[CANCER RESEARCH 59, 962–967, February 15, 1999]

Bombesin Stimulates Adhesion, Spreading, Lamellipodia Formation, and Proliferation in the Human Colon Carcinoma Isreco1 Cell Line[1]

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ABSTRACT

The neuropeptide bombesin and its mammalian homologue, gastrin-releasing peptide (GRP), enhance proliferation in some but not all human tumor cell lines. The pathophysiological relevance of the bombesin/GRP receptor (GRP-R), which is expressed in 30% of human colon tumor cell lines and in 24–40% of native tumors, has not been clearly assessed at this time. We studied the effects of bombesin in the recently characterized human colon carcinoma Isreco1 cell line. Competitive reverse transcription-PCR showed a high GRP-R mRNA level in Isreco1 cells, and binding studies confirmed the expression of bombesin/GRP-subtype receptors (K_d = 0.42 nM; B_{max} = 18,000 sites/cell). Exposure to bombesin resulted in an increase of intracellular calcium concentrations. Bombesin (1 nM) induced cell spreading at 24 h (21.7 ± 1.6% *versus* 6.4 ± 0.8% in control cells; $P < 0.01$) and markedly increased the formation of lamellipodia. In addition, adhesion of Isreco1 cells to collagen I-coated culture dishes was stimulated in the presence of 1 nM bombesin (69 ± 6% *versus* 42 ± 1% in control cells; $P < 0.01$). Finally, bombesin significantly increased [^{3}H]thymidine uptake by Isreco1 cells in a dose-dependent manner, with a first significant response at 0.1 nM and a maximal effect at 100 nM bombesin (192.2 ± 9.7% of control). These results clearly indicate that bombesin exerts morphological, adhesive, and proliferative effects on Isreco1 cells, suggesting that expression of the bombesin/GRP-R may contribute to the malignant properties of colon carcinoma cells.

INTRODUCTION

The amphibian tetradecapeptide bombesin and its mammalian counterpart, GRP,[3] are neurotransmitters and paracrine hormones (for review see Ref. 1). GRP is expressed mainly in nerve fibers throughout the mammalian gut and in the central nervous system, and it exerts several physiological effects, including stimulation of gastrin release from antral G cells and pancreatic exocrine secretion (1). On the other hand, GRP and bombesin are mitogens for several tumor (pancreatic, small cell lung, and mammary) cell lines (2–4). In these cells, as in various types of nontumoral cells, both peptides bind with high affinity to specific GRP-Rs (5–7).

Specific bombesin binding has been demonstrated in 30% of human colorectal cancer cell lines and in 24–40% of membrane preparations of human colorectal tumors (6–8). Interestingly, studies based on specimens of human colonic mucosa showed no specific bombesin binding or GRP-R mRNA expression (7, 9). The abnormal expression of the GRP-R in human colon tumor cells and tumor specimens suggests a specific function for this receptor in tumor cells that has not yet been clearly assessed. Indeed, bombesin did not stimulate cell growth in a variety of human colon or intestinal tumor cells that express functional GRP-Rs (6, 10, 11), with the exception of a murine colon cancer cell line (12). Beside growth-promoting effects, bombesin has been shown to modify cell morphology and the actin cytoskeleton in nontransformed cells (13) and to stimulate motility of human prostatic and small cell lung cancer cells (14, 15). Although these effects of bombesin have thus far not been reported in colon cancer cells, exogenous bombesin was shown to promote *in vivo* the metastasis of colon tumors induced by different carcinogens in rodents (16, 17). Because the metastatic process requires cell locomotion (18), cell adhesion to the extracellular matrix (19), and proliferation of the metastatic cells, it may be postulated that bombesin modulates these dynamic properties of tumor cells, leading to tumor invasion. In this study, the effects of bombesin in the recently characterized human colorectal carcinoma Isreco1 cell line (20) were studied.

We showed, by RT-competitive PCR and binding studies, that this cell line contains high level of GRP-R mRNA and expresses functional bombesin/GRP-Rs. Bombesin induced cell spreading, lamellipodia formation, adhesion to collagen I, and increased [^{3}H]thymidine uptake in Isreco1 cells. These results suggest that, beside the well-known growth-promoting effects of bombesin, activation of the GRP-R in colonic cancer cell lines may contribute to the invasive properties of these cells.

MATERIALS AND METHODS

Materials and Cell Culture. The human colon carcinoma Isreco1 cell line was a gift from Dr. B. Sordat (University of Lausanne, Epalinges, Switzerland) through the courtesy of Dr. Hamelin (Institut National de la Santé et de la Recherche Médicale U434, Paris, France). The human colon carcinoma Lovo E2 clone of the Lovo cell line has been described previously (21). The bombesin antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 (22), was a gift from Dr. D. H. Coy (Tulane University, New Orleans, LA). Carrier-free Na-^{125}I and [methyl-^{3}H]thymidine (specific activity, 80 Ci/mmol) were purchased from Amersham (Les Ulis, France). Bombesin, GRP, neuromedin B, and Fura-2/AM were from Sigma Chemical Co. (St. Quentin Fallavier, France). High-performance liquid chromatography C18 μBondapak columns were obtained from Waters SA (Montigny le Bretonneux, France). Fluorescein phalloidin was purchased from Molecular Probes (Interchim, Montluçon, France). Isreco1 cells were grown in DMEM (Life Technologies, Inc., Cergy Pontoise, France) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, and antibiotics (100 IU/ml penicillin-50 μM streptomycin) and were used at 80% confluency.

