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Bombesin-Like Peptide Receptor Subtypes Promote Mitogenesis, Which Requires Persistent Receptor Signaling

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SUMMARY

Bombesin-like peptides (BLPs) can regulate the growth of normal and transformed cells. To compare the relative activities of the three known human BLP receptor subtypes [i.e., the gastrin-releasing peptide (GRP) receptor, neuromedin B (NMB) receptor, or BLP receptor subtype 3] in growth regulation, we expressed each receptor in a receptor-deficient host, Balb/3T3 cells. None of the receptor agonists used in our study promoted DNA synthesis by quiescent parental, nontransfected Balb/3T3 cells. Using clones stably transfected with the NMB receptor, however, we found that NMB stimulated the incorporation of [^{3}H]thymidine 2.5- to 8-fold over basal levels. The greatest net stimulation of [^{3}H]thymidine incorporation occurred when the medium contained insulin. In quiescent Balb/3T3 cells transfected with the GRP receptor, GRP promoted a 15-fold increase in DNA synthesis in the absence of insulin or other growth factors. GRP also induced the labeling of a large percentage (53%) of the cells with bromodeoxyuridine. To determine the length of time that GRP receptor signaling was required to drive quiescent cells into the S phase of the cell cycle, we blocked GRP receptor signaling by addition of a competitive GRP receptor antagonist at different times after stimulating cells with GRP. Our data demonstrate that persistent GRP receptor signaling throughout a large part of the G_1 phase of the cell cycle is important in the mitogenic effects of GRP in these cells. Hitherto uncharacterized GRP receptor signaling pathways may be important in this process. BLPs also stimulated a mitogenic response by transfectants expressing the BLP receptor subtype 3 if insulin was contained in the medium. Taken together, these studies indicate that all three BLP receptor subtypes may contribute to growth regulation *in vivo*.

NMB and GRP are mammalian members of a family of peptides found in amphibian skin, referred to as BLPs (1). These peptides have diverse biological effects (2), including the stimulation of cell growth, secretion in endocrine and exocrine tissues, contraction of smooth muscle, and the regulation of behavior, appetite, and homeostasis. Of particular interest has been the finding that BLPs stimulate the proliferation of breast, colon, and prostate cancer and small cell lung cancer cells *in vitro* or *in vivo* (3–7). It also has been reported that bombesin antagonists can inhibit the growth of various cancers in animal models, including prostate, pancreatic, colon, breast, and small-cell lung cancer and glioma xenografts in nude mice (8–13).

The physiological effects of BLPs in mammals may be mediated by at least three distinct receptors that are members of the seven-transmembrane domain receptor superfamily that couples to signaling pathways via heterotrimeric G proteins. The GRP receptor and NMB receptor have been cloned from rodents and humans (14–16) and have binding preferences for GRP and NMB, respectively. A third receptor was also cloned with extensive homology to the NMB and GRP receptors and termed BRS-3 (17, 18). A number of naturally occurring BLPs can activate BRS-3 expressed in *Xenopus laevis* oocytes (17) or fibroblast cells (19), but only at relatively high, nonphysiological (micromolar) concentrations, which suggests that the ligand for BRS-3 *in vivo* may be a novel peptide in the BLP family.

In small-cell lung carcinoma cell lines and potentially in other tumors, the expression of BLP receptor subtypes is heterogenous (15). Therefore, it has been of interest to determine which of the BLP receptor subtypes may be responsible for the growth-regulating effects that have been observed with BLPs. There is considerable evidence that the GRP receptor subtype can couple to mitogenic responses in a number of cells, including Swiss 3T3 fibroblasts (20) and HFL cells (21). Furthermore, BLP-stimulated mitogenesis in Swiss 3T3, HFL, and a number of cancer cell lines can be blocked by a selective antagonist of the GRP receptor subtype (8–11, 21). Efforts to directly evaluate mitogenic responses of the NMB receptor or BRS-3 in growth regulation, however,

ABBREVIATIONS: BLP, bombesin-like peptide; NMB, neuromedin B; Ac-NMB(3–10), amino-terminally acetylated neuromedin B (3–10); [Ca^{2+}]$_i$, intracellular calcium concentration; BRS-3, bombesin-like peptide receptor subtype 3; DMEM, Dulbecco's modified Eagle's medium; G418, geneticin; FGF, fibroblast growth factor; GRP, gastrin-releasing peptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFL, human fetal lung cells; MPSV, myeloproliferative sarcoma virus; BrdU, bromodeoxyuridine.

were hampered by the lack of natural, nontransformed cell lines expressing these receptor subtypes without a background expression of other BLP receptor subtypes and the lack of selective antagonists of the NMB receptor and BRS-3.

Another approach to assessing the mitogenic effects of individual BLP receptors is to ectopically express these receptors in a common host cell, which allows a direct comparison of their potential for regulating cell growth. Balb/3T3 fibroblasts, which have been used extensively to characterize effects of growth factors on the cell cycle, do not express BLP receptor subtypes and are suitable for such studies. We (21)[1] and others (22–24) have found that Balb/3T3 cells that stably express rodent or human BLP receptors couple to a number of cellular responses, including the stimulation of ligand-induced internalization, inositol phosphate formation, and the mobilization of intracellular Ca^{2+}, and provide model systems that seem to reflect the activities of BLP receptors in natural cell lines.

In this report, we characterize in detail the mitogenic activity of the GRP and NMB receptors and BRS-3, which were transfected individually into Balb/3T3 cells. All three receptors were found to promote quiescent cells to enter the S phase of the cell cycle. Although naturally expressed and ectopically expressed GRP receptors have been shown previously to promote mitogenic responses, our data provide new evidence of the role of the NMB receptor and BRS-3 in growth regulation. We also describe serum-free and growth-factor-free conditions in which the activation of the GRP receptor in Balb/3T3 cells could drive a large percentage of quiescent cells into the S phase. By use of a selective pure antagonist of GRP receptor-mediated mitogenic effects, F_5-D-Phe^6-D-Ala^{11}-BN(6–13) methyl ester, we were able to demonstrate that prolonged signaling through the GRP receptor, continuing through a large part of the G_1 phase of the cell cycle, was required to promote quiescent cells into the S phase of the cell cycle.

