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Modulation of hepatocyte growth factor-induced scattering of HT29 colon carcinoma cells

Involvement of the MAPK pathway

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SUMMARY

Hepatocyte growth factor (HGF)/scatter factor modulates the motility of HT29 colon carcinoma cells in vitro by inducing morphological changes that depend on the type of extra-cellular matrix (ECM) ligand; HGF-induced scattering of HT29 cells is observed if cells are grown on plastic coated with serum proteins but not laminin. The absence of scattering correlates with a lack of cell spreading on laminin and it is not due to impaired HGF induced tyrosine phosphorylation of the E-cadherin/desmosome component, γ-catenin, or lack of activation of mitogen activated protein kinase (MAPK). Treatment of HT29 cells with phorbol 12-myristate, 13-acetate (PMA), but not arachidonic acid, restored the ability of the cells to spread on laminin in an integrin-dependent manner. Moreover, the addition of both PMA and HGF restored the ability of these cells to scatter on laminin in a synergistic manner. This event correlated with increased tyrosine phosphorylation of paxillin and activation of MAPK. Moreover, when the MEK (MAPK kinase)/MAPK pathway was blocked by the MEK inhibitor PD098059, HGF-induced scattering of HT29 cells was blocked. Thus, HGF modulation of HT29 cell motility is regulated by both integrin and growth factor-dependent signaling and implicates MAPK in the modulation of intercellular adhesion and epithelial cell motility.

Key words: MAPK, Cellular adhesion, Scatter factor, HT29 cell

INTRODUCTION

The ability of tumor cells to metastasize is dependent on several factors that regulate cell-cell and cell-extracellular matrix (ECM)1 interactions (Streit et al., 1996; Matsumoto et al., 1995; Agrez and Bates, 1994; Schwartz, 1993).

Cadherins play a central role in the maintenance of morphology and polarity of epithelial cells (Drubin and Nelson, 1996) and the invasive phenotype of tumor cells (Streit et al., 1996; Vermeulen et al., 1995; Simcha et al., 1996). The functional integrity of E-cadherin and its cytoplasmic-associated proteins, α-, β- and γ-catenin, is required for homotypic cell-cell interactions and association with the actin cytoskeleton (Takeichi, 1990). This protein complex participates in the regulation of the formation and function of junctional complexes and desmosomes of epithelial cells (Mechanic et al., 1991; Citi, 1993; Gumbiner and Simons, 1986; Watabe et al., 1994; Lewis et al., 1997).

The E-cadherin/catenin system is regulated by the integrin-dependent adhesion system as well as by growth factors and oncogenes (Matsumoto et al., 1995; Wang et al., 1990a,b; Shiozaki et al., 1995; Vega-Salas et al., 1987; Pignatelli et al., 1992; Hinck et al., 1994; Takeda et al., 1995; Matsuyoshi et al., 1997; Hamaguchi et al., 1993; Behrens et al., 1997; Kinch et al., 1995). Studies carried out with the epithelial cell line MDCK demonstrated that ECM is required to induce the localization of tight junction proteins (ZO-1) to the apical-lateral membrane boundary (Wang et al., 1990a) and is involved in the modulation of cell polarity (Wang et al., 1990b). In addition, the α2β1 integrin was shown to regulate collagen-mediated epithelial membrane remodeling and tubule formation in MDCK cells (Schwimmer and Ojakian, 1995). Conversely, studies performed in both fibroblasts and keratinocytes showed that expression and function of integrins is regulated by the cadherin system (Finnemann et al., 1995; Hodivala and Watt, 1994).

Growth factor-mediated regulation of the cadherin/catenin complex is best illustrated by the ability of HGF/scatter factor to induce dissociation and morphological changes of the epithelial colonies (Stoker et al., 1987; Weidner et al., 1990). In some instances, direct binding of growth factor receptors to the cadherin/catenin system has been demonstrated (Shibata et al., 1996; Hoschuetzky et al., 1994) and both growth factors and v-src transformation lead to tyrosine phosphorylation of p120CAS, a protein known to be part of the cadherin complex (Reynolds et al., 1994; Daniel and Reynolds, 1995; Mo and Reynolds, 1996; Shibamoto et al., 1995). A regulatory cycle involving the action of tyrosine phosphatases has been

implicated in the dynamic regulation of the cadherin/catenin system (Kypta et al., 1997).

The mechanism by which HGF induces dispersion of responsive epithelial cells is not fully understood. Both the morphological and motogenic effects of HGF are mediated by binding to its receptor, c-Met, a tyrosine kinase (Weidner et al., 1993; Ponzetto et al., 1994; Brinkmann et al., 1995). Scattering of cells in response to HGF is mediated by ligand induced activation of this receptor and subsequent assembly of signaling complexes (Ponzetto et al., 1994). Studies carried out in MDCK cells showed that HGF induced scattering requires the activity of the small GTP-binding protein Ras (Ridley et al., 1995; Hartmann et al., 1994) as well as additional, ras-independent signaling pathways (Ridley et al., 1995). The morphogenic activity of HGF, but not its scattering activity, requires signaling mediated by the Grb2 adapter molecule (Fournier et al., 1996).

The ability of colon cancer cells to invade and disperse is likely due to loss of their adhesive properties (Streit et al., 1996; Agrez and Bates, 1994; Citi, 1993). Defining the common link that results in adhesion-dependent regulation of growth factor induced motility may facilitate our understanding of these events. Here evidence is presented that demonstrates that scattering of the HT29 colon carcinoma cell line on laminin in response to HGF requires integrin mediated, PMA stimulated cell spreading. In addition, a role for the activity of the MEK/MAPK signaling pathway in the regulation of HGF-induced cell scattering has been established.

MATERIALS AND METHODS

Cells

The HT29 human colon carcinoma cell line and MDCK cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM-F12 medium, high glucose, supplemented with 5% fetal calf serum (Gibco Laboratories, Grand Island, NY).

Antibodies

Monoclonal antibodies directed against γ-catenin, E-cadherin, and paxillin were obtained from Transduction Laboratories (Lexington, KY). Rat monoclonal antibody against E-cadherin was purchased from Zymed. Anti-focal adhesion kinase (FAK) antibodies and Protein A/G Plus were purchased from Santa Cruz (Santa Cruz, CA). Monoclonal antibodies directed against α6 (CD49f) and α5 (CD49e) integrin subunits were purchased from PharMingen. Antibody to CD18 (CBRLFA-1/2) was a gift from L. Petruzzelli (University of Michigan). The anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Mouse mAb directed against desmoplakins I and II and fluorescein-conjugated goat anti-rat antibody were purchased from Chemicon International (Temecula, CA). Oregon Green-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rat IgG were obtained from Molecular Probes (Eugene, Oregon). Phospho-specific anti-MAPK antibody was purchased from Promega (Madison, WI).

