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Stimulation of proliferation and migration of a colorectal cancer cell line by amidated and glycine-extended gastrin-releasing peptide via the same receptor

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Abstract

Although amidated forms of gastrin-releasing peptide (GRP) have been identified as autocrine growth factors in small cell lung cancer, their role in the development and progression of colorectal carcinoma is less clear. In addition, the biological activity of non-amidated gastrin-releasing peptide has not been investigated in colorectal carcinoma cells. We therefore investigated the effect of bombesin (a homologue of gastrin-releasing peptide) on proliferation, migration and inositol phosphate production in the human colorectal carcinoma cell line DLD-1, and determined the ability of gastrin-releasing peptide receptor antagonists to inhibit these effects. We also compared the biological activities of amidated and non-amidated GRP in the same assays. Treatment with either bombesin, or amidated or non-amidated GRP resulted in significant increase in proliferation, and in migration in a wound-healing assay. Both the mitogenic and migratory effects of amidated and non-amidated forms were inhibited by the GRP receptor antagonist [D-Phe^{6}, Leu-NHet^{13}, des-Met^{14}]–bombesin(6–13). The presence of GRP receptor mRNA and GRP binding sites in three colorectal carcinoma cell lines was demonstrated by RT-PCR and by binding of radiolabelled bombesin, respectively. Transfection of DLD-1 cells with a dominant negative phosphatidylinositol 3-kinase did not affect bombesin-stimulated cell proliferation, but inhibited bombesin-stimulated cell migration. Bombesin and GRPgly activated phospholipase C, mitogen-activated protein kinase and focal adhesion kinase. We conclude that both amidated and non-amidated forms of gastrin-releasing peptide accelerate proliferation and migration of DLD-1 human colorectal carcinoma cells via the gastrin-releasing peptide receptor, but that phosphatidylinositol 3-kinase is only involved in the cell migration signalling pathway. Our results suggest a potential role for gastrin-releasing peptide receptor antagonists in the management of colorectal carcinoma.

Keywords: Bombesin; GRP; Glycine-extended GRP; Signal transduction; Migration; Proliferation; Amidation

1. Introduction

Gastrin releasing peptide (GRP), named after its first identified bioactivity, is a potent mitogen for a number of tumour types including pancreatic, small cell lung, prostate, renal and breast cancers [1]. The detection of GRP and its cognate receptor (GRP-R) in small cell lung cancers, and the anti-proliferative effect of a GRP antibody, resulted in GRP becoming the prototype autocrine growth factor [2]. A proliferative effect of GRP in colorectal carcinoma (CRC) cell lines, and reversal of the effect by a GRP-R antagonist, have been demonstrated in vitro [3,4] and in vivo [5]. However, the findings were inconsistent [6,7] and were not related to the presence of receptors or endogenous ligand. More recent studies suggest that GRP is a differentiation factor as well as a mitogen in colorectal carcinoma [8,9].

Abbreviations: BRS-3, bombesin receptor subtype 3; DMEM, dulbeccos modified Eagles medium; ERK, extracellular signal regulated kinase; FAK, focal adhesion kinase; GRP, gastrin-releasing peptide; GRP-R, gastrin-releasing peptide receptor; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase

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Peptides of the GRP family also stimulate cell migration. GRP itself has been shown to increase migration of LNCaP and TSU-pr1 prostate cancer cells [10]. The related frog peptide bombesin has been shown to stimulate invasion and migration of Isreco1 colon carcinoma cells [11]. A link between a high level of expression of the GRP receptor and the invasive characteristics of human colon tumours has been hypothesized [12].

The two known GRP receptors, GRP-R and BRS-3, are members of the G-protein-coupled seven transmembrane domain receptor family. GRP binds to GRP-R with high affinity, but binds to BRS-3 with only low affinity. No high-affinity naturally occurring ligand for BRS-3 has been identified. Nearly all human colon cancers express GRP-R mRNA, [12,13] but only 20–40 % have measurable binding sites for iodinated GRP [14]. Similarly, most CRC cell lines express GRP-R mRNA but only 30–50% have detectable binding sites [15,16].

Until recently, it was believed that only the amidated forms of peptide hormones were biologically active. This view is now being questioned as recent studies have demonstrated biological activities of prohormones and prohormone-derived peptides such as glucagon-like peptide (GLP-1) [17], progastrin [18] and glycine-extended gastrins [19]. These non-amidated forms act through distinct receptors from their amidated counterparts. In this context it is relevant to note that glycine-extended forms of $GRP_{18–27}$ and $GRP_{1–27}$ arise during the processing of proGRP, which consists of 125 amino acids. The glycine-extended forms are further processed by the α-amidating monooxygenase, giving rise to amidated forms of GRP. Recently, a glycine-extended form of bombesin has been shown to stimulate proliferation and inositol phosphate production in Swiss 3T3 fibroblasts. Inhibition of the responses by a GRP-R antagonist suggested that both the glycine-extended and amidated forms acted through the same receptor [20].

A number of intracellular mediators of the actions of bombesin/GRP have been identified. ERK1 and ERK2 belong to a family of kinases, collectively known as the mitogen-activated protein kinases (MAPK), that are activated in response to extracellular stimuli such as growth factors and hormones to induce cell proliferation [21]. Bombesin treatment of the U-373MG human adult glioblastoma cell line caused a marked increase in phosphorylation of ERK1 and ERK2, which was both time- and concentration-dependent [22]. Bombesin also stimulated activation of p42ERK2 and $p125^{FAK}$ (focal adhesion kinase) in the pancreatic carcinoma cell line AR42J [23]. In contrast, in the duodenal cancer cell line HuTu 80 bombesin did not activate either ERK isoform, although stimulation of cyclic AMP response element binding protein (CREB) phosphorylation by bombesin was observed [24].

Focal adhesion kinase (FAK) is a second intracellular mediator that colocalizes with the integrins at focal adhesions, and becomes tyrosine phosphorylated in response to many growth factors acting through diverse receptors. Hyperactivation of FAK in response to growth factors correlated with increased motility of squamous cell carcinoma and small cell lung cancer cells, and increased growth and invasive capacity of mammary carcinoma cells [25]. Bombesin and GRP have been shown to increase tyrosine phosphorylation of FAK and paxillin in non-small cell lung cancer cells [26], and bombesin rapidly stimulated tyrosine phosphorylation of FAK in a dose-dependent manner in the prostate carcinoma cell line PC-3 [27]. Immunohistochemical studies have shown that FAK is phosphorylated at tyrosine residues 397 and 407 in well-differentiated colorectal carcinoma cells, and that phosphorylation correlates with the expression of GRP and its receptor [28].

Phosphatidylinositol 3-kinase (PI3K) is a third important intracellular regulator of mammalian cell proliferation [29] and migration [30]. The available clinical evidence for deregulation of the PI3K-pathway in various cancers and the identification of downstream kinases (e.g. AKT, mTOR, PDK1 and ILK) that are involved in mediating the effect of PI3K provide potential targets for the development of small-molecule therapies [31]. To date there are no reports of the involvement of the PI3K pathway in bombesin-activated proliferation or migration in colorectal carcinoma cell lines.

The outstanding issues with regard to the role of GRP in colorectal carcinoma are the presence of endogenous ligand, the relationship between receptor and response to the ligand, and the question whether GRP stimulates both proliferation and migration. Importantly, the relative roles of amidated and non-amidated GRP have not been investigated. Accordingly, we have determined (1) the presence of GRP-R mRNA and binding sites and BRS-3 mRNA in a panel of colorectal carcinoma cell lines, (2) the proliferative and migratory effects of bombesin, GRPamide and GRPgly on two colorectal carcinoma cell lines, (3) the effect of a GRP-R antagonist on bombesin- , GRPamide- and GRPgly-stimulated proliferation and migration, and (4) the role of phosphatidylinositol 3-kinase (PI3K), focal adhesion kinase (FAK), and mitogen-activated protein kinase (MAPK) in the proliferative and migratory effects of amidated and non-amidated GRP.