Experimental Procedures

Materials. GRP(1–27) and NMB were purchased from either Peninsula Laboratories (Belmont, CA) or Bachem California (Torrance, CA). Ac-NMB(3–10) was custom synthesized by Multiple Peptide Systems (San Diego, CA). F_5-D-Phe^6-D-Ala^{11}-BN(6–13) methyl ester was provided by Biomeasure (Milford, MA). G418 was purchased from GIBCO/BRL (Gaithersburg, MD) and Hygromycin B from Calbiochem (La Jolla, CA). All other cell culture media, supplements, and fetal calf serum were purchased from BioWhittaker (Walkersville, MD). Bovine serum albumin (fatty acid free), sulfinpyrazone, bacitracin, polyethyleneimine, and HEPES were purchased from Sigma Chemical (St. Louis, MO). ^{125}I-GRP (~2000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) and [methyl-^{3}H]thymidine (2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals used were of reagent grade.

Expression plasmids and stable transfection of Balb/3T3 cells. Balb/3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Balb/3T3 cells expressing the human GRP receptor that were used in this study (GR1 cells) were generated as described previously (21). cDNAs encoding the human form of the NMB receptor (15) were cloned into an expression vector derived from pCD2 (21), which drives expression via the simian virus 40 promoter. BRS-3 (17) was also cloned into the expression vector pBBS70, described previously (21), which drives expression from the MPSV promoter. Stable transfection of cells with either vector was achieved using a calcium phosphate precipitation procedure and selection with G418 or hygromycin (21). Transfected cells were grown in DMEM, supplemented with 4.5 g/liter glucose, 10% fetal bovine serum, 2 mM glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 270 μg/ml G418 or 100 μg/ml hygromycin at 37° in a humidified atmosphere containing 10% CO_2.

Analysis of intracellular calcium concentration. Intracellular calcium was measured in cells loaded with the dye Fura-2 essentially as described previously (21). To load the dye, cells were incubated with Fura-2 acetoxymethyl ester (2.5 μM) for 45 min in Roswell Park Memorial Institute 1640 culture medium and 25 mM HEPES, pH 7.5, 0.25 mM sulfinpyrazone, and 0.1% bovine serum albumin at a density of 500,000 cells/ml. This medium contained 2 mM $CaCl_2$. Cells were washed and then suspended at a density of 200,000 cells/ml in 50 mM HEPES, pH 7.5, 2 mM EDTA, 5 mM $MgSO_4$, 5 mM KCl, 0.13 M NaCl, 2 mM $CaCl_2$, 0.25 mM sulfinpyrazone, and 0.1% bovine serum albumin. The effect of peptides on Fura-2 fluorescence at 510 nm was determined at 37° using a PTI Deltascan 4000 fluorometer (Photon Technology, South Brunswick, NJ) equipped with dual excitation monochromators set at 340 and 380 nm. The concentration of unchelated extracellular calcium under our experimental conditions ranged from 50 to 100 μM, as determined from the fluorescence of Fura-2 in the presence of Triton X-100.

Mitogenesis assays. The ability of BLP receptors expressed in quiescent Balb/3T3 cells to stimulate [^{3}H]thymidine incorporation was measured by modification of the protocol described previously (21). Briefly, cells (3400 per well) were grown in 96-well plates as described above for 4–5 days, at which time they were confluent. The medium was then changed to assay medium (DMEM/Waymouth's, 1:1), supplemented with 1% glutamine, 1.25 mg/ml bovine serum albumin, 6.3 ng/ml selenious acid, and 6.3 μg/ml transferrin. Where indicated in the figure legends, 6.3 μg/ml insulin or 5.4 μg/ml linoleic acid was also present. Cells were incubated at 37° in an atmosphere containing 5% CO_2 for 24 hr. BLPs, with or without the receptor antagonist BIM-26226, were prepared in assay medium supplemented with antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin) and added to the cells to yield the final concentrations specified in the figure legends. Sixteen hours after addition of BLP, cells were pulse-labeled for 2 hr with [methyl ^{3}H]thymidine (1 μCi/well) at 37° in an atmosphere containing 5% CO_2. The medium was then aspirated and the cells, which were detached by trypsin treatment, were collected onto 96-well glass-fiber filter plates using a Packard Filtermate harvester (Packard, Meriden, CT). Radioactivity on the filters was determined by liquid scintillation counting using a Packard Topcount (Packard). Incorporated counts were less than 2% of the total counts added. All treatments were performed in quadruplicate for each experiment.

BrdU labeling of cell nuclei. Cell growth conditions and assay conditions were essentially the same as those used to assay BLP-stimulated incorporation of [^{3}H]thymidine by quiescent cells, as described above, except that cells were grown onto 2-cm^2 chamber slides. Sixteen hours after treatment of quiescent cells with BLPs, the cells were labeled with 10 μM BrdU (Becton Dickinson, San Jose, CA) for 4 hr. The cells were then washed twice with phosphate-buffered saline and fixed in methanol. Cells were treated in 2 N HCl for 10 min to denature chromatin and, after neutralization, were stained with an anti-BrdU/monoclonal antibody/fluorescein conjugate. Labeled nuclei were counted using fluorescent microscopy. Total nuclei were counted after counterstaining with Hoechst dye.

