Phosphorylation of Focal Adhesion Kinase Tyrosine 397 Critically Mediates Gastrin-Releasing Peptide’s Morphogenic Properties

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We have proposed that gastrin-releasing peptide (GRP) and its receptor (GRP-R) are morphogens that when aberrantly re-expressed in colon cancer promote tumor cell differentiation and retard metastasis. Because circumstantial evidence suggested that these properties were mediated via focal adhesion kinase (FAK), the purpose of this study was to elucidate the role of GRP-induced activation of this enzyme on properties fundamental to metastasis including cell attachment, motility, and deformability. To do this, we studied 293 cells, a non-malignant epithelial cell line that we show expresses GRP and GRPR. To dissect out the role of FAK, 293 cells were modified to inducibly express the dominant negative enzyme FAK-related non-kinase (FRNK) under control of a Tet-On (i.e., doxycycline-sensitive) promoter. Under serum-free conditions, GRP acting in an autocrine manner caused FAK to be phosphorylated at Y397; and this could be completely inhibited either by incubating with the specific GRP-R antagonist D-Phe6(bombesin) methyl ester, or by upregulating FRNK using doxycycline. To measure cell attachment, we designed a cone-plate viscometer that recorded the shear stress required to detach cells from their underlying matrix. To assess motility, confluent cells were wounded and behavior assessed by time-lapse photography. To measure deformability, we recorded the ability of cells to be completely drawn into a micropipette <50% the size of the non-deformed cell. Control 293 cells adhered more avidly to their underlying matrix, rapidly remodeled wounded tissues without any increase in overall proliferation, and were less distensible than cells treated with antagonist or doxycycline. Thus, these findings suggest that expression of GRP/GRPR in cancer inhibits metastasis by enhancing cell attachment to the matrix, regulating motility in the context of remodeling, and decreasing deformability. J. Cell. Physiol. 199: 77–88, 2004.

Most studies of colon cancer focus on the mutational events necessary for normal colonic epithelium to progress to an adenoma and then to a carcinoma (reviewed in, Vogelstein et al., 1988; Fearon and Vogelstein, 1990). In contrast, little is known about the events post neoplastic transformation that regulate tumor progression such as differentiation and metastasis. In the context of solid tumors, differentiation describes the degree to which the tumor cell(s) resemble the non-malignant tissues whence they originated. Clinically, the most important implication of tumor differentiation relates to the fact that it predicts the development of metastases (Birchmeier et al., 1996; Villavicencio et al., 1997; Kowalski and Medina, 1998; Ng, 1998; Nicolson and Moustafa, 1998; Park et al., 2000; Rampaul et al., 2001). Yet the factors regulating tumor cell differentiation, and their role in permitting or inhibiting metastasis, have not been well studied at the cellular and molecular level.

Abbreviations: GRP, gastrin-releasing peptide; GRP-R, GRP receptor.

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We have recently proposed that gastrin-releasing peptide (GRP) and its receptor (GRP-R) are morphogens, critically involved in regulating the differentiation of individual tumor cells in colon cancer. Although mature epithelial cells lining the colon do not normally express GRP/GRP-R, they are upregulated post neoplastic transformation where they act to retain tumor cells in a better differentiated state (reviewed in Jensen et al., 2001). Our study of chemically-induced colon cancers in wild type mice and mice genetically incapable of synthesizing GRP-R (i.e., GRPR−/− mice) suggested a possible mechanism for this action. Although the amount of GRP/GRP-R expressed by tumors occurring in wild type mice tightly correlated with improved differentiation as well as the presence of focal adhesion kinase (FAK), this enzyme was nearly undetectable in tumors developing in GRPR−/− mice (Carroll et al., 2000a).

FAK is a 125-kDa cytoplasmic protein found in focal adhesions, structures primarily responsible for tethering cells to the extracellular matrix. FAK activity is enhanced by tyrosine autophosphorylation, and is typically studied in the context of its regulation by proteins resident in the extracellular matrix (reviewed in, Richardson and Parson, 1995). However, non-matrix-initiated signals, such as those generated by transmembrane-spanning receptors including the GRP-R, are also known to activate this enzyme (Zachary and Rozengurt, 1992; Sinnett-Smith et al., 1993). Recently, we showed that human colon cancer cells expressed total FAK, and FAK phosphorylated at tyrosine 397 (Y397), in relation to both their stage of differentiation as well as to the amount of GRP/GRP-R co-expression (Matkowsky et al., 2003b). Yet the functional consequence(s) of GRP-induced FAK activation on cell behavior remains to be determined, including whether this signaling mediates any of the changes associated with differentiation such as appearance, motility, and metastasis.