2. Materials and methods

2.1. Materials

The four colorectal carcinoma cells lines used were obtained from the following sources: DLD-1 (ATCC), HT-29 (ATCC), LIM1215 (Ludwig Institute for Cancer Research) and LIM1899 (Ludwig Institute). DLD-1 was maintained in DMEM with 10% FBS. HT-29, LIM1215 and LIM1899 were grown in RMPI 1640 with 10% FBS.

PI3K mutants were generated by stable transfection of DLD-1 cells [32] with a plasmid that encodes a dominant-negative ΔSH2 mutant of the p85 subunit of PI3K that is unable to bind the catalytic p110 subunit [33]. Transfected DLD-1 cells were maintained in an appropriate concentration of antibiotic G418 to ensure the continued presence of the transfected gene [32]. Antibodies against total and phosphorylated p42/44 MAPKs were obtained from New England Biolabs. Antibody against total FAK was from BD Biosciences, and antibody against phosphorylated FAK ($pTyr^{397}$) was from Sigma. Bombesin and amidated GRP were obtained from Auspep. Glycine-extended GRP (GRPgly) was custom synthesised by Auspep; peptide composition and purity (>99%) were determined by mass spectrometry and HPLC, respectively. The stocks of GRPamide were monitored and shown to be stable with no change in immunoreactivity over time using a radioimmunoassay specific for GRPamide described elsewhere [34]. GRP-R receptor antagonist [D-Phe^6, Leu-$NHet^{13}$, des-Met^{14}]–bombesin-(6–13) was purchased from Bachem AG. The PI3K inhibitor LY294002 and the MAPK inhibitor PD098059 were obtained from Sigma. ^{125}I-[Tyr^4]bombesin was obtained from Amersham Biosciences.

2.2. Cell proliferation assays

Cell proliferation was measured using the MTT colorimetric assay, which is based on the reduction of MTT to a blue formazan product by mitochondrial dehydrogenases present in viable cells. Briefly, 1×10^4 cells/well were plated into a 96-well plate in DMEM containing 10% FBS. Next day the medium was replaced with serum-free medium and cells were incubated for a further 24 h. On the third day peptides were diluted in medium containing 1% FBS, added to the cells, and incubated for a further 3 days. The total volume in each well was 200 μl. At the end of incubation 15 μl of MTT solution (5 mg/ml in PBS (phosphate-buffered saline)) was added to each well and incubated at 37 °C for 4 h. The medium was then removed carefully without disturbing the insoluble formazan crystals. Two hundred microlitres of 0.04 M HCl in isopropanol was added to each well to solubilise the crystals, and the absorbance was read at 570 nm using a spectrophotometer. Readings from wells that received control medium and no MTT treatment were used as blanks.

Cell proliferation was also measured by [^{3}H]-thymidine incorporation [20]. Briefly, 1×10^5 cells/well were plated into a 24-well plate in DMEM with 10% FBS. Next day the cells were synchronized in G_0 by starving the cells in serum-free medium for 24 h. On the third day, test peptides were diluted in DMEM containing 1% FBS, added to cells, and further incubated for 17 h. The cells were then pulsed with 0.5 μCi of [^{3}H]-thymidine for 4 h, and washed three times with PBS containing 0.2% BSA. DNA was precipitated by incubating the cells in 5% TCA for 20 min at 4 °C, washed with 95% ethanol, and dissolved in 1 M NaOH. Lysates were then transferred to scintillation vials, neutralized with 1 M HCl and radioactivity was counted in a β-counter after the addition of 5 ml of Pico-fluor 40 solution (Packard).

2.3. Wound healing assay

To study the migratory effect of peptides, wound healing assays were performed as described previously [35]. Briefly, cells were seeded in 12-well plates in DMEM containing 10% FBS, and grown until 90% confluent. Cells were then serum starved for 24 h, and a linear wound was created in the confluent monolayer using a 20 μl pipette tip. Cells were then washed with PBS and treated with or without different peptides diluted in DMEM containing 1% FBS. Wounds were photographed at 0, 17 and 24 h, and the wound size was measured at five random sites perpendicular to the wound.

2.4. Inositol phosphate production

Production of total inositol phosphates was measured using a modification of a previously reported method [20]. 5×10^4 cells/well were seeded in DMEM containing 10% FBS in a 24-well plate and allowed to attach overnight. Next day cells were labeled with [^{3}H]-myo-inositol for 24 h in serum-free medium. The cells were then incubated with or without peptides diluted in assay buffer (20 mM Hepes, 135 mM NaCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, 0.5% BSA, pH 7.45) for 1 h at 37 °C. The reaction was stopped by adding 1 ml of acidified ethanol (1/2000, v/v conc. HCl/ethanol), and the solution was then loaded on to a Dowex AG-1-X8 column (BioRad). Columns were washed with 4 ml of distilled water followed by 4 ml of 40 mM ammonium formate. Inositol phosphates were eluted with 4 ml of 1 M ammonium formate and radioactivity was measured after addition of 5 ml of scintillation fluid to the eluates.

2.5. Reverse transcriptase PCR

DLD-1, HT-29, LIM1215 and LIM1899 colorectal cancer cells were grown in media containing 10% FBS, seeded into a 10 cm Petri dish and allowed to attach overnight. The cells were then synchronised by growth for 24 h in media with no FBS. Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. 2.5 μg DNAse I-treated total RNA was used to synthesise first-strand cDNA using Superscript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen). A duplicate sample of total RNA was also subjected to the reverse transcriptase process without Superscript II enzyme, to act as a control for genomic contamination. The cDNA was tested to see if it was a viable template using primers for the β-actin gene. The expected product was

observed for all samples, except the genomic controls and water control (data not shown). GRP-R mRNA was amplified using primers 5′-ATCTTCTGTACAGTCAAGTCC-3′ (nucleotides 588–608, Genbank sequence XM010317) and 5′-GCTTTCCTCATGGAAGGGATG-3′ (reverse and complement of nucleotides 942–962). The oligomer 5′-GATGCCAGCAGGTACCTGGCT-3′ (nucleotides 687–707) was used as a hybridisation probe. BRS-3 mRNA was amplified using primers 5′-CTGCGTCTGGATC-GTGTCTA-3′ (nucleotides 656–676, Genbank sequence NM001727) and 5′-GGGATTCAATCTGCTTACGG-3′ (reverse and complement of nucleotides 925–945). The oligomer 5′-TTCAAATGTATACACTTTTCGAGATCC-3′ (nucleotides 704–730) was used as a hybridisation probe. The cDNA was amplified using *Taq* polymerase (Geneworks) in a 50 μl reaction mix, which contained 1 μl of cDNA, 2 U polymerase, 100 ng of each of the forward and reverse primers, 1× *Taq* polymerase buffer (supplied by manufacturer), 0.2 mM of each deoxynucleotide triphosphate (dGTP, dATP, dCTP, dTTP) and 1.5 mM magnesium chloride. To avoid sample-to-sample variation, all components except cDNA were pooled in a premix, which was aliquoted into PCR tubes containing template cDNA. Amplification consisted of 30 (GRP-R) or 35 (BRS-3) cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C, and extension of primers for 30 s at 72 °C. The products were held at 72 °C for 10 min for DNA extensions to occur. One-fifth of each reaction was analyzed by electrophoresis through a 2% agarose gel, and the products were visualised by staining with 0.5 μg/ml ethidium bromide. The nucleic acids were transferred to Hybond N+ nylon membrane (Amersham Pharmacia Biotech) by capillary action in 0.4 M sodium hydroxide. ^{32}P-labelled oligonucleotides were labelled using terminal transferase according to the manufacturer's instructions (Roche), and were used for Southern hybridisation of the blot in rapid-hyb buffer (Amersham Pharmacia Biotech) at 42 °C for 1–2 h. Unhybridised probe was removed by rinsing blots twice in 0.5× SSC containing 0.1% SDS at room temperature and then washing for 15 min (twice) in the same solution at 42 °C. The products were visualised after exposure to Kodak Biomax MR X-ray film (Amersham Pharmacia Biotech) for 2–12 h.