Cell culture on laminin

Cells were cultured on laminin coated dishes (Collaborative Research) blocked with 0.5% BSA prior to the addition of the cells. For cells used for immunohistochemistry, plastic slide chambers (LabTeck) were coated with 100 μg/ml laminin in PBS for eight hours, followed by blocking with BSA (0.5%) in PBS prior to the addition of the cells.

Cell adhesion and spreading

Cells were removed from the tissue culture dish with trypsin/EDTA, washed with PBS (Mg^{2+}/Ca^{2+} free), rinsed with 0.5% BSA in DMEM and resuspended as a single cell suspension in serum-free DMEM. Equal numbers of cells were allowed to bind to either collagen I or laminin coated dishes (Collaborative Research) in DMEM containing 0.5 mM Mn^{2+} for 30 minutes. The percentage of cells spread was calculated from five low power microscope fields by dividing the number of cells spread by the total number of cells (n=100-200) per field. Cells were observed and recorded using a Nikon Diaphot 300 microscope equipped with an Optronic Engineering video camera.

Immunoprecipitations and immmunoblots

Cell extracts were prepared by detergent solubilization (1% Triton X-100, 50 mM Hepes, pH 7.5, 10% glycerol, 100 mM sodium phosphate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 3 mM sodium orthovanadate, 50 mM β-glycerol phosphate and 2 μg/ml of aprotinin and leupeptin) for 10 minutes on ice. After centrifugation (15 minutes, 12,000 rpm in an Eppendorf microfuge) protein concentration was determined. Equal amounts of protein were immunoprecipitated with specific antibodies as described in the legend to the figures. Immunecomplexes were separate by SDS-PAGE, transferred to nitrocellulose and immunobloted as described previously (Petruzzelli et al., 1996).

Immunohistochemistry

Cells cultured on plastic slides were fixed with 4% paraformaldehyde in PBS containing 0.3 mM $CaCl_2$ for twenty minutes at room temperature, permeabilized with 0.2% Triton X-100 in PBS/0.3 mM $CaCl_2$ for five minutes and incubated with the specific antibodies in 1% ovalbumin and 1% BSA in PBS as described in the legend to the figures. Fluorescent secondary antibody and phalloidin staining were carried out as described previously (Herrera and Shivers, 1994). Photographs were taken using an Axiovert 135 Zeiss microscope and Kodak Ektar 400 film. Processing was performed digitally using Adobe Photoshop software.

RESULTS

HT29 cell spreading on extracellular matrix

HT29 cells grown under standard conditions (see Materials and Methods) exhibit epithelial morphology with a growth pattern characterized by isolated, flat islets and extensive cell-cell contact. Immunohistochemical staining of cells with mAb to γ catenin (Fig. 1A) yielded strong labeling along the contact between cells thus confirming the presence of adherent junctions along the contact regions. Since γ-catenin can also be found as a component of desmosomes (Citi, 1993; Lewis et al., 1997; Mechanic et al., 1991) the labeling pattern is likely a mixture of both adherent junctions and desmosomes. The cell surface that is not in contact with an adjacent cell, is poorly labeled by the anti-γ catenin mAb. In contrast, HT29 cells cultured on laminin coated surfaces exhibit a markedly different morphological pattern (Bouziges et al., 1991) in that the cells tend to grow as spheres of aggregated cells (Fig. 1B).

The pattern of HT29 cells spreading on basement membrane components is an intrinsic property of the adhesion receptors involved. As shown in Fig. 1C, HT29 cells rapidly and extensively spread on collagen coated surfaces but failed to spread on laminin coated surfaces indicating that the morphology of cells grown long term on laminin could be explained, in part, by their inability to spread on laminin.

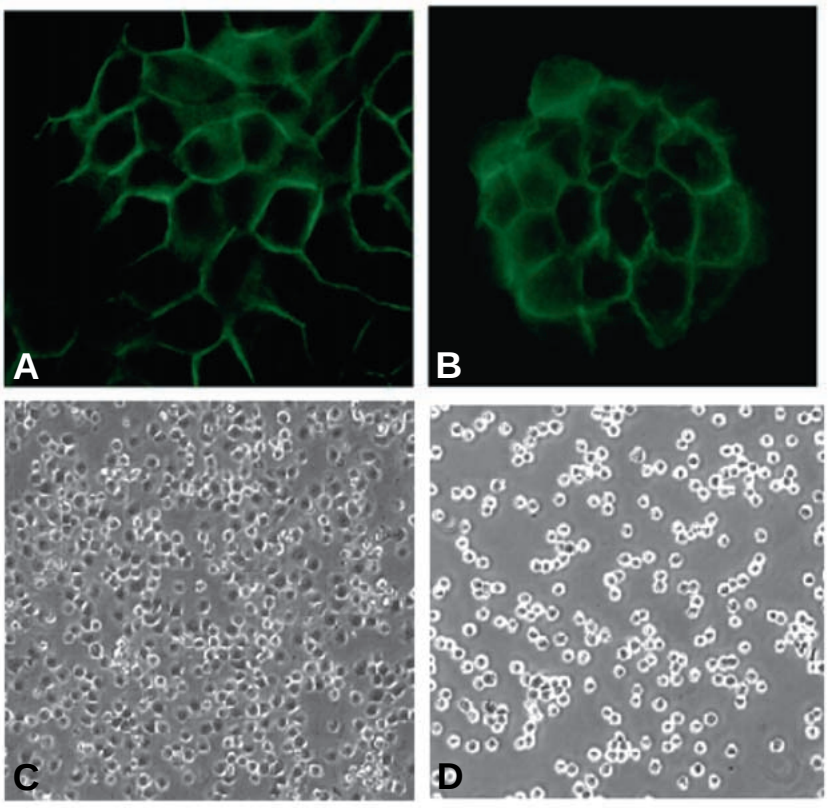

Fig. 1. HT29 cells spread on collagen I but not laminin. HT29 cells were cultured on plastic slides coated with fetal calf serum (A) or laminin (B) followed by immunostaining with mAb to γ-catenin as described in Materials and Methods. Alternatively, HT29 cells were removed from the culture dish by trypsin/EDTA treatment and allowed to spread on dishes coated with collagen I (C) or laminin (D) for 1 hour. Cells were photographed as described in Materials and Methods.