RT and Competitive PCR. RT and competitive PCR amplification were performed as described previously (23). Briefly, first-strand DNA synthesis was performed from 1 μg of total RNA with 100 pmol of a GRP-R mRNA-specific oligonucleotide ($_{1210}$5′-TTCCTGTCTAGCCATAAAGC-3′$_{1191}$) and 200 units of reverse transcriptase (MMLV RT Superscript; Life Technologies, Inc.) in corresponding buffer. One μl of the RT medium was added with known concentrations of a 470-bp DNA competitor construct in 97 μl of PCR mix [50 pmol of primers (sense, $_{-115}$5′-TTAAAGAAGGCAAAGAGC-3′$_{-98}$; antisense, $_{469}$5′-ATCTTCATCAGGGCATGGGA-3′$_{450}$), 0.2 mM dNTPs, and 1 unit of *Taq* polymerase (Appligene, Illkirch, France) in corresponding buffer]. The GRP-R cDNA amplified sequence was 584 bp (nucleotides −115 to 469; Ref. 5). One μl of the RT product was amplified with decreasing amounts of competitor (from 4×10^6 to 25 amol/tube) in separate tubes, each subjected to 30 cycles of amplification (Perkin-Elmer Corp. thermal cycler), including denaturation (94°C, 1 min), hybridization (59°C, 1 min), and elon-

Received 9/18/98; accepted 12/8/98.

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[1] This work was supported in part by Programme Hospitalier de Recherche Clinique Grant CA-3359.

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[3] The abbreviations used are: GRP, gastrin-releasing peptide; GRP-R, GRP receptor; RT, reverse transcription.

gation (72°C, 1 min). PCR products were separated on 1.5% agarose gels, blotted to nitrocellulose-nylon filters, heat-cross-linked, and hybridized with a specific 10^6 cpm/ml ^{32}P-labeled oligonucleotide ($_{323}$5′-AAATAGCCATCT-GTCAGCCAGG-3′$_{302}$), as described (23). Intensity of hybridization signals obtained with the two PCR products (GRP-R mRNA and competitor) was compared visually. The amount of GRP-R mRNA in the tested sample was estimated from the amount of competitor yielding an equivalent hybridization signal after amplification (competition equivalence point).

Radioiodination of GRP and Binding Studies. Five μg of GRP were dissolved in 10 μl of 0.5 M ammonium acetate (pH 5.5), added with 1 mCi of Na-^{125}I, 5 μg of lactoperoxidase (Sigma), and 5 μl of 0.06% hydrogen peroxide for 15 min. The radiolabeled peptide was purified by reverse-phase high-performance liquid chromatography (C18 μBondapak) with a linear gradient of acetonitrile (10–50%, v/v) in 0.1% trifluoroacetic acid. The flow rate was 1 ml/min. The specific activity of the radioactive ligand was ~2000 Ci/mmol.

For binding studies, cells from stock cultures were seeded into 2-cm^2 (24-well) cell culture dishes (Falcon, Becton Dickinson, Le Pont de Claix, France) at a concentration of 1×10^5 cells/well in DMEM-10% FCS. After 48 h of culture, the monolayers were washed twice with PBS (pH 7.4) containing 0.2% BSA and then incubated in 240 μl of buffer [50 mM HEPES, 0.25 M sucrose, 10 mM NH_4Cl, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM $MgCl_2$, 1% BSA, 0.1% bacitracin, 1 mM phenantroline, and 0.2% soybean trypsin inhibitor (pH 7.5)] and 50 pM ^{125}I-labeled GRP, in the presence or absence of different concentrations of unlabeled agonists or of the specific antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 (22). Incubation was performed during 90 min at 22°C (equilibrium binding conditions). The reaction was stopped on ice by two washing steps in PBS-0.2% BSA at 4°C. The bound radioactivity was released using 1 N NaOH and measured in a gamma counter. Specific binding was calculated as the difference between the amount of ^{125}I-labeled GRP bound in the absence (total binding) and the presence (nonspecific binding) of 10^{-6} M unlabeled GRP. Dissociation constants were determined by the Curve-Expert Version 1.3 curve-fitting program (provided by D. Hyams, Starkville, MS). The inhibition constant value (K_i) of the antagonist was calculated by the method of Cheng and Prusoff (24). The number of binding sites was determined by the method of Akera and Cheng (25). In each experiment, duplicate wells were used to determine the cell number using a hemacytometer.

Measurement of Intracellular Calcium ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was measured as described previously (26). Briefly, cells (10^6/ml) were loaded with 1 μM Fura-2/AM, under continuous stirring for 30 min at 37°C, in DMEM containing 1% FCS and 10 mM HEPES. Cells were then washed twice in a modified Krebs-Ringer bicarbonate buffer, and a 2-ml cell suspension (2×10^6 cells) was added to a cuvette and placed in a Kontron SFM 25 spectrofluorometer (Zürich, Switzerland) equipped with a warming block kept at 37°C and a stirring apparatus for constant mixing of the suspension. The fluorescence signal (F) of Fura-2 was recorded, with excitation and emission at 340 and 510 nm, respectively.

