Transient upregulation of GRP and its receptor critically regulate colon cancer cell motility during remodeling

Sarah Glover, Rajkumar Nathaniel, Lubna Shakir, Cecile Perrault, Rebecca K. Anderson, Roger Tran-Son-Tay, and Richard V. Benya

Department of Medicine, University of Illinois at Chicago and Chicago Veterans Administration Medical Center (West Side Division), Chicago, Illinois; Department of Mechanical and Aerospace Engineering, University of Florida, Gainesville, Florida; Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, Illinois

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Glover, Sarah, Rajkumar Nathaniel, Lubna Shakir, Cecile Perrault, Rebecca K. Anderson, Roger Tran-Son-Tay, and Richard V. Benya. Transient upregulation of GRP and its receptor critically regulate colon cancer cell motility during remodeling. Am J Physiol Gastrointest Liver Physiol 288: G1274–G1282, 2005; doi:10.1152/ajpgi.00108.2004.—Gastrin-releasing peptide (GRP) is typically viewed as a growth factor in cancer. However, we have suggested that in colon cancer, GRP acts primarily as a morphogen when it and its receptor (GRP-R) are aberrantly upregulated. As such, GRP/GRP-R act(s) primarily to modulate processes contributing to the assumption or maintenance of tumor differentiation. One of the most important such processes is the ability of tumor cells to achieve directed motility in the context of tissue remodeling. Yet the cellular conditions affecting GRP/GRP-R expression, and the biochemical pathways involved in mediating its morphogenic properties, remain to be established. To study this, we evaluated the human colon cancer cell lines Caco-2 and HT-29 cells. We found that confluent cells do not express GRP/GRP-R. In contrast, disaggregation and plating at subconfluent densities results in rapid GRP/GRP-R upregulation followed by their progressive decrease as confluence is achieved. GRP/GRP-R coexpression correlated with that of focal adhesion kinase (FAK) phosphorylation of Tyr397, Tyr407, Tyr861, and Tyr925 but not Tyr576 or Tyr577. To more specifically evaluate the kinetics of GRP/GRP-R upregulation, we wounded confluent cell monolayers. At t = 0 h, GRP/GRP-R were not expressed, yet cells immediately began migrating into the gap created by the wound. GRP/GRP-R were first detected at ~2 h, and maximal levels were observed at ~6 h postwounding. The GRP-specific antagonist [n-Phe⁶]-labeled bombesin methyl ester had no effect on cell motility before GRP/GRP-R expression. In contrast, this agent increasingly attenuated cell motility with increasing GRP-R expression such that from t = 6 h onward no further cell migration into the gap was observed. Overall, these findings indicate the existence of GRP-independent and -dependent phases of tumor cell remodeling with the latter mediating colon cancer cell motility during remodeling via FAK.

bombesin; morphogen; motogen

GASTRIN-RELEASING PEPTIDE (GRP) and its receptor (GRP-R) are transiently expressed during gut development where they are involved in regulating villous development in humans and mice (2). After this period of normal expression, epithelial cells lining the adult colon no longer express GRP/GRP-R unless aberrantly upregulated postmalignant transformation. Although commonly considered to be mitogens, we have shown that in colon cancer GRP and its receptor recapitulate their normal developmental role by promoting the tumor’s assumption of a better-differentiated phenotype (reviewed in Ref. 11). As such, GRP/GRP-R act as morphogens, proteins that ultimately serve to improve patient survival by retarding the development of metastases. Yet the context in which GRP/GRP-R are upregulated, along with the biochemical mechanism by which they mediate their morphogenic properties, remains to be determined.