Modulation of HT29 cell spreading in response to PMA and arachidonic acid

Studies carried out in other cell systems have shown that the ability of cells to spread on a given ligand-coated surface can be modulated by the lipid second messenger arachidonic acid (Lefkowith et al., 1991; Auer and Jacobson, 1995; Teslenko et al., 1997). In some instances, this event correlates with activation of PKC (Auer and Jacobson, 1995; Chun et al., 1996). The ability of HT29 cells to spread on laminin upon stimulation with arachidonic acid or by direct activation of PKC by PMA was tested. As shown in Fig. 2A, treatment of HT29 cells with arachidonic acid (1-15 μM) failed to induce significant cell spreading on laminin. However, stimulation of HT29 cells with PMA (20 nM) induced marked spreading and extensive rearrangement of the actin cytoskeleton (Fig. 2B). Morphologically, spreading of PMA-stimulated HT29 cells on laminin resembles the phenotype of cells spread on collagen I.

Binding of HT29 cells to ECM components is largely mediated by integrins (Schreiner et al., 1991; Simon-Assmann et al., 1994). HT29 cells express several integrins that are known to be receptors for laminin; however, α_6 containing integrins are the main receptors involved in binding of HT29 cells to laminin (Simon-Assmann et al., 1994; Schreiner et al., 1991). A blocking mAb directed against α6 was used to test whether PMA-induced cell spreading on laminin was dependent on this integrin. As depicted in Fig. 2C, mAb to α6, but not α5, strongly inhibited PMA-induced cell spreading indicating that this event is an integrin-dependent process. It is important to mention that a significant number of cells that were incubated in the presence of the mAb to α6 antibody detached from the coated surface during the 30 minute assay. Nevertheless, the remaining attached cells did not spread.

Integrin-mediated signal transduction in response to adhesion of HT29 cells to laminin

The data presented above indicate that HT29 cells spread on laminin in an integrin-dependent manner after stimulation with PMA. It is presumed that the PMA-mediated effects occur through activation of PKC (Nishizuka, 1992). To determine whether the failure of HT29 cells to spread on laminin was due to a lack of early integrin signaling events, the adhesion-dependent tyrosine phosphorylation of paxillin, FAK and MAPK activation was measured in cells attached to either collagen or laminin coated surfaces. Analysis of the induction of tyrosine phosphorylation of proteins upon adhesion of HT29 cells to both collagen and laminin demonstrates the appearance of a broad migrating band (Fig. 3A) that resembles the pattern seen in other cells (Burridge et al., 1992; Bockholt and Burridge, 1993). A component of this band is paxillin as shown by direct immunoprecipitation with anti-paxillin antibody (Fig. 3B). As has been demonstrated in other cell types (Chen et al., 1994; Wary et al., 1996; Schlaepfer et al., 1994; Zhu and Assoian, 1995), adhesion of HT29 to ECM components also induces activation of MAPK (Fig. 3C) indicating that the early signaling events associated with HT29 cell adhesion to these ligands are present under both binding conditions.

Since it has previously been shown that PMA-mediated spreading of hamster CS-1 melanoma cells on vitronectin correlated with a 2.3-fold increased tyrosine phosphorylation of FAK (Lewis et al., 1996), the pattern of FAK

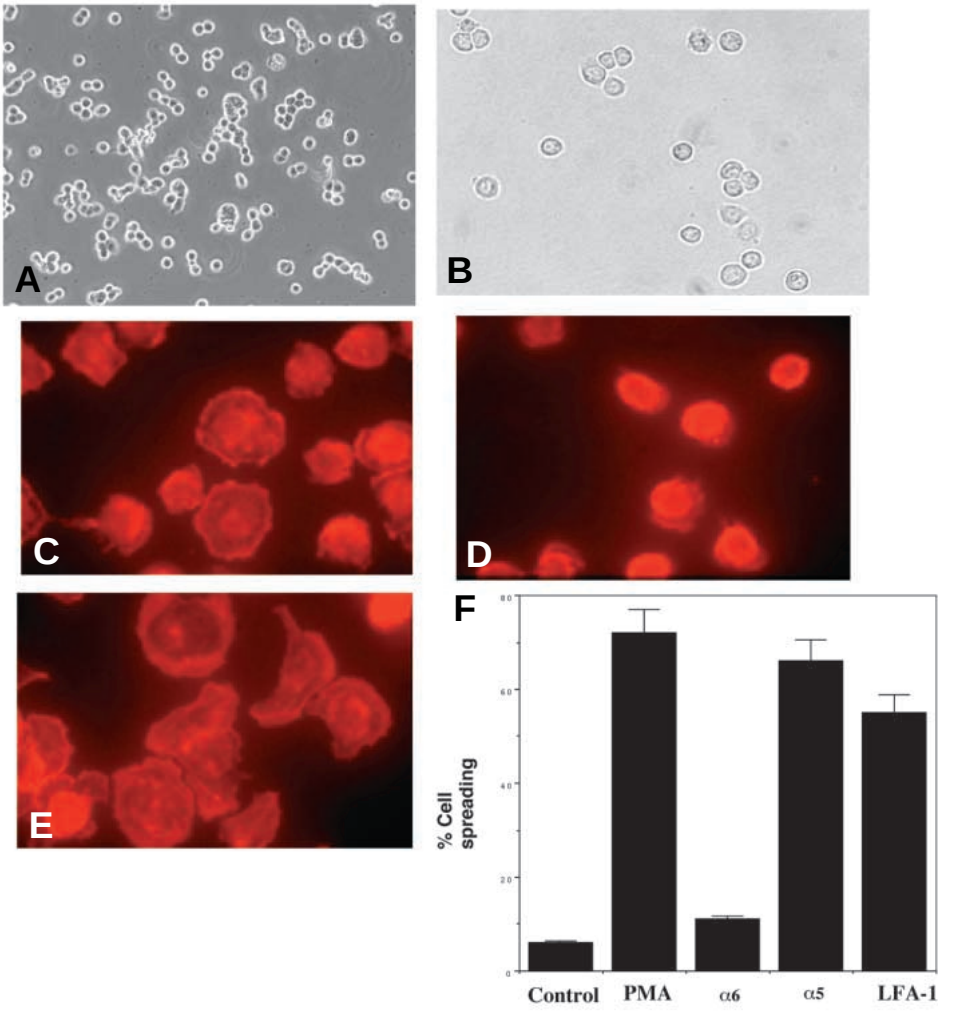

Fig. 2. PMA, but not arachidonic acid, induces α_6 integrin-dependent HT29 cell spreading on laminin coated surfaces. HT29 cells were prepared as a single cell suspension, as described in Materials and Methods, allowed to adhere to a laminin coated surface in the absence (A) or presence of 15 μM arachidonic acid (B) and photographed as described. Alternatively, cells were allowed to adhere to collagen I (C) or laminin (D and F) coated plastic slides and were treated with PMA (20 nM) for 30 minutes as indicated. The slides were processed for actin staining as described in Materials and Methods. For mAb inhibition of cell spreading, cells were allowed to bind to laminin coated dishes for 1 hour followed by incubation with the indicated antibodies (10 μg/ml) for 10 minutes prior to the addition of PMA. Spreading was measured after 20 minutes of PMA stimulation as described in Materials and Methods. Ten low power fields (20×) were scanned and a minimum of 100 cells were counted per field.

