Involvement of Focal Adhesion Kinase in Hepatocyte Growth Factor-induced Scatter of Madin-Darby Canine Kidney Cells*

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Focal adhesion kinase (FAK) has been implicated to play a critical role in integrin-mediated control of cell behavior. However, it is unclear whether FAK also participates in the regulation of growth factor-elicited cellular functions. In this study, we have demonstrated that although overexpression of FAK in Madin-Darby canine kidney cells did not alter their growth property or ability to form tubules within collagen gel upon hepatocyte growth factor (HGF) stimulation, it apparently enhanced HGF-induced cell scattering. This enhancement was largely because of an increase in the third phase (i.e. cell migration) of cell scattering rather than the first two phases (i.e. cell spreading and cell-cell dissociation). Conversely, the expression of FAK-related nonkinase significantly (−60%) inhibited HGF-induced cell migration. Moreover, we have found that the effect of FAK on promoting HGF-induced cell motility was greatly dependent on cell-matrix interactions. We showed that HGF treatment selectively increased the expression of integrins α5 and, to a lesser extent, αc in Madin-Darby canine kidney cells and that a monoclonal antibody against integrin α5 efficiently blocked HGF-enhanced cell migration on collagen. In our efforts to determine the mechanism by which FAK promotes HGF-induced cell migration, we found that FAK mutants deficient in phosphatidylinositol 3-kinase or p130Cas binding failed to promote HGF-induced cell migration. Interestingly, cells expressing a FAK mutant defective in Grb2 binding exhibited a rate of migration ~50% lower than that of cells expressing wild type FAK in response to HGF stimulation. Taken together, our results suggest a link between HGF-increased integrin expression, FAK activation, and enhanced cell motility and implicate a role for FAK in the facilitation of growth factor-induced cell motility.

Focal adhesion kinase (FAK),1 a 125-kDa cytoplasmic tyrosine kinase localized in focal adhesions, has been implicated to play an important role in regulating integrin-mediated cellular functions, including cell spreading (1, 2), cell migration (3, 4), cell cycle progression (5, 6), and cell survival (7–9). The ability of FAK to regulate these cellular functions is believed to be dependent on its ability to interact with several intracellular signaling molecules including Src family kinases (10, 11), phosphatidylinositol 3-kinase (PI3K; Ref.12), adapter protein Grb2 (13), and docking protein p130Cas (14, 15). Tyr-397 has been identified as the major site of FAK autophosphorylation (16), and the binding site for the Src homology 2 domains of Src (10, 11) and PI3K (17). A FAK mutant deficient only in PI3K binding has recently been introduced by a substitution of Asp-395 with Ala (18). The proline-rich sequence region of FAK (residues 712–718) has been identified as the major binding site for the SH3 domain of p130Cas (14, 19). Upon binding to FAK, Src phosphorylates FAK at Tyr-925 (20), creating a binding site for the complex of Grb2 and Sos. Because Sos functions as a guanine nucleotide exchange factor for Ras (21), it has been proposed that FAK may link integrin-initiated signals to the Ras/mitogen-activated protein kinase cascades (13, 20, 22).

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 (23). The diverse biological effects of HGF are transmitted through activation of its transmembrane receptor encoded by the c-met proto-oncogene (24, 25). The Met receptor is a heterodimer composed of a 45-kDa α chain that remains entirely extracellular and a 145-kDa β chain that traverses the plasma membrane and contains the intracellular tyrosine kinase domain (26–29). Upon HGF binding, the intrinsic tyrosine kinase of the receptor is activated resulting in autophosphorylation on specific tyrosine residues in the β chain (30, 31). The phosphorylated tyrosine residues can then associate with molecules containing Src homology 2 and phosphotyrosine-binding domains that act to transduce extracellular signals to the cell interior (32).