Morphological Analysis and Cell Spreading Assays. Cells from stock cultures were seeded into 2-cm^2 (24-well) cell culture dishes at a concentration of 5×10^4 cells/well in 1 ml of DMEM-0.1% BSA. The cells were incubated with or without bombesin for 24 or 48 h. The specific antagonist (D-Phe^6, Cpa^{14},ψ13–14)Bn6–14, when indicated, was added to the medium 20 min prior bombesin. At time intervals, wells were washed once with PBS, and the percentage of spreading cells was determined under a phase-contrast microscope (Diaphot microscope; Nikon, Tokyo, Japan). The quantification of spreading cells was performed using the following criteria (27, 28): rounded refringent cells, resistant to washing with PBS, were included in the nonspreading group, and cells with a flattened round base or with lamellae or filopodia were included in the spreading group. The percentage of spreading cells in five high-power microscopic fields (×20) per well was calculated, and the mean percentage of four different wells was determined for each experiment. In some cases, cell morphology was assessed by fixing and staining cells, after two washing steps with cold PBS, for 5 min in the following buffer: 0.026% (w/v) Coomassie Blue, 9.33% (v/v) acetic acid, and 50% (v/v) methanol.

For filamentous actin (F-actin) localization, cells were seeded into eight-chamber glass culture slides (Falcon) at a concentration of 4×10^4 cells/chamber in 400 μl of DMEM-0.1% BSA with or without bombesin. At 24 h, cells were fixed in a freshly prepared solution of 4% paraformaldehyde-PBS for 5 min, permeabilized with 0.1% Triton X-100 for 5 min in PBS at room temperature, and incubated with fluorescein-phalloidin (0.16 μM), following the manufacturer's instructions. The coverslips were examined on a Leitz fluorescence microscope using Leitz 25/0.75 and 50/1.00 immersion objectives.

Adhesion Assays and DNA Count. Cells from stock cultures were suspended in DMEM-0.1% BSA at a concentration of 3×10^5 cells/ml and kept in suspension for 45 min at 37°C. Cells were then seeded into 2-cm^2 (24-well) collagen I-coated cell culture dishes (Becton Dickinson) and allowed to adhere for 15 min, as described previously (14), in the presence or absence of the indicated concentrations of bombesin. The specific bombesin antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 was added immediately before bombesin, when indicated. After 15 min, the wells were washed once with DMEM-0.1% BSA, adherent cells were lysed with 120 μl H_20 for 15 min, and a 40-μl aliquot of the homogenate was used for DNA determination following the method of Labarca and Paigen (29).

Proliferation Assays. The growth of Isreco1 cells in culture was assessed from [^{3}H]thymidine uptake. Cells from stock cultures were seeded into 2-cm^2 (24-well) cell culture dishes at a concentration of 5×10^4 cells/well in 1 ml of DMEM-0.1% BSA and were incubated for 48 h with or without increasing concentrations of bombesin. The medium was changed after 24 h, and cells were pulsed with [^{3}H]thymidine (20 μCi/well) for 24 h before the end of the incubation time. The reaction was stopped on ice, and wells were washed twice with ice-cold PBS, incubated 30 min with cold 5% trichloroacetic acid, and then washed twice with ethanol. One ml 1 M NaOH was added to each well for 30 min. An aliquot was neutralized, and the radioactivity was determined in a liquid scintillation counter (1600CA Tri-carb liquid scintillation analyzer; Packard, Rungis, France).

Statistical Analysis. Results were analyzed by one-way ANOVA followed by Fisher post hoc comparison. Differences between two means with a $P < 0.05$ were regarded as significant. All values were expressed as means ± SE of at least three experiments.

RESULTS

Bombesin/GRP-Rs Are Expressed in Isreco1 Cells. First, we used RT-competitive PCR to study expression of the GRP-R mRNA in Isreco1 cells (Fig. 1), as compared to the Lovo E2 cell line, shown previously to contain GRP-R mRNA and to express ~2500 GRP-Rs/cell (23). RT-competitive PCR revealed that GRP-R mRNA was expressed in Isreco1 cells and that the mRNA level in this cell line was ~1 log level higher than that of Lovo E2 cells (Fig. 1). We then

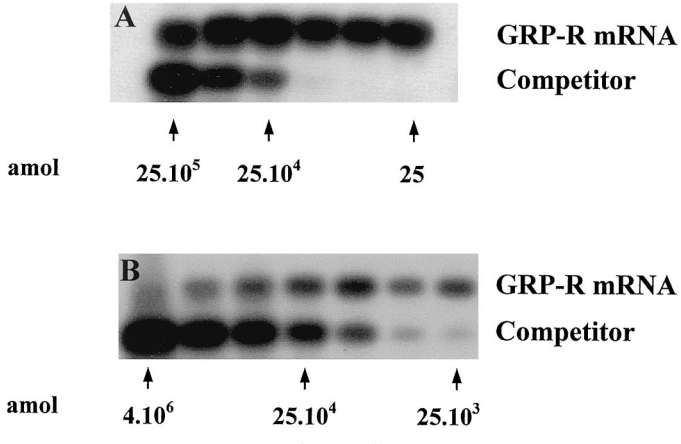

Fig. 1. RT-competitive PCR amplification of the GRP-R mRNA in Isreco1 (*A*) and Lovo E2 (*B*) cell lines. The reverse-transcription product of 1 μg of total RNA was coamplified with decreasing concentrations of the competitor. The PCR product was blotted and hybridized with a specific ^{32}P-labeled oligonucleotide. The two signals correspond to the GRP-R cDNA (584 bp, *top signal*) and the competitor (470 bp, *bottom signal*). Different amounts of competitor in reaction tubes are indicated (in amol). Equal intensities of the hybridization signals represent equal starting amounts of GRP-R cDNA and competitor in the PCR. The blots are representative of five separate experiments.