Other methods. ^{125}I-GRP and ^{125}I-Tyr^{4}BN binding assays were performed as described previously (25). The binding medium contained 50 mM HEPES, pH 7.5, 2 mM EDTA, 0.13 M NaCl, 5 mM $MgSO_4$, 5 mM KCl, 10 mg/ml bovine serum albumin, and 30 μg/ml bacitracin. Binding reactions were initiated by the addition of 0.02 nM ^{125}I-GRP or ^{125}I-Tyr^4-BN and were carried out at 37° for 1 hr. Data from competitive displacement experiments were used to cal-

[1] J. M. Wu and R. I. Feldman, unpublished observations.

culate K_D and K_I values using the computer program LIGAND (provided by Dr. Peter J. Munson, National Institutes of Health, Bethesda, MD) (26).

Results

NMB receptor coupling to DNA synthesis in Balb/3T3 cells. Human NMB receptor cDNA was expressed stably in Balb/3T3 cells using the expression vector pCD2. Before transfection, Balb/3T3 cells expressed levels of neither GRP, NMB receptor, nor BRS-3 mRNA detectable by Northern analysis, nor high affinity binding sites for ^{125}I-GRP and ^{125}I-Tyr^4-bombesin. Furthermore, a variety of BLPs, including NMB, GRP, and Ac-NMB(3–10), failed to stimulate an increase in mobilization of intracellular calcium in nontransfected Balb/3T3 cells, which is a response characteristically promoted by BLP receptors in fibroblast cells and other cell types.

To identify transfectants expressing the NMB receptor, Balb/3T3 clones were assayed for high affinity ^{125}I-Tyr^4-bombesin binding sites. Two clones, termed NR2 and NR4, obtained from this procedure were used for subsequent experiments. Based on analysis of competitive binding data using the computer program LIGAND (26), intact NR2 and NR4 cells expressed 4.0×10^5 and 4.5×10^5 high affinity ^{125}I-Tyr^4-bombesin binding sites per cell, respectively (K_D = 4.2 nM). Furthermore, NMB (100 nM) triggered a rapid increase in Ca^{2+} mobilization in NR2 cells and NR4 cells (data not shown), which was monitored by the fluorescence of the dye Fura-2. Taken together, these data demonstrate that NR2 and NR4 cells express abundant levels of functional NMB receptors.

To determine whether activation of the NMB receptor leads to mitogenic responses in Balb/3T3 cells, we examined the effect of NMB on [^{3}H]thymidine incorporation by confluent, growth-factor-fasted NR4 cells (Fig. 1). For these experiments, cells were grown to confluence in DMEM containing 10% fetal calf serum and then switched to a serum-free assay medium containing insulin and linoleic acid. As shown in Fig. 1, three BLPs with high affinity for the NMB receptor, NMB, bombesin, and GRP, promoted a dose-dependent, 6- to 8-fold stimulation of [^{3}H]thymidine incorporation by NR4 cells. The rank order potency of the peptides [i.e., NMB (EC_{50} = 0.11 nM) > bombesin (EC_{50} = 0.14 nM) > GRP (EC_{50} = 1.3 nM)] was consistent with the relative potencies of these peptides for the NMB receptor established previously. As shown in Fig. 1B, NMB and two other BLP peptides [GRP and Ac-NMB(3–10)] used in the studies discussed below had no effect on [^{3}H]thymidine incorporation by nontransfected Balb/3T3 host cells. Vector-alone transfectants were also unresponsive to BLPs (data not shown). Taken together, the data indicate that mitogenic effects of BLPs observed on NR4 cells were mediated through the human NMB receptor.

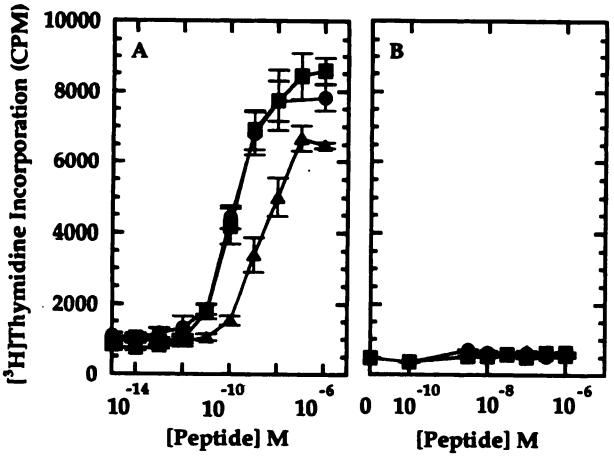

Fig. 1. BLPs stimulate [^{3}H]thymidine synthesis by quiescent NR4 cells expressing the NMB receptor. A, The dose response for NMB (●), bombesin (■), or GRP (▲) on [^{3}H]thymidine incorporation by confluent and serum-fasted NR4 cells in medium containing insulin and linoleic acid. B, Effect of NMB (●), GRP (■), or Ac-NMB(3–10) (▲) on [^{3}H]thymidine incorporation on control, nontransfected Balb/3T3 cells under the same conditions used for A. [^{3}H]thymidine incorporation was measured as described in Experimental Procedures.

Using NR2 cells, we also determined the effect of insulin on NMB-stimulated mitogenesis. NMB stimulated [^{3}H]thymidine incorporation by NR2 cells in medium with or without insulin roughly 3-fold and 6-fold, respectively (Fig. 2). Furthermore, control experiments demonstrate that NMB had no significant effect on [^{3}H]thymidine incorporation by nontransfected Balb/3T3 cells under either condition used (Figs. 1B and 2C). In terms of net incorporation of [^{3}H]thymidine, however, NMB promoted a roughly 3-fold higher response in medium containing insulin. In experiments using NR4 cells, we also found that the net incorporation of [^{3}H]thymidine was increased significantly in the presence of insulin.