2.6. Western blot

Cells were grown in 10 cm Petri dishes in DMEM containing 10% FBS until 90% confluent. Following serum starvation, the cells were stimulated for the indicated times with 10 nM GRPgly or bombesin diluted in DMEM containing 0.2% FBS. Cells were lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 0.5 mM DTT, and protease inhibitors (Sigma) in 20 mM Tris, 150 mM NaCl, pH 7.6). Twenty micrograms of total protein lysates was then mixed with loading buffer, denatured, and separated by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane using a semi-dry blotting system (BioRad). Membranes were then incubated with the appropriate primary antibodies, and detection performed with alkaline phosphatase-coupled anti-rabbit or anti-mouse IgG followed by incubation with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution, pH 9.2 (Sigma). Membranes were scanned using a Hewlett-Packard ScanJet 5200C, and densitometric analysis of protein bands was performed with Fuji BAS software. Band densities were determined by taking the ratio of densities of phosphorylated protein over total protein.

2.7. Receptor binding assays

Specific binding of ^{125}I-[Tyr^4] bombesin to colon cancer cells or to human GRP-R-transfected BALB3T3 cells was measured in competition experiments. Briefly 1.5×10^4 cells per well for each cell line were plated in medium containing 10% FBS. Cells were serum starved for 24 h. Next day, cells were washed once in binding buffer (200 mM Tris–HCl, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine, 0.1% BSA, pH 7.2). Cells were incubated with 50 pM of ^{125}I[Tyr^4] bombesin for 45 min at 37 °C with or without 1 μM unlabelled bombesin. At the end of the incubation cells were washed three times with PBS containing 2% BSA. Cells were then lysed with 1 M NaOH, and radioactivity measured using a γ-counter (LKB-Wallac). To determine the affinity of GRPgly for the GRP-R receptor, binding experiments were carried out in the presence of different concentrations of unlabelled peptide as mentioned in the figure legend. IC_{50} values were determined by regression of the data to the equation for one site competition using the program Sigma Plot.

2.8. Statistics

Results are expressed as the mean ± S.E. of at least three separate experiments. Results were analyzed by one-way analysis of variance followed by Dunnett's or Bonferroni methods. Differences with p values of <0.05 were considered significant.

3. Results

3.1. Growth-promoting effect of bombesin on colorectal carcinoma cell lines

The proliferative effect of increasing doses of bombesin on two colorectal carcinoma cell lines (HT29 and DLD-1) was determined using the MTT colorimetric assay. When proliferation was assayed in the presence of a low concentration of serum (0.5%), no stimulation by bombesin

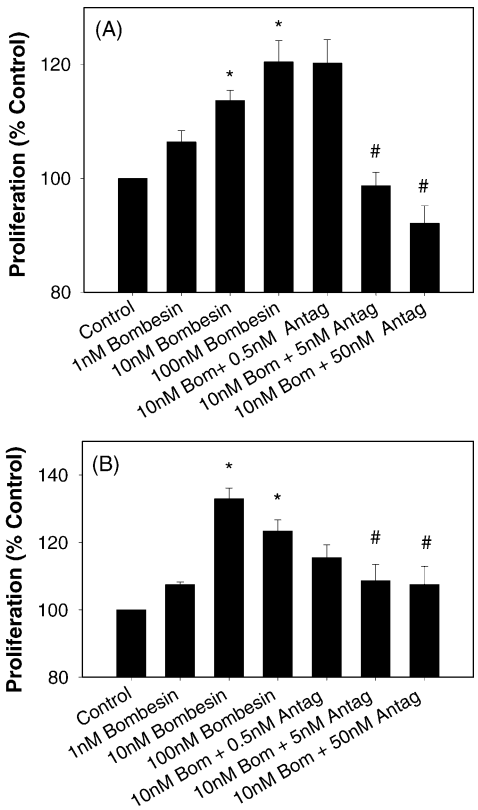

Fig. 1. Bombesin stimulates proliferation of colorectal carcinoma cell lines. The effect on cell proliferation of treatment of HT-29 (A) and DLD-1 (B) cells with increasing doses of bombesin was assessed by a colorimetric assay. 10 nM Bombesin significantly stimulated the growth of both cell lines. The GRP receptor antagonist [D-Phe^6, Leu-NHet^{13}, des-Met^{14}]–bombesin(6–13) inhibited bombesin-stimulated growth in a dose-dependent manner. Data are the mean ± S.E.M. of at least three separate experiments each performed in quadruplicate, and are expressed as a percentage of the control OD value from wells without stimulants. In a typical experiment the control OD value was 0.385 as compared to 0.477 after 10 nM bombesin treatment. Statistical significance was assessed by one-way analysis of variance ($^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to 10 nM bombesin treatment).

was observed after 72 h (data not shown). However when proliferation was assayed in the presence of 2% serum in the case of HT-29 cells, or 1% serum in the case of DLD-1 cells, bombesin produced an increase in cell proliferation in both cell lines (Fig. 1A and B). For HT-29 cells the maximum increase in proliferation (120 ± 4% of control, $p < 0.05$) was observed at 100 nM bombesin (Fig. 1A). For DLD-1 cells treatment with 10 nM bombesin resulted in maximum stimulation (133 ± 3% of control, $p < 0.05$) (Fig. 1B). The GRP-R antagonist [D-Phe^6, Leu-NHet^{13}, des-Met^{14}]–bombesin-(6–13) at concentrations of 5 and 50 nM inhibited the stimulation of proliferation by 10 nM bombesin in both cell lines. Antagonist alone had no significant effect on cell proliferation (data not shown). Because compounds of the [D-Phe^6]–bombesin-(6–13)-alkylamide family are potent antagonists of the GRP-R receptor [36], but have much lower affinity for the BRS-3 and NMB-R receptors [37], we conclude that the stimulatory effects of bombesin on proliferation of HT-29 and DLD-1 cells are mediated by the GRP-R receptor.

3.2. Bombesin stimulates migration of DLD cells

A wound-healing assay was used to study whether bombesin stimulated migration of DLD-1 cells. Bombesin caused a time-dependent decrease in wound size (Fig. 2A). For example, after treatment with bombesin in the presence of 1% serum for 24 h, the wound was completely closed, while in the untreated control, the wound was still 46 ± 7% of its original size (Fig. 2B). At a lower serum concentra-

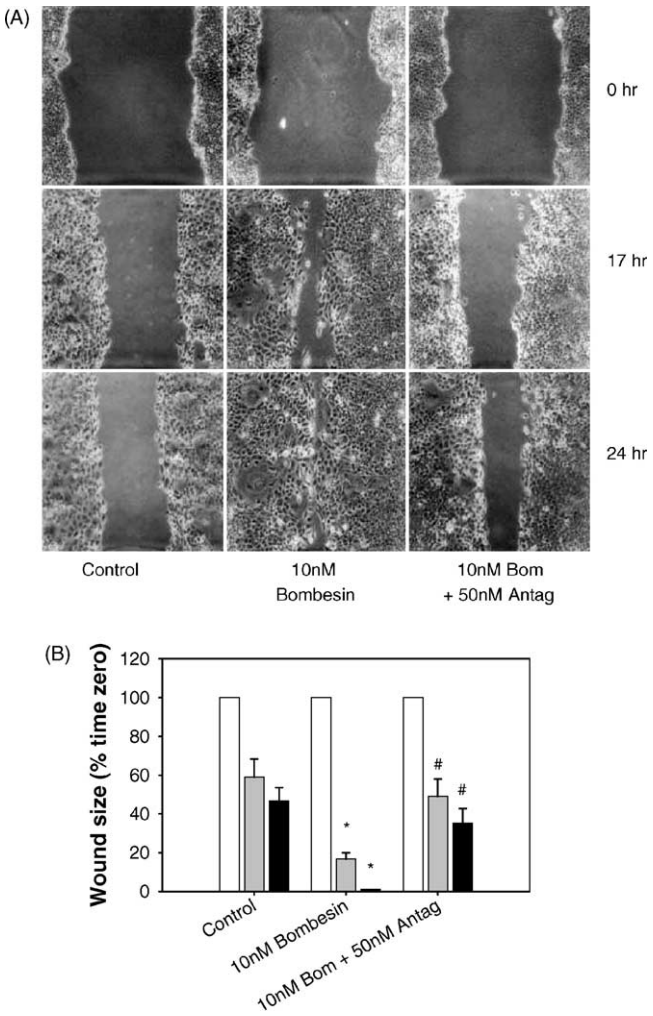

Fig. 2. Bombesin stimulates migration of DLD-1 colorectal carcinoma cells. A linear wound was created on a confluent monolayer of serum-starved DLD-1 cells, which were then treated with the indicated concentrations of bombesin and GRP-R receptor antagonist for 24 h. (A) Microphotographs of a similar randomly chosen field for each one of the wounded monolayers were taken at 0, 17 and 24 h. (B) Histogram representing the change in wound size over time in untreated or treated cells. The width of the wound was measured at five distinct sites at 0 h (white bars), 17 h (grey bars) or 24 h (black bars). Data are the mean ± S.E.M. of at least three separate experiments. Statistical significance was assessed by one-way analysis of variance ($^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to 10 nM bombesin treatment).

tion (0.5%) the rate of wound closure was reduced, but was still significantly stimulated by bombesin (data not shown). The stimulation of wound repair by 10 nM bombesin was inhibited by a GRP-R receptor antagonist at a concentration of 50 nM.

