migratory behavior of undifferentiated HT29-D4 cells towards various purified ECM proteins. We observed that, whatever the protein used, HT29-D4 cells had no migratory behavior in the absence of any stimulation, although cells strongly attached to the four proteins tested and spread on three of them. However, migration of HT29-D4 cells could be promoted by PMA treatment. PMA-induced cell migration could only be observed in a narrow range of protein concentration. Ligand concentration allowing maximum migration depended on the protein. Moving away from this optimal concentration resulted in a substantial decrease in cell motility. Such biphasic curves in cell migration have already been reported (Palecek et al., 1997). As cell migration requires the formation of new attachments at the cell front and the break of attachments at the rear, maximum cell speed is predicted to occur at an intermediate ratio of cell-substratum adhesiveness to intracellular contractile force (Palecek et al., 1997). Low ligand concentrations decrease the probability of formation of

stable attachments by the lamellipods, while high ligand concentrations probably hinders cell migration by obstructing the release of adhesions at the rear.

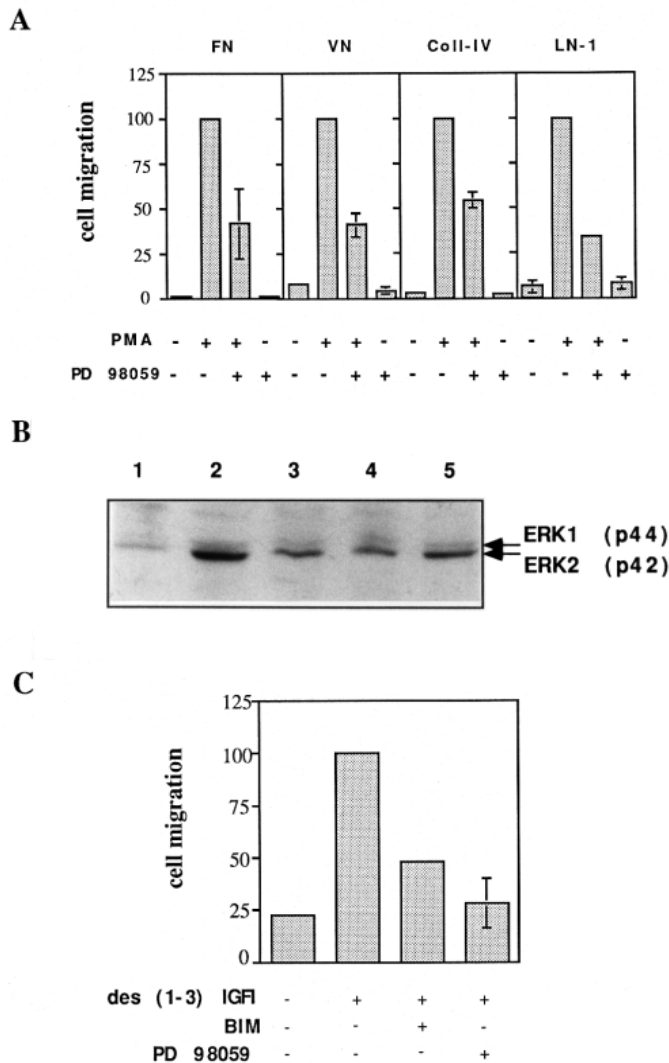
Cell migration depends on cell adhesion, as well as deadhesion of cell surface receptors (Lauffenburger and Horwitz, 1996). Adhesion assays performed on purified ECM

proteins indicated that PMA stimulation had very little or no influence on HT29-D4 attachment. However, stimulation by PMA resulted in an accelerated rate of spreading on type IV collagen, laminin-1 and vitronectin when compared to control cells. Moreover, we observed that spreading of HT29-D4 cells on fibronectin required stimulation by PMA. This indicates that PMA-induced inside/out signalling processes are essential for spreading on fibronectin via  $\alpha v \beta 6$ , the major fibronectin receptor of HT29-D4 cells (Lehmann et al., 1994).

In this study, we used two independent straightforward approaches to determine whether PKCs are involved in the migration of HT29-D4 cells. We first used 4 $\alpha$ -PMA, a phorbol ester that does not activate PKCs. We observed that PMA, which is known to stimulate PKCs, accelerated cell spreading and induced a migratory behavior of HT29-D4 cells, whereas the inactive ester 4 $\alpha$ -PMA had no effect. In the second approach, we inhibited the enzymatic activity of PKCs in intact cells by two compounds with different action sites on the kinase: calphostin C, which inhibits PKCs by competing with diacylglycerol or phorbol esters at the binding site (Kobayashi et al., 1989), and bisindolylmaleimide GF109203X, which interacts with the ATP-binding site (Toullec et al., 1991). Both compounds completely prevented the PMA-induced spreading and migration of HT29-D4 cells. Thus, these data point out the role of PKC activation in the pathway leading to spreading and migration of HT29-D4 cells.