We have previously demonstrated that HGF stimulates tyrosine phosphorylation of FAK and its association with the Grb2-Sos complex, which further contributes to the activation of extracellular signal-regulated kinase (ERK) by HGF in human embryonic kidney 293 cells (33). However, the role of FAK in HGF-elicited cellular functions has not been clarified. In this study, we have found that overexpression of FAK in Madin-Darby canine kidney (MDCK) cells apparently enhanced HGF-induced cell scattering. In contrast, the expression of FAK-related nonkinase (FRNK) significantly inhibited HGF-induced cell motility. Moreover, our results suggest that, in addition to PI3K and p130Cas, the Grb2 binding may also contribute to the ability of FAK to promote cell migration. Taken together, these results strongly implicated a role for FAK in the facilitation of growth factor-enhanced cell motility.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human HGF was purchased from Sigma. Fetal bovine serum and LipofectAMINE were purchased from Life Technologies.
Technologies, Inc. Collagen type I, fibronectin, and vitronectin were purchased from Collaborative Biomedical Products (Bedford, MA). The MEK inhibitor PD98059 and G418 sulfate were purchased from Calbiochem (San Diego, CA). The monoclonal anti-hermagglutinin (HA) epitope was purchased from Roche Molecular Biochemicals. The rabbit polyclonal anti-ERK (sc-94) and anti-phosphoERK (1:1500), and the monoclonal anti-FAK (clone 77) and monoclonal anti-phosphotyrosine (PY20) were purchased from Transduction Laboratories (Lexington, KY). The rabbit polyclonal anti-ERK (sc-94) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal anti-phosphoERK was purchased from Cell Signaling Technology, Inc. (Beverly, MA).

The monoclonal antibody (GE8) recognizing α5 integrin was kindly provided by Dr. R. B. Bankert (Roswell Park Cancer Institute, Buffalo, NY) and was described previously (34). The pKH3 expression plasmid encoding HA epitope-tagged FRNK was provided by Dr. J.-L. Guan (Cornell University, Ithaca, NY) and described previously (5).

For scatter assays, MDCK cells were allowed to grow as discrete colonies and then treated with 10 ng/ml HGF as described above. 12 h after HGF stimulation, cells were collected by trypsinization for cell number (\(N_c\)) measurement. The cell dissociation index was expressed as \(N_d/N_c \times 100\%\).

For cell migration assays, MDCK cells were allowed to grow as discrete colonies and then treated with 10 ng/ml HGF as described above. 12 h after HGF stimulation, cells were collected by trypsinization for cell number (\(N_c\)) measurement. The cell dissociation index was expressed as \(N_d/N_c \times 100\%\).

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on collagen was ~2-fold ofNeo cells, supporting a role of FAK in promoting cell spreading on matrix proteins, as described previously (1, 2). The HGF treatment induced a similar increase (~10%) in cell spreading for both control and WT cells. This result was consistent with our observation that HGF induced colonies of control and WT cells to spread with a similar extent during first 3 h after HGF stimulation (Fig. 1C). Thus, these results suggest that the effect of HGF on cell spreading is likely to be independent of FAK expression. Next we determined the degree of cell-cell adhesion using cell dissociation assays. As shown in Fig. 2B, 4-h HGF treatment induced a similar increase (~10%) in the degree of cell dissociation for both control and WT cells. However, FAK overexpression did not appear to have any effect on this event. This conclusion was further supported by our observations that the expression and tyrosine phosphorylation of E-cadherine or β-catenin, two proteins known to be involved in cell-cell adhesion (38), were not affected by FAK overexpression in MDCK cells (data not shown).