To further understand these effects, we analyzed the efficiency of NMB-stimulated mitogenesis in the presence and absence of insulin by measuring the percentage of NR2 cell nuclei labeled with BrdU. In the absence of NMB, with and without insulin, about 5–7% of the cells were labeled with BrdU (Table 1). In the presence of insulin, NMB stimulated labeling of an additional 9% of the cell nuclei, whereas in the absence of insulin, NMB stimulated a 13% increase in labeled nuclei (Table 1). These data indicate that somewhat more cells progress into S phase of the cell cycle in media not containing insulin than in media containing insulin. Because there was a lower net incorporation of [^{3}H]thymidine in the absence of insulin, the data further suggest that progression of cells through S phase may become slowed or blocked when insulin is not present. Because cells were transferred into assay medium 24 hr before addition of NMB, NR2 and NR4 cells may require some growth factors in the medium to maintain their competence for cell division.

GRP receptor coupling to DNA synthesis. Previously, we generated Balb/3T3 cells stably transfected with the human GRP receptor (21). These cells, designated as GR1 cells, express 46,000 ^{125}I-GRP binding sites per cell (K_D = 10 pM) (21). In addition, we found that GRP promotes an increase in $[Ca^{2+}]_i$ in GR1 cells. In the presence of insulin, GRP also stimulates DNA synthesis by confluent, serum-fasted GR1 cells about 3- to 5-fold (21). We also observed that GRP stimulated DNA synthesis in six other isolated Balb/3T3 clones, which ectopically expressed from 1×10^5 to 1.3×10^6 GRP receptors per cell (data not shown) (25). In these clones, which expressed higher levels of GRP receptor than GR1 cells, the dose response for GRP was bell-shaped because of growth inhibitory effects of higher levels of GRP receptor activation (25).

Because GR1 cells are not sensitive to the growth inhibitory effects of GRP, we used this cell line to further investigate conditions important for GRP-stimulated mitogenic ef-

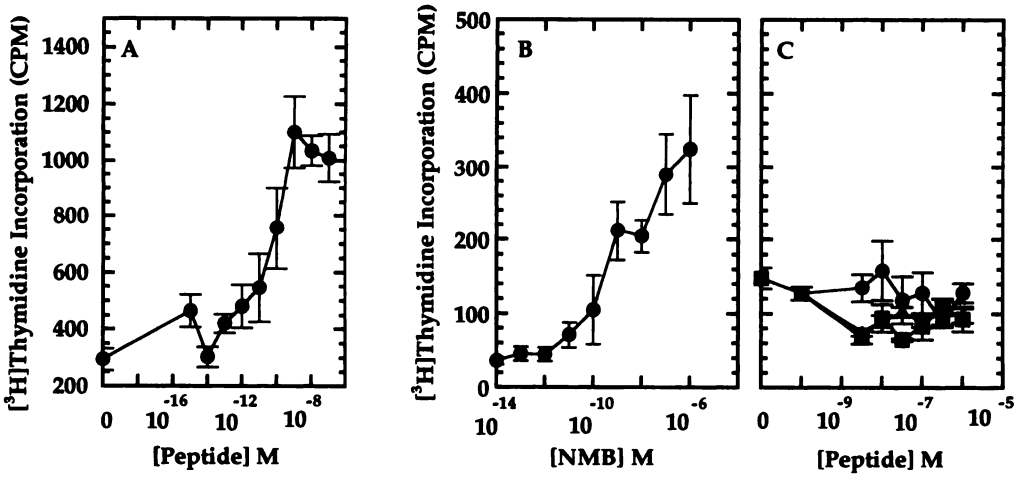

Fig. 2. Effect of insulin and linoleic acid on NMB-stimulated [^{3}H]thymidine synthesis by quiescent NR2 cells expressing the NMB receptor. A dose response of NMB-stimulated [^{3}H]thymidine incorporation by confluent and serum-fasted NR2 cells was performed either in medium containing insulin and linoleic acid (A) or in medium lacking these components (B). C, The effect of NMB (●), GRP (■), or Ac-NMB(3–10) (▲) on [^{3}H]thymidine incorporation on control, nontransfected Balb/3T3 cells in a medium not containing insulin and linoleic acid.

TABLE 1

Percentage of quiescent Balb/3T3 cell nuclei labeled with BrdU in response to activation of ectopically expressed GRP, NMB receptors or BRS-3

The synthesis of DNA in individual nuclei of Balb/3T3 clones expressing different BLP receptor isoforms was determined as described in Experimental Procedures. Where indicated, insulin (6.3 μg/ml) and linoleic acid (5.4 μg/ml) were added when cells were switched to serum-free media 24 hr before stimulation with BLPs.

Balb/3T3 transfectant	Receptor expressed	Additions	Nuclei synthesizing DNA
			%
GR1	GRP receptor	Vehicle	3.7 ± 1.4
		GRP[a]	53 ± 3.5[e]
		GRP[a] + BIM 26226[b]	5.3 ± 0.73
NR2	NMB receptor	Vehicle	6.8 ± 0.16
		NMB[c]	20 ± 1.6[e]
		Vehicle + insulin	5.2 ± 1.0
		NMB[d] + insulin	14 ± 3.0[e]
BR2	BRS-3	Vehicle	0.75 ± 0.75
		Ac-NMB (3–10)[d]	2.8 ± 0.61
		Vehicle + insulin	19 ± 3.0
		Ac-NMB (3–10)[d] + insulin	36 ± 4.1[e]

[a] added at 1 nM.
[b,d] added at 1 μM.
[c] added at 100 nM.
[e] $p < 0.01$, to vehicle control.

fects. Using the basal conditions employed previously (21) (i.e., media containing insulin and linoleic acid), we found that BrdU labeled 20% of the cell nuclei, which indicates that a relatively high percentage of cells were not quiescent. Consistent with this, insulin also promoted a dose-dependent increase in [^{3}H]thymidine incorporation by confluent, serum-fasted GR1 cells (data not shown). In contrast, without the presence of insulin and linoleic acid, a much larger number of cells seemed to be quiescent, because only 3.7% of the cell nuclei were labeled with BrdU in the absence of GRP (Table 1). Therefore, we used these conditions to examine the mitogenic effects of GRP on GR1 cells. Without insulin or linoleic acid, GRP induced more than 50% of the cell population to become labeled with BrdU. Without other growth factors, therefore, GRP can efficiently drive quiescent GR1 cells into the S phase of the cell cycle. Unlike its effect on GR1 cells, however, insulin did not increase the basal labeling of NR2 cells with BrdU, as discussed above. Furthermore, NMB had greater mitogenic effects in medium containing insulin using NR2 and NR4 cells (Figs. 1 and 2). Therefore, some clonal variation was apparent among our collection of BLP receptor transfected Balb/3T3 cell lines.