The PKC-induced cell motility was integrin-dependent as shown both by the absence of migration of cells plated on membranes coated with poly-L-lysine, a non specific substratum, and by the inhibitory effect of antibodies directed against functional epitopes on integrin subunits. Our results demonstrate the implication of the  $\alpha 2 \beta 1$  and  $\alpha 3 \beta 1$  integrins in migration towards type IV collagen. When using laminin-coated membranes, an antibody directed against  $\alpha 3$  had little inhibitory effect indicating that  $\alpha 3 \beta 1$ , a putative laminin receptor, is not probably involved in migration towards laminin. On the contrary, antibodies against  $\alpha 2$ ,  $\alpha 6$  and  $\beta 1$  subunits affected PMA-induced migration. Although  $\alpha 6$  and  $\beta 1$  are the most expressed integrin chains on HT29-D4 cells (see Figs 6 and 7), immunoprecipitation experiments performed in our laboratory and by others on the parental cell line HT29 (Phillips et al., 1991; Schreiner et al., 1991; Simon-Assmann et al., 1994), indicate that  $\alpha 6$  is preferentially associated with  $\beta 4$ . Thus, it is likely that  $\alpha 2 \beta 1$  and  $\alpha 6 \beta 4$  integrins are important for the migratory behavior of HT29-D4 cells on laminin-1. In the case of fibronectin and vitronectin, only the anti- $\alpha v$  antibody blocked migration, suggesting that the  $\alpha v \beta 5$  and  $\alpha v \beta 6$  integrins, the main receptors for vitronectin and fibronectin, respectively (Lehmann et al., 1994), mediate cell migration. Thus, taken together our results show that interactions between  $\alpha 2 \beta 1$ ,  $\alpha 3 \beta 1$ ,  $\alpha 6 \beta 4$ ,  $\alpha v \beta 5$ ,  $\alpha v \beta 6$  integrins and their specific ECM ligands are necessary for the PKC-mediated migration.

Conflicting reports have been published on altered integrin expression upon PKC activation by PMA. Using human T cell leukemia cells (Jurkat), Wilkins et al. (1991) reported enhanced adherence to matrix upon PMA stimulation, but showed there was no modulation of  $\beta 1$  integrins expression. However, in a study also performed on Jurkat cells, Stoolman et al. (1993) showed an up-regulation of the  $\alpha 6 \beta 1$  integrin and increased binding to laminin. Several reports on other cell



**Fig. 8.** MAPK is involved in PKC-induced cell migration. (A) Cells were pretreated for 2 hours with 25  $\mu$ M PD98059 before performing cell migration assays using 2.5  $\mu$ g/ml vitronectin (VN) or 8  $\mu$ g/ml type IV collagen (Coll-IV), laminin-1 (LN-1) or fibronectin (FN). Results (mean  $\pm$  s.d.) are expressed as percent of the migration in the presence of PMA from three experiments. (B) Cells were incubated in DMEM 0.1% BSA alone (lane 1) or with 10 nM PMA for 5 minutes (lane 2), 10 minutes (lane 3), 20 minutes (lane 4) or with 8 nM des(1-3)IGF-I for 30 minutes (lane 5). After cell lysis, 75  $\mu$ g of extracted proteins were resolved by SDS-PAGE and blotted onto a nitrocellulose sheet. Active MAP kinases (EKR1 and EKR2) were revealed by specific anti-active MAP kinase antibody. (C) Migration assays were performed using filters coated with 2.5  $\mu$ g/ml of type IV collagen. Cells (70,000 cells/chamber) were treated with 8 nM des(1-3)IGF-I in the presence or not of 5  $\mu$ M bisindolylmaleimide (BIM) or 25  $\mu$ M of PD98059. Kinase inhibitors were added 2 hours before the experiment. Results are means ( $\pm$ s.d.) from three experiments.

types have also mentioned an enhanced integrin expression in response to PMA stimulation (Ferreira et al., 1991; Eguchi and Horikoshi, 1996). In our study, cell sorter analyses demonstrated that the migration and the increased spreading of HT29-D4 observed in response to PMA did not result from a modulation of integrin chains expression level at the membrane surface. Moreover, PMA did not modify neither the labeling intensity, nor the  $\alpha\beta$  integrin combinations on HT29-D4, as determined by immunoprecipitation experiments. Whether PMA effect could be explained by a modification of integrin-mediated transduction signals or by integrin clustering remains to be determined.

MAP kinases (EKR1 and EKR2) have been recognized as a major system by which cells transduce a variety of extracellular signals and thus represent the convergence point for many signalling pathways. While the MAP kinase pathway has been associated with the transcriptional control of genes important for cell proliferation and differentiation, it is now clear that MAP kinases can also promote cell migration on the ECM in a transcription-independent manner (Graf et al., 1997; Klemke et al., 1997). In this work we report that stimulation of cells by PMA was able to rapidly activate ERK2. We also show that the impediment of MAP kinase activation resulted in the loss of cell migration. This suggests that activation of MAP kinase by PKCs may be a prerequisite in the pathway leading to HT29-D4 cell motility. In agreement with this conclusion, we observed that stimulation of the IGF signalling pathway also led to MAP kinase activation and to the induction of cell migration. Moreover, the growth factor-induced motility of HT29-D4 was affected both by PKC and MAP kinase cascade inhibitors.

It thus appears that both integrin engagement and MAP kinase activation by PKCs are required for migration of HT29-D4 cells. Taken separately, none of these stimuli was able to promote cell motility, suggesting the existence of a cross-talk between these signalling pathways.

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