To examine whether FAK participates in the third phase (i.e., cell migration) of HGF-induced cell scattering, cells from control and WT clones were trypsinized 12 h after HGF stimulation and then subjected to cell migration assays using collagen as an attractant (Fig. 3C). In the absence of HGF stimulation, WT cells had exhibited a higher (~5-fold) rate of cell migration in comparison with the control cells. This is consistent with previous studies (4, 18) showing that FAK overexpression in Chinese hamster ovary (CHO) cells promoted their migration on fibronectin. Significantly, cells overexpressing FAK dramatically increased their migration on collagen upon HGF stimulation, indicating that FAK overexpression synergized with the effect of HGF to promote cell migration. To further confirm the role of FAK in HGF-enhanced cell migration, FRNK, a FAK carboxyl-terminal construct known to function as a dominant negative mutant of FAK (1, 2), was overexpressed in MDCK cells. As shown in Fig. 3, approximately 60% of HGF-induced cell migration was inhibited by FRNK expression. Together, these results indicate that FAK is involved in regulating MDCK cell motility induced by not only collagen but also HGF.

To examine whether the effect of FAK overexpression on promoting HGF-induced cell motility is matrix-dependent, control and WT cells were subjected to cell migration assays with or without using a matrix protein as an attractant (Fig. 4A). Although the HGF treatment generally increased MDCK cell motility, the effect of FAK overexpression on promoting HGF-induced cell motility was much more prominent when a matrix protein, especially collagen, was present. These data indicated that the ability of FAK to promote HGF-induced cell migration is mainly matrix-dependent. It is known that integrin α2β1 is the major receptor for collagen on MDCK cells (39). To examine the involvement of integrin α2β1 in HGF-enhanced cell migration on collagen, a monoclonal antibody 5E8 (34), which recognizes integrin α2 subunit and prevents integrin α2β1 from its ligand binding, was applied to the migration assays (Fig. 4B). The treatment of WT cells with this blocking antibody, but not a control antibody (monoclonal anti-HA), resulted in a dose-dependent inhibition of HGF-induced migration, indicating that the effect of FAK on promoting HGF-induced cell migration on collagen is mediated by integrin α2β1. It is noteworthy that at
the concentration of 5 μg/ml, this blocking antibody was able to completely inhibit WT cell migration in the absence of HGF stimulation but reached only a partial (60%) inhibition in the presence of HGF stimulation. These results suggested that HGF might increase the expression of integrins α2β1 and/or alter their conformation, leading to a less efficiency for this antibody to exert its blocking function. To examine the effect of HGF on integrin expression, control and WT cells were treated with or without HGF for 12 h, and their whole cell lysates were prepared for immunoblotting analysis. As shown in Fig. 4C, HGF induced a marked (2-fold) increase in the expression of integrin α2 and, to a lesser extent, α3. Conversely, the expression of integrin αV (Fig. 4C) or FAK (data not shown) did not appear to be affected by HGF stimulation. Together, these results suggest a link between HGF-increased integrin expression, FAK activation, and enhanced cell motility.

To investigate the mechanisms by which FAK promotes HGF-induced cell migration, stable MDCK cell lines overexpressing FAK mutants including D395A, P712A/P715A, and Y925F, deficient in binding to PI3K, p130Cas, and Grb2, respectively, were subjected to cell migration assays. The expression levels of these FAK mutants were slightly higher than that of WT FAK (Fig. 5A). In addition, HGF treatment was able to induce an increase in tyrosine phosphorylation of these exogenously expressed FAK proteins (Fig. 5B). Similar to prior experiments using CHO cells (18), FAK D395A and P712A/P715A mutants failed to promote MDCK cell migration in the absence of HGF stimulation (Fig. 5C). Interestingly, these two FAK mutants also failed to promote HGF-induced cell migration in the presence of HGF stimulation.
tion (Fig. 5C). These results suggest that direct bindings of both PI3K and p130Cas are required for FAK to promote cell migration induced by matrix proteins alone or together with HGF stimulation.