3.3. Role of PI3K in migratory and proliferative effects of bombesin

To establish the role played by PI3K in bombesin-stimulated proliferation and migration, DLD-1 cells expressing a dominant negative mutant of the p85 regulatory subunit of PI3K [32] were compared to DLD-1 vector-only cells. The observation that 10 nM bombesin significantly stimulated thymidine incorporation in both the dominant negative PI3K and vector-only cells (Fig. 3A) suggested that PI3K was not essential for bombesin-stimulated proliferation. Similarly, the specific PI3K inhibitor LY294002 had no effect on the thymidine incorporation following treatment of the wild-type DLD-1 colorectal cells with 10 nM bombesin (data not shown). In contrast, the observation that bombesin was unable to stimulate wound healing in the PI3K mutant cells (Fig. 3B) suggested that PI3K was essential for bombesin-stimulated migration. As before, bombesin treatment of DLD-1 vector-only cells resulted in complete wound repair.

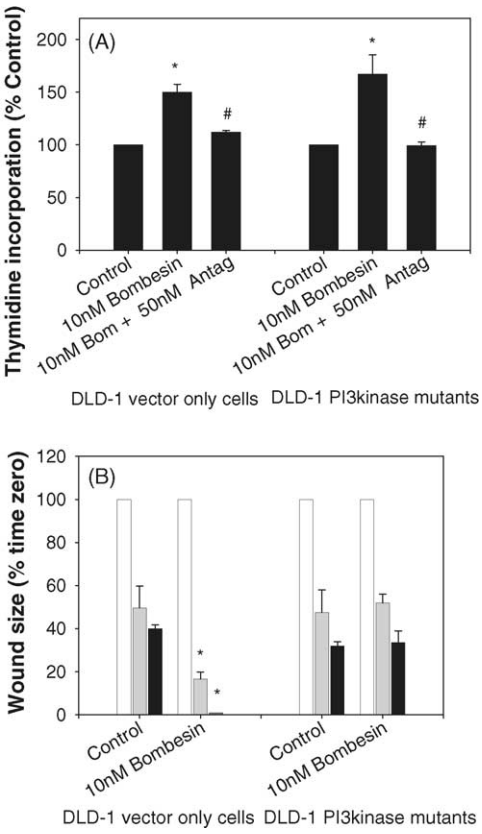

Fig. 3. PI3K is essential for migratory but not proliferative effects of bombesin. [^{3}H]-thymidine incorporation (A) and wound healing (B) assays were performed on DLD-1 colorectal carcinoma cells that had been stably transfected with vector only or with vector encoding a dominant negative PI3K mutant as described in materials and methods. Cells were stimulated with 10 nM bombesin with or without 50 nM GRP-R receptor antagonist. In the wound healing assay the width of the wound was measured at five distinct sites at 0 h (white bars), 17 h (grey bars) or 24 h (black bars). Data are the mean of ± S.E.M. of at least three separate experiments. Statistical significance was assessed by *t*-test ($^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to 10 nM bombesin treatment).

3.4. Effect of amidation on activity

The proliferative effects of the glycine-extended and amidated forms of GRP were compared using DLD-1 colorectal carcinoma cells. As shown in Fig. 4A, GRPgly significantly stimulated thymidine incorporation to a similar extent to bombesin or GRP amide. The observation that a GRP-R antagonist inhibited the stimulatory effects of both GRPgly and bombesin indicated that the effects of both peptides were mediated by the GRP receptor. The fact that the response to bombesin and the inhibition by the GRP-R antagonist were similar to the effects seen with the MTT assay (Fig. 1) indicated that both thymidine incorporation and MTT assays were valid measures of proliferation. The glycine-extended and amidated forms of GRP were both able to stimulate cell migration in a wound-healing assay (Fig. 4B and C). As with the effects on proliferation, the observation that a GRP-R antagonist inhibited the stimulatory effects of both GRPgly and bombesin indicated that the effects of both peptides were mediated by the GRP receptor.

3.5. Stimulation of inositol phosphate production by GRP and GRPgly

Inositol phosphate production is one of the early events that occur following the binding of a peptide to its receptor. Bombesin, GRP and GRPgly all significantly stimulated inositol phosphate production in DLD-1 colorectal carcinoma cells (Fig. 5). The maximum stimulation was obtained at a 1 nM concentration of each peptide, and was similar in magnitude for all three peptides.

3.6. MAPK kinase activation by bombesin and GRPgly

The mitogen-activated protein kinases (MAPKs) are key components of the intracellular signaling pathways that control cell proliferation [21]. Phosphorylation of both the 42- and 44-kDa forms of MAPK by MAPK kinase was stimulated rapidly and in a time-dependent manner by treatment of the DLD-1 colorectal carcinoma cell line with GRPgly (Fig. 6). Maximal phosphorylation (170 ± 9%) was observed 3 min after addition of 10 nM GRPgly (Fig. 6B). Similarly, 10 nM bombesin treatment of DLD-1 colorectal cells for 3 min caused a significant increase (190 ± 26%) in phosphorylation of both the isoforms of MAPK