Consistent with the BrdU labeling data using GR1 cells, treatment of GR1 cells with GRP in the absence of insulin resulted in a roughly 15-fold increase in the amount of [^{3}H]thymidine incorporated over basal levels (EC_{50} = 88 pM) (Fig. 3). GRP had no effect on the parental Balb/3T3 cells under these conditions (Fig. 2C), indicating that the response of GR1 cells measured was dependent upon expression of the GRP receptor. In terms of both the net amount of [^{3}H]thymidine incorporated and the fold stimulation of [^{3}H]thymidine incorporated over basal levels, GRP gave a better response in the absence of insulin and linoleic acid. This is probably because GR1 cells were better synchronized in a quiescent state under growth-factor-free conditions.

The GRP antagonist BIM-26226 [F_5-D-Phe^6-D-Ala^{11}-BN (6–13) methyl ester], which has been shown to block GRP-stimulated amylase release from AR4-2J rat pancreatic car-

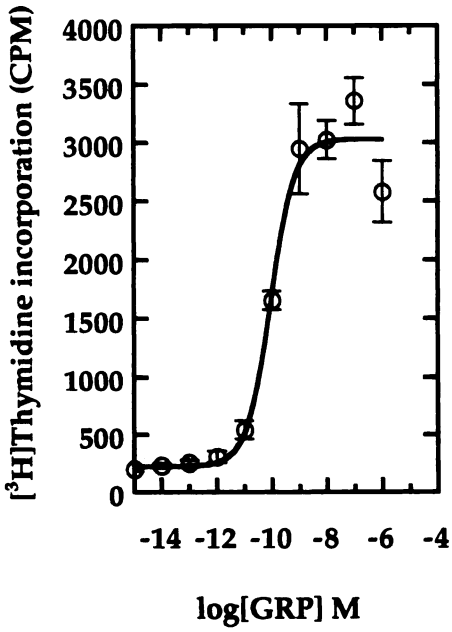

Fig. 3. GRP stimulates [^{3}H]thymidine incorporation by quiescent Balb/3T3 cells expressing the GRP receptor. [^{3}H]thymidine incorporation in response to GRP was measured in confluent and serum-fasted GR1 cells as described in Experimental Procedures.

cinoma cells (27), completely blocked GRP-stimulated incorporation of BrdU (Table 1), which further demonstrates that the response was mediated through GRP receptors. BIM-26226 also potently blocked DNA synthesis stimulated in quiescent GR1 cells by 1 nM GRP (IC_{50} = 10 nM) but on its own exhibited no agonist activity in this assay (data not shown). Thus, BIM-26226 was also a pure antagonist of the human GRP receptor. Furthermore, at a concentration of 30 nM, BIM-26226 shifted the EC_{50} for stimulation of DNA synthesis by GRP about 30-fold while having no effect on the size of the maximal GRP response (Fig. 4). This result indicates that BIM-26226 acts as a competitive antagonist of GRP-stimulated mitogenesis, consistent with studies of similar compounds in the literature (28). Taken together, our studies demonstrate that the GRP receptor promoted quiescent Balb/3T3 cells to enter the S phase of the cell cycle with a high degree of efficiency, even in the absence of other growth factors.

Mitogenic responses required prolonged activation of the GRP receptor. A large number of cellular signaling responses have been correlated with the promotion of mitogenesis by GRP and other growth factors. In most cases, these responses are triggered within minutes and are transient. GRP, without other growth factors, can efficiently drive both the entry of quiescent GR1 cells into the cell cycle and their progression into the S phase of the cell cycle; however, what duration of direct GRP receptor signaling was required to promote this response? To address this question, we selectively blocked the GRP receptor at various times after stimulation with GRP by addition of the antagonist BIM-26226. As discussed above, we found BIM-26226 to be a potent blocker of mitogenic effects mediated by the human GRP receptor in Balb/3T3 cells.

The effect of adding BIM-26226 to quiescent GR1 cells at various times after adding GRP is shown in Fig. 5. Addition of BIM-26226 at early times after stimulating cells with GRP resulted in a near total inhibition of GRP-mediated mitogenic effects. When added at times up to 10 hr after stimulating quiescent cells with GRP, however, BIM-26226 continued to have some detectable inhibitory effect on progression of cells into S phase of the cell cycle (Fig. 5). This period corresponds roughly to the time required for quiescent cells to enter the S phase of the cell cycle after stimulation with GRP (data not shown). From these experiments, it is apparent that progression of cells into the S phase of the cell cycle requires that continuous GRP receptor signaling occur throughout a large percentage of the G_1 phase of the cell cycle. This is consistent with the BrdU labeling data presented above, which demonstrates that GRP, in the absence of other growth factors, can efficiently stimulate quiescent GR1 cells to enter the S phase of the cell cycle and suggests that direct receptor-mediated signaling events drive Balb/3T3 cells through critical G_1 cell cycle checkpoints.

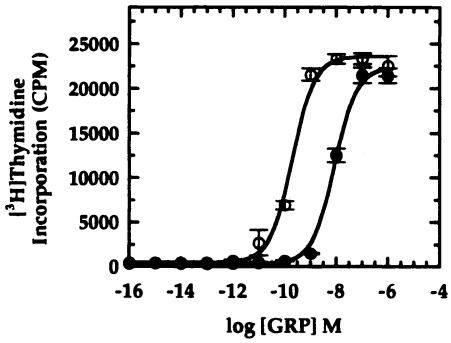

Fig. 4. The effect of BIM-26226 on the dose response of gastrin-releasing peptide (GRP) stimulated [^{3}H]thymidine incorporation by quiescent Balb/3T3 cells expressing the GRP receptor. The effect of various doses of GRP in the presence (●) or absence (○) of BIM-26226 (30 nM) on [^{3}H]thymidine incorporation by quiescent GR1 cells was determined as described in Experimental Procedures.