Cary et al. (40) reported that the mutation at Tyr-925 had no effect on the ability of FAK to promote CHO cell migration. In contrast, we found that MDCK cells expressing FAK Y925F mutant exhibited a rate of cell migration 50% lower than that of cells expressing WT FAK in the condition with or without HGF treatment (Fig. 5C). These results suggest that the direct Grb2 binding and its downstream signals (e.g., activation of ERKs) may also contribute to FAK-promoted cell migration. In fact, we have previously demonstrated that the tyrosine phosphorylation of FAK increased by HGF stimulation leads to ERK activation (33). To examine the potential role of ERKs in FAK-promoted cell migration, control and WT cells were subjected to cell migration assays in the presence of the selective MEK inhibitor PD98059. At the concentration of 100 μM, PD98059 efficiently inhibited both ERK1 and ERK2 (Fig. 6A) and decreased ~50% of cell migration promoted by FAK overexpression in WT cells in the presence or absence of HGF treatment (Fig. 6B). Together, these results indicate that the Grb2 binding and subsequent ERK activation contribute partially to the ability of FAK to promote cell migration.

**DISCUSSION**

In this study, we have used MDCK cells as a model to examine the effect of FAK overexpression on the biological functions of HGF. First we showed that although FAK overexpression did not affect MDCK cell proliferation or tubulogenesis, it apparently enhanced HGF-induced cell scattering. The scatter response of MDCK cells to HGF stimulation can be
visualized first as centrifugal spreading of cell colonies (after 2–4 h) followed by cell dissociation (after 4–6 h) and subsequent cell migration (from 6 h). Consistent with the role of FAK in promoting integrin-mediated cell migration (3, 4), FAK overexpression appeared only to affect the third phase (i.e., cell migration) of HGF-induced cell scattering (Fig. 1C). Furthermore, we showed that FAK overexpression prominently (−6-fold) enhanced the effect of HGF on cell migration (Fig. 2C). Conversely, the expression of FRNK, which functions as a dominant negative mutant of FAK, significantly (−60%) inhibited HGF-induced cell migration (Fig. 3B). Taken together, our results strongly suggest a role for FAK in the facilitation of HGF-induced cell motility.

FAK has previously been shown to play a role in promoting the G_{1} phase progression of the cell cycle in NIH-3T3 and human foreskin fibroblasts (5, 6). However, in this study, we do not observe any differences in the proliferation of MDCK epithelial cells by FAK overexpression. In fact, even the constitutively active form of FAK (CD2-FAK) by anchoring it to the plasma membrane was unable to promote MDCK cell proliferation (7). These results suggest that the function of FAK in cell cycle regulation may be cell type-dependent. Moreover, consistent with previous reports (41, 42), we found that HGF at 1 ng/ml did not stimulate the proliferation of MDCK cells (Fig. 1A), although it is capable of stimulating endothelial cell proliferation (43). Surprisingly, a higher concentration (10 ng/ml) of HGF, which induces cells to scatter, appears to reduce the growth rate of MDCK cells (data not shown).

The tubulogenesis is a complicated process involving various cellular activities including cell proliferation, apoptosis, and cell migration (44–46). Despite the fact that FAK overexpression enhances HGF-induced cell migration, we did not observe any apparent differences in the tubulogenesis of MDCK cells by FAK overexpression (Fig. 1B). A possible explanation for this is that HGF promotion of tubulogenesis involves the coordinate regulation of a number of intracellular signaling pathways such as the STAT (47), PI3K (48), and mitogen-activated protein kinase (48, 49) pathways; therefore a single molecule like FAK may not be sufficient to disturb the balance among these signaling pathways. Alternatively, it could be simply because the enforced expression of FAK in MDCK cells was not high enough to manifest its effect on the tubulogenesis. Nevertheless, our results do not exclude the possibility that FAK may play a permissive role for tubulogenesis.