To evaluate GRP, its receptor, and their role in regulating differentiation by activating FAK, we were unable to study colon cancer cell lines because we previously have shown that both the GRPR gene (Glover et al., 2005) and its mRNA (Carroll et al., 2000b) are ubiquitously mutated in this tumor type. We, therefore, studied 293 cells, a transformed human fetal renal epithelial cell line, were transfected to stably express the dominant negative inhibitor of FAK, FRNK, under the control of a doxycycline inducible promoter as recently described (Kornberg and Fleigel, 2003). Cells were cultured in Opti-MEM containing HEPES buffer, sodium bicarbonate, and L-glutamine supplemented with 10% tetracycline-free FBS, 600 μg/ml G-418, and 200 μg/ml hygromycin B. Cells were maintained at 37°C in a 5% CO2 atmosphere.

**MATERIALS AND METHODS**

**Materials**

All cell culture reagents were obtained from Gibco BRL (Carlsbad, CA) except for tetracycline-free fetal bovine serum (FBS), which was from Clonetech (Palo Alto, CA) and antibiotic G-418, which was from Fisher (Pittsburgh, PA). 293 cells were obtained from ATCC (CRL-1573; Rockville, MD). All immunohistochemical supplies were from DAKO (Carpentaria, CA) except for antibodies to total FAK (Upstate Biotechnology; Lake Placid, NY) and to FAK phosphorylated at tyrosine 397 (Y397) (Biosource, Camarillo, CA). Western blot analysis was performed using peroxidase conjugated goat anti-rabbit IgG from BioRad, Hercules, CA) and the ECL Plus detection system from Amersham (Piscataway, NJ). Bombesin was from Bachem (Torrance, CA); 125I-Tyr4-bombesin was kindly provided by Dr. Robert Jensen (National Institutes of Health, Bethesda, MD); and D-Phe6(bombesin) methyl ester was kindly provided by Dr. David Coy (Tulane University, New Orleans, LA). All other supplies were molecular biology grade and were from Sigma (St. Louis, MO).

**Cell culture**

293 cells, an adenovirally-transformed human fetal renal epithelial cell line, were transfected to stably express the dominant negative inhibitor of FAK, FRNK, under the control of a doxycycline inducible promoter as recently described (Kornberg and Fleigel, 2003). Cells were cultured in Opti-MEM containing HEPES buffer, sodium bicarbonate, and L-glutamine supplemented with 10% tetracycline-free FBS, 600 μg/ml G-418, and 200 μg/ml hygromycin B. Cells were maintained at 37°C in a 5% CO2 atmosphere.

**GRPR gene sequencing**

We previously showed that GRP-R mRNA was aberrantly expressed and mutated in all human colon cancer cell lines studied to date (Carroll et al., 2000b). We therefore cloned the GRPR gene from 293 cells as previously described (Glover et al., 2003). Briefly, each of the GRPR gene’s 3 exons were amplified using gene-specific primers located in non-coding sequence ~50 nt away from the intron–exon boundary, or in the 5’- or 3’- untranslated regions, as appropriate. The 1st exon was amplified using the following primer set: forward, 5'-CTA GAG ATG GCT CTA AAT GAC TG-3' and reverse 5'-TCC CGG AAG CGA CTT-3'. The second exon was amplified using forward, 5'-ATT GTC CGG CCA ATG GAT ATC C-3' and reverse 5’-CCC TTC CAC GGG AAG ATT GTA A-3'. The third and final exon was amplified using forward, 5'-TCC CGG AAG AGA CTT GCC AAG-3' and reverse 5’-CCC CAC CTA CAC TCA GGA-3'. For all three primer sets, the following conditions were employed: hot start; 94°C for 5 min; followed by 40 cycles of 94°C for 30 sec, 60°C for 60 sec and 72°C for 60 sec; concluding with 72°C for 7 min and then held at 4°C thereafter.

**Binding studies**

In all instances, binding studies were performed by suspending 3 × 10^6 of disaggregated cells per milliliter in PBS containing [125I-Tyr4]bombesin for 30 min at 22°C.
Non-saturable binding of radiolabeled peptide was defined as the amount of radioactivity associated with cells incubated with 1-μM bombesin. Non-saturable binding was <10% of total binding in all experiments. Receptor number was determined by Scatchard analysis of the binding data using the least-squares regression program LIGAND (Muson and Robard, 1980).

**Immunohistochemistry**

Cells were seeded on uncoated, multi-well LabTek II slides (Fisher) in complete media for 24 h. For analysis of gastrin releasing peptide (GRP), cells were washed with Tris-buffered saline (TBS) and then serum starved for 24 h prior to immunohistochemistry. Cells were then fixed in 3.7% formaldehyde for 20 min at 37°C. Slides were processed using the Envision Plus system (DAKO). Briefly, fixing solution was removed and cells were washed with TBS supplemented with 0.1% Tween 20. Endogenous peroxidase activity was blocked using 0.03% hydrogen peroxide in TBS for 5 min at room temperature in the dark, followed by washing the cells and then incubating with primary antibody for 1 h at room temperature. Cells were probed with a polymer labeled anti-rabbit secondary antibody for 30 min, followed by DAB+ for 5 min. Slides were then counterstained with Gill’s hematoxylin for 1 min. The rabbit polyclonal antibody to GRP-R was synthesized as previously described (Carroll et al., 1999) and was used at a dilution of 1:1,000; whereas the commercially available rabbit polyclonal antibody to GRP (DAKO) was used at a concentration of 0.045 μg/ml.