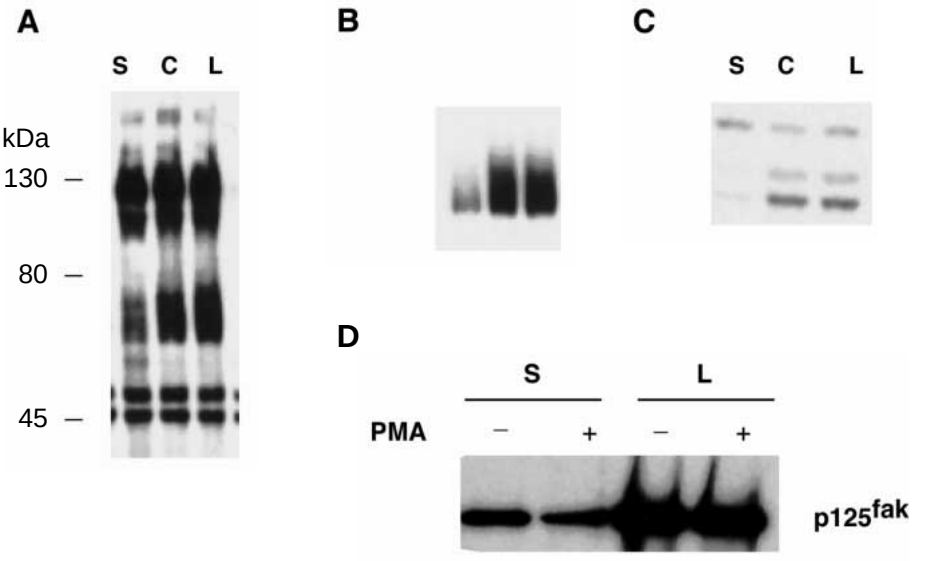

Fig. 3. Adhesion of HT29 cells to collagen I or laminin induces paxillin and FAK phosphorylation and MAPK activation. A single cell suspension of HT29 cells (S) was allowed to bind to collagen I (C) or laminin (L) coated dishes and the pattern of tyrosine phosphorylated proteins (A) or phosphorylated paxillin (B) were detected by antiphosphotyrosine inmmunoblot as described in Materials and Methods. The activation of MAPK (C) was estimated by an anti-active MAPK antibody as described in Materials and Methods. (D) HT29 cells were kept in suspension (S) or allowed to bind to laminin (L) for 1 hour followed by the addition of PMA (20 nM). After 20 minutes, cells were lysed, FAK was immunoprecipitated and phosphotyrosine content of FAK was estimated by immunoblot as described in Materials and Methods.

phosphorylation of HT29 cells undergoing spreading on laminin in response to PMA was investigated. As seen in Fig. 3D, binding of HT29 cells to laminin-coated plastic induced tyrosine phosphorylation of FAK. However, the addition of

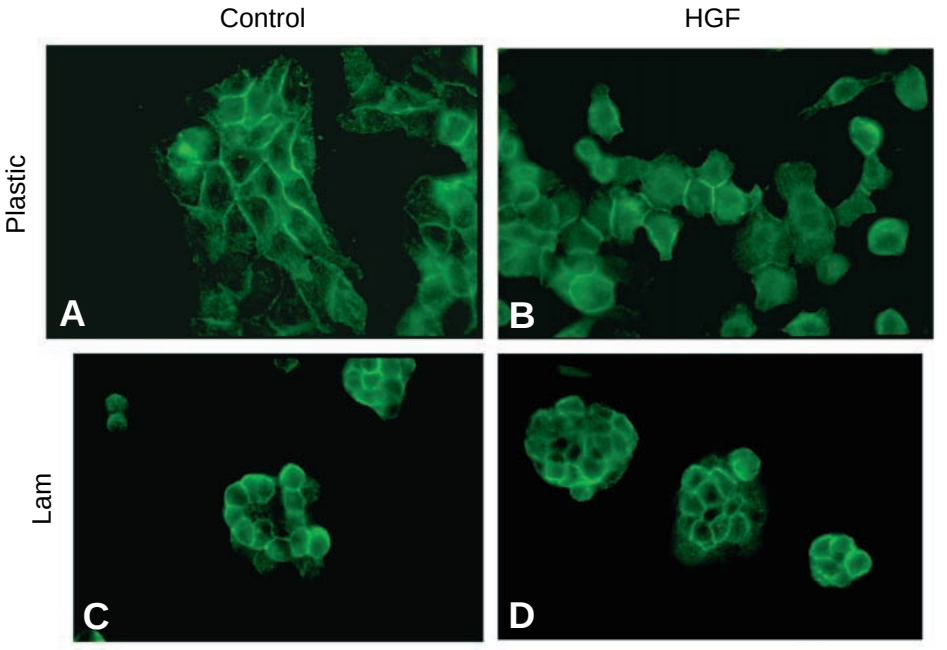

Fig. 4. ECM modulation of HGF-induced scattering of HT29 cells. HT29 cells were cultured on plastic slides coated with either serum proteins (Plastic) or laminin (Lam). HGF (40 ng/ml) was added and the scattering was monitored by immunostaining as described in Materials and Methods.

PMA to the bound cells, which initiates spreading, did not result in increased phosphorylation of FAK. Reprobing the blot with a mAb directed to FAK demonstrated that a similar amount of protein was present in each lane (data not shown).

Spreading is required for scatter factor activity

The cohesive epithelial morphology of many cell lines can be modulated by the action of hepatocyte growth factor or scatter factor (Brinkmann et al., 1995; Weidner et al., 1990, 1993; Stoker et al., 1987); however, the details of the molecular mechanism of this event is not fully understood. Regulation of HGF action on HT29 cells by ECM was studied in order to establish whether cell spreading was necessary for its scatter activity. The α6 integrin subunit was used as a marker for the morphological changes induced by HGF. The staining pattern of this integrin subunit shows that it is present prominently at the boundaries of cell-cell contact (Fig. 4). A more diffuse pattern is observed over the cell body as has been reported previously (Simon-Assmann et al., 1994). The staining at the cell boundaries was not modified by growth conditions (laminin or plastic).

HT29 cells grown on plastic (Fig. 4A) scatter upon the addition of HGF (40 ng/ml) (Fig. 5B) whereas cells grown on a laminin coated surface (Fig. 4C and D) remain as tight round colonies, suggesting that HGF scatter activity is not fully supported by laminin as an ECM ligand. The scattering of these cells induces loss of the α6 integrin from the cell periphery suggesting that its distribution is dependent upon intact cell-cell contact.