performed binding studies to confirm at the protein level the expression of the GRP-R in Isreco1 cells (Fig. 2). Indeed, Isreco1 cells exhibited a specific ^{125}I-labeled GRP binding. To characterize the subtype of receptor present on cells, the agonists bombesin, GRP, and neuromedin B and the antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14, specific for bombesin/GRP subtype receptors (22), were tested for their abilities to inhibit binding of ^{125}I-labeled GRP. Bombesin and GRP were more potent than neuromedin B at displacing binding of the tracer, thus suggesting the presence of a bombesin/GRP subtype receptor in Isreco1 cells. Computer analysis of the dose-inhibition curve of ^{125}I-labeled GRP binding by GRP revealed a single class of binding sites. The analysis demonstrated ~18,000 binding sites/cell (n = 4) and a K_d of 0.42 nM. The specific antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 also displaced binding of the tracer with a K_i of 4.5 nM. Comparable results were obtained using ^{125}I-labeled Tyr^4-bombesin instead of ^{125}I-labeled GRP in binding studies (data not shown), suggesting that bombesin receptors expressed by Isreco1 cells are of the bombesin/GRP and not neuromedin B subtype.

Bombesin Increases Cytosolic Calcium Concentration. $[Ca^{2+}]_i$ mobilization by bombesin was detected at 1 nM and 100 nM but not at 0.01 nM (Fig. 3*A*). Bombesin caused a rapid and transient increase in $[Ca^{2+}]_i$, which peaked promptly after the addition of the peptide and then faded within 2 min. To establish that bombesin was, in fact, mediating its effects on $[Ca^{2+}]_i$ by interacting with bombesin receptors, we tested the ability of the specific bombesin receptor antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 to inhibit the action of bombesin (Fig. 3*B*). The addition of 1 μM (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 alone did not modify $[Ca^{2+}]_i$ but totally blocked the increase in $[Ca^{2+}]_i$ caused by 1 nM and 100 nM bombesin. In all conditions, the ionomycin-induced $[Ca^{2+}]_i$ increase was preserved.

Bombesin Stimulates Cell Spreading and Lamellipodia Formation. Modifications of cell morphology and the cytoskeleton are frequent effects of growth factors, and they play a role in cell adhesion, locomotion, and invasion (30, 31). Bombesin can induce cell shape changes such as membrane ruffling and stress fibers formation as reported in bombesin-treated Swiss 3T3 fibroblasts (13, 32). The effect of bombesin on Isreco1 cell morphology was, therefore, investigated. After 24 h of culture with 100 nM bombesin, Isreco1 cells, stained with Coomassie Blue, showed increased spreading as compared to controls and frequent large veil-like structures (lamellipodia) or short filaments (filopodia) protruding from the cell surface (Fig. 4, *b versus* controls in *a*). These features were also observed in some cells cultured without bombesin, but their frequency was dramatically increased in the presence of bombesin. In a second step, we used fluorescein-phalloidin to assess whether bombesin could induce F-actin reorganization in Isreco1 cells. As shown in Fig. 4, *c* and *d*, bombesin (100 nM) induced no stress fiber formation at 24 h, but an extended meshwork of actin filaments was observed at the cell periphery in protruding structures, as compared to control cells. Comparable although less pronounced effects were observed at 1 nM bombesin (data not shown).

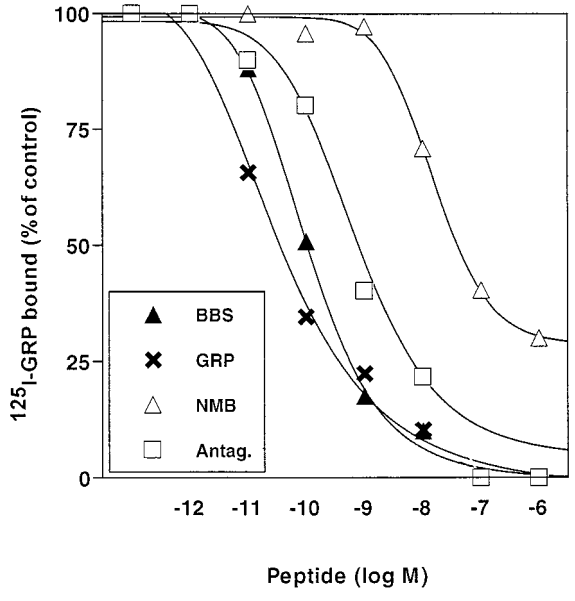

Fig. 2. Inhibition of ^{125}I-labeled GRP binding by GRP, bombesin (*BBS*), neuromedin B (*NMB*), and the antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 (*Antag.*) in Isreco1 cells. Cell monolayers were incubated for 90 min at 22°C with 50 pM ^{125}I-labeled GRP and increasing concentrations of unlabeled peptides. *Data points*, percentages of specifically bound ^{125}I-labeled GRP, expressed as means of at least three experiments performed in duplicate.