A potential mechanism of action was suggested by our study of colon cancer formation in wild-type mice, and mice genetically incapable of synthesizing GRP-R (i.e., GRP-R−/− mice) (3). Whereas tumor differentiation in wild-type mice correlated with the extent to which GRP/GRP-R was coexpressed, tumors in GRP-R−/− mice progressively de-differentiated. In wild-type tumors, GRP/GRP-R coexpression correlated with the presence of focal adhesion kinase (FAK), whereas this enzyme was barely detectable in GRP-R−/− mice (3). In human colon cancers, the magnitude of GRP/GRP-R coexpression correlates with tumor cell differentiation as well as with the amount of total FAK and FAK phosphorylated at Tyr397 and Tyr407 (13). Most recently, we showed in HEK-293 cells (a human fetal renal epithelial cell line) that FAK phosphorylation at Tyr397 critically mediated GRP’s ability to modulate biophysical properties required for cells to metastasize (8). However, the link between GRP and FAK in human colon cancer, and their role in altering tumor cell behavior, is yet to be determined.

In this paper, we study human colon cancer cell lines Caco-2 and HT-29. We herein demonstrate that both cell lines transiently secrete GRP and express GRP-R as a function of tumor cell confluence. When present, GRP autocrine activation of its receptor drives FAK phosphorylation at Tyr397, Tyr407, Tyr461, and Tyr925. Phosphorylation of FAK at these sites dramatically regulates cell motility and size, parameters consistent with GRP’s action as a morphogen, while having little to no effect on rates of cell proliferation or apoptosis. Importantly, GRP/GRP-R expression is eliminated once Caco-2 or HT-29 cells achieve confluence, resulting in decreased tumor cell motility. Overall, these findings are consistent with GRP acting as a morphogen in colon cancer and indicate that this occurs via FAK phosphorylation.

MATERIALS AND METHODS

Materials. All cell culture reagents were obtained from Fisher (Pittsburgh, PA) except Opti-MEM, which was from Invitrogen (Carlsbad, CA). Caco-2 and HT-29 cells were obtained from Amer-
ian Type Culture Collection (Nos. HTB 37 and HTB 38; Manassas, VA). Cells were maintained in dishware from Nunc (Rochester, NY). All immunohistochemical supplies including a polyclonal antibody to GRP were from DakoCytomation (Carpentaria, CA) except for antibodies to the NH2 terminus of FAK (Upstate Biotechnology, Lake Placid, NY) or antibodies recognizing Ki-67, a marker of cell proliferation, and FAK phosphorylated at Tyr907, Tyr1067, Tyr576, Tyr577, Tyr861, and Tyr925 (Santa Cruz Biotechnology, Santa Cruz, CA). The rabbit polyclonal antibody to GRP-R was synthesized as previously described (10). Western blot analysis was performed by using peroxidase-conjugated goat anti-rabbit IgG from Santa Cruz Biotechnology and the enhanced chemiluminescence (ECL) Plus detection system from Amersham Biosciences (Piscataway, NJ). [d-Phe6]-labeled bombesin methyl ester was kindly provided by Dr. David Hoy (Tulane University, New Orleans, LA). Bombesin was purchased from Bachem (San Carlos, CA). All other supplies were molecular biology grade and were from Sigma (St. Louis, MO).

Cell culture. Caco-2 and HT-29 cells were cultured in DMEM/F-12 containing glucose and L-glutamine supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a 5% CO2 atmosphere in all instances.

Immunohistochemistry. Cells were seeded on uncoated, multiwell Permanox (Nunc) slides in complete medium. To achieve confluence by day 4 postplating, 400,000 cells were plated per 4.5 cm2 well of a two-well slide. To evaluate for GRP and GRP-R expression, cells were washed with PBS and then serum starved for 24 h before immunohistochemistry. Cells were then fixed in 3.7% formaldehyde in PBS for 30 min at 37°C. Slides were processed by using the Envision Plus system (DakoCytomation). Briefly, fixing solution was removed, and cells were washed with Tris-buffered saline supplemented with 0.05% Tween 20. Endogenous peroxidase activity was blocked by using 0.3% hydrogen peroxide for 5 min at room temperature followed by washing the cells and then incubating with primary antibody for 1 h at room temperature. (For detection of Ki-67, cells were incubated overnight in a humidity chamber at 4°C). Cells were probed with a polymer-labeled anti-rabbit secondary antibody for 30 min, followed by dianaminobenzidine plus for 3 min. Slides were then counterstained with Gill’s hematoxylin for 2 min. The rabbit polyclonal antibody to GRP-R was synthesized as previously described (1) and was used at a dilution of 1:1,000, whereas the commercially available rabbit polyclonal antibody to GRP (DakoCytomation) and rabbit polyclonal antibody to Ki-67 (Santa Cruz Biotechnology) were used at concentrations of 45 and 1 µg/ml, respectively.

