Focal Adhesion Kinase, a Downstream Mediator of Raf-1 Signaling, Suppresses Cellular Adhesion, Migration, and Neuroendocrine Markers in BON Carcinoid Cells

Li Ning, Herbert Chen, and Muthusamy Kunnimalaiyaan

Abstract

We have recently reported that activation of the Raf-1/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2)/ERK1/2 signaling cascade in gastrointestinal carcinoid cell line (BON) alters cellular morphology and neuroendocrine phenotype. The mechanisms by which Raf-1 mediates these changes in carcinoid cells are unclear. Here, we report that activation of the Raf-1 signaling cascade in BON cells induced the expression of focal adhesion kinase (FAK) protein, suppressed the production of neuroendocrine markers, and resulted in significant decreases in cellular adhesion and migration. Importantly, inactivation of MEK1/2 by 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene or abolition of FAK induction in Raf-1-activated BON cells by targeted siRNA led to reversal of the Raf-1-mediated reduction in neuroendocrine markers and cellular adhesion and migration. Phosphorylation site-specific antibodies detected the phosphorylated FAK_{Tyr407}, but not FAK_{Tyr397}, in these Raf-1-activated cells, indicating that FAK_{Tyr407} may be associated with changes in the neuroendocrine phenotype. Overexpression of constitutively active FAK plasmids (wild-type FAK or FAK_{Tyr397} mutant) into BON cells reduced neuroendocrine markers, whereas the FAK_{Tyr397} mutant plasmid did not show any decrease in the levels of neuroendocrine markers, indicating that phosphorylation of FAK at the Tyr407 residue may be important for these effects. Our results showed for the first time that FAK is an essential downstream effector of the Raf-1/MEK1/2/ERK1/2 signaling cascade and negatively regulated the neuroendocrine and metastatic phenotype in BON cells.

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Introduction

Gastrointestinal carcinoids are neuroendocrine neoplasms that are characterized by their highly metastatic behavior and their propensities to secrete bioactive markers and peptides (1, 2). More than 75% of gastrointestinal carcinoid tumors are metastatic at the time of diagnosis. Furthermore, patients with metastatic carcinoid tumors often suffer from carcinoid syndrome due to the high levels of serotonin, bradykinin, and other neuroendocrine markers secreted by the tumors (2, 3). The mechanisms that control the metastatic behavior as well as the production of bioactive markers and peptides in gastrointestinal carcinoid cells are poorly understood. We and others have shown that activation of the Raf-1/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2)/ERK1/2 signaling cascade is associated with alteration in cellular morphology and neuroendocrine phenotype (4-6), suggesting that this signaling cascade may regulate the metastatic and neuroendocrine phenotypes of BON carcinoid tumor cells. However, the downstream mediators of the Raf-1/MEK1/2/ERK1/2 signaling cascade are unknown.

Focal adhesion kinase (FAK) is a cytoplasmic protein-tyrosine kinase that localizes to focal adhesions, sites of integrin engagement with the extracellular matrix (7). FAK comprises a central catalytic domain flanked by a large NH2 terminal domain containing the FERM region (band four point one, ezrin, radixin, and moesin) and a COOH terminal domain harboring the focal adhesion targeting sequence (7). The focal adhesion targeting sequence contains a binding site for tyrosine-phosphorylated adaptor protein that recruits multiple signaling molecules into complex via a Src homology (SH2) domain mediated interaction (8). In many cell types, FAK activation leads to the SH2 domain-mediated binding of Src family protein-tyrosine kinases to the motif surrounding the FAK_{Tyr397} phosphorylation site (9). Src binding to FAK promotes...
increased Src kinase activity, and in turn, Src-mediated phosphorylation of FAK within the kinase domain activation loop at Tyr^{576/577} is needed for maximal FAK-associated activity (10). Src also phosphorylates FAK at Tyr^{407}, Tyr^{861}, or Tyr^{925}, creating docking sites for other SH2 domain–bearing molecules, such as growth factor receptor binding protein 2, which links FAK to activation of the MAPK cascade (7, 10, 11).