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 epithelial-mesenchymal transition. Several intracellular signaling proteins have been implicated to act downstream of the HGF receptor to mediate scatter response. For example, PI3K and Ras have been shown to be essential for cell dissociation and migration following stimulation of MDCK cells with HGF (36, 37, 51). Although GTP-bound Ras interacts with PI3K and may contribute to its activation (52, 53), recent studies suggested that Ras and PI3K might act on different signal transduction cascades (i.e., Ras/ERK and PI3K/Rac) to facilitate HGF-induced cell motility (54, 55). We found that the overexpression of FAK in MDCK cells is able to increase the extent of the activation of ERK (Fig. 6A) and PI3K (data not shown) upon HGF stimulation, suggesting that the role of FAK in HGF-induced cell motility may be because of its contribution to both pathways. This assumption was also supported by our observation that FAK enhancement of HGF-induced cell motility could be completely inhibited by the PI3K inhibitor LY294002 (data not shown) and partially (−50%) inhibited by the MEK inhibitor PD98059 (Fig. 6B).

We have previously demonstrated that HGF induces a rapid (within 10 min) increase in FAK phosphorylation, which likely results from the activation of Src upon HGF stimulation (33). Importantly, this immediate effect of HGF on FAK activation was independent of integrin-mediated cell adhesion (33). In this report, we show the first time that a prolonged (12 h) stimulation of HGF selectively increases the expression of integrins α_{3} and, to a lesser extent, α_{5} in MDCK cells (Fig. 4C), which presumably further leads to the activation of FAK upon cell adhesion to collagen. It is therefore likely that the effect of HGF on FAK activation can be through an immediate (integrin-independent) response and a delayed (integrin-dependent) response. Moreover, it has previously been shown that integrin α_{3}β_{1} is essential for the HGF promotion of tubulogenesis (45). Here we show that a blocking antibody against integrin α_{3}β_{1} efficiently inhibits HGF-enhanced cell migration on collagen (Fig. 4B). Together with our observation that HGF induced an increase in integrin α_{3}β_{1} expression (Fig. 4C), it is possible that *de novo* synthesis of integrin α_{3}β_{1} is required for the long term cellular responses to HGF, such as cell scattering and tubulogenesis. Consistent with this, we found that cycloheximide, a translation inhibitor, blocked HGF-induced cell scattering and tubulogenesis (data not shown).

Similar to previous studies using CHO cells (4, 18), we showed that overexpression of WT FAK, but not its mutant deficient in PI3K or p130Cas binding, in MDCK cells promoted their migration on collagen (Fig. 5C). In addition, our results indicated that the bindings of PI3K and p130Cas were essential for the ability of FAK to promote HGF-induced cell migration. Together with our finding that HGF-induced cell migration on collagen depends on the expression of integrin α_{3}β_{1} (Fig. 4B), it...
is likely that P13K and p130Cas act downstream of FAK to facilitate the HGF-regulated integrin-mediated cell migration. Furthermore, we showed that MDCK cells expressing the Y925F FAK mutant defective in the Grb2 binding exhibited a rate of migration ~50% lower than that of cells expressing WT FAK (Fig. 5C). In addition, the MEK inhibitor PD98059 at 100 μM, which efficiently inhibited the activation of ERKs, significantly (~50%) decreased the migration of MDCK cells overexpressing FAK and their control cells in the condition with or without HGF stimulation (Fig. 6). Taken together, our results suggest that, in addition to PI3K and p130Cas, the Grb2 binding and the ERK signaling pathway may also contribute, at least in part, to the ability of FAK to promote cell migration.

However, Cary et al. (40) has shown previously that CHO cells expressing the Y925F FAK mutant exhibited an increased level of migration comparable with that of cells expressing WT FAK. In addition, they found that ectopically expressed FAK from CHO cells did not bind to the Src homology 2 domain of Grb2 in vitro, nor did it activate ERKs in CHO cells. Based on these observations, they concluded that the Grb2 binding and the ERK signaling pathway are not involved in FAK-mediated cell migration. The discrepancy between their work and ours is likely that PI3K and p130Cas act downstream of FAK to isotype PI3K and p130Cas, the Grb2 binding and the ERK signaling pathway may also contribute, at least in part, to the ability of FAK to promote cell migration.
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