Apoptosis assay. To determine the number of apoptotic cells around the region of a wound, cell monolayers were fixed at the indicated time points with 3.7% formaldehyde in PBS for 30 min at 37°C and then stained with 300 nM 4,6-diamidino-2-phenylindol (DAPI; Molecular Probes, Eugene, OR) in PBS for 5 min in a light-free environment. Coverslips were attached by using Pristine Permanox (Nunc) slides in complete medium. Cells were then cultured for an additional 18 h in serum-free medium. At that point sufficient [d-Phe6]-bombesin methyl ester to achieve a final concentration of 1 µM was added, and cells were cultured for an additional 18 h, at which point total cell lysates were obtained, and Western blot analysis was performed as described above.

Wounding, time lapse microscopy, and morphometric analysis. Cells were plated and cultured until 2 days postconfluent. Cells were washed and then cultured in serum-free medium for an additional 18 h. A sterile scalpel blade was used to create a linear gap across the cell monolayer after which the cells were mounted in a custom-designed Leica inverted tissue culture microscope that allowed cells to be maintained at a constant temperature of 37°C. Cell images were acquired every 5 min for 20 h in the absence of any exogenous peptide or in the presence of either 1 µM bombesin or 1 µM [d-Phe6]-bombesin methyl ester using a Hamamatsu 1.3 mega pixel digital camera (Hamamatsu City, Japan). All evaluations of recorded cell behavior were performed by using Open Lab (Improvision, Coventry, UK).

Cell motility was defined as the percentage of the original wound size or gap remaining over time. Three separate values were obtained for each image, and each experiment was repeated a minimum of 3 separate times. Images were converted into QuickTime files at native size (640 x 480) using the “Video” codec, edited by using Final Cut Pro 4.0 (Apple), and exported for viewing using the “JPEG A” codec at reduced size (320 x 240) at 10 frames/s. The final videos can

Western blot analysis. Cells were counted by using a Z2 Coulter counter (Beckman-Coulter, Miami, FL) so that 1.5 x 106 cells were plated in a 25 cm2 flask in complete medium. Cells were then harvested at the indicated time points or stages of confluence. Protein concentrations were determined by using the bicinchoninic acid 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 polyvinylidene difluoride membranes (Fisher). Immunoreactive bands were visualized by using a horseradish peroxidase-conjugated goat anti-rabbit IgG and the ECL Plus detection system.

For analysis of GRP-R, cell monolayers were rinsed with PBS and then lysed with ice-cold Tris-EDTA buffer supplemented with 1:20 mammalian protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 15,000 g, and the supernatant was subsequently analyzed by Western blot. GRP-R (molecular mass of the glycosylated form: ~80 kDa) were detected by using rabbit polyclonal antibodies at a concentration of 1:1,000.