The MAPK cascade that activates ERK, c-Jun NH2-terminal kinase, and p38 kinases plays an important role in modifying the morphogenetic and motile responses of cells (12). Recently, we have shown that activation of the Raf-1 pathway regulates cell–cell contact in medullary thyroid cancer cells (13). Hunger-Glaser et al. reported that ERK phosphorylates FAK both in vitro and in vivo (14). Although the precise role and the mechanism remain to be clarified, these emerging data implicate the MAPK signaling cascade in the control of focal adhesion formation and turnover required for cellular morphogenesis and motility. Given the biological properties of FAK and its potential association with the Raf-1 signaling cascade, we hypothesized that FAK may play an important role in these Raf-1–mediated events. In the present study, we show that FAK is an essential downstream effector of the Raf-1/MEK1/2/ERK1/2 signaling cascade in BON cells.

Materials and Methods

Cell culture and treatment. Metastatic human pancreatic carcinoid cell line (BON), a gift of Dr. Mark Evers and Dr. Courtney Townsend, Jr. (University of Texas, Galveston, TX), was maintained in DMEM: nutrient mixture F-12 in a 1:1 ratio (Invitrogen), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Cultured cells were detached from culture plates with 0.25 mg/mL of soybean trypsin inhibitor, heat-inactivated bovine serum albumin (Sigma-Aldrich)–containing media, and then centrifuged. The cells were resuspended in SFM containing 0.25 mg/mL of soybean trypsin inhibitor, and the cell lysates were prepared for Western blot.

siRNA transfection. FAK siRNA, ERK1 siRNA, ERK2 siRNA, and nonspecific control siRNA were purchased from Santa Cruz Biotechnology and transfected into BON-raf cells using Lipofectamine (Invitrogen), as described previously (15). Briefly, BON-raf cells were plated at a density of 50% to 60% confluence in six-well plates. On the next day, transfection with siRNA using Lipofectamine was carried out. On the following day, the medium containing the transfection complexes was replaced with fresh medium with E2 or ethanol, and the cells were incubated for another 48 hours. The cell lysates were prepared for Western blot or the cells were removed by trypsinization from culture plates for adhesion and migration assays.

FAK expression vector transfection. Constitutively active plasmids pKH3-FAK/WT (encoding full-length wild-type FAK protein), pKH3-FAK/397F (encoding mutant FAK at Tyr^{397}), and pKH3 empty vector were generous gifts of Dr. Patricia Keely (University of Wisconsin, Madison, WI). The constitutively active plasmid pKH3-FAK/407F (encoding mutant FAK at Tyr^{407}) was a generous gift of Dr. Eok-Soo Oh (Ewha Womans University, Seoul, Korea). BON cells were plated in 60-mm dishes and grown to 80% confluence for 24 hours. The cells were transfected with a mixture of 15 μL of Lipofectamine and 4 μg of plasmid DNA. The transfection mixture was replaced with fresh medium on the next day, and the cells were incubated for another 24 hours. The cell lysates were prepared for Western blot.

Western blot analyses. Cells were harvested and lysed, and the cell lysates were prepared as described previously (15). Total protein concentration was quantified with a bicinchoninic acid assay kit (Pierce). Denatured cellular extracts (20–40 μg) were resolved by 8% or 10% SDS-PAGE, transferred onto nitrocellulose membrane (Bio-Rad Laboratories), blocked in 5% w/v nonfat dry milk, and incubated with appropriate antibodies. Antibodies were diluted as follows: ERK1/2, phosphorylated ERK1/2, and phosphorylated MEK1/2 (1:1,000, Cell Signaling Technology); mammalian achaete scute homologue-1 (ASCL1; 1:1,000, BD Pharmingen); chromogranin A (CgA; 1:1,000, Zymed Laboratories); FAK (1:200, Santa Cruz Biotechnology, Inc.); polyclonal antibodies to a phosphorylation site specific against FAK^{tyr^{397}} (1:200, Santa Cruz); polyclonal antibodies to a phosphorylation site specific against FAK^{tyr^{407}} (1:1,000, BioSource Quality Controlled Biochemicals, Inc.); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000, Trevigen). Horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Cell Signaling) were used depending on the source of the primary antibody. The membranes were visualized with Immuno-star (Bio-Rad) or SuperSignal West Femto kit (Pierce).

