Sustained Activation of Extracellular Signal-regulated Kinase Stimulated by Hepatocyte Growth Factor Leads to Integrin α2 Expression That Is Involved in Cell Scattering*

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We have previously shown that hepatocyte growth factor (HGF) selectively increases the expression of integrin α2 in Madin-Darby canine kidney (MDCK) cells. In this study, we have further investigated the signal transduction pathways responsible for the event and its role in HGF-induced cell scattering. We found that the level of integrin α2/β1 expression induced by HGF correlated with the extent of cell scattering and that a functional blocking antibody against integrin α2, at the concentration of 25 μg/ml partially (40%) inhibited the HGF-induced cell scattering. However, in the presence of the specific phosphatidylinositol 3-kinase inhibitor LY294002 or the selective Src family kinase inhibitor PP1, although cells retained their response to HGF for increasing integrin α2 expression, they failed to scatter, indicating that increased expression of integrin α2 alone is not sufficient for cell scattering. Moreover, epidermal growth factor, which induced a transient (1 h) activation of extracellular signal-regulated kinase (ERK) in MDCK cells, only slightly increased integrin α2 expression and failed to trigger cell scattering. Conversely, HGF induced a sustained (at least 12 h) activation of ERK in the cells. Expression of constitutively active ERK kinase (MEK) in MDCK cells led to increased expression of integrin α2 even in the absence of HGF stimulation. In contrast, expression of ERK phosphatase or dominant negative MEK inhibited HGF-induced integrin α2 expression. Taken together, our results suggest that the increased expression of integrin α2/β1 by HGF is at least partially required for cell scattering and that the duration of MEK/ERK activation is likely to be a crucial determinant for cells to activate integrin α2 expression and cell scattering.

Hepatocyte growth factor (HGF), also known as scatter factor, is a multifunctional growth factor that elicits mitogenic, motogenic, and morphogenic activities in various cell types (1). The diverse biological effects of HGF are transmitted through activation of its transmembrane receptor encoded by the c-met proto-oncogene (2, 3). Although a number of growth factors are known to modulate cell motility, HGF is unique because of the intensity with which it stimulates motility and induces the epithelial-mesenchymal (E-M) transition. The scatter response of Madin-Darby canine kidney (MDCK) cells to HGF stimulation has been used extensively as a model to study the E-M transition, characterized by the loss of epithelial polarity, the disruption of E-cadherine-mediated cell-cell adhesions, and the acquisition of a migratory mesenchymal cell phenotype (4–6). Upon HGF stimulation, the scatter of MDCK cells can be visualized first as centrifugal spreading of cell colonies (after 2–4 h) followed by cell-cell dissociation (after 4–6 h) and subsequent cell migration (from 6 h) (4, 7).

Several intracellular signaling pathways have been implicated to act downstream of the HGF receptor to mediate scatter response. For example, both phosphatidylinositol 3-kinase (PI3K) and small GTPase Ras have been shown to be essential for cell dissociation and migration following stimulation of MDCK cells with HGF (4, 5, 8). Although GTP-bound Ras interacts with PI3K and may contribute to its activation (9, 10), recent studies suggested that Ras and PI3K might act on different signal transduction cascades to facilitate HGF-induced cell scattering (6, 11). It has been shown that PI3K acts upstream of Tiam1, an activator of the small GTPase Rac, to modulate both E-cadherine-mediated cell adhesion and cell migration (6). More recently, Zondag et al. (12) showed that the expression of oncogenic Ras permanently suppresses Rac activity through down-regulation of Tiam1 expression, which leads to up-regulation of the Rho activity and the E-M transition of MDCK cells. In addition, inhibition of HGF-induced cell scattering by dominant negative Ras or the specific inhibitor for extracellular signal-regulated kinase (ERK) kinase (MEK) has implicated an essential role for the Ras/ERK cascade in cell scattering (13–15).