**Western blot analysis**

Cells were grown to at least 60% confluence and then incubated in serum-free media for 24 h. To evaluate the effect of GRP-R agonists or antagonists, cells were treated with the indicated peptide for an additional 1 h. To induce FRNK, cells were exposed to 2 μg/ml doxycycline (Clonetech) for at least 18 h. Cell monolayers were then rinsed in phosphate-buffered saline (PBS) and lysed in ice-cold RIPA (50 mM HEPES, pH 7.4; 150 mM NaCl; 1% Triton X-10; 0.1% SDS; 0.5% sodium deoxycholate; 1 μM sodium orthovanade; 5 μM EDTA; 5 μM sodium fluoride) containing a 1:20 dilution of mammalian protease inhibitor cocktail (Sigma). Protein concentrations were determined using the BCA reagent (Fisher), with 15 μg of each extract electrophoresed per lane on a 10% polyacrylamide gel under denaturing and reducing conditions. The resolved proteins were electrophoretically transferred to PVDF membranes (Fisher). FAK (125 kDa) and FRNK (44 kDa) were detected using a rabbit polyclonal antibody directed against the c-terminus of human FAK at a concentration of 0.1 μg/ml. The major phosphorylation site of FAK, tyrosine 397 (Eide et al., 1995; Touitou et al., 2000), was detected using an antibody to human FAK397 at 0.35 μg/ml. Immunoreactive bands were visualized using a horseradish peroxidase conjugated goat anti-rabbit IgG and the ECL Plus detection system.

**Cell proliferation and size measurements**

Fifty thousand cells per well were plated in 24-well plates for 24 hr. Cells were then washed two times, and the number of cells remaining attached determined after removing using 500 μl/well Accutase (Innovative Cell Technologies, San Diego, CA) and counting in a Z2 counter (Beckman-Coulter, Miami, FL). This represented the index number of cells against which the effect of other agents was determined. Cells were then incubated in serum-free media in the presence or absence of 1-μM GRP, 1 μM of the antagonist bombesin methyl ester, or 2-μM doxycycline and counted at the indicated time points.

**Wounding, time lapse photomicroscopy, and morphometric analysis**

Cells were plated and cultured until >80% confluent. Cells were washed and then cultured in serum-free media for an additional 24 h. A sterile razor blade was used to create a linear gap across the cell monolayer, after which the cells were mounted in a custom-designed Leica inverted microscope that allowed the cells to be maintained at 37°C. Cell images were acquired every 5 min with a Hamamatsu 1.3 mega-pixel digital camera (Hamamatsu City, Japan) using Open Lab (Improvision, Coventry, UK). Images were converted into QuickTime files at native size (640 x 480) using the “Video” codec, edited using Final Cut Pro 3.0 (Apple Computer, Cupertino, CA), and exported for viewing using the “JPEG A” codec at reduced size (320 x 240) at 10 frames/sec. The resultant videos can be viewed at: http://www.uic.edu/com/dom/gastro/labvideos.

Determination of cell area was determined at each video frame using the Open Lab software by calculating the percent of the total area under visualization that was occupied by cells. Cell number in time-lapse experiments was determined by counting the total number of cells present in each video frame over the course of a 24-h experiment. Cell motility was determined by tracing the path of randomly selected cells on a frame-by-frame basis.

**Measurement of cell adhesion**

Cell adhesion was determined using a custom-designed rheoscope. The rheoscope is a cone-plate viscometer coupled to a microscope that provides a direct approach for accurately determining the shear stress required to detach cells from a glass coverslip or other matrix (Schmid-Schonbein et al., 1973; Tran-Son-Tay et al., 1987). Briefly, the fluid moving in the rheoscope has a velocity profile that is linear. At the bottom plate, the fluid has a velocity of zero, while at the cone there is maximum tangential velocity (i.e., the angular velocity multiplied by the radius). Because of this velocity profile, the shear stress at the bottom plate is equal at all points along radius r. Adjusting the speed of the motor changes the angular velocity of the cone allowing for the specific level of shear stress on the bottom of the plate to be acquired. As a result, the shear stress field remains nearly stationary and can be studied without the aid of high-speed cinematography. Shear stress ($T_w$, dynes/cm²) is calculated as:

$$T_w = (μΩr)/h_r$$

where $μ$ is the viscosity of fluid between the plates, $Ω$ is the angular velocity, $r$ is the radius and $h_r$ is the height of the gap between the cone and the plate.
Cells were plated on standard glass microscope slides to achieve a density of 5–10 cells/mm² in serum-free media alone or with 1 μM GRP, 1 μM of the antagonist d-Phe⁶(bombesin) methyl ester, or 2 μg/ml doxycycline for 24 h. Slides were then placed in the rheoscope and actions viewed through a microscope at 100×. Cells were exposed to the indicated shear force for 15 sec and the number of cells remaining attached determined and expressed as a function of those initially present.