This effect is not due to the inability of laminin-bound cells to express functional HGF receptors since addition of HGF induces tyrosine phosphorylation of E-cadherin associated proteins in cells grown both on plastic and laminin (Fig. 5A). The phosphotyrosine containing protein that migrates at a molecular mass of 80 kDa in the E-cadherin immunoprecipitate corresponds to the molecular mass of γ-catenin. Immunoprecipitation of detergent extracts of HT29 cells grown on laminin and treated with HGF revealed time dependent phosphorylation of γ-catenin (Fig. 5B). Unlike what has previously been described (Shibamoto et al., 1995) there was no oscillation in the content of tyrosine phosphorylated p120CAS in serum-starved HT29 cells (data not shown), nor was it detected in E-cadherin immunoprecipitates. This may be due to the low abundance of the E-cadherin/p120CAS complex (Shibamoto et al., 1995).

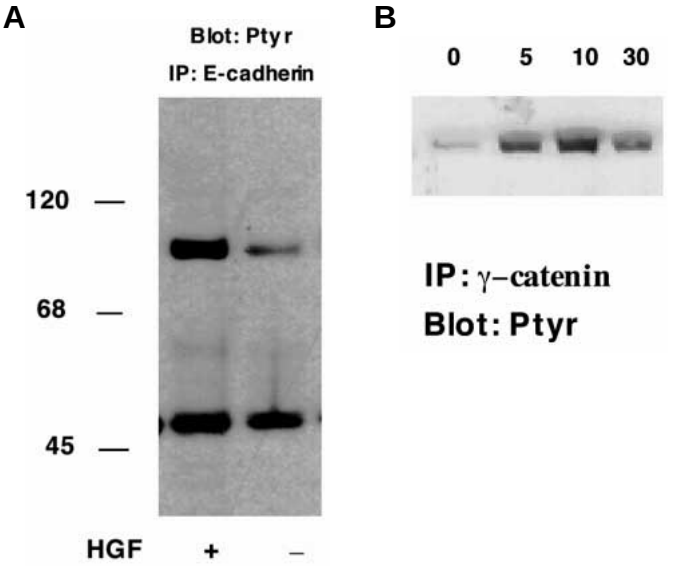

Fig. 5. HGF signaling in HT29 is supported by laminin. HT29 cells were cultured on laminin coated dishes, serum starved overnight and stimulated by the addition of HGF (40 ng/ml) for 20 minutes (A) or for the indicated times (B). Cells were lysed and immunoprecipitated with anti-E-cadherin (A) or anti-γ-catenin (B) antibodies.

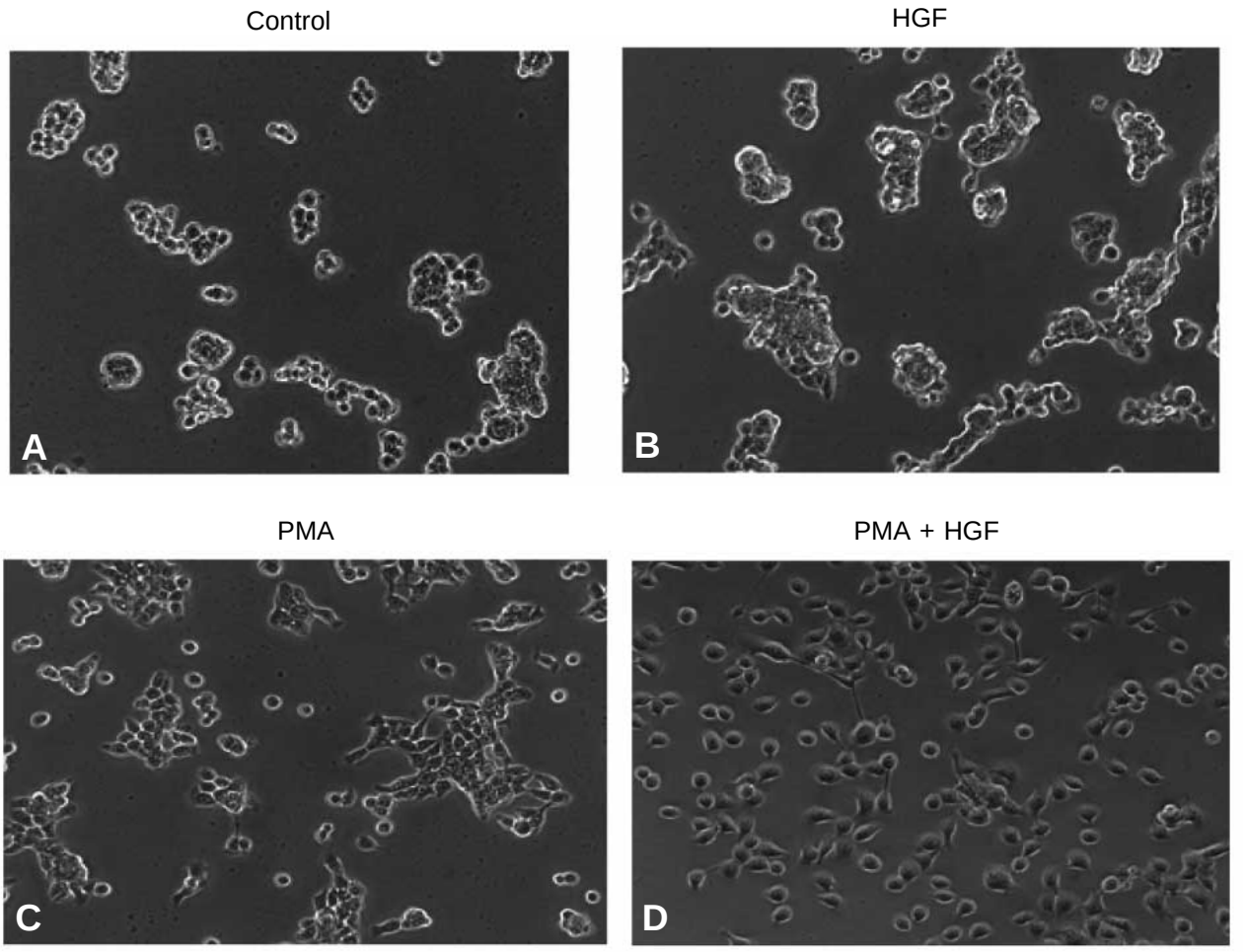

Fig. 6. PMA and HGF synergize to induce HT29 scattering on laminin. Cells were cultured on laminin coated dishes, serum starved overnight and stimulated with HGF, PMA or both, as indicated. After 24 hours of treatment, the cells were directly photographed as described in Materials and Methods.