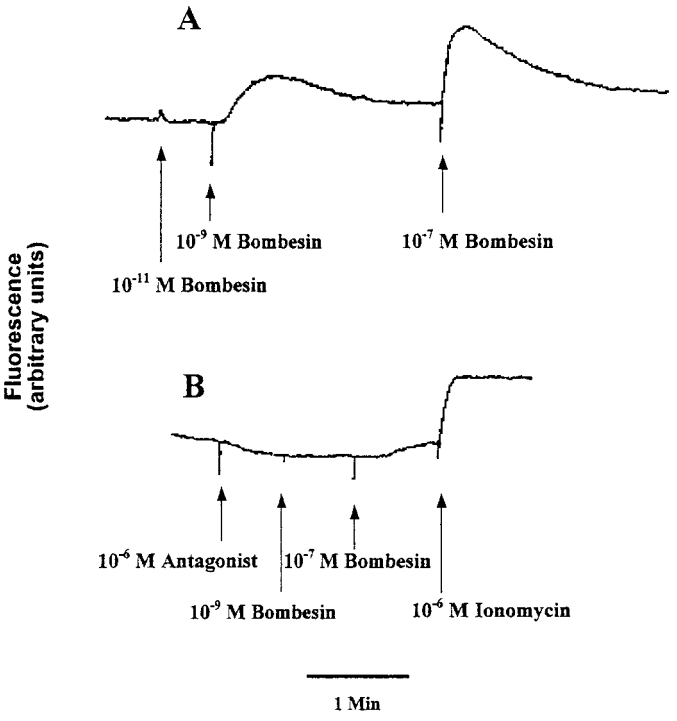

Fig. 3. Effect of bombesin on cytosolic free ionized calcium in Isreco1 cells. *A*, cells were loaded with 1 μM Fura-2/AM and assayed fluorometrically for the increase in $[Ca^{2+}]_i$ in response to 10^{-11}, 10^{-9}, and 10^{-7} M bombesin. *B*, inhibition of the bombesin-induced cytosolic free calcium mobilization by the specific antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14. Ionomycin was used in each case to check the effects of cell permeabilization to extracellular calcium. Recordings are representative of three separate experiments.

We then determined the percentage of spreading cells in the absence or presence of bombesin for 24 h (Fig. 5). The mean percentage of spreading cells in six separate experiments was 6.4 ± 0.8% in control Isreco1 cells and increased to 21.7 ± 1.6% in the presence of 1 nM bombesin ($P < 0.05$). Treating cells with 100 nM bombesin increased this value to 27 ± 2.0% ($P = 0.15$). Increasing incubation time up to 48 h with 1 nM bombesin gave similar results (control, 19.3 ± 1.6%; bombesin-treated, 39.6 ± 2.3%; $P < 0.05$). To determine whether bombesin stimulation of cell spreading in Isreco1 resulted from the interaction with bombesin receptors, we tested the ability of (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 to inhibit this effect (Fig. 5). The antagonist alone (1 μM) had no effect on the spreading of Isreco1 cells (data not shown) but significantly reduced the percentage of spreading cells induced by 100 nM bombesin (14.9 ± 2.4% *versus* 27.2 ± 2.0% without antagonist).

Bombesin Stimulates Cell Adhesion to Collagen I. The spreading process reflects the coordinated activity of actin-driven membrane