Integrin α2/β1 is known to serve as the major receptor for collagen on MDCK cells (16) and play a crucial role in HGF-elicted cell motility and tubulogenesis (7, 17). We have previously demonstrated that HGF selectively enhances the expression of integrin α2 and to a lesser extent α1 in MDCK cells (7). In this study, we have further investigated the signal transduction pathways responsible for increased integrin α2 expression and its role in HGF-induced cell scattering.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human HGF and epidermal growth factor (EGF) were purchased from R&D Systems, Inc. Fetal bovine serum and LipofectAMINE Plus were purchased from Life Technologies, Inc. G418, Bisindolylmaleimide, genistein, PD98059, LY294002, cycloheximide, and phorbol-12-myristate-13-acetate (PMA) were purchased from Calbiochem (San Diego, CA). PP1 was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). EZ-Link sulfo-NHS-biotin and avidin-immobilized agarose beads were purchased from Pierce. The polyclonal (AB1944) and monoclonal (clone P1E6) anti-integrin α2 and the polyclonal anti-integrin β1 (AB1952) were pur-
chased from Chemicon (Temecula, CA). The polyclonal anti-ERK (sc-94) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The polyclonal anti-phosphoERK was purchased from New England Biolabs, Inc. (Beverly, MA). The cDNA of human integrin α2 was purchased from the American Type Culture Collection. The plasmids encoding constitutively active MEK1 (S218E/S222E), dominant negative MEK1 (S218A/S222A), and ERK phosphatase HVH2 were kindly provided by Dr. Kun-Liang Guan (University of Michigan, Ann Arbor, MI) and described previously (18, 19).

**Cell Culture and Transfections—**MDCK II 3B5 cells were maintained in Dulbecco's Modified Eagle Medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air atmosphere. To induce cell scattering and analyze the expression of integrin α2, MDCK cells were seeded at 10^4/60-mm dish and allowed to grow as discrete colonies for 2 days. For HGF stimulation, the culture medium was replaced by fresh medium containing 5% serum and 25 ng/ml HGF. In some experiments, HGF was substituted by EGF (100 ng/ml) or PMA (100 nM). After washed in phosphate-buffered saline (PBS), cells were incubated in PBS containing 500 μg/ml sulfo-NHS-biotin at room temperature for 30 min, washed once with cold PBS, and incubated in PBS containing 0.1 mM glycine (pH 7.4) on ice for 15 min. After several washes in PBS, the cells were lysed in Nonidet P-40 lysis buffer containing protease inhibitors. Aliquots (500 μg) of lysates were incubated with avidin-immobilized agarose beads. Integrin α2 in avidin-precipitated complexes was detected by immunoblotting with anti-integrin α2. Values are expressed as -fold increase relative to the level of cells without HGF treatment. For cell surface biotinylation, whole cell lysates or avidin-precipitated complexes were boiled for 3 min in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk, probed with the antibody at 37 °C for 30 min before loading them to a Neuroblot fluorescent system for detection.

**Antibody Inhibition of HGF-induced Cell Scatter and Cell Migration—**MDCK cells were allowed to grow as discrete colonies on 60-mm dishes and then treated with 25 ng/ml HGF in the presence or the absence of 25 μg/ml monoclonal anti-integrin α2 (clone P1E6). 12 h later, cells were washed in PBS, fixed in methanol for 10 min, and stained by modified Giemsa stain (Sigma) for 1 h. The percentage of scattered cells was measured in the total number of counted (≈1000) cells from 50 colonies under a light microscope. A cell was judged as a scattered cell when it has lost contact with its neighbors and exhibited a fibroblast-like phenotype.

For the cell migration assay, the MDCK cells were treated with 25 ng/ml HGF for 12 h, collected by trypsinization, and suspended in serum-free medium at 5 × 10^5 cells/ml with or without 25 μg/ml monoclonal anti-integrin α2 (P1E6) antibody. The cells were incubated with the antibody at 37 °C for 30 min before loading them to a Neuro Probe 48-well chemotaxis chamber (Cabin John, MD). Cells in the upper chamber were allowed to migrate through a porous (8-μm pore size) membrane toward the lower chamber containing 10 μg/ml collagen.
as an attractant for 5 h. The migrated cells were fixed, stained, and enumerated as described previously (21).