HGF induces HT29 cell scatter on plastic and collagen, but not on laminin. Since spreading may be required to achieve HGF induced cell scattering, the combined effect of both PMA and HGF was studied. As shown in Fig. 6, cells grown on laminin undergo the characteristic spreading but fail to scatter in response to PMA. Although the addition of HGF had no effect, the addition of both stimuli induced a synergistic response. As early as six hours post treatment a scatter response can be seen and, by twenty-four hours post stimulation, there is a complete dissociation of the colonies.

Tyrosine phosphorylation of paxillin and MAPK activation was analyzed in response to PMA and HGF under conditions where synergy to induce HT29 cell scattering takes place. As seen in Fig. 7, paxillin phosphorylation was seen in those cells treated with PMA, but not HGF, and phosphorylation correlated with cell spreading. In contrast, MAPK activation was increased by the combined addition of both PMA and HGF, conditions that lead to both spreading and scattering. These observations suggest that scattering of these cells in response to HGF is dependent on the ECM component and requires a PKC dependent signal to fully induce the epithelial cell motility.

Requirement for MAPK activation during cell scattering

The HGF receptor (c-Met) is a tyrosine kinase that initiates signaling by the formation of a protein complex containing both adapter and signaling proteins (Ponzetto et al., 1994; Weidner et al., 1993; Hartmann et al., 1994). Activation of the Ras pathway has been implicated in HGF induced scattering activity (Hartmann et al., 1994) thus it was of interest to determine whether HGF mediated scattering required the activity of MAPK, a downstream effector of Ras. The MEK/MAPK pathway can be selectively blocked by PD098059, an inhibitor shown to inhibit the activation of MEK in response to a variety of stimuli, including growth factors (Alessi et al., 1995; Dudley et al., 1995). Treatment of HT29 cells with HGF induces a measurable increase in level of activated MAPK that can be completely blocked by incubating the cells with PD098059 (Fig. 8A). Since cell spreading correlated with the ability of HGF to induce HT29 cell scattering on laminin, the effect of PD098059 on PMA induced spreading of these cells on laminin was measured. As depicted in Fig. 8B, PMA-induced spreading of HT29 cells on laminin was not blocked by the MEK inhibitor.

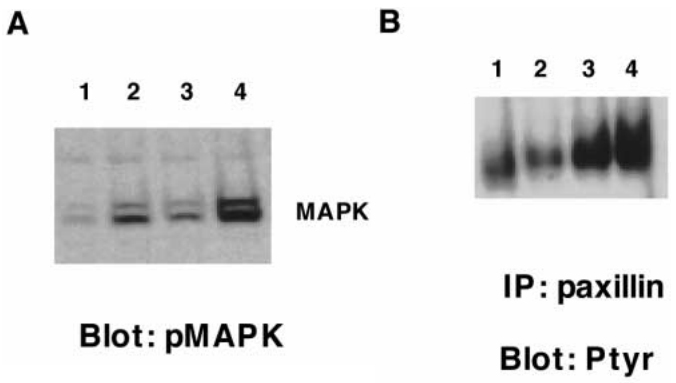

Fig. 7. MAPK activation correlates with PMA/HGF induce scattering of HT29 cells on laminin. Cells were cultured on laminin coated dishes, serum starved overnight and stimulated with HGF (lane 2), PMA (lane 3) or both (lane 4). Lane 1 represents unstimulated cells. (A) Cells were lysed and 100 μg of protein were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with a mAb directed against the active MAPK as described in Materials and Methods. (B) Cell extracts were immunoprecipitated with an antibody directed against paxillin and the immunocomplex was separated by SDS-PAGE, transferred to nitrocellulose and probed with the anti-phosphotyrosine antibody (4G10) as described in Materials and Methods.

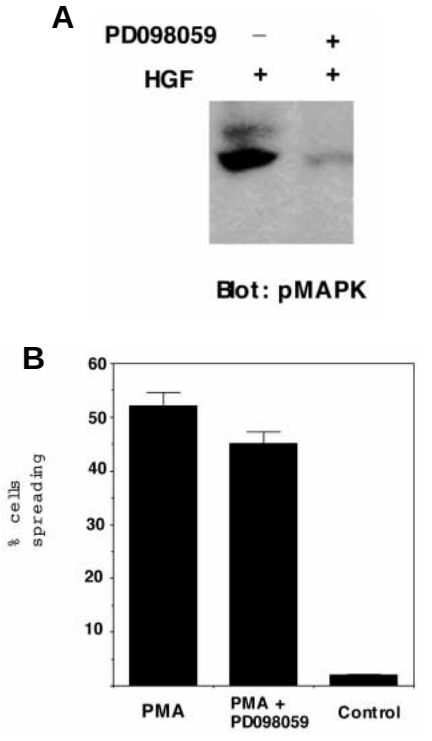

Fig. 8. Inhibition of MAPK activation does not alter HT29 spreading. (A) HT29 cells were treated with HGF in the presence (+) or absence (-) of PD098059 and the level of dually phosphorylated MAPK was estimated as described in the legend to Fig. 8. (B) HT29 cells were allowed to spread on laminin in response to PMA in the presence or absence of PD098059 as described in the legend to Fig. 3. The results are presented as a percentage of cells spread under the assay conditions. Spreading was estimated as described in the legend to Fig. 2.

In order to analyze the role of MAPK activation in HT29 scattering induced by HGF we followed the rate of change of the actin-based cytoskeleton or the localization of desmoplakins and E-cadherin upon HGF addition in the presence or absence of the MEK inhibitor, PD098059. As shown in Fig. 9, one hour after addition of HGF, a measurable actin rearrangement is observed that is characterized by an increase in the content of F-actin along the cell body and a reduction in polymerized actin beneath the plasma membrane at the sites of cell-cell contact. By six hours following HGF addition, most of the polymerized actin at cell-cell boundaries has disappeared and concomitant with this is the beginning of colony dispersion and involves disappearance of E-cadherin from intercellular contact points (data not shown). The above described events are partially blocked by the MEK inhibitor. The early actin rearrangement (1 hour) is minimally affected by this compound whereas the total disruption of colony integrity is markedly inhibited. This observation suggests that the MEK/MAPK pathway is not involved in the early events associated with actin rearrangement in response to HGF but it is required for HGF induced scattering. This is confirmed by the results depicted in Fig. 10A-C, where inhibition of the MEK/MAPK pathway is shown to prevent the down regulation of desmosomal integrity as measured by the disappearance of desmoplakin I and II from intercellular contact areas as early as one hour post stimulation. The disruption of adherent junctions, as measured by staining the distribution of E-cadherin (Fig. 10D-F), is a slower process. This result suggests that the disruption of the desmosome is the initial event upon HGF addition and is followed by the disappearance of adherent junctions. After six hours of HGF addition, a clear effect on the integrity of cell colonies is observed and individual cells begin to move apart from each other. By twenty-four hours after addition of HGF, full scattering of the cells is observed (data not shown). Specificity of PD098059 inhibition is supported by the observation that EGTA-induced colony disassembly is not prevented by this agent (data not shown). The requirement for MAPK activity in HGF-induced cell scattering is not unique to HT29 cells since similar results were obtained using MDCK cells (data not shown).