Cell adhesion assays. Fibronectin (Sigma-Aldrich) was diluted to 10 μg/mL in serum-free medium (SFM) and dispensed to 12-well plates that were incubated at room temperature for at least 1 hour to allow adsorption. The plates were then washed with PBS, blocked with 0.2% heat-inactivated bovine serum albumin (Sigma-Aldrich) for 1 hour, and then washed with SFM (2 × 10 minutes). Cultured cells were detached from culture plates with 0.05% trypsin and 0.53 mmol/L EDTA, suspended in SFM containing 0.25 mg/mL of soybean trypsin inhibitor, and then centrifuged. The cells were resuspended in SFM, plated onto fibronectin–coated plates, and incubated for the indicated durations at 37°C. The wells were washed with PBS, and the numbers of adherent cells per high power microscope field were counted. All experiments were conducted in quadruplicate.

Cell migration assays. The assays were conducted using 8.0-μm pore size and 6.5-mm diameter transwell filters (Corning Corp.). The undersurface of the polycarbonate membrane of the chambers was coated with fibronectin (10 μg/mL) for 2 hours at 37°C. The membrane was washed in PBS to remove excess ligand, and the lower chamber was filled with 500 μL of 10% fetal bovine serum–containing
media. Cells were seeded at a density of $5 \times 10^4$ per well in SFM in the upper chamber of the insert, and cells were allowed to migrate through the fibronectin-coated chamber for 8 hours at 37°C. Nonmigrated cells on the upper membrane were removed with a sterile cotton swab. Migrated cells (located on the lower surface of the filters) were fixed for 5 minutes in methanol, stained with 0.6% hematoxylin (Fisher Scientific Co.) and 0.5% eosin (Fisher), and then counted. All experiments were conducted in quadruplicate.

**Statistical analysis.** ANOVA with Bonferroni post hoc testing (SPSS software version 10.0, SPSS) was used for statistical comparisons. A $P$ value of $<0.05$ was considered significant. Data were represented as means ± SD.

**Results**

**Activated Raf-1 induces FAK expression and regulates neuroendocrine and metastatic phenotypes.** We used BON-raf cells to study the relationship between Raf-1 and FAK. Creation and treatment of BON-raf cells were described elsewhere (6). BON-raf cells were treated with either ethanol (control) or estradiol (E2) for 48 hours, and the total cellular extracts were isolated and analyzed by Western blot for downstream targets of the Raf-1/MEK1/2/ERK1/2 signaling cascade and FAK expression. As shown in Fig. 1A, activation of raf-1 by E2 in BON-raf cells resulted in an increase in FAK protein expression compared with control cells, suggesting that the increase in the levels of FAK protein is due to the activation of the raf-1 pathway. Phosphorylation of FAK at specific sites has been reported to be associated with different tumor types. In most types of tumor cells, when FAK signaling is activated, FAK is recruited to focal contacts and autophosphorylated at Tyr397 (16). Thus, Western blot was done to determine the phosphorylation of FAK at the Tyr397 residue by site-specific antibodies in this study. As shown in Fig. 1A, no phosphorylated FAK Tyr397 was detected in both control and E2 treatment. Interestingly, we found that phosphorylated FAK Tyr407 was present at high levels in E2-treated BON-raf cells compared with the levels in control treatment. It is not clear whether raf-1 activation induced the phosphorylation of FAK at Tyr407 or overexpression of FAK by raf-1 activation resulted in phosphorylation. Surprisingly, raf-1 activation did not induce phosphorylation of FAK at the Tyr397 residue, and the mechanism is not known.