For FAK, cell monolayers were rinsed in PBS and lysed in ice-cold RIPA buffer (in mM: 50 HEPES, pH 7.4, 150 NaCl, 1 sodium orthovanadate, 5 EDTA, and 5 sodium fluoride, plus 1% Nonidet P-40, 0.5% sodium deoxycholate) containing a 1:20 dilution of mammalian protease inhibitor cocktail (Sigma). Freezing the samples in a dry ice/100% ethanol bath and then thawing them in water kept at room temperature served to remove the nuclear material. After five cycles of freeze-thaw, lysates were centrifuged at 15,000 g for 30 min at 4°C. In all instances ~120 µg protein was loaded into a preparative well of a 10% polyacrylamide and run under denaturing and reducing conditions. The presence of FAK phosphorylated at specific tyrosine residues was determined by using antibodies at the following concentrations (in µg/ml): 1 Tyr907, 0.8 Tyr407, 0.4 Tyr576, 0.4 Tyr577, 0.4 Tyr861, 0.4 Tyr925, whereas total NH2-terminal FAK present was detected by using specific antibody at a concentration of 0.1 µg/ml.

To equilibrate loading and to permit relative phosphotyrosine assessments, each blot was probed by using a multiscreen apparatus (Bio-Rad, Hercules, CA), a device that allows for identical amounts of total protein in a single gel to be simultaneously evaluated by using multiple different antibodies.

In experiments performed by using the GRP-R antagonist [d-Phe6]-bombesin methyl ester, cells were initially plated in total medium containing serum for 24 h. Thereafter cells were washed and cultured for an additional 18 h in serum-free medium. At that point sufficient [d-Phe6]-bombesin methyl ester to achieve a final concentration of 1 µM was added, and cells were cultured for an additional 18 h, at which point total cell lysates were obtained, and Western blot analysis was performed as described above.
be accessed at the online supplement (http://ajpgi.physiology.org/cgi/content/full/00108.2004/DC1).

Statistical analysis. All evaluations were performed by using Excel (Microsoft, Redmond, WA), with data reported as means ± SE. Data were evaluated by unpaired t-test or by ANOVA, as appropriate, using built-in statistical functions provided by Excel.

RESULTS

Caco-2 and HT-29 cells express GRP and GRP-R as a function of confluence. We have previously shown (1) that GRP/GRP-R is transiently expressed postneoplastic transformation such that it is highly expressed in well-differentiated colon cancer cells and absent in poorly differentiated colon cancer cells and colon cancer metastases. Caco-2 and HT-29 cells were used in this study because they are well-accepted models that do not detach and die postconfluence, particularly in studies relating to differentiation (17). Cells were plated at subconfluent densities and every 24 h thereafter were evaluated immunohistochemically using primary antibodies to GRP or GRP-R. To allow for cell attachment, cells were allowed 24 h postplating before being studied. Whereas preconfluent cells expressed both GRP and GRP-R at the earliest time point evaluated (Fig. 1, B and D), confluent cells did not (Fig. 1, A and C). Indeed, expression levels of GRP/GRP-R progressively decreased with increasing confluence of both Caco-2 cells (Fig. 2) and HT-29 cells (data not shown).

GRP/GRP-R expression correlates with FAK phosphorylated at specific tyrosines. Because GRP’s morphogenic properties may be mediated via FAK (3, 8, 12), we were curious to know whether GRP/GRP-R expression correlated with this enzyme’s phosphorylation at specific sites. To do this, we studied preconfluent Caco-2 and HT-29 cells 24 h after passage and confluent cells ~5 days after passage by Western blot analysis using antibodies that recognize FAK phosphorylated at specific tyrosines. Both preconfluent and confluent cells were exposed to 1 μM [D-Phe⁶]-bombesin methyl ester, a GRP-R-specific antagonist (4, 5). In this fashion, we could determine which tyrosine residues were specifically phosphorylated in response to GRP-R autocrine activation. In preconfluent Caco-2 cells (Fig. 3A) and HT-29 cells (data not shown), the amount of FAK phosphorylated at Tyr²⁹⁷, Tyr⁴⁰⁷, Tyr⁸⁶¹, and Tyr⁹²⁵ was significantly decreased in the presence of 1 μM [D-Phe⁶]-bombesin methyl ester for 24 h (Fig. 3A). In contrast, antagonist did not alter the amount of FAK phosphorylated at Tyr⁵⁷⁶ or Tyr⁵⁷⁷ in either Caco-2 (Fig. 3) or HT-29 (data not shown) cells. The specificity of GRP-induced FAK phosphorylation was confirmed by studying the phosphorylation profile in postconfluent Caco-2 (Fig. 3B) and HT-29 (data not shown) cells. For all phosphotyrosines studied, [D-Phe⁶]-bombesin methyl ester had no effect on the amount of FAK phosphorylation.
lated at any tyrosine, consistent with postconfluent cells not expressing GRP/GRP-R (Fig. 1).