Northern Hybridization—The total cellular RNA from MDCK cells that had been treated with or without 25 ng/ml HGF for 12 h was extracted by TRIzol reagent (Life Technologies, Inc.) following the manufacturer’s instructions. To analyze the mRNA level of integrin \( \alpha_2 \) in MDCK cells, an equal amount (25 \( \mu \)g) of total cellular RNA was fractionated through 1% agarose gels containing 2.2 \( \times \) formaldehyde and transferred to nitrocellulose membrane (Schleicher & Schuell). A fluorescein-labeled DNA probe from human integrin \( \alpha_2 \) was prepared using Renaissance random primer fluorescein labeling kit (PerkinElmer Life Sciences). The hybridization was carried out at 55 °C. After several washes, the membrane was incubated with peroxidase-conjugated anti-fluorescein (1:1000) at room temperature for 1 h, and then exposed to 25 ng/ml HGF for various times before harvest. Coincident with the scatter of cell colonies (Fig. 1A), the effect of PMA on promoting both integrin \( \alpha_2 \) and \( \beta_1 \) expression is regulated at transcriptional level, total RNA extracted from MDCK cells was analyzed by Northern hybridization using human integrin \( \alpha_2 \) cDNA as a probe (Fig. 1D). The result showed that a 12-h treatment of HGF apparently (−3-fold) increased the level of integrin \( \alpha_2 \) transcripts, indicating that the promoter activation of the integrin \( \alpha_2 \) gene is involved in its increased expression induced by HGF.

To examine whether the HGF induction of integrin \( \alpha_2 \) expression is regulated at transcriptional level, total RNA extracted from MDCK cells was analyzed by Northern hybridization using human integrin \( \alpha_2 \) cDNA as a probe (Fig. 1D). The result showed that a 12-h treatment of HGF apparently (−3-fold) increased the level of integrin \( \alpha_2 \) transcripts, indicating that the promoter activation of the integrin \( \alpha_2 \) gene is involved in its increased expression induced by HGF.

To examine whether HGF-induced cell scattering was partially (−40%) inhibited by the integrin \( \alpha_2 \) blocking antibody at the concentration of 25 \( \mu \)g/ml. However, at the same concentration, this antibody was unable to efficiently inhibit HGF-induced cell migration toward collagen in a chemotax chamber (Fig. 2B), indicating a successful inhibition of integrin \( \alpha_2 \) function by this antibody. Thus, these results indicated that increased expression of integrin \( \alpha_2 \) induced by HGF contributes only partially to cell scattering.

PMA, a strong activator for conventional and novel sub-
PMA-induced cellular effects. In contrast, bisindolylmaleimide did not appear to inhibit HGF-induced cell scattering and only modestly (−25%) decreased the level of integrin α2 induced by HGF, indicating that the effect of HGF on promoting integrin α2 expression and cell scattering is mainly PKC-independent.

To identify signal transduction pathways required for HGF to induce integrin α2 expression, inhibitors including genistein (a general tyrosine kinase inhibitor), PP1 (a selective Src family kinase inhibitor), PD98059 (a specific MEK inhibitor), LY294002 (a specific PI3K inhibitor), and cycloheximide (a translation inhibitor) were applied to the experiments (Fig. 3B). Of these inhibitors, genistein, PD98059, and cycloheximide were found to efficiently inhibit the effect of HGF on integrin α2 induction. In contrast, PP1 and LY294002 had no such effect. These results indicate that MEK, but not Src or PI3K, is required for HGF to induce integrin α2 expression. It should be noted that in the presence of PP1 or LY294002, although cells retained their response to HGF for increasing integrin α2 expression, they failed to scatter, indicating that the elevated expression of integrin α2 alone is not sufficient for cell scattering.

As suggested in Fig. 3, the HGF induction of integrin α2 expression is mainly dependent on the MEK pathway. However, in addition to HGF, many other growth factors such as EGF are known to potently activate MEK and its downstream targets, ERK 1 and 2. To examine whether EGF induces integrin α2 expression and/or cell scattering, MDCK cells were incubated with EGF or HGF for 24 h. As shown in Fig. 4A, although EGF induced a slight increase in integrin α2 expression and lamellipodium formation around cell colony, it failed to trigger cell scattering. Next, we measured the phosphorylation of ERK upon HGF or EGF stimulation, which serves as an indicator for ERK activation (Fig. 4B). The phosphorylation of ERK induced by EGF was transient; it declined by 30 min and returned to the basal level by 1 h post-stimulation. In contrast, HGF induced a sustained phosphorylation of ERK, which lasted for at least 12 h post-stimulation. Furthermore, the sustained phosphorylation of ERK induced by HGF coincided with its persistent nuclear accumulation (Fig. 4C).