To determine the effects of Raf-1 activation on the metastatic phenotype, cell adhesion assays were done. BON and BON-raf cells were treated with ethanol or E2 for 48 hours. The cells were detached and plated on fibronectin-coated plates and observed for spreading to measure cell adhesion. As shown in Fig. 1B, by 30 minutes, ~30% of ethanol-treated BON cells spread onto fibronectin. E2-treated BON cells and ethanol-treated BON-raf cells had similar levels of adhesion. In contrast, activation of Raf-1 by E2 treatment in BON-raf cells led to a significant reduction in cell adhesion. Similar results were
observed at 60 and 90 minutes. We next examined the adhesion-mediated migration property of the gastrointestinal carcinoid cells after Raf-1 activation. BON and BON-raf cells treated with ethanol or E2 for 48 hours were seeded in the upper chamber of the insert and allowed to migrate through the fibronectin-coated chamber for 8 hours. As shown in Fig. 1C, the cell migration rate of ethanol-treated BON cells was ~55%. E2-treated BON cells and ethanol-treated BON-raf cells had similar levels of migration. By contrast, E2-treated BON-raf cells showed a significant decrease in cell migration. Taken together, these results indicate that Raf-1 activation decreases carcinoid cell adhesion and migration, suggesting a reduction in metastatic potential.

**Raf-1–mediated decrease of cellular adhesion and migration are dependent on the MEK1/2/ERK1/2 cascade.** To determine whether Raf-1–associated events proceed through the MEK1/2/ERK1/2-dependent cascade, we used the selective MEK1/2 inhibitor U0126, which has been shown to inhibit the activity of MEK1/2, causing an inability to phosphorylate ERK1/2. BON-raf cells were incubated for 1 hour in the presence of U0126 (10 μmol/L) or DMSO (control) and then treated with either ethanol or E2 for 48 hours. Western blot was done to investigate the effect of U0126 on FAK expression. As shown in Fig. 2A, as predicted, ERK1/2 activation was associated with an increase in the levels of FAK protein. On the other hand, MEK1/2 activation due to the Raf-1 activation by E2 treatment in BON-raf cells was blocked by prior exposure to U0126 (Fig. 2A). Inhibition of MEK1/2 activation by U0126 treatment abrogated ERK1/2 phosphorylation and most importantly FAK induction.

To substantiate the results obtained with U0126, we investigated if the abolition of ERK1/2 activation could recapitulate the effect of U0126. BON-raf cells were transfected with nonspecific siRNA (NS siRNA; control) or ERK1/2-targeted siRNA (ERK1/2 siRNA) and subsequently treated with ethanol or E2 for 48 hours. Western blot was done to detect Raf-1 activation and establish the extent to which the siRNA treatments yielded an effective knockdown. As shown in Fig. 2B, ERK1/2 siRNA transfection had no effect on the Raf-1–induced adhesion suppression in E2-treated BON-raf cells, but it led to a significant reduction in both total ERK1/2 and phosphorylated ERK1/2. Importantly, siRNA treatment against ERK1/2 in the presence of Raf-1 activation resulted in a marked reduction in the level of FAK protein, suggesting that ERK1/2 activation was associated with FAK induction in these cells. As predicted, NS