To confirm that the amount of phospho-FAK related to GRP-R amount, as well as to determine whether these changes were associated with alterations in the amount of total FAK present, we studied Caco-2 cells every 24 h after plating at subconfluent density. We restricted our study of phospho-FAK to that phosphorylation at Tyr397, because this particular residue showed the greatest amount of change when preconfluent cells were exposed to the antagonist [d-Phe6]-bombesin methyl ester (Fig. 3A). When Caco-2 cells were harvested every 24 h, Western blot analysis showed a progressive decrease in the amount of GRP-R expressed (Fig. 4) with kinetics similar to what we observed immunohistochemically (Fig. 1). This decrease was associated with an identical decrease in the amount of FAK phosphorylated at Tyr397 without any significant change observed in the amount of total FAK present (Fig. 4). A similar decrease in GRP-R and FAK phosphorylation at Tyr397, without change in total FAK, was seen in HT-29 cells as a function of increasing confluence (data not shown).

**Kinetics of GRP-R upregulation.** After passage, cells require ∼24 h before developing sufficient adhesiveness to permit their in situ study by immunohistochemistry. Thus to better evaluate the kinetics of GRP/GRP-R expression, as well as to evaluate the contribution of these proteins to modulating cell behavior, we studied Caco-2 cells postwounding with a sterile scalpel blade. In these experiments, cells were cultured to confluence, were washed, and were exposed only to medium without serum or other supplements for 24 h before wounding.

As shown earlier (Fig. 1), confluent cells did not show immunohistochemical evidence of GRP or GRP-R expression immediately (Fig. 5A) or 1 h postwounding (Fig. 5B). However, 6 h after wounding, GRP/GRP-R were maximally expressed (Fig. 5C). Expression of these proteins was not uniform or homogenous in nature; rather, individual cells, including many away from the wound edge, showed evidence of GRP/GRP-R expression, whereas others did not. Yet by 8 h postwounding, GRP/GRP-R expression decreased while remaining heterogeneous in nature (Fig. 5D). Similar kinetics of expression were seen in HT-29 cells such that at 1 h postwounding no GRP/GRP-R expression could be detected (Fig. 5E), whereas at 6 h postwounding expression was maximal (Fig. 5F).

Because each immunohistochemical experiment used an entire set of cells postwounding, it was not possible to track GRP/GRP-R expression in any individual cell. Thus to quantify the kinetics of GRP-R expression, we used our technique of quantitative immunohistochemistry (14) to calculate the amount of relevant chromogen present in only immunohistochemically positive cells. Because GRP and GRP-R are invariably coexpressed, we limited our evaluation to the amount to receptor seen. In Caco-2 cells (Fig. 6) and HT-29 cells (data not shown) GRP-R expression peaked at \( t = 6 \) h, followed by an equally rapid downregulation such that by 18 h postwounding, the point in time at which the gap has been completely filled in by migrating cells, GRP-R was no longer detectable.

