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Gastrin-Releasing Peptide Receptor Signaling Resulting in Growth Inhibition

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SUMMARY

We demonstrate that gastrin-releasing peptide (GRP) can inhibit the proliferation of human immortal nontumorigenic (184-B5) mammary epithelial cells ectopically expressing the human GRP receptor. Growth of Balb 3T3 cells ectopically expressing relatively high levels of the GRP receptor was also inhibited by GRP; however, growth of transfectants expressing lower levels of the receptor was not inhibited. Compared with Balb 3T3 cells, mammary epithelial cells could be rendered sensitive to growth inhibition by GRP by the expression of fewer GRP receptors. GRP also stimulated DNA synthesis in quiescent, serum-starved Balb 3T3 transfectants. In clones that were sensitive to growth inhibition by GRP by virtue of their expression of relatively high levels of the GRP receptor, the dose-response curve for GRP-stimulated DNA synthesis was bell shaped. This is consistent with our conclusion that the growth-inhibiting activity of GRP required the activation of a relatively large pool of receptors in Balb 3T3 cells. Significantly, prostaglandin H synthase inhibitors, which block the production of prostaglandins from arachidonic acid, reduced GRP-inhibitory effects on DNA synthesis. We also compared a number of GRP-stimulated signaling pathways in Balb 3T3 clones that were sensitive or insensitive to growth inhibition by GRP, including cAMP formation, phospholipase C activation, calcium mobilization, and arachidonic acid formation. Taken together, these results demonstrate a novel GRP receptor-coupled signal pathway promoting growth inhibition in which prostaglandin H synthase plays a significant role.

The amphibian tetradecapeptide BN, or its two known mammalian counterparts, GRP and NMB (1), have pleiotropic effects on cell growth. BLPs are autocrine growth factors for some small cell lung cancer cell lines (2–4), and they promote the proliferation of a number of other cancers, including breast (5), colon (6), pancreatic (7, 8), and prostate (9) cancers. In some cases, however, BLPs elicit growth-inhibitory rather than proliferative responses. For example, chronically administered GRP inhibited the growth of SKI and H2T human ductal pancreatic adenocarcinoma xenografts in nude mice (10, 11). Furthermore, although BLPs were found to stimulate the growth of a number of small cell lung cancer lines *in vitro*, doses of peptide of >50 nM promoted a growth-inhibitory response (12). In light of these results, further study is warranted to understand both the mitogenic and growth-inhibitory responses of GRP in different cell types.

Two distinct receptors responding to BLPs have been cloned from rodents and humans (13, 14). The GRP receptor and the NMB receptor have binding preference for either GRP or NMB, respectively, and are members of the seven-transmembrane domain receptor superfamily coupled to signaling pathways via heterotrimeric G proteins. A third receptor in the BLP receptor family has also been cloned, BN receptor subtype 3 (15, 16). This subtype displays very weak affinity for currently known BLPs, suggesting that its natural ligand is a new member of the BLP family.

The intracellular signaling pathways coupled to the GRP receptor have been studied extensively in Swiss 3T3 fibroblasts. BLPs exert a potent mitogenic effect on these cells (17). The signaling events promoted by BLPs in Swiss 3T3 cells include the activation of PLC, tyrosine kinases, and mitogen-activated protein kinase; arachidonic acid formation; mobilization of intracellular calcium; and induction of c-*myc* and c-*fos* expression (18–24).

To further understand the positive and negative growth regulatory signals coupled to the human GRP receptor, we stably expressed this receptor in two BLP receptor-deficient, nontransformed cell hosts: Balb 3T3 fibroblast cells and chemically immortalized 184-B5 human mammary epithelial cells. We demonstrated that GRP strikingly inhibited the growth of both Balb 3T3 and 184-B5 transfectants. Among a panel of stable Balb 3T3 transfectants expressing a wide range of receptor levels, there was a correlation between the number of GRP receptors expressed and the sensitivity of

ABBREVIATIONS: BN, bombesin; DMEM, Dulbecco's modified Eagle's medium; GRP, gastrin-releasing peptide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGHS, prostaglandin H synthase; IP, inositol phosphate; NMB, neuromedin B; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; BLP, bombesin-like peptide; PLC, phospholipase C; EGF, epidermal growth factor; IBMX, 3-isobutyl-1-methylxanthine.

cells to growth inhibition by GRP. To examine the mechanism of GRP-mediated growth inhibition in Balb 3T3 transfectants, we determined the effect of GRP on cell signaling pathways that may be involved in growth inhibition, including the formation of arachidonic acid, cAMP, and soluble IPs, and the mobilization of intracellular calcium. We also examined the effect of a number of inhibitors of arachidonic acid metabolism. The results suggest that the formation of arachidonic acid and its metabolism through PGHS are important for the GRP-mediated growth-inhibitory effects that we observed.

Experimental Procedures

Materials. The growth supplement ITS$^+$ containing insulin, transferrin, and selenium was obtained from Collaborative Research (Bedford, MA). Geneticin (G418) was purchased from GIBCO-BRL (Gaithersburg, MD), and Hygromycin B was obtained from Calbiochem (La Jolla, CA). All other cell culture media, supplements, and fetal calf serum were purchased from Whitaker Biochemicals (Walkersville, MD). Bovine serum albumin (fatty acid free), bacitracin, polyethyleneimine, sulfinpyrazone, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR). ^{125}I-GRP (2000 Ci/mmol), *myo*-[1,2-^{3}H]inositol (80–100 Ci/mmol), and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid were purchased from Amersham (Arlington Heights, IL), and [*methyl*-^{3}H]thymidine (2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). 184-B5 cells (25) were a generous gift from Dr. Martha Stampfer (Lawrence Berkeley Laboratory, Berkeley, CA). All other chemicals were of reagent-grade quality.