To further examine whether sustained activation of ERK leads to integrin α2 expression, constitutively active MEK was expressed in MDCK cells (Fig. 5A). As expected, the expression of constitutively active MEK resulted in ERK activation, and importantly, even in the absence of HGF stimulation, it enhanced the expression of integrin α2. Conversely, the expression of an ERK phosphatase HVH2 or a dominant negative MEK suppressed both HGF-induced ERK activation and integrin α2 expression (Fig. 5B). These results together suggest that sustained activation of the MEK/ERK signal cascade may be required and sufficient for induction of integrin α2 expression.

**DISCUSSION**

In this study, we have used MDCK cells as a model to investigate the role of up-regulation of integrin α2β1 in HGF-induced cell scattering and the signal transduction pathways responsible for this up-regulation. We showed that the level of integrin α2β1 expression increased by HGF stimulation correlated with the extent of cell scattering (Fig. 1) and that block of HGF-induced integrin α2 expression by inhibitors including genistein, PD98059, and cycloheximide all accompanied an inhibition in cell scattering (Fig. 3B). However, only 40% of HGF-induced cell scattering could be inhibited by the functional blocking antibody against integrin α2 (Fig. 2A), suggesting that increased integrin α2β1 may contribute only partially to cell scattering likely through promoting the third phase of
creased the expression of integrin α2, which led to sustained activation of ERK, inactivation of ERK. Moreover, the expression of constitutively active MEK, which does not cause MDCK cells to scatter, led to transient activation of ERK as judged by the phosphorylation of ERK and its accumulation in the nucleus (Fig. 4). In contrast, EGF, which activates the MEK/ERK cascade may be generally required for extracellular stimuli to activate the integrin α2 gene. The mechanism of transcriptional regulation of the integrin α2 gene is currently unknown. York et al. (33) have suggested that the duration of ERK activation is due to the activation of another small GTPase, Rap1. Thus, it will be of interest to examine whether sustained activation of ERK is required for MDCK cell scattering, associated with morphologic transformation to a mesenchymal cell phenotype. The mechanism by which MDCK cells maintain ERK in an active status for a long period upon HGF stimulation is currently unclear. York et al. (32) showed that in PC12 cells, the early phase of nerve growth factor-stimulated ERK activation is mediated by the small GTPase Ras, but the sustained phase of the ERK activation is due to the activation of another small GTPase, Rap1. Thus, it will be of interest to examine whether

Although the ERK signaling pathway has been implicated to be essential for HGF-induced cell scattering (13–15), here we propose for the first time that the duration of MEK/ERK activation is likely to be the major determinant for cells to activate integrin α2 expression and cell scattering upon HGF stimulation. Treatment of MDCK cells with HGF led to prolonged activation of ERK as judged by the phosphorylation of ERK and its accumulation in the nucleus (Fig. 4). In contrast, EGF, which does not cause MDCK cells to scatter, led to transient activation of ERK. Moreover, the expression of constitutively active MEK, which led to sustained activation of ERK, increased the expression of integrin α2 even in the absence of HGF stimulation (Fig. 5A). In contrast, the expression of ERK phosphatase or dominant negative MEK blocked HGF-induced integrin α2 expression (Fig. 5B). Together, these data support a model in which the different consequences of transient versus sustained activation of ERK are because sustained activation leads to persistent nuclear accumulation of ERK, resulting in phosphorylation of transcription factors and changes in gene expression.

We showed in Fig. 1 that the increased expression of integrin α2 by HGF in MDCK cells was a consequence of increased α2 mRNA likely because of transcriptional activation of the integrin α2 gene. The mechanism of transcriptional regulation of the canine integrin α2 gene by HGF is currently unknown. However, characterization of the 5′-flanking region of the human integrin α2 gene revealed that the promoter region contains consensus binding sites for several transcription factors, including Sp1, AP1, and AP2 (24). The binding of phosphorylated Sp1 proteins to two Sp1-binding sites in the core promoter region of the human integrin α2 gene has been shown to be required for full promoter activity (25). It is known that ERK activation increases AP1 activity via c-Jun activation and increased c-Fos synthesis, leading to an increase in c-Jun/c-Fos heterodimerization and DNA binding (26, 27). In addition, a recent study showed that ERK was able to phosphorylate Sp1 and induced its DNA binding activity (28). Therefore, it is possible that persistent nuclear accumulation of ERK induced by HGF stimulation may target Sp1 and AP1, leading to activation of the integrin α2 gene. Furthermore, the MEK inhibitor PD98059 inhibits the up-regulation of integrin α2 expression induced by PMA in MDCK cells (data not shown) or by phorbol dibutyrate in human leukemia K562 cells (29), suggesting that the MEK/ERK cascade may be generally required for extracellular stimuli to activate the integrin α2 gene.