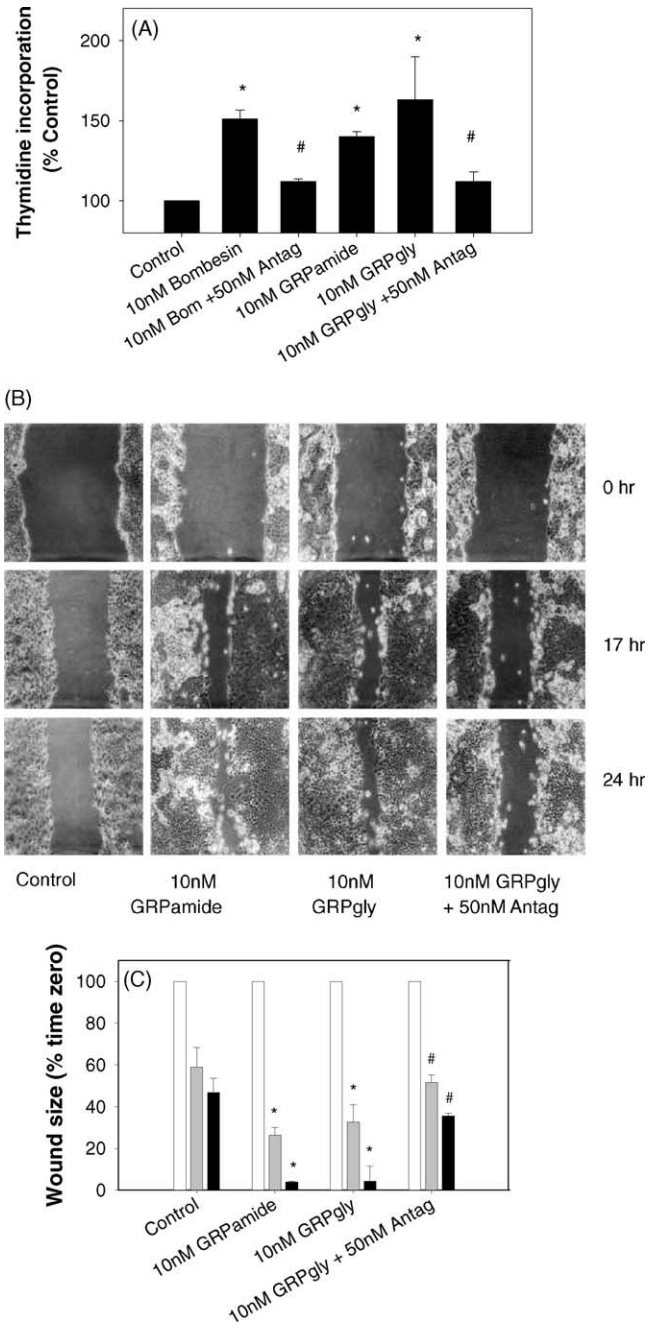

Fig. 4. Amidation is not required for GRP activity. DLD-1 colorectal carcinoma cells were treated with 10 nM amidated bombesin, GRP amide or non-amidated GRPgly. The effect on proliferation (A) and migration (B, C) was assessed as described in Section 2. In the wound healing assay the width of the wound was measured at five distinct sites at 0 h (white bars), 17 h (grey bars) or 24 h (black bars). Data are the mean ± S.E.M. of at least three separate experiments each performed in triplicate. In a typical proliferation assay the counts per minute incorporated in control wells were 32353 ± 485 as compared to 50810 ± 893 in wells stimulated with 10 nM GRPgly. Statistical significance was assessed by one-way analysis of variance ($^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to 10 nM bombesin treatment).

as compared to untreated cells (Fig. 6A). The MAPK kinase inhibitor PD098059 at a concentration of 50 μM completely blocked bombesin-induced thymidine incorporation in DLD-1 cells (data not shown).

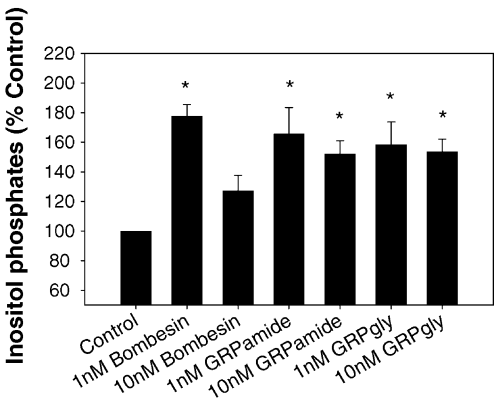

Fig. 5. Stimulation of inositol phosphate production by GRP and GRPgly. Production of inositol phosphates by DLD-1 human colorectal carcinoma cells after incubation with 1 or 10 nM bombesin, GRPamide or GRPgly was measured as described in Section 2. Data are the mean ± S.E.M. of at least three separate experiments each performed in duplicate. Statistical significance compared to unstimulated controls was assessed by one-way analysis of variance ($^{*}p < 0.05$).

3.7. Focal adhesion kinase activation by bombesin and GRPgly

GRPgly also caused a time-dependent phosphorylation of the focal adhesion kinase $p125^{FAK}$ (Fig. 7A). Maximal phosphorylation (175 ± 11%) on Tyr397 was observed 5 min after addition of 10 nM GRPgly (Fig. 7B). In comparison, 10 nM bombesin also caused an increase (132 ± 11%) in phosphorylation after 5 min treatment (Fig. 7A).

3.8. Detection of GRP-R and BRS-3 in colorectal carcinoma cell lines

GRP-R and BRS-3 mRNAs were detected in cDNA prepared from DLD-1, HT-29, LIM1215 and LIM1899 cell lines by PCR. The identity of the ethidium bromide-stained band was confirmed by Southern hybridisation using an internal oligonucleotide probe for the appropriate receptor mRNA (Fig. 8A). GRP-R mRNA was present in all four cell lines, with the highest mRNA expression in LIM1215 cells, and the lowest in DLD-1 and LIM1899 cells. BRS-3 mRNA was detected in all four cell lines, although the expression in LIM1899 was very low compared to the other cell lines.

In order to confirm the expression of GRP-R at the protein level in the cell lines in which significant quantities of GRP-R and/or BRS-3 mRNA had been detected, we then performed binding studies using iodinated bombesin (Fig. 8B). Binding sites for $^{125}I[Tyr^4]$ bombesin were detected on LIM 1215, DLD-1 and HT29 cells. The specificity of GRP-R binding was confirmed using 1 μM cold bombesin, which completely abolished the binding of $^{125}I[Tyr^4]$ bombesin. LIM 1215 cells had the highest level of bound radioactivity, and the highest level of GRP-R mRNA. Interestingly, DLD-1 cells had a greater number of

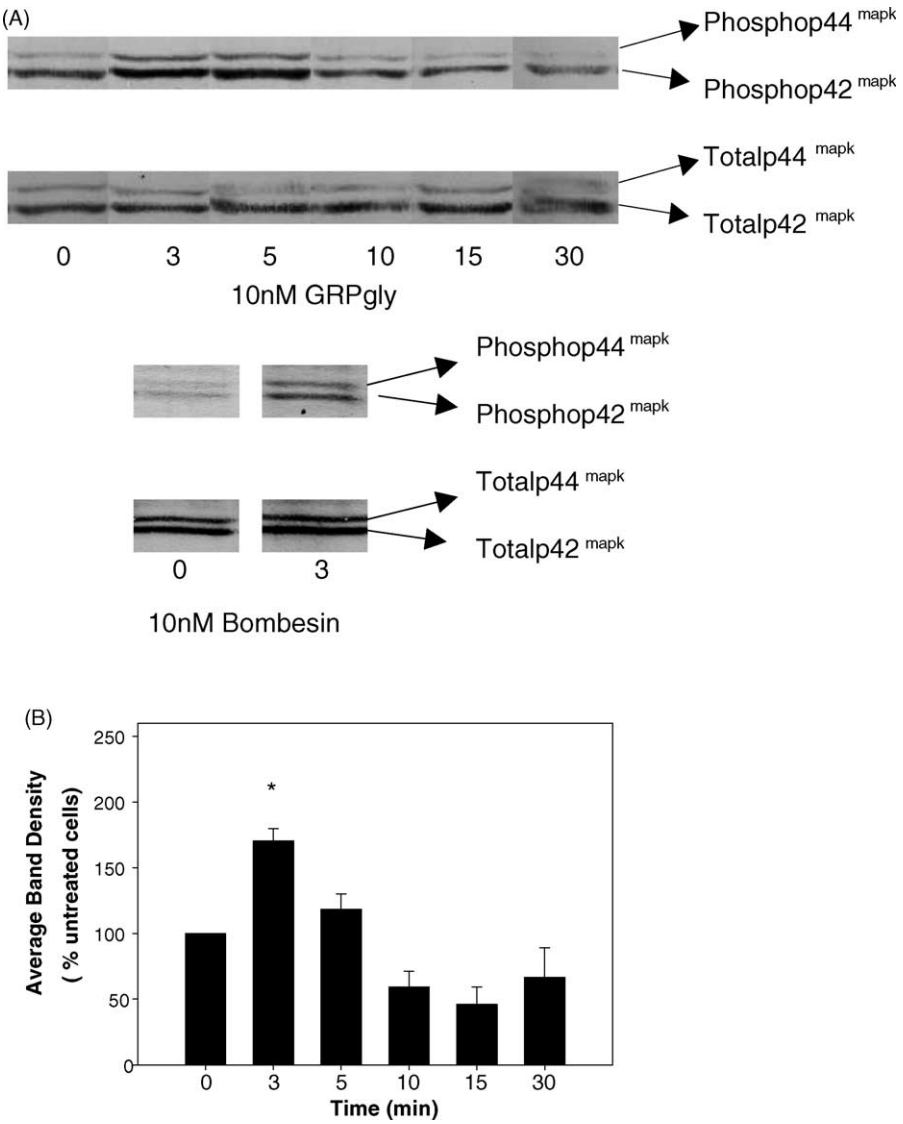

Fig. 6. Bombesin and GRPgly activate MAPK kinase. Phosphorylation of MAPK in DLD-1 human colorectal carcinoma cells after incubation with 10 nM GRPgly or 10 nM bombesin was measured as described in Section 2. (A) Cell lysates were electrophoresed on SDS-polyacrylamide gels and Western blotted. The blots were probed with an anti-phosphorylated p42/44 MAPK antibody to measure MAPK activation, and an anti-total p42/44 MAPK antibody to control for equal loading of samples. (B) Results of densitometric analysis of phosphorylated p42/44 MAPK in three independent experiments following GRPgly stimulation were corrected for variation in protein loading, and are expressed as a percentage of unstimulated control. Error bars represent the standard error of the mean. Statistical significance compared to unstimulated controls was assessed by one-way analysis of variance ($^{*}p < 0.05$).

binding sites than HT-29 cells, although the latter had higher expression of GRP-R mRNA.