**GRP-independent and -dependent effects on Caco-2 cell motility.** We previously showed that GRP-induced phosphorylation of FAK regulates the motility of the human fetal renal epithelial cell line HEK-293 (8). Thus we were interested to know whether transient GRP/GRP-R expression similarly reg-
ulated the motility of colon cancer cells. Caco-2 cells were cultured to confluence, washed, placed in serum-free medium for 24 h, and then wounded by using a sterile scalpel. Cells were then tracked by time-lapse photography in the presence (Fig. 7, A–C) or absence (Fig. 7, D–F) of the GRP-R-specific antagonist [D-Phe⁶]-bombesin methyl ester (the entire videos can be accessed at http://ajpgi.physiology.org/cgi/content/full/00108.2004/DC1). The degree to which migrating Caco-2 cells engaged in remodeling was quantified as the percentage of the original gap between the cells induced by wounding that remained as a function of time (Fig. 8).

Immediately postwounding, Caco-2 cells rapidly migrated into the gap before GRP/GRP-R upregulation and without being affected by exposure to the antagonist [D-Phe⁶]-bombesin methyl ester (Fig. 8). However, with increasing GRP-R expression, Caco-2 cells exposed to antagonist became progressively more restricted in their ability to migrate into the gap such that by ~6 h postwounding, the point of maximal GRP-R expression, Caco-2 cells were completely immotile (Fig. 7, B and C and Fig. 8). In contrast, adding pharmacological concentrations of exogenous bombesin (i.e., 1 µM) accelerated wound repair by ~50%. As early as 2 h after bombesin addition, the effects of this agonist could be demonstrated (P = 0.04 for control vs. bombesin-treated cells at 2 h, unpaired t-test). Thus these findings identify the existence of GRP-dependent and -independent phases of colon cancer cell motility in the context of tumor remodeling.

Effect of GRP/GRP-R expression of cell proliferation and apoptosis. Because GRP is commonly perceived to act as a mitogen in cancer (and despite significant evidence to the
contrary as reviewed in Ref. 11), we studied cell proliferation and apoptosis postwounding (Fig. 9). Using the same conditions for wounding confluent cells as described above, we then stained them using an antibody for Ki-67, a marker of cell proliferation, or using DAPI to identify cells with condensed chromatin and/or DNA fragmentation, markers of cell apoptosis. Both proliferating and apoptotic cells increased, with maximal levels observed at $t = 6$ h, the same time point at which GRP/GRP-R levels peaked (Fig. 6). At $t = 6$ h, there were 2.2-fold more cells than were present at $t = 2$ h ($P = 0.287$, unpaired $t$-test; $P = 0.286$, ANOVA). This increase, however, was nearly identically balanced by a similar increase in apoptotic cells, which increased by 1.8-fold over the same time period ($P = 0.025$, unpaired $t$-test; $P = 0.023$, ANOVA). Thus, although these data are consistent with GRP acting as a weak mitogen, this effect was not statistically significant and was associated with a similar yet significant increase in apoptotic cells. Thus the remodeling of the wounded cells primarily represents their migration into the gap without being accompanied by significant alterations in cell number.

**DISCUSSION**

Epithelial cells lining the adult colon do not normally express GRP or its cognate receptor (1). When aberrantly expressed in human colon cancer, these proteins typically have been viewed to act as mitogens (reviewed in Ref. 11). However, this mitogenic effect on at least human colon cancer cell lines has not been dramatic. Whereas exogenous agonist increases Isreco-1 cell proliferation 1.9-fold (21), GRP has no effect on Colo-320 or HCT-116 cells (19). In contrast, GRP-specific antagonists have been shown to decrease the proliferation of HT-29 cells grown as explants in nude mice by as little as 15% (18, 19).