Measurement of cell deformability

Cell deformability was assessed using a micropipette as previously described (Needham et al., 1991). Briefly, micropipettes of ~8 μm in diameter were formed from 1 mm/0.5 mm (outer diameter/inner diameter) capillary tubes using a micropipette puller (Tran-Son-Tay et al., 1991). Pipettes were pre-treated with FBS and connected to a manometer. Pipette pressure was applied using the displacement from two water-filled reservoirs, and cells aspirated at a constant pressure of 600 Pa. Experiments were recorded using an interference contrast video microscope, and deformability defined as whether cells could completely flow into the micropipette without pausing for more than 5 sec under constant negative pressure.

Statistical analysis

All evaluations were performed using StatView (Abacus Concepts, Berkeley, CA), with data reported as means ± standard error. Data were evaluated by unpaired Student’s t-test or by analysis of variance (ANOVA) as appropriate. Data shown in Figure 9 were evaluated using a Bernoulli (binomial) test, with significance assumed when z > 1.65.

RESULTS

293 cells secrete GRP and synthesize GRP-R

To determine if 293 cells expressed GRP and its receptor, cells were processed immunohistochemically using specific polyclonal antibodies directed against GRP (McDonald et al., 1979; Fraser et al., 1994) and its cognate receptor (Kroog et al., 1995; Carroll et al., 1999). Diffuse non-nuclear staining for both proteins was detected in 293 cells (Fig. 1A,B), with chromogen quantity unchanged as a function of confluence. To ascertain the number of receptors expressed by these cells, we performed quantitative immunohistochemistry as previously described (Matkowskyj et al., 2000, 2003a). By this technique, we determined that each cell expressed approximately 125,000 GRP-R/cell. To confirm this finding, and to gain insight into the pharmacological characteristics of the expressed receptor, we performed competitive displacement binding studies using [125I]-GRP as previously described (Benya et al., 1995). Increasing concentrations of GRP were effective in competing with [125I]-GRP such that half-maximal binding of radio-ligand was observed with ~1-nM GRP and no specific binding was observed with 1-μM GRP (Fig. 1C). Evaluating the data using a least-squares regression analysis according to the method of Scatchard (Muson and Robard, 1980) allowed us to determine that the data were best fit by a single binding site model with a Ki = 2.1 ± 0.4 nM. We next studied whether GRP/GRP-R expressed by 293 cells were functional by studying FAK phosphorylation in the presence and absence of a specific and selective receptor antagonist.

Autocrine stimulation of GRP-R expressed by 293 cells phosphorylates FAK at Y397

Multiple studies have shown that GRP activates focal adhesion kinase (FAK) (Zachary and Rozengurt, 1992; Sinnett-Smith et al., 1993; Leopoldt et al., 2000; Leyton et al., 2001). Furthermore, we have recently shown that FAK is expressed in murine (Carroll et al., 2000a) and human (Matkowskyj et al., 2003b) colon cancers co-expressing GRP/GRP-R. In human cancers, we specifically demonstrated that GRP/GRP-R co-expression was associated with increased total FAK, and of FAK phosphorylated at Y397 (Matkowskyj et al., 2003b). We, therefore, determined the amount of these two FAK forms that were present in 293 cells. Using specific antibodies against total cell proteins extracted from

Fig. 1. 293 cells express GRP and GRP-R. GRP (A) and GRP-R (B) were detected by immunohistochemistry using polyclonal antibodies as described in Materials and Methods. Chromogen specificity was determined in all instances by processing control cells identically except for the absence of primary antibody (insets, A,B). Pharmacological characterization of GRP-R expressed by 293 cells was determined by competitive inhibition of [125I]-GRP binding in the face of increasing concentrations of GRP (C). In all instances, 3 x 10⁶ cells/ml were incubated with 50 μM [125I]-GRP for 30 min at 22 °C and the indicated concentrations of GRP. Results are expressed as the means ± SE of at least three separate experiments, with each experiment having been performed in triplicate.
confuent control 293 cells, we determined by Western blot assay that FAK (Fig. 2A, top) and FAK phosphorylated at Y397 (Fig. 2A, bottom) were both prominently present under basal conditions. Treating 293 cells with 1 μM of the GRP-R antagonist D-Phe⁶(bombesin) methyl ester completely eliminated this enzyme from being phosphorylated at Y397 (Fig. 2A, bottom). In contrast, addition of exogenous GRP to achieve a final concentration of 1 μM did not alter the levels of Y397 phosphorylation (Fig. 2A, bottom), indicating that endogenous ligand secreted by 293 cells maximally activated FAK.