DISCUSSION

The regulation of cell motility by both ECM and growth factors is a critical event in the evolution of colon carcinoma (Agrez and Bates, 1994; Schwartz, 1993; Matsumoto et al., 1995; Streit et al., 1996; Vermeulen et al., 1995). In this study, HT29 cells were used to evaluate the role of specific signaling events associated with cell spreading and growth factor induced cell motility. As shown in Figs 1 and 2, HT29 cells fail to spread on laminin although these cells bind to laminin in an α6 integrin dependent manner (Simon-Assmann et al., 1994). The lack of laminin supported cell spreading is not due to a failure of adhesion dependent signaling since binding of these cells to laminin induced integrin mediated signaling such as tyrosine phosphorylation of paxillin and activation of MAPK (Fig. 3). These results suggest that extensive HT29 cell flattening is not required to induce paxillin phosphorylation and, in this case, may be associated with point of contact instead of classical focal adhesion formation (Tawil et al., 1993). Similarly, MAPK

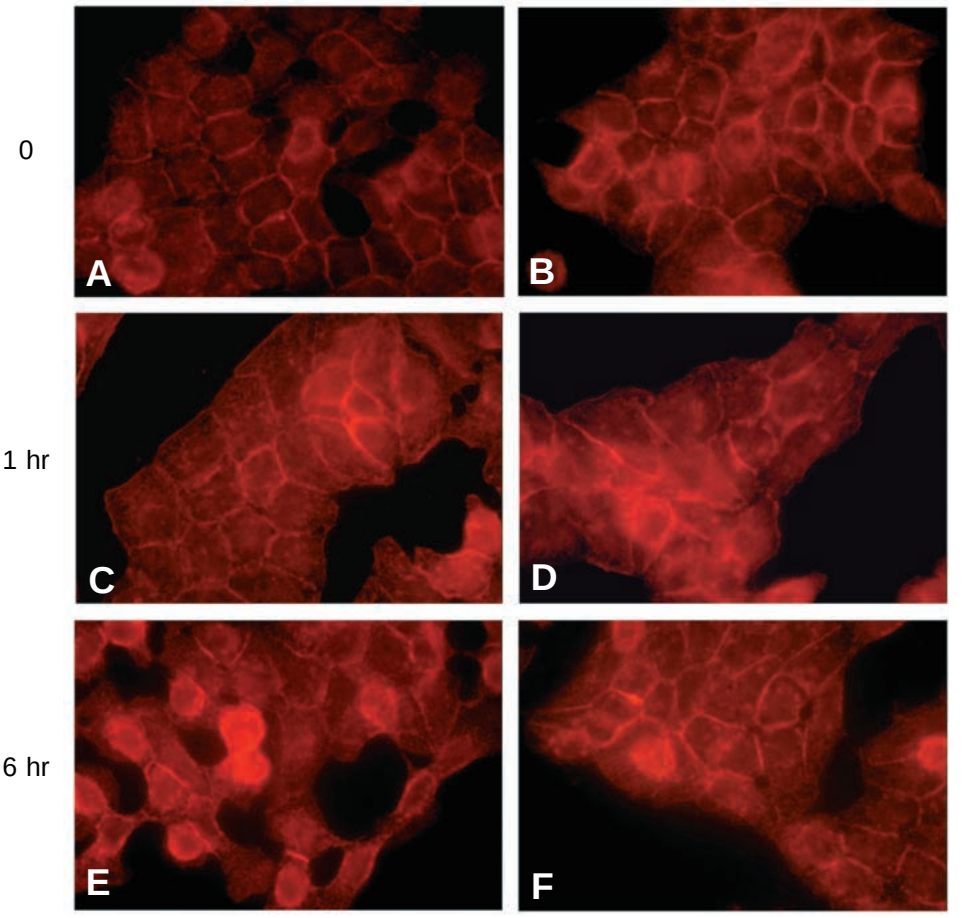

Fig. 9. Actin rearrangement in response to HGF stimulation of HT29 cells. HT29 cells were grown on plastic slides, serum starved overnight and incubated with HGF in the presence (B,D,F) or absence (A,C,E) of PD098059. At the indicated times, the cells were fixed and processed for labeling of actin as described in Materials and Methods.

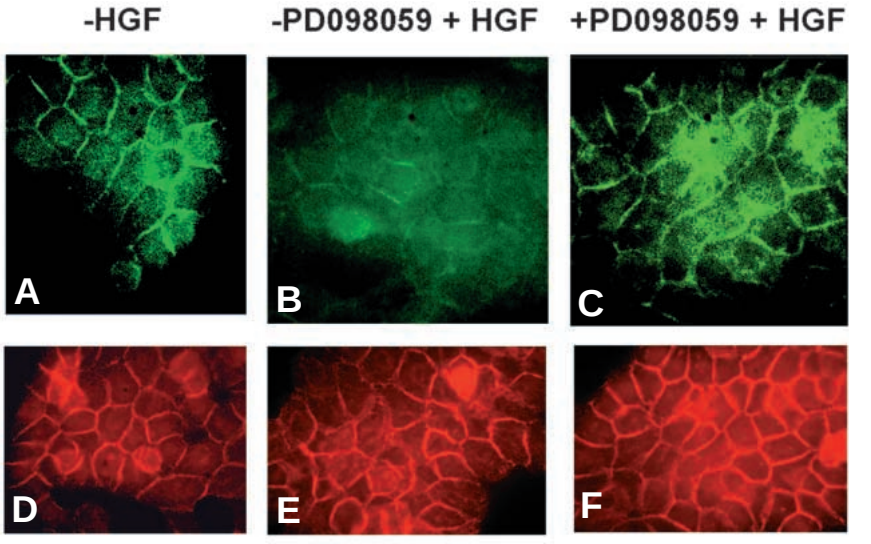

Fig. 10. HGF induced desmosomal turnover requires MAPK activation. HT29 cells were cultured on plastic slides, serum starved overnight and treated with HGF in the presence or absence of the MEK inhibitor, PD098059, for one hour. The cells were fixed and immunostained with anti-desmoplakins I and II mAb (A-C) or anti-E-cadherin mAb (D-F) as described in Materials and Methods.

activation does not appear to require cell spreading in contrast to what has been described in other cell systems (Zhu and Assoian, 1995).