**FIGURE 2.** Raf-1–induced neuroendocrine marker reduction and decrease in cell adhesion and migration are dependent on the ERK cascade. Western blot analyses were done on lysates from various treatments. A, MEK1/2 inhibition by U0126 treatment abrogated FAK induction. B, ERK1/2 siRNA transfection blocked total ERK1/2 expression and subsequently prevented FAK induction in raf-1–activated BON-raf cells. GAPDH was used to confirm equal protein loading. C and D, BON-raf cells were incubated with U0126 (10 μmol/L) or DMSO for 1 hour and subsequently treated with either ethanol or E2 for 48 hours. Assays for cell adhesion and migration were done to measure cell motility. C, E2 treatment induced Raf-1 activation in DMSO-pretreated BON-raf cells, leading to a significant reduction in cell adhesion. By contrast, U0126 blocked Raf-1–induced adhesion suppression in E2-treated BON-raf cells. D, treatment of DMSO-pretreated BON-raf cells with E2 resulted in a significant decrease in cell migration. In contrast, U0126 blocked Raf-1–induced migration inhibition in E2-treated BON-raf cells. Data were represented as means ± SD. *, P < 0.05.
siRNA transfection had no effect on either the ERK1/2 phosphorylation or the total ERK1/2 proteins in all treatment groups.

We next explored the effects of inactivated MEK1/2 by U0126 in the presence of Raf-1 activation on cell adhesion. As shown in Fig. 2C, ~30% of the BON-raf cells, pretreated with either DMSO or U0126 and subsequently treated with ethanol, spread onto the fibronectin by 30 minutes. In contrast, Raf-1 activation by the addition of E2 to DMSO-pretreated cells led to a significant reduction in cell adhesion, as shown previously. Importantly, the presence of U0126 blocked Raf-1–induced adhesion suppression in E2-treated cells. Similar results were obtained at 60 and 90 minutes. We next examined the effects of U0126 on cell migration. As shown in Fig. 2D, ~53% of the BON-raf cells, pretreated with either DMSO or U0126 and subsequently treated with ethanol, migrated through the fibronectin-coated chamber at indicated time. Addition of E2 to DMSO-pretreated cells led to a significant reduction in cell migration. As predicted, U0126 treatment prevented the decrease of cell migration in E2-treated cells.

Taken together, these results indicate that Raf-1–mediated FAK induction and decrease in cellular adhesion and migration are all dependent on the phosphorylation of ERK1/2. Furthermore, these data illustrate that FAK is a downstream target of ERK1/2 in the Raf-1/MEK1/2/ERK1/2 signaling cascade.

Raf-1–mediated neuroendocrine marker reduction, decrease in cellular adhesion and migration are dependent on FAK induction. Earlier we have reported that activation of raf-1 in BON cells resulted in significant reduction of neuroendocrine markers, such as ASCL1, CgA, and serotonin (6). Although we showed here that the activation of the Raf-1/MEK1/2/ERK1/2 signaling cascade upregulated FAK expression, it is unclear whether or not FAK plays a role in the reduction of neuroendocrine markers. To assess the potential role of FAK in modulating neuroendocrine and metastatic phenotypes in BON cells, BON-raf cells were transiently transfected with NS siRNA (control) or FAK-targeted siRNA (FAK siRNA). On the next day, transfected cells were treated with either ethanol or E2 for 48 hours, and cell lysates were analyzed by Western blot. As shown in Fig. 3A, neither NS siRNA nor FAK siRNA transfection had an effect on Raf-1 activation in E2-treated BON-raf cells, as manifested by the same marked increase in the levels of phosphorylated ERK1/2 after E2 treatment in both transfections. This observation was expected because the ERK1/2 siRNA experiments (Fig. 2B-D) clearly put FAK as downstream of ERK1/2. Treatment of NS siRNA–transfected cells with E2 resulted in a marked increase of FAK expression and a decrease in the levels of ASCL1 and CgA. FAK siRNA effectively abrogated the FAK protein, even after Raf-1–induced expression (Fig. 3A, lane 4). More importantly, absence of FAK in these cells abolished Raf-1–induced reduction in the levels of ASCL1 and CgA, indicating that FAK plays an important role in the regulation of neuroendocrine markers.