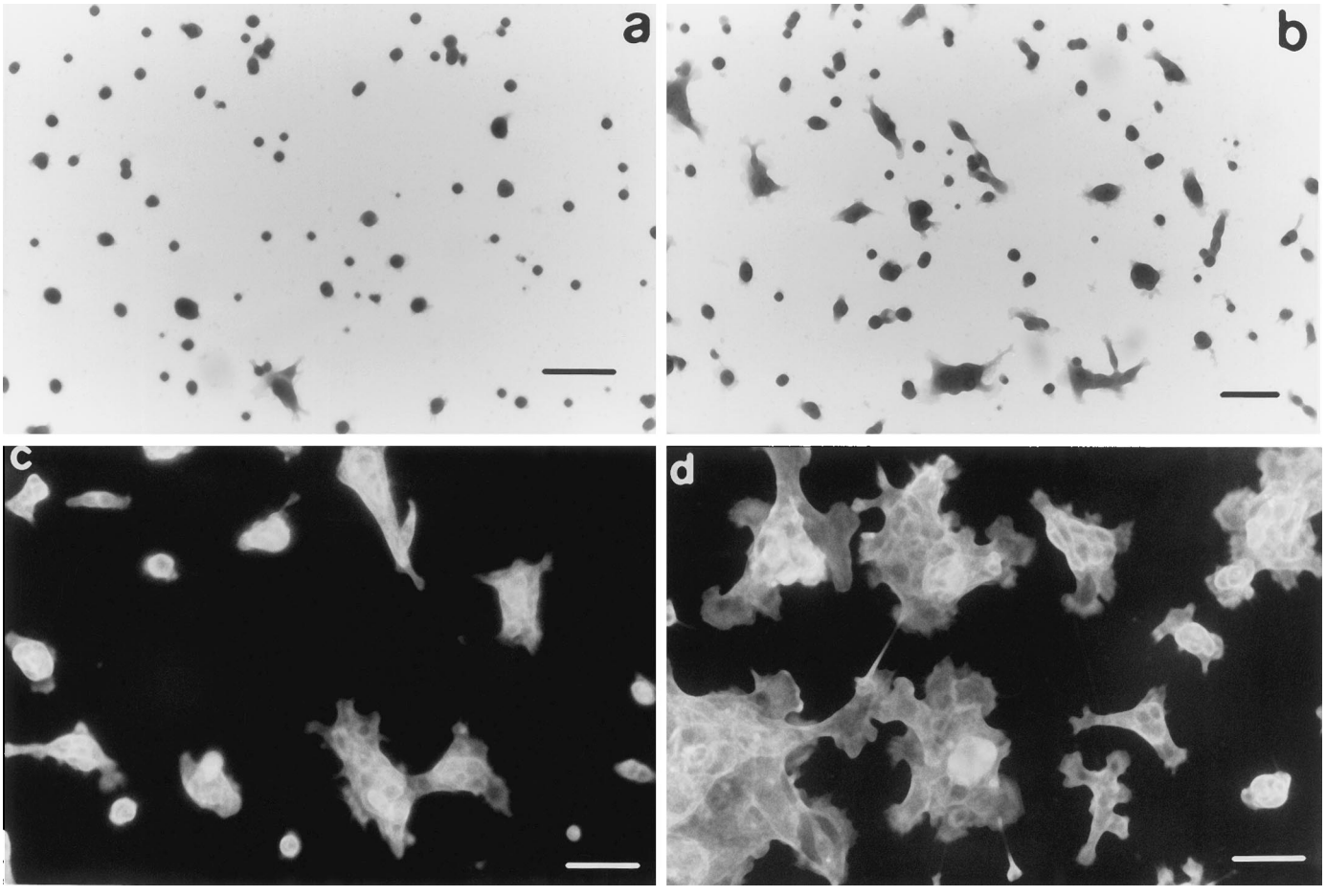

Fig. 4. Bombesin-stimulated spreading of Isreco1 cells. Cells (5×10^4/well) were seeded in 24-well plates and cultured for 24 h in the absence (*a* and *c*) or the presence (*b* and *d*) of 100 nM bombesin. *a* and *b*, Coomassie blue staining, ×125 (*scale bars* = 30 μm). Note the frequent veil-like protrusions (lamellipodia) in cells cultured with bombesin. *c* and *d*, fluorescein-phalloidin staining, ×200 (*scale bars* = 20 μm). Note the absence of stress fibers and the concentration of actin in multiple veil-like protrusions at the periphery of the cell in cells cultured with 100 nM bombesin.

protrusion and integrin-dependent cell adhesion to the substratum (33, 34). Thus, we tested the effects of bombesin on adhesion of Isreco1 cells to collagen I, an extracellular matrix component (Fig. 6). Fifteen min after seeding, bombesin increased in a dose-dependent fashion the number of adherent cells resistant to PBS washing, as assessed by DNA determination in cell homogenates. The maximal effect was observed at 1 nM (148.1 ± 8.9% over control) and a lower (supramaximal) effect at 100 nM (124.7 ± 7.2%). By estimating cell numbers from DNA counts, we calculated that 42 ± 1% of control cells had adhered to collagen I-coated culture dishes after 15 min *versus* 69 ± 6% in the presence of 1 nM bombesin (data not shown). The bombesin receptor antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 alone, at a concentration of 1 μM, had no effect on Isreco1 cell adhesion (data not shown) but completely inhibited the effect of 1 nM bombesin on Isreco1 cell adhesion to collagen I-coated culture dishes (97.2 ± 6.1% of control value; Fig. 6).

Bombesin Stimulates DNA Synthesis. Bombesin was previously shown to stimulate proliferation in several cancer cell lines (3, 4), which did not include human carcinoma lines of colonic origin (10). We measured [^{3}H]thymidine uptake by Isreco1 cells in the presence or absence of increasing concentrations of bombesin (Fig. 7). Bombesin increased [^{3}H]Thymidine uptake by the cells over the 0.1–100 nM concentration range. We then tested the ability of the bombesin antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 to inhibit bombesin-induced growth of Isreco1 cells (Fig. 7). (D-Phe^6, Cpa^{14},ψ13–14)Bn6–14 alone, at a concentration of 1 μM, had no effect on

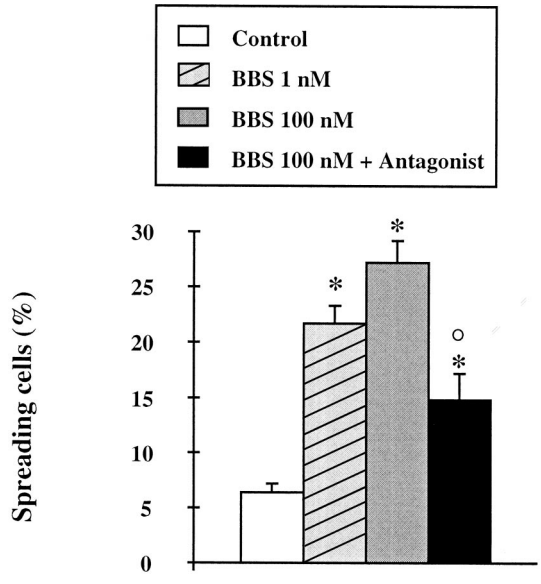

Fig. 5. Effect of bombesin (*BBS*) on the percentage of spreading Isreco1 cells at 24 h. Cells (5×10^4/well) were seeded in 24-well plates in the presence or absence of 10^{-9} M and 10^{-7} M bombesin. Spreading and nonspreading cells were counted independently after 24 h of culture. The antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 was used at a concentration of 10^{-6} M. *Columns*, means of six separate experiments, each performed in quadruplicate; *bars*, SE. *, significantly different from control; ○, significantly different from 10^{-7} M bombesin.