Ectopic expression of FRNK eliminates FAK phosphorylation at Y397

To modulate the amount of active FAK present, we created 293 cells expressing the dominant negative inhibitor, FRNK, under the control of a doxycycline-sensitive promoter as previously described (Kornberg and Fleigel, 2003) (Fig. 2B). In the absence of doxycycline, negligible promoter leak was evident (Fig. 2A, top). In contrast, addition of 2 μg/ml doxycycline for 18 h resulted in a brisk upregulation of FRNK in these cells, with a concomitant decreases in total FAK (Fig. 2A, top) and FAK phosphorylated at Y397 observed (Fig. 2A, bottom). To confirm that our manipulation of 293 cells to create the “293-FRNK” clone did not alter their ability to secrete GRP or express GRP-R, both were quantified by Q-IHC as described above. Briefly, 293-FRNK cells expressed similar quantities of GRP and GRP-R (data NS) as native, unmanipulated 293 cells. Hence, these modified 293-FRNK cells permit us to study the role of GRP-induced activation of FAK on cell behavior at both the level of the receptor by using a GRP-R antagonist and downstream by upregulating FRNK (Fig. 2B).

**GRP does not affect 293 cell proliferation**

GRP is widely perceived to act as a mitogen in cancer, despite significant evidence to the contrary (reviewed in Jensen et al., 2001). We, therefore, studied the effect of GRP on the proliferation of both 293 cells and 293-FRNK cells by evaluating them in the presence and absence of either the GRP-R specific antagonist D-Phe⁶(bombesin) methyl ester. To study whether FAK could possibly be involved in 293-FRNK cell proliferation, we also evaluated the effect of culturing these cells in the presence or absence of doxycycline. Both 293 cells and 293-FRNK cells (Fig. 3) survived and proliferated, albeit modestly, for as long as 4 days in the absence of serum. After 48 h in serum-free media, 293-FRNK cells increased in number by 1.1 ± 0.2-fold, and after 96 h 2.4 ± 0.5-fold (Fig. 3); with similar changes observed under the same conditions as for unmodified 293 cells. Addition of pharmacological concentrations of GRP increased overall proliferation by <15% at 96 h, a difference that was not statistically significant for either 293 cells (P = 0.25) or 293-FRNK cells (P = 0.30). Likewise, addition of D-Phe⁶(bombesin) methyl ester or doxycycline decreased proliferation similarly in both cell lines, but in a non-statistically significant manner (293 cells: P = 0.35 for antagonist and 0.30 for doxycycline as compared to control cells processed in parallel; 293-FRNK cells: P = 0.44 for either agent as compared to control cells processed in parallel). Overall, then, these data indicate that autocrine activation of GRP-R,
expressed by either 293 cells or 293-FRNK cells, does not cause increased cell proliferation.

**GRP regulates cell motility and size but not number during remodeling**

Assessing the rapidity by which confluent cell monolayers are restored post-wounding is an oft-used marker of tumor cell invasiveness (Saurin et al., 2002). Yet this assay only measures the ability of cells to replace those lost in wounding, and as such more accurately reflects their ability to contribute to tissue healing and remodeling.

Wounding of wild type 293 cells or 293-FRNK cells resulted in equally rapid wound closure (Fig. 4A, panel A vs. panel B). In contrast, concomitant administration of the antagonist D-Phe⁶(bombesin) methyl ester to inhibit GRP-R activation by endogenous ligand (Fig. 4C), or addition of doxycycline to cause FRNK to be upregulated (Fig. 4D), equally and completely inhibited wound closure in 293-FRNK cells. The actions of antagonist were identical in 293 cells, whereas doxycycline had no effect in this cell line (data NS). Wound closure in untreated wild type 293 cells or 293-FRNK cells was due to both rapid cell movement (Fig. 5A) into the gap and away from adjacent cells (see Video #1 at: http://www.uic.edu/com/dom/gastro/labvideos). This was associated with a near doubling in cell size (Fig. 6A) without a corresponding change in cell number (Fig. 6B). In contrast, incubating 293-FRNK cells with doxycycline to inhibit GRP-activated FAK dramatically attenuated cell motility (Fig. 5B and Video #2) and growth (Fig. 6A) while having no effect on cell proliferation (Fig. 6B). Identical effects on motility (data NS), growth (Fig. 6A), and number (Fig. 6B) were observed when these cells were cultured in the presence of the antagonist D-Phe⁶(bombesin) methyl ester as when incubated in the presence of doxycycline. Finally, antagonist had similar effects in 293 cells as on 293-FRNK cells in altering motility, growth, and proliferation (data NS). In contrast, doxycycline had no effect on these parameters in 293 cells (data NS). Hence, these findings indicate that GRP activation of its cognate receptor, resulting in FAK activation and phosphorylation, promotes cell motility and growth without affecting proliferation in the context of wound healing and tissue remodeling.