In the present study, we show that GRP/GRP-R upregulation in confluent Caco-2 cells postwounding results in a modest 2.2-fold increase in proliferating cells (Fig. 9), consistent with this peptide hormone acting as a mitogen. Yet this increase in proliferation was balanced by a nearly equivalent increase in the number of apoptotic cells (Fig. 9). This finding is similar to what we observed in our study of colon cancers developing in wild-type and GRP-R$^{-/-}$ mice (3), such that there was no significant difference in tumor size or weight in age-matched mice of either genotype subsequent to carcinogen exposure. Rather, our most striking observation has been that when aberrantly expressed in human colon cancers, GRP/GRP-R coexpression is limited to better-differentiated tumor cells but is not observed in poorly differentiated cells or in distant metastases (1). We confirmed that GRP/GRP-R act to regulate tumor cell differentiation by observing that colon cancers in...
GRP-R<sup>&1−/−</sup> mice develop tumors that progressively de-differentiate, whereas those in wild-type mice are well differentiated (3). Given that GRP/GRP-R act to regulate normal intestinal differentiation during organogenesis (2), we suggested that these proteins act as morphogens.

Morphogens are developmentally expressed proteins that regulate organogenesis and normal tissue differentiation (7). In this capacity, morphogens modulate host cell motility to influence tissue modeling and remodeling. In cancer, we have suggested that morphogens are proteins that are aberrantly reexpressed postmalignant transformation where they recapitulate, albeit dysfunctionally, their ability to modulate tissue remodeling. Results obtained studying wild-type and GRP-R<sup>&1−/−</sup> mice indicate that GRP likely mediates its effects via FAK. Whereas FAK expression in colon cancers in wild-type mice is a function of their differentiation, and by contrast, the GRP-R-specific antagonist [d-Phe<sup>6</sup>]-bombesin methyl ester as well as upregulation of FRNK had identical effects in completely ablating HEK-293 cell motility (8). However, until the present paper, nothing was known as to whether GRP acted as a motogen in human colon cancer cells, and if so, whether this effect was mediated via FAK phosphorylation.

In this paper, we show that GRP/GRP-R: 1) are transiently upregulated by Caco-2 and HT-29 cells as a function of their confluence, 2) act to increase cell motility but not cell number in remodeling confluent monolayers postwounding; and 3) mediate their effects via FAK phosphorylated at Tyr<sup>397</sup>, Tyr<sup>407</sup>, Tyr<sup>961</sup> and/or Tyr<sup>925</sup>. Overall, these data provide the first direct evidence that GRP and its receptor can be transiently expressed in a human colon cancer cell line and that when present, function primarily as morphogens regulating tissue remodeling. Because assessing the rapidity with which confluent monolayers are restored postwounding is commonly used as a marker of tumor cell invasiveness (for example, see Ref. 20), it could be argued that our findings actually implicate GRP/GRP-R as promoting metastasis and a worse patient outcome. However, it needs to be appreciated that the wounding assay itself only measures cells’ ability to remodel, especially because truly invasive cells would continue advancing past the wound gap itself, such that cells from both margins would intermingle with one another. As demonstrated herein (Fig. 7; and as shown at http://ajpgi.physiology.org/cgi/content/full/00108.2004/DC1), this does not happen. Furthermore, we have no evidence from human studies that patients whose colon cancers express GRP/GRP-R do any worse than those whose tumors do not express these proteins (1). Thus our findings most likely reflect GRP/GRP-R acting as morpho-

**Fig. 8.** GRP-dependent and -independent phases of Caco-2 cell motility correlate with GRP-R expression. Cells were treated as described in Fig. 7. The amount of remaining wound or gap between cell margins was then determined as the %remaining compared with the size of the original wound (●) using OpenLab for cells treated with (●) or without (▼) GRP-R antagonist [d-Phe<sup>6</sup>]-bombesin methyl ester; or with 1 μM bombesin (□). In separate experiments performed in parallel, cells were treated identically; the amount of GRP-R expression was determined immunohistochemically and quantified as described in MATERIALS AND METHODS. Results are expressed as the means ± SE of at least 3 separate experiments, with each experiment having been performed in triplicate. Asterisks identify time points at which gap closure in the presence of antagonist [d-Phe<sup>6</sup>]-bombesin methyl ester or exogenous bombesin differed significantly from what was observed for untreated cells (P < 0.05, ANOVA).