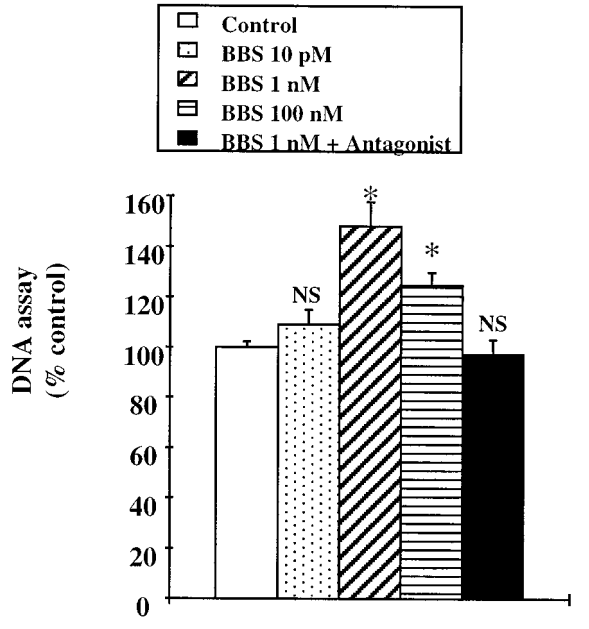

Fig. 6. Effect of bombesin (*BBS*) on Isreco1 cell adhesion to collagen I. Cells (3×10^5/well) were seeded onto collagen I-coated 24-well plates and allowed to adhere for 15 min at 37°C. Total DNA was measured from PBS washing-resistant cell lysates. The specific antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 was used at a concentration of 10^{-6} M. *Columns*, means of three separate experiments, each performed in triplicate; *bars*, SE. *, significantly different from control; *NS*, nonsignificant *versus* control.

[^{3}H]thymidine incorporation by Isreco1 cells but inhibited the [^{3}H]thymidine incorporation induced by 0.1 μM bombesin by 40% and that induced by 10 nM bombesin by 70%.

DISCUSSION

The neuropeptide bombesin is a potent growth factor, activating specific receptors in human small cell lung (3), prostate (35), mammary (4), pancreatic (2), and murine colorectal cancer cell lines (12) but not in human colon (10) or intestinal (11) tumor cells. In nontransformed murine fibroblasts Swiss 3T3 cells, bombesin also modifies the cell cytoskeleton and induces the formation of membrane ruffles, stress fibers, and focal adhesions that anchor stress fibers to the cell membrane (13, 32). In contrast, these effects of bombesin on cell morphology and cytoskeleton have not been reported previously in transformed cells. This work shows that bombesin induces cell spreading and lamellipodia formation and stimulates cell adhesion to collagen I and DNA synthesis by activating specific bombesin/GRP-Rs in the human colon cancer cell line Isreco1.

In vitro, 30% of human colorectal cancer cell lines were previously shown to express bombesin/GRP-Rs in plasma membranes (6, 10). In Isreco1 cells, a high level of GRP-R mRNA was correlated with the expression of high affinity bombesin/GRP surface receptors, positively linked to the mobilization of intracellular calcium. The density of membrane GRP-Rs in Isreco1 cells was higher than that reported in other human colon cancer cells (6), including the Lovo E2 cell line (23). A low density of GRP-R may explain why bombesin did not stimulate the proliferation of human colorectal cancer cell lines in other studies (10) because a growth effect of bombesin was only observed in cells expressing a high density of GRP-Rs (35–37). Moreover, different intracellular coupling to second messengers may also explain the lack of growth-promoting effects of bombesin in colon cancer cell lines. Indeed, the lack of growth-promoting effect of bombesin in BALB/c 3T3 fibroblasts overexpressing the GRP-R, which contrasts with the growth-promoting effects of bombesin in nontransfected Swiss 3T3 fibroblasts, was attributed to a deficient coupling to adenylate cyclase (38). The Isreco1 cell line may prove useful to study the intracellular cascade of events involved in the bombesin-stimulated growth of colon cancer cells. Indeed, this effect was observed in serum-free medium, avoiding the known interactions of bombesin with serum growth factors such as insulin and platelet-derived growth factor (39). However, we cannot exclude that autocrine growth factors may interact with the bombesin-induced proliferative effects.