**GRP enhances the avidity of 293 cell attachment to the extracellular matrix**

Cell detachment is traditionally assayed by measuring sensitivity to enzymatic digestion (i.e., trypsin) or by studying the inverse process of cell attachment. Yet digestion assays do not mimic that which occurs in vivo; while attachment assays erroneously assume that attachment and detachment are biologically equivalent processes. To measure the propensity of cultured cells to detach from the extracellular matrix under physiological conditions, we designed a rheoscope (Fig. 7A). As described in Materials and Methods, this cone-plate viscometer allows for cells under microscopic visualization to be subject to stable, known shear forces similar to that which would be found in a venule or a lymphatic vessel (Byers et al., 1995; Li et al., 2001).

Wild type 293 cells and 293-FRNK cells attached with equal avidity to the extracellular matrix. When exposed to a shear force of 0.055 dynes/cm² for 15 sec, ~60–75% of cells remained attached when exposed to a force of 1 dyne/cm². In contrast, co-culture with the antagonist D-Phe⁶(bombesin) methyl ester to block endogenous ligand action equally and markedly reduced cell attachment by ~50% for both wild type 293 cells and 293-FRNK cells at all shear forces considered. Although doxycycline had no effect on the ability of wild type 293 cells to attach (Fig. 7B, left graph), this compound markedly decreased the avidity of 293-FRNK cells to remain attached to the matrix when exposed to increasing shear force (Fig. 7B, right graph). Indeed, the effect of doxycycline on decreasing 293-FRNK cell adhesiveness to the matrix was indistinguishable from that caused by GRP-R antagonist (Fig. 7B, right graph).

We did appreciate a ~15% difference in attachment under control conditions between 293 cells and 293-FRNK cells (Fig. 7B). This difference was constant.
across all shear forces considered, and likely reflects
the low-level promoter leak (Fig. 2A). However, the
effects of doxycycline and GRP-R antagonist were
identical in 293-FRNK cells, indicating nonetheless
that autocrine stimulation of GRP-R in 293 cells causing
FAK to be phosphorylated at Y397 has no effect on cell
proliferation while regulating cell size, motility, and
attachment.

Fig. 4. Response of “293-FRNK” cells to wounding. Cells were cultured in standard media until they
were 90% confluent followed by washing and then culturing for another 24 h in serum-free media in the
absence (A,B) or presence (C,D) of doxycycline. Cells were washed again followed by wounding using a
sterile razor blade (A,C, with wound edge indicated by the dashed line). In the absence of doxycycline cells
rapidly migrated to close the wound (B), whereas this was completely attenuated in the continued
presence of doxycycline (D). Movies showing this motility can be viewed at http://www.uic.edu/com/dom/
gastro/labvideos (Video 1: absence of doxycycline; Video 2: presence of doxycycline).

Fig. 5. Hourly position of randomly selected 293-FRNK cells in serum-free media (A) and in the presence
of doxycycline (B). Cells were wounded as described in the legend to Figure 4, images captured digitally,
and the position of representative cells identified each hour using the OpenLab software (Improvision,
Coventry, UK).
GRP decreases cell deformability

For cells to metastasize they must be able to transmigrate across tight spaces such as exist between endothelial cells. Cell deformability is thus an accepted viscoelastic marker of greater malignancy (Ward et al., 1991; Dong et al., 1994). To measure this, we aspirated wild type 293 cells and 293-FRNK cells into micropipettes designed so that the internal diameter was ~50% of the size of the cell being evaluated (Fig. 8). Control 293 and 293-FRNK cells aspirated with difficulty into the micropipette (Fig. 8A; and Video #3). In contrast, exposure of 293 cells to the GRP-R antagonist D-Phe6(bombesin) methyl ester (Fig. 8B) or 293-FRNK cells to this same agent or doxycycline (Fig. 8C; and video #4) markedly increased the ability for these cells to be aspirated completely.

Under basal conditions, ~20% of 293 cells completely entered the micropipette, and this was not significantly different from that observed in the presence of doxycycline (Fig. 9; \( z = 1.6 \)). Pre-incubation with the antagonist D-Phe6(bombesin) methyl ester significantly increased 293 cell deformability such that ~70% could achieve micropipette entry (\( z = 5.14 \)). Approximately 15% of 293-FRNK cells entered the micropipette under basal conditions, a value that was not significantly different from that observed for 293 cells (\( z = 0 \)). In contrast, doxycycline and D-Phe6(bombesin) methyl ester equally increased 293-FRNK cell deformability (Fig. 9), with the effect of antagonist being indistinguishable from that observed in unmodified 293 cells (\( z = 0.7 \)). Overall, then, these findings indicate that GRP-induced activation of FAK hinders 293 cell deformability, consistent with its actions as a morphogen and serving to inhibit metastasis.