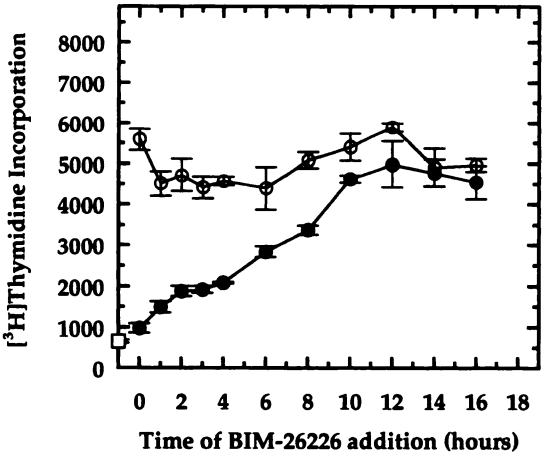

Fig. 5. The effect of BIM-26226, added at various times after stimulation of quiescent Balb/3T3 cells expressing the GRP receptor with GRP, on GRP stimulated [^{3}H]thymidine incorporation. Stimulation of [^{3}H]thymidine incorporation by quiescent GR1 with 1 nM GRP was determined as described in Experimental Procedures, except that the GRP antagonist BIM-26226 (1 μM) (●) or vehicle (○) was added at the various times after addition of GRP, as indicated in the figure. □, Basal level of [^{3}H]thymidine incorporation.

BRS-3 coupling to DNA synthesis in Balb/3T3 cells. To address the ability of BRS-3 to promote cell growth, we followed the strategy used for the GRP and NMB receptors of transfecting BRS-3 into Balb/3T3 cells and examining its mitogenic effects. BRS-3 expressing clones were identified using Northern analysis. Because a high affinity BRS-3 ligand that was suitable for receptor-binding studies has not been discovered, we were not able to directly measure BRS-3 receptor-binding activity in these cells. Nevertheless, the two representative clones chosen for further study, termed BR1 and BR2, expressed abundant levels of BRS-3 mRNA.

Before characterizing the functional activities of BRS-3, it was necessary to identify a BRS-3 agonist. At micromolar concentrations, NMB can activate BRS-3 expressed in Balb/3T3 cells, as assessed by its ability to promote an increase in the $[Ca^{2+}]_i$. NMB was also shown to activate BRS-3 expressed in *X. laevis* oocytes (17). In addition, we found that an analog of NMB, Ac-NMB(3–10), in which an acetyl moiety replaced the first two amino acids at the amino terminus, was a significantly more potent stimulator of BRS-3-stimulated calcium mobilization in BRS-3-transfected Balb/3T3 cells (EC_{50} = 200 nM) than was full-length NMB (19).

The ability of Ac-NMB(3–10) to promote Ca^{2+} mobilization by BR2 cells, as assessed by Fura-2 fluorescence, is shown in Fig. 6. Ac-NMB(3–10) also promoted Ca^{2+} mobilization by BR1 cells (data not shown). These studies confirmed that BRS-3 was expressed in a functional form in BR1 and BR2 cells and provided a suitable BRS-3 agonist, Ac-NMB(3–10), to assess the effect of BRS-3 activation on DNA synthesis by

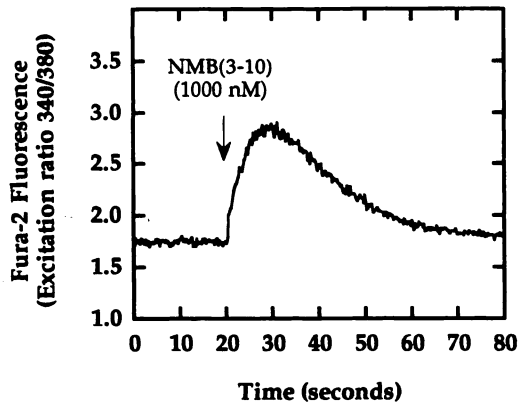

Fig. 6. Ac-NMB(3–10) promotes an increase in $[Ca^{2+}]_i$ in Balb/3T3 cells expressing the BRS-3. $[Ca^{2+}]_i$ was monitored with the dye Fura-2 as described in Experimental Procedures. Shown is the effect of 1 μM Ac-NMB(3–10) on the concentration of intracellular calcium in BRS-3 receptor transfected Balb/3T3 cells (BR2 cells) as a function of time.

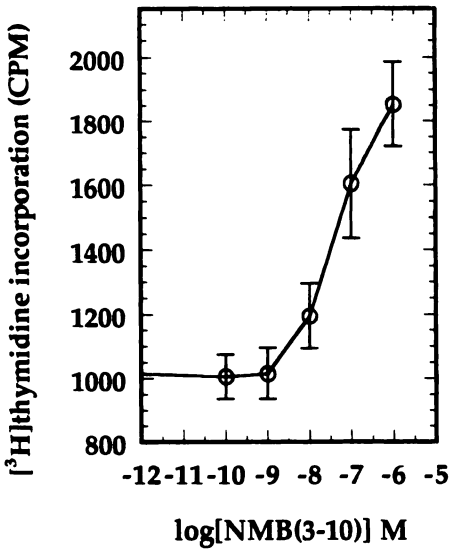

Fig. 7. Ac-NMB(3–10) stimulates [^{3}H]thymidine synthesis by quiescent Balb/3T3 cells expressing BRS-3. [^{3}H]thymidine incorporation in response to Ac-NMB(3–10) was measured in confluent and serum-fasted BR2 cells in an insulin-containing media as described in Experimental Procedures.

quiescent Balb/3T3 cells. Because we demonstrated previously that fewer GRP receptors are required to promote receptor-mediated mitogenic effects than calcium mobilization in Balb/3T3 cells (21), the data suggest that ample levels of BRS-3 receptors are expressed in BR1 and BR2 cells to study their mitogenic responses.