The exact molecular mechanism of integrin mediated cell spreading is not fully understood. Studies carried out in hamster melanoma (Lewis et al., 1996), Chinese hamster ovary (Vuori and Ruoslahti, 1993) and HeLa cells (Chun et al., 1996) have shown that PKC is a common link between adhesion and cell spreading, and may involve gene expression mediated by NF-κB (Yebra et al., 1995). The results presented in Fig. 2 demonstrate that PKC activation also modulates spreading of HT29 colon carcinoma cells. This event is associated with an

extensive actin cytoskeletal rearrangement as shown by the appearance of polymerized cortical actin and formation of lamellipodia.

The biochemical changes associated with cell spreading correlate with changes in the phosphorylation state of several proteins. Thus, Colo 201 cell spreading in response to staurosporine correlates with tyrosine phosphorylation of Src and tensin (Yoshimura et al., 1995), and hamster CS-1 melanoma cell spreading in response to PMA stimulation correlates with increased FAK phosphorylation (Lewis et al., 1996). Paxillin phosphorylation has been shown to be associated with spreading of SW1116 colon cancer cells and macrophages (Sakamoto et al., 1996; Li et al., 1996). The results described here (Figs 3 and 7) show that HT29 cell spreading on laminin in response to PMA stimulation correlates with paxillin, but not FAK, phosphorylation. Although adhesion of HT29 cells to laminin correlates with activation of MAPK, this event is not sufficient to induce cell spreading on laminin nor is it necessary for cell spreading in response to PMA (Figs 3 and 8). This is in agreement with a recently published report demonstrating that MAPK is not required for cell spreading (Klemke et al., 1997).

The results presented in this paper show that the in vitro motogenic action of scatter factor on HT29 cells is dependent on the type of ECM ligand to which the cells bind. This effect is not due to inhibition of early events associated with HGF action since treatment of the laminin-bound cells with HGF result in biochemical changes such as tyrosine phosphorylation of γ-catenin (Fig. 5) and activation of MAPK (Fig. 8A). Tyrosine phosphorylation of E-cadherin associated proteins such as β- and γ-catenins has been implicated in the modulation of cell-cell adhesive interactions (Hamaguchi et al., 1993; Behrens et al., 1997; Kinch et al., 1995; Hinck et al., 1994; Takeda et al., 1995) and, by implication, may play a role in HGF-mediated epithelial colony dispersion. It appears that one critical component is cell spreading since restoration of cell spreading through activation of PKC by PMA enabled HGF to induce scattering of HT29 cells grown on laminin. Cell spreading by itself is not sufficient to induce scattering since PMA-treated HT29 cells grown on laminin do not scatter in response to PMA. Studies carried out in MDCK cells have shown that HGF mediated scattering requires, in addition to cell spreading, a secondary cellular event (Ridley et al., 1995).

Modulation of HGF activity by ECM has been observed previously (Fournier et al., 1996; Clark, 1994). Thus, the morphogenic events induced by HGF on MDCK cells are modulated by the type of ECM used. Incubation of MDCK cells in a 3-dimensional basement membrane gel (as opposed to collagen I gel) blocked HGF-induced tubulogenesis (Santos and Nigam, 1993). Similarly, scattering of these cells in response to HGF is positively regulated by adhesion to fibronectin (Clark, 1994). The results presented here extend this observation to colon carcinoma cells and demonstrate that growth factor regulation of the migratory properties of a given tumor cell are dependent, in part, upon the extracellular and basement membrane matrices that surround the cells.

The motogenic effects induced by HGF are initiated by its interaction with its cell surface receptor, a protein tyrosine kinase encoded by the protooncogene c-Met (Schwimmer and Ojakian, 1995; Ponzetto et al., 1994; Weidner et al., 1993), which result in activation of Ras, Rac and Rho as well as MAPK (Ponzetto et al., 1994; Pelicci et al., 1995). Molecular analysis of the signaling pathways involved in the motogenic activity induced by HGF showed that Ras activation is essential (Hartmann et al., 1994) but binding of one of the known upstream activators of Ras, Grb2, to the phosphorylated receptor is dispensable (Fournier et al., 1996). Since SHC is associated with signaling by the Met protein, activation of Ras could be accomplished by the SHC/Grb2/SOS complex (Pelicci et al., 1995). A downstream effector of Ras activation is the MEK/MAPK pathway (Cobb and Goldsmith, 1995; Blenis, 1993; Marshall, 1995), however, its contribution to the scattering activity by HGF is unknown. The results presented here (Figs 9 and 10) show that HGF induced scattering of HT29 cells requires the activity of the MEK/MAPK pathway since its effects were blocked by the specific MEK inhibitor PD098059. This blockade is not due to a general inhibition of cell spreading since PMA stimulated spreading was not altered by this inhibitor.

The requirement for actin reorganization that leads to cell spreading in response to HGF has been described in MDCK cells. This event requires the activity of the small GTP-binding protein Rac1 but its activation is not sufficient to induce cell scattering (Ridley et al., 1995). On the other hand, the activity of the small GTP-binding protein Rho is required for the maintenance of cadherin-dependent cell-cell contact (Braga et al., 1997) and its activation inhibits HGF-induced scattering in MDCK cells (Ridley et al., 1995). It remains to be determined whether the inhibitory activity of PD098059 is due to selective interference with Rho-dependent signaling events. Morphologically, the inhibitor prevented the initial event associated with the disruption of desmoplakin containing structures, desmosomes. This suggests that remodeling of the adherent junction/desmosome structures by HGF requires permissive activity provided by the MEK/MAPK pathway and illustrates cross-talk that appears to take place between desmosomes and adherent junctions, especially those containing γ-catenin (Lewis et al., 1997).

The scatter activity of HGF has been shown to require both RNA and protein synthesis (Ridley et al., 1995) thus suggesting that the activity of MAPK could be required for one of these events. Indeed, a known target for MAPK dependent regulation of gene expression is the c-fos gene, a protein implicated in the regulation of epithelial polarity and epithelial-mesenchymal cell conversion (Reichmann et al., 1992). A role for the MAPK family of proteins in the regulation of cellular adhesion, motility and migration has been described. Epithelial sheet movement in *Drosophila* is regulated by the HEP gene, a JNKK homologue (Glise et al., 1995), and the migration of FG carcinoma cells is dependent on MAPK activity (Klemke et al., 1997). While this manuscript was in preparation, a report was published demonstrating that the epithelial-mesenchymal transition of MDCK cells could be triggered by expression of activated MEK1, a direct regulator of MAPK (Schramek et al., 1997).

In summary, the data presented in this paper support two main conclusions: (1) the scatter activity of HGF on HT29 cells is modulated by the ECM surrounding the cell. This modulation is reflected as a requirement for integrin-mediated cell spreading in order for HGF to induce the morphological changes associated with the epithelial-mesenchymal transition.

(2) The activity of at least two signaling pathways, PKC and MAPK, are required for HGF induced scattering of HT29 cells on laminin and the MEK/MAPK pathway is involved in the initial phase of growth factor induced desmosome disassembly.

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