To determine the role of FAK on cellular metastatic phenotype, adhesion and migration assays were carried out in FAK siRNA–treated cells. As shown in the Fig. 3B controls, ~30%, 55%, and 75% of the BON-raf...
FIGURE 4. Tyr407 phosphorylation alters the neuroendocrine phenotype of BON cells. BON cells were transfected with plasmids pKH3-FAK/WT, pKH3-FAK/397F, or pKH3-FAK/407F; pKH3 (vector control); or Lipofectamine (Lipo, vehicle control) alone. Western blot analyses were done to detect the expression of FAK, the phosphorylation of FAK at site-specific residues, and the levels of neuroendocrine markers. GAPDH was used to confirm equal protein loading.

Ayaki et al. found that FAK protein expression decreased in human metastatic liver tumor samples compared with their matched primary colorectal adenocarcinoma samples (23) in contrast to overexpression of FAK mRNA and/or protein has been observed in cancers of the breast, colon, thyroid, prostate, liver, stomach, rectum, cervix, oral epithelium, and ovary (24-27). Because FAK controls the dynamic regulation of cellular adhesions and peripheral actin structures, thus contributing to cell migration (7, 8, 28),
we investigated the role of FAK in Raf-1–mediated changes in the metastatic phenotype in BON cells. We found that Raf-1 activation led to a significant reduction in the capability of cellular adhesion and migration in these cells and the metastatic phenotype was abrogated with depletion of FAK protein. Although FAK has been identified as a substrate of ERK to regulate cell motility (18-20), our finding will prove intriguing, as many reports have implicated FAK as a positive regulator of cell motility (7, 8, 28). The contradictory results are not simply explained by cell type differences, as inhibition of FAK expression by siRNA also impairs cell motility in both human fibroblasts and HeLa cells (29).

Given that the ability of FAK to transduce downstream signals is dependent on its phosphorylation (8), the phosphorylation of FAK at different tyrosine residues could contribute to the different roles of FAK in cell motility in different contexts. Recently, Earley and Plopper showed that phosphorylation of FAK at Tyr397 and Tyr407, but not at Tyr925, contributed to transendothelial migration of metastatic breast cancer cell lines MDA-MB-231 and AU-565 (30). Moreover, the migration of highly metastatic MDA-MB-231 cells expressing the FAKTyr861 mutant is significantly decreased, and the reduction is even more substantial than that caused by the FAKTyr925 mutant in these cells (30). Conversely, the migration of less aggressive AU-565 cells expressing the FAKTyr861 mutant is not diminished at all, suggesting that FAKTyr861 may play a more prominent role in transmigration of more invasive cancer cells (30). These results confirmed the notion that phosphorylation of FAK at different tyrosine residues may play distinct roles during motility of cancer cells.

In this study, we found that activating the Raf-1 signaling cascade induced phosphorylation of FAK at Tyr407 in BON cells, suggesting that FAKTyr407 phosphorylation may play an important role in Raf-1–induced effects. Overexpression of FAK plasmids containing mutations of specific tyrosine sites (407 or 397) substantiated the importance of phosphorylation of Tyr407 residue in the regulation of neuroendocrine phenotype in carcinoid cells. The importance of Tyr407 phosphorylation in FAK function has also been observed by other investigators. A recent study by Lim et al. showed that FAKTyr407 phosphorylation negatively regulated the enzymatic and biological activities of FAK in cell proliferation, spreading, adhesion, and migration (17). The mechanisms underlying this novel type regulation of FAK is not fully understood.

In summary, this is the first description suggesting that FAK is a downstream effector of the regulation of neuroendocrine markers and cell motility by the Raf-1/MEK1/2/ERK1/2 signaling cascade in BON cells. Furthermore, we show that FAKTyr407 phosphorylation may play an important role in the regulation of neuroendocrine phenotype in carcinoid cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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