Fig. 7. Effect of increasing shear force on cell attachment. A: Cross-sectional diagram of the rheoscope designed to generate a stable pan-directional shear force. As described in Materials and Methods, the rheoscope is a cone-plate viscometer that permits a stable, defined shear force perpendicular to the axis of rotation of a plexiglass cone. Cell attachment is determined by counting those remaining after exposure to the indicated shear force for 15 sec. B: Effect of increasing shear force on 293 cells under basal conditions (○), after exposure to doxycycline (■), or the antagonist D-Phe6(bombesin) methyl ester (▲). C: Effect of increasing shear force on 293-FRNK cells under basal conditions (○), after exposure to doxycycline (■), or the antagonist D-Phe6(bombesin) methyl ester (▲). In all instances, results are expressed as the means ± SE of at least three separate experiments, with each experiment having been performed in triplicate. Error bars cannot be visualized due to their relatively small size.
DISCUSSION

Morphogens are developmentally expressed proteins responsible for promoting tissue differentiation during organogenesis (1997). In cancer, morphogens are proteins involved in regulating tumor cell appearance, or differentiation, that act by recapitulating—albeit dys-functionally—their normal role in organogenesis. In other words, since "oncogeny is blocked ontogeny" (Potter, 1969), the ability to modulate tumor cell remodelling represents a property fundamental to morphogens in malignancy.

By promoting the assumption of a better-differentiated phenotype, morphogens in cancer also inhibit the development of metastases. The basis for this statement derives from the fact that for homogeneously differentiated cancers, patients with better-differentiated tumors survive longer (Birchmeier et al., 1996; Villavicencio et al., 1997; Kowalski and Medina, 1998; Ng, 1998; Nicolson and Moustafa, 1998; Park et al., 2000; Rampaul et al., 2001). This statement remains true even for heterogeneously differentiated colon cancers, providing that the differentiation status of specific cell populations closest to the tumor margin is considered.

Fig. 8. Cells in micropipette after exposure to constant negative pressure of 600 Pa. A: Wild type 293 cells, (B) doxycycline-treated 293-FRNK cells, and (C) wild type 293 cells treated with D-Phe^6(bombesin) methyl ester. White bar in each panel identifies the extent to which the cell has been aspirated into the micropipette. Images acquired at 400×.

Fig. 9. Percentage of total number of 293 cells (□) and 293-FRNK cells (■) that were evaluated completely entering the micropipette (internal diameter 8 μm). Cells were evaluated under basal conditions, or after pre-exposure to 1 μM doxycycline (Dox; □□□) or 1 μM D-Phe^6(bombesin) methyl ester (methyl ester; □□□). The data were evaluated using the Bernoulli's test, with significance assumed for $z > 1.65$. The $z$ values for the comparisons indicated above are (a) 0, (b) 2.5, (c) 0.7, and (d) 1.6.
(Wood et al., 1981; Hase et al., 1993). For a tumor cell to metastasize, it must be capable of: (1) detaching from its original environment; and (2) deforming so that it can transmigrate through tight spaces as it negotiates the vascular or lymphatic compartments. Thus we propose that morphogens in cancer possess three critical biophysical properties including promoting tumor cell remodeling as local circumstances require, restraining tumor cell detachment, and inhibiting tumor cell deformation.

We have proposed that GRP and its receptor act as morphogens when aberrantly expressed in colon cancer (reviewed in, Jensen et al., 2001). Both proteins are transiently expressed during, and contribute to, normal gut development in humans and mice (Carroll et al., 2002). After this period of natural expression during organogenesis, GRP/GRP-R are no longer present unless aberrantly upregulated post malignant transformation whereupon they drive the assumption of a better differentiated phenotype (Carroll et al., 1999; Carroll et al., 2000a). Our study of mice genetically incapable of GRP-R expression suggested, but did not prove, that this protein’s morphogenic properties were mediated via its ability to activate focal adhesion kinase (Carroll et al., 2000a); while our study of resected human colon cancers raised the possibility that GRP’s morphogenic effects might occur by promoting FAK phosphorylation at tyrosine 397 (Matkowskyj et al., 2003b).

To determine whether GRP/GRP-R’s morphogenic properties could be mediated via FAK phosphorylation, we herein studied 293 cells, a non-malignant epithelial cell line that we show natively expresses these proteins. By manipulating this cell line to inducibly express FRNK, the dominant negative regulator of FAK (Richardson and Parsons, 1996), we show for the first time that GRP binding to its cognate receptor drives FAK phosphorylation at the tyrosine located at amino acid position 397. Furthermore, we show that in this non-malignant cell system GRP is not a mitogen, but rather is a morphogen that via FAK activation promotes cellular remodeling, restrains cell detachment from the underlying substratum, and inhibits deformation.