Several growth factors are known to modulate cell morphology and induce rearrangements of the cytoskeleton (13, 33). In the nontransformed murine fibroblast Swiss 3T3 cell line, bombesin induced the formation of actin stress fibers and of focal adhesions, as well as the polymerization of actin within ruffles at the plasma membrane (13, 32). However, to the best of our knowledge, these effects of bombesin have not been demonstrated in transformed cells. The alterations of the cytoskeleton and cell morphology induced by bombesin in Isreco1 cells differed from that described in Swiss 3T3 cells, in that bombesin did not promote the formation of stress fibers. Interestingly, such

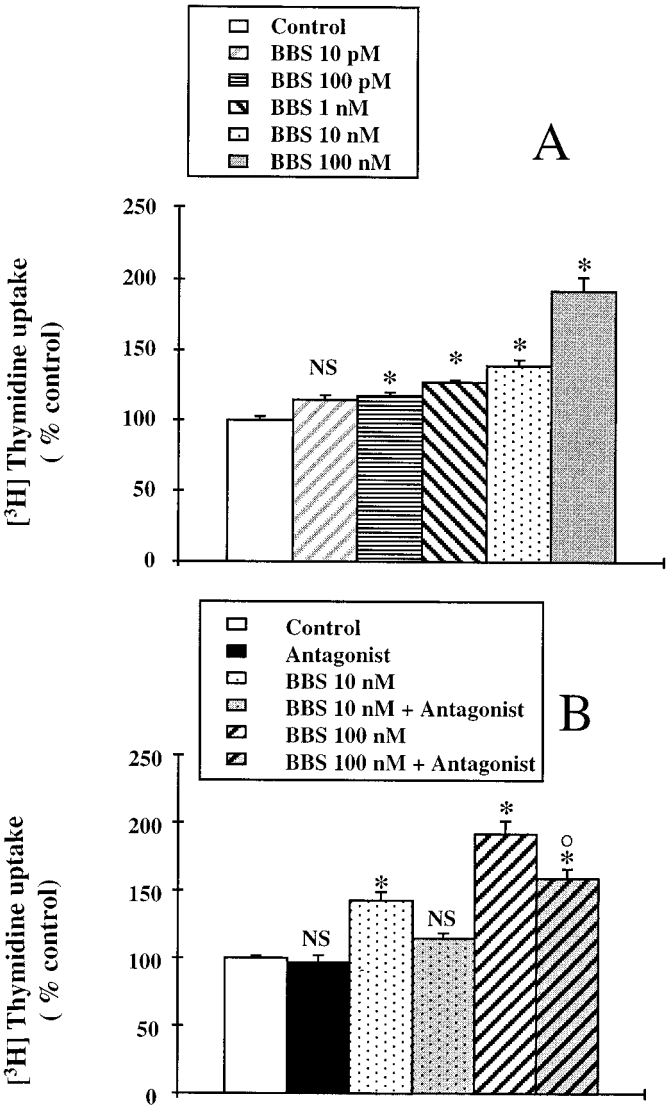

Fig. 7. Effect of bombesin (*BBS*) on Isreco1 cell proliferation. *A*, cells (5×10^4/well) were seeded in 24-well plates, cultured for 24 h in the absence or presence of increasing concentrations of bombesin, and then pulsed with [^{3}H]thymidine for 24 h. *B*, effect of the specific antagonist (D-Phe^6,Cpa^{14},ψ13–14)Bn6–14 (1 μM) on the [^{3}H]thymidine uptake induced by 10 and 100 nM bombesin. *Columns*, means of four separate experiments, each performed in duplicate; *bars*, SE. *, significantly different from control; ○, significantly different from 100 nM bombesin; *NS*, nonsignificant *versus* control.

changes in actin organization are known to be regulated by small G proteins of the Ras family (40), which are frequently altered in colon tumors (41). A mutation at codon 12 of the K-*ras* gene has been identified in Isreco1 cells, and the wild-type K-*ras* sequence was absent.[4] We showed that bombesin could induce the formation of lamellipodia in Isreco1 cells. Lamellipodia are considered to play a major role in cell adhesion and locomotion (33) and have been linked in transformed chicken fibroblasts to an increase in motility and extracellular matrix proteolysis (18).

The formation of lamellipodia and the cell adhesion process were previously shown to be closely linked to each other, and both processes have been implicated in tumor cell metastasis (18, 19). Indeed, lamellipodia formation and cell adhesion are associated with common intracellular mechanisms, such as phosphorylation of the $p125^{FAK}$ protein and formation of focal adhesions (31, 33, 42). Bombesin was shown to rapidly trigger $p125^{FAK}$ phosphorylation and/or focal adhesion formation in transformed and nontransformed cells (14, 43). In this study, the kinetics of bombesin-stimulated cell adhesion and lamellipodia formation were, however, clearly different. Cell adhesion was increased at 15 min, whereas lamellipodia formation was observed only after 4–6 h of culture in the presence of bombesin. This suggests that different, yet undetermined, intracellular mechanisms are responsible for these two effects of bombesin in Isreco1 cells. The underlying mechanisms, including the potential role of the focal adhesion-associated protein $p125^{FAK}$, remain to be clarified in further studies.

In conclusion, we showed that bombesin, in the human colon cancer cell line Isreco1, stimulates proliferation but also cell spreading and lamellipodia formation and adhesion to collagen I. This suggests that bombesin/GRP-R, which is expressed in at least 20–40% of native human colon tumors, may play a significant role in the growth of human colonic tumor cells but may also be involved in the invasion and metastatic process.

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[4] Unpublished results.