In repeated attempts, however, we failed to demonstrate that Ac-NMB(3–10), in the absence of other growth factors, had a significant effect on [^{3}H]thymidine incorporation by Balb/3T3 cells expressing BRS-3. Various doses of Ac-NMB(3–10) were used, to account for the possibility that the activation of too large a number of receptors can possibly produce a growth inhibitory effect, as has been observed in studies of transfected GRP receptors (25). In addition, we did not detect a significant effect of Ac-NMB(3–10) on the percentage of cells entering the S phase of the cell cycle under these conditions, which we assessed by labeling individual cell nuclei synthesizing DNA with BrdU (Table 1). In the absence of Ac-NMB(3–10), less than 1% of the cells were in the S phase of the cell cycle during the labeling period, which shows that the cells were quiescent.

In contrast to these results, treatment of BRS-3-transfected Balb/3T3 cells (BR2 cells) with Ac-NMB(3–10) in the presence of insulin and linoleic acid (Fig. 7) resulted in a relatively small but significant 1.5- to 2-fold increase in [^{3}H]thymidine incorporation (apparent EC_{50} = 500 nM). The potency of Ac-NMB(3–10) in stimulating thymidine incorporation was similar to its potency in elevating Ca^{2+} mobilization in Balb/3T3 cells expressing BRS-3 (19). Furthermore, control experiments showed that Ac-NMB(3–10) did not have a significant effect on [^{3}H]thymidine incorporation by nontransfected Balb/3T3 cells under the conditions used (Fig. 1B). These experiments confirm that the mitogenic effect of Ac-NMB(3–10) was mediated by BRS-3 receptors.

We also found that Ac-NMB(3–10), in the presence of insulin and linoleic acid, stimulated a 1.9-fold increase in the labeling of BR2 nuclei with BrdU, consistent with the [^{3}H]thymidine incorporation data presented above. In the presence of Ac-NMB(3–10), 36% of the BR2 cell nuclei became labeled with BrdU, compared with 19% of the BR2 nuclei in the absence of Ac-NMB(3–10). This relatively high basal labeling of nuclei in the presence of insulin, compared with the absence of insulin (<1%), was consistent with experiments showing that insulin promoted DNA synthesis in quiescent nontransfected Balb/3T3 host cells. Taken together, our experiments indicate that BRS-3 activation can have a significant mitogenic effect on Balb/3T3 cells if another growth factor (i.e., insulin) is also present.

Discussion

In this study, we demonstrated that the three known human BLP receptor subtypes can promote entry of quiescent cells into the S phase of the cell cycle when expressed in Balb/3T3 cells. The use of a common host cell for assessing the activity of each BLP receptor subtype minimized signaling differences found in different cell types and allowed us to gain insight into the relative activities of BLP receptor subtypes.

Consistent with previous results, we demonstrate that the GRP receptor can stimulate quiescent Balb/3T3 cells to enter the S phase of the cell cycle. These effects were not limited to the single cell line described herein (i.e., GR1 cells), because GRP stimulated mitogenesis in six other Balb/3T3 transfectants we tested. In many of these other clones, however, growth inhibitory effects were seen at high GRP concentrations that could be attributed to a level of GRP receptor expression above 10^5 receptors per cell (data not shown) (25). GRP robustly stimulated [^{3}H]thymidine incorporation (15-fold) by quiescent GR1 cells, which are not sensitive to the growth inhibitory effects of GRP, in serum-free medium that lacked other growth factors. By labeling cells with BrdU, we further showed that GRP stimulated a large percentage of quiescent GR1 cells (about 50%) to enter the S phase of the cell cycle under these conditions. Our finding that the GRP receptor can efficiently provide both the competence and progression signals required to drive quiescent fibroblast cells through critical G_1 checkpoints and into the S phase of the cell cycle has not been demonstrated previously.

Consistent with these findings, we found that the mitogenic activity of GRP depended on continued GRP receptor

signaling throughout most of the G_1 phase of the cell cycle. This was demonstrated by selectively blocking the GRP receptor at various times after addition of GRP using the competitive GRP receptor antagonist BIM-26226. Similar studies using cells expressing the NMB receptor or BRS-3 may be performed if suitable antagonists for these receptors are developed. Our results are analogous to those obtained for FGF-1 on Balb/3T3 cells, which express FGF receptors. The continued presence of FGF-1 is required throughout the 12-hr G_1 phase of the cell cycle to achieve a maximal mitogenic effect of the growth factor (29). FGF-1 was further shown to stimulate the phosphorylation of a number of proteins on tyrosine residues that persisted late into the G_1 phase of the cell cycle (29, 30).

We have also found that GRP, in the absence of other growth factors, can stimulate DNA synthesis in quiescent, nontransformed HFL cells, which also naturally express the GRP receptor. Under these conditions, GRP stimulated a response in roughly 10–15% of the HFL cells (unpublished data). Therefore, our results obtained in cells transfected with the GRP receptor appear to reflect the activity of the GRP receptor in nontransfected human fibroblast cells.