We used this cell line for these studies because 293 cells natively express both GRP and its receptor, and because it is derived from non-cancerous cells. With respect to the first criterion, the GRP-R is similar to other heptaspanning receptors such as those for tachykinins (Gether et al., 1992) insofar as its pharmacology can be altered when ectopically expressed. For example, we have shown that stably transfecting the non-malignant human colon epithelial cell line NCM460 with the cDNA for the human GRP-R results in a constitutively activated receptor (Ferris et al., 1997). In contrast, GRP-R ectopically expressed by Balb 3T3 cells fail to activate the same intracellular pathways when natively expressed by the highly homologous Swiss 3T3 cell line (Benya et al., 1994). With respect to the second criterion, using human colon cancer cell lines is limited by the fact that GRP-R mRNA is ubiquitously mutated such that receptors are either non-functional or have altered pharmacology (Carroll et al., 2000b). Hence, 293 cells are invaluable since as they natively express non-mutated GRP-R that are pharmacologically identical to that which has been previously described (Benya et al., 1995).

We then modified these cells so as to inducibly express FRNK, the dominant negative inhibitor of FAK (Richardson and Parsons, 1996). Native and modified cells bound agonist similarly, and in so doing indistinguishably caused FAK to be phosphorylated at Y397. The amount of unstimulated promoter leak in these modified 293-FRNK cells was minimal, the effect of which appeared to be limited to altering basal attachment to plastic matrix (Fig. 7). In contrast, there were no differences between 293 cells and 293-FRNK cells in terms of proliferation rates, motility, or deformability under basal conditions. Likewise, addition of the GRP-R antagonist β-Phe₃(bombesin) methyl ester equally affected the behavior of 293 cells and 293-FRNK cells; while addition of doxycycline to upregulate FRNK had no effect on 293 cells but impacted 293-FRNK cells similarly as when exposed to antagonist. Overall, these findings show for the first time that GRP activation of its receptor causing FAK phosphorylation regulates key morphogenic properties including promoting cell remodeling, inhibiting cell detachment, and antagonizing cell deformability.

These observations may seem surprising. FAK has traditionally been viewed as a contributor to tumor invasion and metastasis, and by extension, to greater malignancy (Sieg et al., 2000; Hauck et al., 2002). Yet the literature evaluating FAK expression in resected whole human colon cancers is both limited and conflicting. An immunohistochemical study indicated that FAK is present in greater amounts in invasive as compared to non-invasive cancers (Owens et al., 1995). But this observation only indicates the presence of this enzyme in tumors that have breached the muscularis propria, and thus includes all cancers staged Dukes B or greater. Conflictingly, some have reported the presence of FAK in colon cancer metastases (Han et al., 1997), while others have specifically failed to find this enzyme in sites distant from the primary lesion (Ayaki et al., 2001).

As an enzyme, FAK activity is markedly enhanced by tyrosine autophosphorylation, particularly at amino acid position 397 (Schaller et al., 1994). Unfortunately studies of this enzyme in cancer have focused only on the presence or absence of total FAK, ignoring both its phosphorylation status as well as its specific location within a colon cancer. To address this, we recently evaluated FAK expression and phosphorylation in resected human colon cancers as a function of tumor cell differentiation (Matkowskyj et al., 2003b). We found that total FAK, and FAK phosphorylated at Y397, strongly correlated with GRP/GRP-R co-expression; with all entities co-localizing to better differentiated tumor cells within any particular colon cancer (Matkowskyj et al., 2003b). In combination with our observation that FAK expression in murine colon cancer is a function of tumor cell differentiation, and that this enzyme is not present in the poorly differentiated tumors arising in GRPR mice (Carroll et al., 2000a), these findings suggest a role for GRP-mediated FAK activation in regulating tumor cell appearance in colon cancer.

In so doing, FAK in cancer may well be recapitulating a role in regulating normal development, a role only recently appreciated. Embryos in FAK-deficient mice cannot progress beyond late gastrulation, a phenomenon that appears to be related to this enzyme's
regulation of cell migration and adhesion (reviewed in, Ridyard and Sanders, 1999). An immunohistochemical study performed on human abortuses identified transient FAK expression in many epithelia including the lung and kidney (Tani et al., 1996). Although, this study did not find evidence of FAK expression in fetal GI tissues, no information was presented as to the ages of the abortuses. We have previously found GRP/GRP-R co-expression in human fetal tissues, but only in those aged 14–20 weeks of development (Carroll et al., 2002). Thus it remains possible that FAK is normally expressed during development of the GI tract. If not, however, its role in other developmental processes nonetheless suggests that this enzyme may be acting as a morphogen when upregulated in colon cancer.

In summary, this study describes a number of novel biophysical assays for assessing the propensity of a cell to metastasize. We demonstrate that GRP activation of its receptor causes FAK to be phosphorylated at tyrosine 397, and use these assays to show that this results in enhanced cell motility during remodeling, enhanced cell attachment to the extracellular matrix, and attenuation of a cell’s ability to undergo mechanical deformation. These findings are consistent with GRP/GRP-R expression in colon cancer promoting tumor cell differentiation via FAK, and retard metastasis.

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LITERATURE CITED