3.9. Affinity of GRPgly for the GRP-R receptor

In order to determine the affinity of GRPgly for the human GRP-R the abilities of GRPgly and GRPamide to displace ^{125}I[Tyr^4] bombesin from BALB 3T3 cells transfected with the human GRP-R (Fig. 9A), and from untransfected DLD-1 cells (Fig. 9B), were determined. Amidated GRP exhibited a 50-fold higher receptor affinity (IC_{50} 11 $\pm$ 4 nM) than GRPgly (IC_{50} 560 $\pm$ 180 nM) on BALB 3T3 cells (Fig. 9A), and a 20-fold higher receptor affinity (IC_{50} 26 $\pm$ 9 nM) than GRPgly (IC_{50} 470 $\pm$ 180 nM) on DLD-1 cells (Fig. 9B).

4. Discussion

It is increasingly recognized that many neuropeptides play an important role as autocrine/paracrine growth factors for human cancers. Bombesin and its mammalian equivalent GRP bind to GRP-R and exert various biological effects. However, the role played by GRP in the development and progression of colorectal carcinoma is less clear with active debate over the presence and type of GRP, the frequency and nature of receptors, and the biological activities of GRP. Human colorectal cancer cell lines were previously shown to express bombesin/GRP-R [15,16]. In Isreco1 colon cells, a high level of GRP-R mRNA was correlated with the expression of high-affinity bombesin/GRP surface receptors [3]. In this study we have

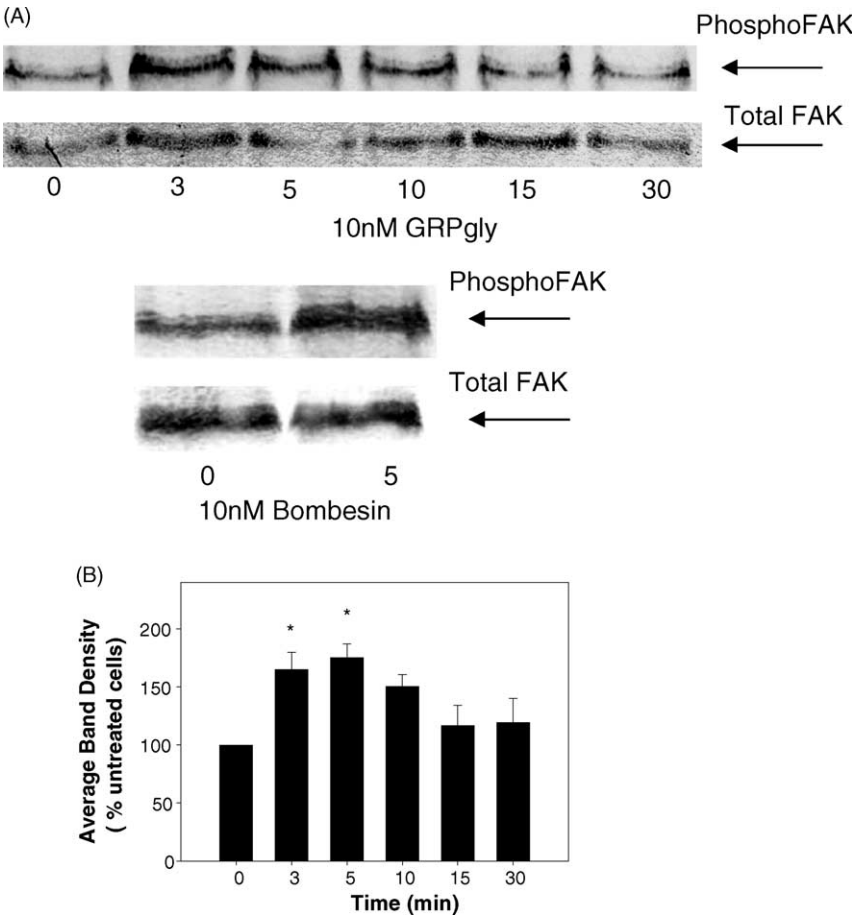

Fig. 7. Bombesin and GRPgly activate FAK. Phosphorylation of FAK in DLD-1 human colorectal carcinoma cells after incubation with 10 nM GRPgly or 10 nM bombesin was measured as described in Section 2. (A) Cell lysates were electrophoresed on SDS-polyacrylamide gels and Western blotted. The blots were probed with an anti-phosphorylated p125 FAK antibody to measure FAK activation, and an anti-total p125 FAK antibody to control for equal loading of samples. (B) Results of densitometric analysis of phosphorylated p125 FAK in three independent experiments following GRPgly stimulation were corrected for variation in protein loading, and are expressed as a percentage of unstimulated control. Error bars represent the standard error of the mean. Statistical significance compared to unstimulated controls was assessed by one-way analysis of variance ($^{*}p < 0.05$).

provided further evidence that two bombesin receptor subtypes GRP-R and BRS-3 are expressed by a number of colorectal carcinoma cell lines including HT-29, DLD-1, LIM1899 and LIM 1215 at the mRNA level as well as the protein level.

We have also demonstrated that GRP-R in HT29 and DLD-1 cells is functional. In both cell lines bombesin in the presence of intermediate concentrations of serum caused an increase in proliferation, which was blocked by a GRP-R-selective antagonist. These observations are in agreement with the finding of Cassona and coworkers, who demonstrated that the HT29 CRC cell line proliferated in response to bombesin-like peptides in medium containing 1% FBS [4]. In DLD-1 cells Carroll et al. reported that the cDNA encoding the GRP-R carried three mutations, two of which (H36N and I209V) did not affect binding when expressed singly in CHO cells. In contrast, the V317E mutation completely abolished binding when expressed singly in CHO cells [16]. To our surprise PCR amplification and sequencing of the appropriate region of the GRP-R gene from our DLD-1 cells, which were obtained from the ATCC in July 1999, did not reveal any trace of the V317E mutation (data not shown). We conclude that the difference between our observation of a functional GRP-R in DLD-1 cells, and the report by Carroll et al., may be the result of clonal variation.

The proposal that the tyrosine kinase activity of the epidermal growth factor (EGF) receptor is required for induction of cell cycle progression by bombesin in Rat-1 and Swiss 3T3 cells may in part explain the need for the presence of serum to observe the stimulatory effect [38]. However, it is clear that the GRP-R receptor is also involved in proliferation, since the effects of both GRPamide and GRPgly were blocked by a specific antagonist that does not alter EGF receptor signalling, and since the EGF receptor inhibitor tyrphostin 25 did not affect bombesin-stimulated proliferation (data not shown). These observations support the hypothesis of multiple growth factors acting in conjunction with one another to drive cancer progression.