**Fig. 9.** Effect of GRP/GRP-R expression on Caco-2 cell proliferation and apoptosis. Cells were treated as described in Fig. 7. At the indicated time points, cells were fixed and evaluated immunohistochemically for Ki-67, a marker of cell proliferation (open bars); or by counting 4,6-diamidino-2-phenylindole-stained cells for chromatin condensation and/or DNA fragmentation, measures of apoptosis (solid bars). Results are expressed as the means ± SE of at least 3 separate experiments, with each experiment having been performed in triplicate. Statistical comparisons, along with the test employed, are between values at t = 6 h, the time of maximal GRP/GRP-R expression, and t = 2 h, a time at which GRP/GRP-R are not expressed. HPF, high-powered field (×400).
gens, with remodeling being a prime component property of being a morphogen.

This paper raises a number of interesting and ultimately unanswerable questions. First, the heterogeneous nature of GRP/GRP-R upregulation in both Caco-2 and HT-29 cells postwounding (Fig. 5) was unexpected. Although it has been previously reported that, at least for Caco-2 cells, various hydrolases typically located in the brush border are heterogeneously expressed (22, 23), we found GRP/GRP-R expression to be homogeneously present in preconfluent cells (Fig. 1). Indeed, we would have predicted that cells immediately proximate to the wound would have uniformly expressed these proteins, given their predominant function as motogens critically regulating at least a portion of the gap closure postwounding. However, it is possible that for the entire mass of Caco-2 cells to expand and migrate into the gap, a more diffuse upregulation, similar to that shown herein, is in fact necessary.

Second, the regulators of GRP and GRP-R expression are not known. Although in vitro studies have identified functional corticosteroid response elements and cAMP binding sites in the GRP-R gene’s promoter region (25), this information does not shed any light as to how this protein’s expression is regulated in vivo. Intriguingly, both GRP and GRP-R appear to be coexpressed simultaneously in Caco-2 cells, suggesting that a common factor may be involved, whereas the heterogeneous nature of expression suggests that GRP/GRP-R transcriptional regulation is possibly regulated by some unknown soluble factor(s).

Third, the role of various phospho-FAKs in mediating GRP’s motogenic effects is not clear. FAK phosphorylation at Tyr\(^{397}\) (pTyr\(^{397}\)) is well appreciated as generically important for enhancing this enzyme’s activity. However, a recent study has implicated pTyr\(^{861}\) in mediating VEGF-induced cell migration as well as epithelial-to-mesenchymal transdifferentiation (16). In contrast, the roles of pTyr\(^{407}\) and pTyr\(^{925}\) in mediating cell motility and/or morphogenesis have not, to our knowledge, been previously described.

Finally, the profile of FAK phosphorylation we noted in Caco-2 cells differs from what we have previously described in resected human colon cancers. Whereas we show that GRP-R activation in Caco-2 cells increases pTyr\(^{397}\), pTyr\(^{407}\), pTyr\(^{861}\), and pTyr\(^{925}\), we only observed differences for pTyr\(^{925}\) and pTyr\(^{407}\) in human colon cancers. This may be due to the fact that Caco-2 cells may be a better model for the study of normal intestinal development than reflect that which occurs in colon cancer. Alternatively, as a line clonally expanded from a single tumor cell, the phosphorylation of FAK at Tyr\(^{861}\) and Tyr\(^{925}\) in Caco-2 cells may simply be specific to this particular cell line.

In summary, we herein demonstrate variable expression of GRP and its cognate receptor in two colon cancer cell lines and show that this expression critically mediates a GRP-dependent phase of cell motility by causing FAK to be phosphorylated at multiple specific sites. The transient expression of GRP/GRP-R contributes to the ability of these tumor cells to remodel in a proliferation-independent manner. Overall, these findings support GRP/GRP-R acting as morphogens, along with a mechanism by which they act, when aberrantly expressed in colon cancer.

GRANTS
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REFERENCES