Although the role of the ERK singling pathway in cell proliferation has been well established (30, 31), increasing evidence has suggested that the duration of ERK activation may be a crucial determinant for cells to proceed certain cellular functions other than proliferation. For example, sustained ERK activity has been shown to be required for nerve growth factor-induced neuronal differentiation of the phaeochromocytoma PC12 cells (32), fibroblast growth factor-induced angiogenesis of the endothelial cells on chick chorioallantoic membrane (33), and PMA-induced macrophage-like differentiation of the myeloid leukemia TF-1a cells (34). In this study, we demonstrate that HGF-mediated sustained activation of ERK is required for MDCK cell scattering, associated with morphological transformation to a mesenchymal cell phenotype. The mechanism by which MDCK cells maintain ERK in an active status for a long period upon HGF stimulation is currently unclear.
Rap1 is involved in HGF-induced sustained activation of ERK. Alternatively, the de novo expression of integrin α₂β₁ stimulated by HGF may contribute to the sustained phase of ERK activation. It is known that integrin-mediated cell adhesion to extracellular matrix proteins activates the ERK cascade and often potentiates the effect of growth factors on activating the cascade (35). Eliceiri et al. (33) showed that the sustained phase but not the initial phase of ERK activation by the fibroblast growth factor depends on integrin α₂β₁ in endothelial cells. The potential role of de novo synthesized integrin α₂β₁ in HGF-induced sustained activation of ERK cascade is currently under investigation.

It is known that in cells transformed by oncogenic Ras or Raf, the relative ability of external stimuli to activate ERK is usually diminished. However, we showed in this study that the expression of constitutively active MEK in MDCK cells weakly activated ERK compared with HGF and did not negatively impact on subsequent activation of ERK by HGF (Fig. 5A). The explanation for this discrepancy could be simply the low expression of constitutively active MEK in our selected MDCK cells, in which a fraction of ERK remains inactive and susceptible to HGF stimulation. This assumption was based on our observation that consecutive culture of active MEK-expressed MDCK cells, especially those with higher expression level of it, resulted in apoptosis. These results suggest that severely aberrant activation of MDCK by constitutively active MEK may be cytotoxic to MDCK cells and that those with a lower expression level of active MEK can survive. Alternatively, it is possible that in addition to the Ras/Raf/MEK pathway, other signal transduction pathways may collaborate to activate ERK upon HGF stimulation. For example, ERK has been shown to be activated by protein kinase C 3 and 6 via Ras-independent pathways (36, 37) and inactivated by protein phosphatases such as PP2A (38) and ERK phosphatases (39) in other systems. Therefore, it is plausible that activation of protein kinase C and/or inactivation of ERK/MEK phosphatases by HGF may further activate ERK in MDCK cells expressing constitutively active MEK. Recently, the protein-tyrosine phosphatase SHP-2 was shown to be required for HGF-induced activation of ERK in MDCK cells (40), although the detailed mechanism is currently unclear.

In this study, we have found that PMA was able to induce the E-M transition similar to that induced by HGF (Fig. 3). However, unlike HGF, the effect of PMA on induction of the E-M transition is mainly PKC-dependent (Fig. 3). In addition, stable expression of constitutively active MEK in MDCK cells has been shown to lead to the E-M transition (41), suggesting that sustained activation of ERK signaling cascade may be sufficient for triggering this process. Zondag et al. (12) reported recently that expression of oncogenic Ras leads to the E-M transition of MDCK cells through regulating the balance between the activation state of two GTPase Rho family proteins, Rac and Rho. They showed that oncogenic Ras-mediated sustained ERK signaling decreases Rac activity through transcriptional down-regulation of the Rac-specific exchange factor Tiam1 and, by contrast, increases Rho activity through an unknown mechanism. It remains to be tested whether HGF, PMA, and constitutive active MEK act in the same fashion as oncogenic Ras to induce the E-M transition.

Acknowledgment—We thank Dr. K.-L. Guan for the plasmid encoding constitutively active MEK, dominant negative MEK, and phosphatase HVH2.

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² C.-C. Liang and H.-C. Chen, unpublished data.
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J. Biol. Chem. 2001, 276:21146-21152. doi: 10.1074/jbc.M010669200 originally published online April 3, 2001

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