An interesting result that emerged from the binding studies was that DLD-1 cells seem to express a higher

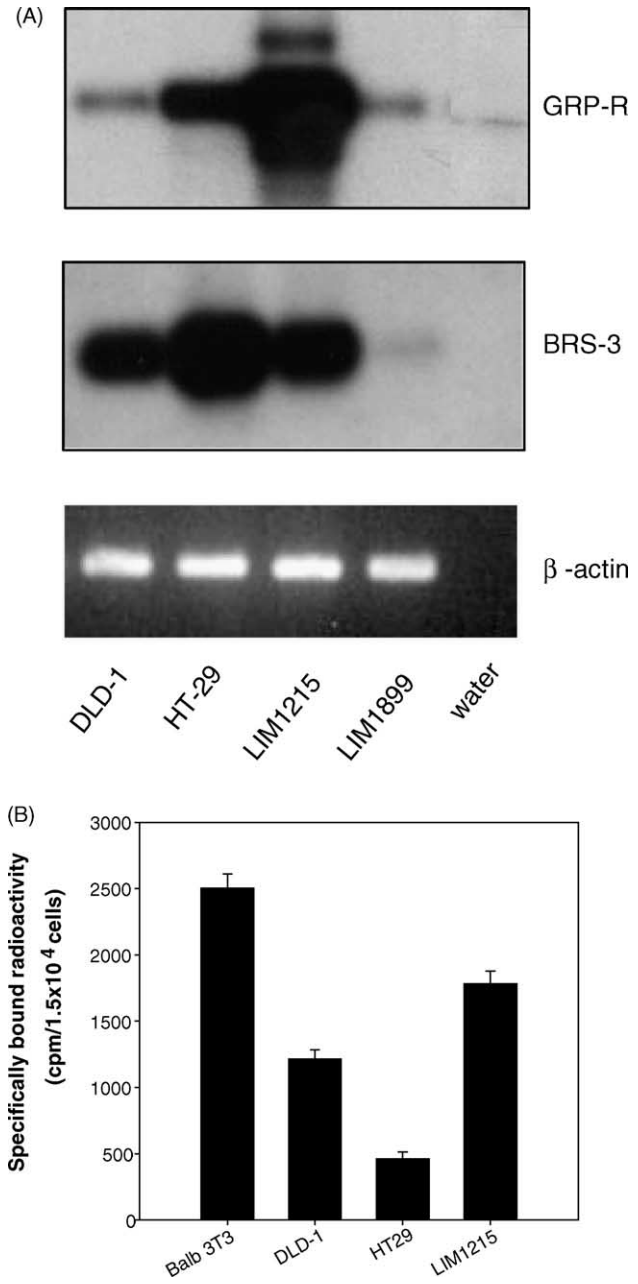

Fig. 8. Expression of GRP receptors GRP-R and BRS-3 in colorectal carcinoma cell lines. (A) Total RNA was isolated from DLD-1, HT-29, LIM1215 and LIM1899 colorectal carcinoma cell lines. Template cDNA prepared from RNA, or water as a control, was subjected to PCR with primers specific for GRP-R (30 cycles) or BRS-3 (35 cycles) mRNA. PCR products were separated on an agarose gel and visualised by Southern hybridisation with radiolabelled internal oligonucleotides specific for GRP-R or BRS-3. As an internal control to check the amount of starting material and also the integrity of RNA, template cDNA (1 μl) was subjected to PCR with β-actin primers under similar conditions, and the products were detected by ethidium bromide staining. (B) Binding of 50 pM ^{125}I-labeled bombesin to 1.5×10^4 DLD-1, HT-29 or LIM1215 colorectal carcinoma cells, or to GRP-R-transfected BALB 3T3 cells, was determined as described in Section 2. Data are expressed as the specifically bound radioactivity (i.e. the difference in counts per minute (cpm) bound per 1.5×10^4 cells in the absence and presence of 1 μM unlabelled bombesin. For each experiment, each value was determined in triplicate and the results are mean ± S.E.M. of three experiments.

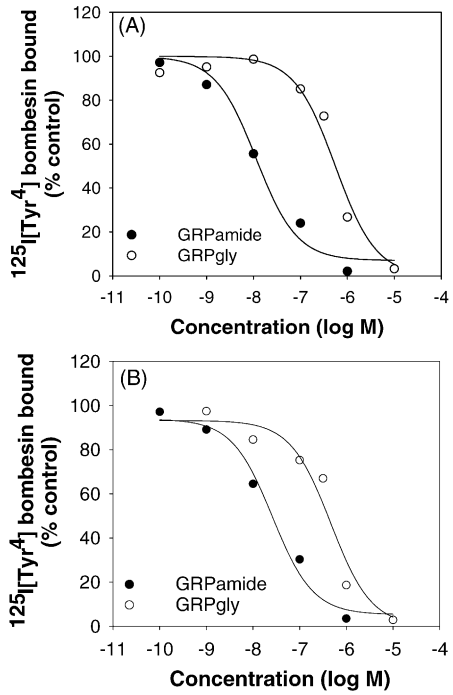

Fig. 9. Comparison of affinities of GRPamide and GRPgly for the GRP-R. Binding of 50 pM ^{125}I-labeled bombesin to 2.0×10^4 GRP-R-transfected BALB 3T3 cells (A) or to untransfected DLD-1 cells (B) was measured in the presence of increasing concentrations of unlabeled amidated GRP or glycine-extended GRP as described in Section 2. Experimental data are expressed as a percentage of the radioactivity bound in the absence of unlabelled peptide (i.e. percentage control), and lines of best fit were determined by regression of the data to the equation for one site competition using the program Sigma Plot. Amidated GRP exhibited a 50-fold higher receptor affinity (IC_{50} 11 ± 4 nM) than GRPgly (IC_{50} 560 ± 180 nM) on transfected BALB 3T3 cells (A), and a 20-fold higher receptor affinity (IC_{50} 26 ± 9 nM) than GRPgly (IC_{50} 470 ± 180 nM) on DLD-1 cells (B).

number of receptors than HT-29 cells even though the latter had higher receptor mRNA expression. This difference in receptor number may have been reflected in a difference in the effect of bombesin on proliferation. In the case of DLD-1 cells, 100 nM bombesin stimulated proliferation to a lesser extent than 10 nM bombesin, but in the case of HT-29 cells, 100 nM bombesin treatment caused a greater increase in proliferation than 10 nM bombesin. This effect may be attributed in part to receptor desensitization. Tsuda and coworkers have shown that in cells expressing high numbers of GRP-R, the receptor undergoes chronic desensitization as compared to low GRP-R-expressing cells [39]. Tsuda and coworkers also observed that high GRP-R expressing cells had higher inositol phosphate production as compared to low GRP-R expressing cells [39]. Our study is in line with this observation as DLD-1 cells had a maximum increase in proliferation of 133 ± 3% of control as compared to 120 ± 4% in HT-29 cells.

A second feature of the binding studies was that in DLD-1 cells only slight ligand competition was observed at a concentration of GRPgly (10 nM, Fig. 9B) that significantly stimulated proliferation (Fig. 4A). This phenom-

enon may well be due to the high-dose inhibition that we observed in DLD-1 cells (Fig. 1B), and that Cassano and coworkers reported with GRPamide and bombesin in HT29 colorectal carcinoma cell lines [4]. Because maximal stimulation is observed when the receptor is only fractionally occupied, in the presence of high-dose inhibition the dose–response curve for proliferation will not mirror the binding curve. The complete inhibition of GRPgly-stimulated proliferation by the GRP-R antagonist confirms that the observed effect is indeed mediated by the GRP-R (Fig. 4A).

It has previously been shown that bombesin increases the migration of prostate cancer [10] and Isreco1 colon cells [11]. In this study, we observed a stimulatory effect of 10 nM bombesin on the migration of DLD-1 colorectal carcinoma cells using a wound-healing assay. The fact that a GRP-R antagonist blocked the stimulatory effect of bombesin indicated that migration was initiated by binding of the peptide to GRP-R. Since metastasis is one of the major causes of mortality in cancer, the observation that occupation of the GRP-R stimulates migration of colorectal carcinoma cell lines has potential clinical relevance.

The phosphatidylinositol 3-kinase (PI3K) pathway regulates various processes, such as proliferation, apoptosis [31] and migration [30], in cancer cells. As a first step in mapping the pathways by which bombesin stimulates proliferation and migration of DLD-1 colorectal carcinoma cells, we investigated the role played by PI3K in these biological effects. DLD-1 cells expressing a dominant negative mutant of the p85 regulatory subunit of PI3K proliferated, but failed to migrate, when stimulated with 10 nM bombesin. We concluded that PI3K plays a crucial role in bombesin-stimulated migration of DLD-1 colorectal carcinoma cells, but not in their proliferation.