Further studies using GR1 cells may allow elucidation of the signaling pathways activated by the GRP receptor that are necessary for transit of cells past critical G_1 checkpoints. To date, GRP receptor signaling has been studied most extensively in murine Swiss 3T3 cells. In these cells, GRP stimulates early responses to GRP receptor activation, including the stimulation of phospholipase C, protein kinase C, intracellular calcium elevation, arachidonic acid formation, MAP kinase activation, phosphorylation of focal adhesion kinase and other proteins on tyrosine residues, and the induction of c-*fos* and c-*myc* (31–37). These responses reach a maximum within minutes of GRP treatment, and it is not clear to what extent they persist into the G_1 phase of the cell cycle. Therefore, it seems likely that other signal pathways coupled to the GRP receptor may be important in driving cells through critical G_1 checkpoints and into the S phase of the cell cycle. The GRP receptor offers two advantages as a model receptor in which to study the coupling of cell surface receptors to cell cycle control: GRP alone can stimulate a mitogenic response in a large percentage of the cells, and the GRP receptor can be shut down at different times after stimulation with GRP by the simple addition of a potent and selective GRP receptor antagonist.

In light of studies demonstrating that GRP receptor agonists trigger rapid GRP receptor internalization (22, 38), it is interesting that the mitogenic response of the GRP receptor results from receptor signaling that lasts for many hours. It may be possible that a relatively small pool of GRP receptors remaining in an active state at the cell surface is responsible for this long-lived activity. Alternatively, if receptors are recycled to the cell surface in a dynamic process, signaling may be promoted by receptors returned to the cell surface, or indeed, from receptors within intracellular compartments.

Although our studies and others demonstrate a role for BLP receptors in promoting growth, we have also found that GRP can greatly inhibit the rate of proliferation of Balb/3T3 cells ectopically expressing the GRP receptor, provided that the expression level of the GRP receptor exceeded about 10^5 human GRP receptors per cell. We did not observe such inhibitory effects in this study because we used GR1 cells, which express the GRP receptor at a level below this threshold value. Growth inhibitory effects of GRP are not restricted to cells transfected with GRP receptors. For example, high levels of BLPs were found to inhibit [^{3}H]thymidine incorporation by Swiss 3T3 cells (39) and the colony plating efficiency of small-cell lung cancer cell lines in soft agar (5). Furthermore, the growth of several pancreatic tumor cell lines in nude mice was inhibited in those animals that were infused with GRP (40, 41). These observations indicate that the mitogenic effects of BLP receptors can depend greatly on their cellular context.

Another significant finding of this study is that activation of the BRS-3 in quiescent host Balb/3T3 cells can promote the entry of cells into the S phase of the cell cycle. To study BRS-3-mediated mitogenesis, we first had to discover a suitable ligand. Interestingly, Ac-NMB(3–10) displayed a relatively high potency for activation of BRS-3, as assessed by measuring a calcium mobilization response, compared with other BLPs (19). Although Ac-NMB(3–10) stimulated a significant level of [^{3}H]thymidine incorporation and promoted BrdU labeling of quiescent BR2 cells, the response was relatively small compared with that promoted by GR1 cells. It is possible that this response can be increased through optimization of conditions, BRS-3 expression levels, or the use of more potent agonists. Nevertheless, our data provide the strongest evidence reported that BRS-3 may have growth regulatory roles *in vivo*.

Our data also further establish a potential role of the human NMB receptor in growth regulation. We found that NMB increased the basal rate of [^{3}H]thymidine incorporation by Balb/3T3 cells expressing the human NMB receptor by up to 8-fold. No growth inhibitory effects of NMB were observed at concentrations up to 1 μM. In previous studies, 10 nM NMB promoted a 1.5- to 3-fold stimulation of [^{3}H]thymidine incorporation by Balb/3T3 cells transfected with the rat NMB receptor (39). At higher levels of NMB, however, [^{3}H]thymidine incorporation was inhibited greatly. This may have been caused by the activation of the larger pool of receptors that were expressed on these cells [i.e., 8×10^5 receptors per cell (39) versus 4×10^5 receptors per cell expressed by NR2 and NR4 cells]. It is also possible that differences in human and rat NMB receptors, host cell clones, or the conditions employed account for differences in the results. We also found that in the absence of insulin, the NMB receptor can stimulate entry of cells into the S phase of the cell cycle, although the efficiency of [^{3}H]thymidine incorporation by NR2 or NR4 cells was reduced under these conditions.

Compared with the GRP receptor, little is known about the signaling pathways coupled to the NMB receptor or BRS-3. In C-6 rat glioblastoma cells, or in Balb/3T3 cells transfected with the NMB receptor, activation of the NMB receptor couples to stimulation of phospholipase C activity and calcium mobilization (22–24). In this article, we show that BRS-3 also couples calcium mobilization in Balb/3T3 cells. When expressed in *X. laevis* oocytes, activation of BRS-3 promotes a chloride current (17). Further elucidation of the intracellular signaling pathways of each BLP receptor in our Balb/3T3-transfected cell lines may provide insight into the differences we observe in their mitogenic activity.

BLPs have been found to stimulate the growth of a number of different cancers. Our finding that each of the three known BLP receptor subtypes can stimulate a mitogenic response in

model Balb/3T3 cells means that any of these receptors could potentially mediate these effects. Although limited information is available on the expression of different BLP receptor subtypes in different cancers, the expression of GRP and NMB receptor and BRS-3 mRNA has been studied in small-cell lung cancer. mRNA for each of the receptor subtypes was detected in a subset of small-cell lung cancer cell lines examined (15, 17). We have also observed that GRP and NMB receptor mRNA but not BRS-3 mRNA were expressed in a high percentage of human prostatic carcinoma tumor biopsy specimens examined.[2]

In summary, we have used Balb/3T3 cells transfected with the human GRP and NMB receptors and BRS-3 to investigate the mitogenic responses of activating these receptors in a single, well characterized, nontransformed host cell type. The GRP receptor produced the most robust mitogenic responses, although all three receptors could promote significant mitogenic responses, depending on the conditions. We also demonstrated that prolonged activation of the GRP receptor was required until late in the G_1 phase of the cell cycle to achieve a maximal mitogenic response in Balb/3T3 cells. These studies suggest that hitherto uncharacterized receptor signaling pathways may be critical in the growth-regulating activity of the GRP receptor.

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