We next investigated the hypothesis that the C-terminal amidation of GRP and related peptides is not required for biological activity. Comparison of GRPgly with the amidated forms GRP and bombesin revealed that the glycine-extended form was as effective as the amidated forms in stimulation of proliferation (Fig. 4A) and migration (Fig. 4B, C) in DLD-1 colorectal carcinoma cells. Interestingly, a GRP receptor antagonist was able to block the effects of GRPgly on proliferation and migration. In BALB 3T3 cells transfected with GRP-R cDNA, competition binding experiments yielded an IC_{50} value of 560 ± 180 nM for GRPgly, 50-fold higher than the IC_{50} value of 11 ± 4 nM for GRPamide (Fig. 9). The apparent discrepancy between the binding studies and the observation that both peptides at 10 nM concentration stimulated proliferation and migration in DLD-1 cells to a similar extent (Fig. 4) may simply indicate that only a small fraction of receptors need to be occupied to generate maximal biological activity. In contrast to gastrin [19] and secretin [40], in which glycine-extended forms of the hormones act through different receptors from their amidated counterparts, GRPgly appears to act through the same receptor as its amidated form. These observations are in agreement with the observations of Oiry and coworkers, who were able to block the effects of glycine-extended bombesin on Swiss 3T3 cells with a GRP-R antagonist [20]. These workers also demonstrated that the K_i of 500 ± 21 nM for bombesin-gly was significantly higher than the K_i of 1.6 ± 0.7 nM for GRPamide [20].

Processing and secretion of peptides in tumors is often incomplete and unregulated [41]. Yang and coworkers have shown that the anti-bombesin monoclonal antibody 2A11 is unable to recognize GRPgly [42]. When this antibody was used in a phase I trial to treat patients with small cell lung carcinoma, no significant anti-tumor response was observed [43], consistent with the hypothesis that non-amidated forms of GRP were the stimulants of tumour development. The observation that non-amidated forms of GRP such as GRPgly may be biologically active, and may act through the same receptor as their amidated counterparts, suggests that the different forms of GRP secreted by colorectal carcinomas should be investigated. Studies performed some 15 years ago reported that proGRP and its fragments were present in higher concentrations than amidated GRP in small cell lung carcinomas and cell lines [44,45]. These studies have not been extended to colorectal carcinomas or cell lines. Characterisation of the GRP forms produced by colorectal carcinomas would permit the design of better therapeutic candidates for treatment of cancers expressing GRP receptors.

The binding of a peptide to its receptor triggers activation of multiple signal transduction pathways that act in a synergistic and combinatorial fashion to relay mitogenic signals to the nucleus and hence promote biological responses. To further investigate the signal tranduction pathways activated by GRPgly we looked at the phosphorylation of two MAPK isoforms $p42^{MAPK}$ (ERK-2) and $p44^{MAPK}$ (ERK-1), that activate AP-1 family transcription factors to modulate target gene expression. It has previously been shown that bombesin treatment causes rapid activation of both isoforms, and that activation is essential for mitogenesis, in Swiss 3T3 fibroblasts [46], non-small cell lung cancer cells [47], and pancreatic cancer cells [21]. In a duodenal cancer cell line HuTu 80, although bombesin stimulated CREB (cyclic AMP response element binding protein) phosphorylation, there was no activation of these MAPK isoforms and no change in cell proliferation [24]. In the present study, we have shown for the first time that treatment of colorectal carcinoma cells with GRPgly causes a robust, time-dependent activation of both isoforms. The activation peaked at 3 min (the first time tested) and subsequently reverted to control levels. A similar change was seen with bombesin. The transient activation of MAPK by bombesin is likely to be an important event in bombesin-stimulated proliferation, since the MAPK kinase

inhibitor (PD98059) completely abolished bombesin-stimulated thymidine incorporation in DLD-1 cells (data not shown). This observation is in line with the results of Douziech and coworkers that MEK1 inhibition by PD98059 abrogated bombesin-stimulated proliferation of MIA PaCa-2 pancreatic cancer cells [21].

Focal adhesion kinase (FAK) is a tyrosine kinase which is phosphorylated on various residues when stimulated by bombesin [48] or other neuropeptides. The observation that activation of FAK takes place at focal adhesions suggested that it might have a primary role in the formation of focal contacts and in the regulation of cell motility and migration. As GRPgly stimulates migration of DLD-1 colorectal carcinoma cells, we also investigated the involvement of FAK, by looking at the phosphorylation of FAK on Tyr397 after stimulation with GRPgly. Treatment of DLD-1 cells with 10 nM GRPgly or bombesin caused increased phosphorylation of FAK from as early as 3 min (the first time point measured), with a peak at 5 min. A further interesting result was the high level of FAK phosphorylation on Tyr397 in untreated quiescent DLD-1 cells. Since Tyr397 has been identified as the binding site for both members of the Src family, and for the p85 subunit of PI3K [49], this observation suggested that the availability of Src or PI3K, as well as the phosphorylation state of Tyr 397, might regulate the ability of this enzyme to integrate multiple pathways [50].

The ability of FAK to transmit signals to downstream targets is dependent on its ability to interact with several other molecules. As GRP-R activation causes increased phosphorylation of FAK at Tyr397, and as PI3K plays an important role in migration stimulated by GRP-R activation, it is possible that interaction of the p85 subunit of PI3K with Tyr397 might be involved in bombesin-stimulated migration of DLD-1 cells. In support of this suggestion, there are reports that substitution of Tyr397 in FAK with Phe abolished cell migration, and that CHO cells expressing a FAK mutant capable of binding to Src but not to PI3K failed to stimulate cell migration [51]. Similarly, it has been shown that inhibition of FAK-mediated PI3K activation prevented vascular endothelial growth factor-induced actin reorganization and migration in porcine aortic endothelial cells [52].

The results from the present paper may be summarised as follows (Fig. 10). GRPgly, GRPamide and its homologue bombesin stimulate both proliferation and migration of the human colorectal carcinoma cell line DLD-1. Although mRNAs for both GRP-R and BRS-3 receptors were detected by RT-PCR, the complete inhibition of the effects of bombesin and GRPamide by a selective GRP-R antagonist clearly demonstrated that all the effects of the amidated peptides are mediated by the GRP-R receptor. The similar blockade of the effects of GRPgly by the same antagonist indicated that non-amidated forms of GRP utilise the same GRP-R receptor. The observed stimulation of FAK phosphorylation following treatment of DLD-1 cells with GRPgly or GRPamide may then lead to recruitment and activation of PI3K as outlined in the previous paragraph. The observation that bombesin significantly stimulated both thymidine incorporation and wound healing in vector-only transfected cells, but was only able to stimulate thymidine incorporation in DLD-1 cells that had been transfected with a dominant negative PI3K, suggested that PI3K was essential for bombesin-stimulated migration but not for bombesin-stimulated proliferation. The observed stimulation of MAPK phosphorylation following treatment of DLD-1 cells with GRPgly or GRPamide is likely to play an important role in proliferation, as it does in other cell types [21,46,47]. Future work in our laboratories will be directed to extending our results with colorectal carcinoma cell lines to an investigation of the role played by both amidated and non-amidated forms of GRP in the development of colorectal carcinoma.

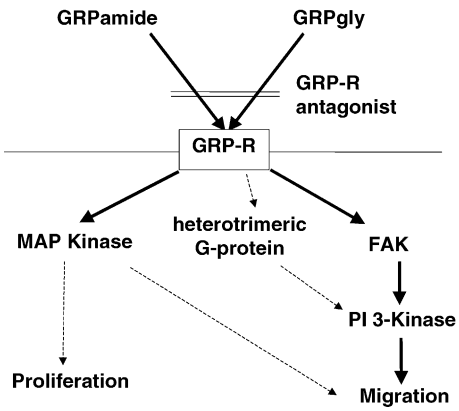

Fig. 10. A flow diagram showing GRP signalling in DLD-1 cells The stimulatory effects of both GRPamide and GRPgly on proliferation and migration of DLD-1 human colorectal carcinoma cells are mediated by the GRP-R receptor. The observation that transfection with a dominant negative mutant of PI3K blocked the effects of bombesin on wound healing but had no effect on cell proliferation indicated that PI3K was only involved in the cell migration signalling pathway. Both GRPamide and GRPgly activate phospholipase C (as measured by increased inositol phosphate production), MAPK and FAK, but the relative importance of these enzymes in signalling colorectal carcinoma cell proliferation and migration remains to be established. In several other cell types, GRPamide-dependent activation of MAPK is essential for proliferation [21,46,47]. Dotted arrows indicate mechanism not tested in the present study. Continuous arrows indicate experimental data.

Acknowledgements

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