Cell culture and transfection. Balb 3T3 cells, obtained from the American Type Culture Collection (Rockville, MD), were grown in DMEM supplemented with 4.5 g glucose/l, 10% fetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate, and 2 mM glutamine at 37° in a humidified atmosphere containing 10% CO_2. 184-B5 cells were grown in DFCI-1 medium, which has been defined previously (26). Expression plasmids pGRP3 and pGRP100 contain the human GRP receptor open reading frame under the control of the Simian virus 40 early promoter or the myeloproliferative sarcoma virus promoter, respectively, as described previously (27). Stable gene expression was achieved by transfection of Balb 3T3 cells with pGRP3 or pGRP100 and transfection of 184-B5 cells with pGRP3 with a calcium phosphate precipitation procedure and selection with either G418 (pGRP3) or puromycin plus hygromycin (pGRP100) (28).

^{125}I-GRP binding to cells. Confluent cells were removed from flasks by agitation after incubation of the cells in PBS with 0.04% EDTA and 50 mM glucose for 10–20 min at 37°. Cells were pelleted and resuspended in ice-cold binding buffer (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, 30 μg/ml bacitracin). Final binding reactions contained binding buffer (3,000–40,000 cells), 0.02 nM ^{125}I-GRP, and various concentrations of cold GRP in a volume of 0.5 ml. After an incubation of 60 min at 37°, binding was terminated by cooling the reaction mixtures on ice and then rapidly filtering them through polyethyleneimine-treated Whatman GF/B glass fiber filters. The filters were then washed four times (4 ml each) with ice-cold 50 mM Tris-Cl and 0.25 M sucrose, pH 7.5. Radioactivity on the filters was determined with a γ-counter. The total number of receptors (B_{max}) and K_D values were determined from competitive displacement data with the use of the computer program LIGAND (29). Each experiment included 10 experimental points, and K_D and B_{max} values represent the average ± standard error of three determinations.

Growth assays. To determine the effect of GRP on cell growth curves, cells were plated at a density of ~10^4 cells/well onto 24-well plates (Corning) in growth medium as indicated above. Treatment with or without GRP was initiated 24 hr after plating cells. Thereafter, the medium with or without fresh 100 nM GRP was changed every 48 hr. At the times indicated, cells were harvested with EDTA treatment and counted with a Coulter counter. The data are presented as the average ± standard error of three replicates. To measure the effect of different concentrations of GRP on growth, cells were counted after treatment with GRP for 2 days. The percent inhibition of cell growth was calculated with the following equation: $100 \times [(C_3 - C_1) - (R_3 - C_1)]/(C_3 - C_1)$, where C_1 is the cell number immediately before GRP treatment (day 1), R_3 is the cell number after 2 days of GRP treatment (day 3), and C_3 is the cell number at day 3 without GRP treatment.

Assays of [^{3}H]thymidine incorporation. Cells (2×10^4/well) were grown in 24-well plates (Corning) as described above until confluent (4–5 days). The medium was then changed to assay medium [DMEM/Waymouth's (1:1), 1% glutamine, 1% ITS$^+$], and the cells were incubated at 37° in a humidified atmosphere containing 5% CO_2 for 24 hr. GRP prepared in 1 ml of assay medium supplemented with antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin) was added to the cells to yield the final concentrations. After 16 hr, cells were incubated for 2 hr with [*methyl*-^{3}H] thymidine (5 μCi/well), the medium was aspirated, and the cells were detached from the plate by trypsinization and collected onto glass fiber filters with the use of a PhD cell harvester (Cambridge Technologies, Watertown, MA). Filters were washed with PBS, 10% (w/v) trichloroacetic acid, and 95% ethanol, and radioactivity on the filters was determined by liquid scintillation counting. Incorporated counts were <2% of the total counts added. To test the effect of arachidonic acid metabolism inhibitors on [^{3}H]thymidine incorporation, inhibitors were added to cells 5–10 min before stimulation with GRP.

Arachidonic acid release. To determine GRP-mediated arachidonic acid release, cells were grown to confluence in 12-well plates (Corning), switched to assay medium [DMEM/Waymouth (1:1) containing 1% glutamine and 1% ITS$^+$ supplement], and labeled with 1 μCi/ml of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid for 24 hr. Labeled cells were washed three times with DMEM supplemented with 50 mM HEPES, pH 7.2, and treated with or without GRP at 37° in 1 ml of DMEM plus 50 mM HEPES, pH 7.2. After 30 min of treatment, the medium was removed and cleared of sedimentable material by centrifugation (12,000 × *g* for 1 min), and the level of tritium in the medium was determined by liquid scintillation counting.

Intracellular cAMP concentration. Cells grown to confluence in six-well plates (Corning) were switched into assay medium [DMEM/Waymouth (1:1) containing 1% glutamine and 1% ITS$^+$] for 24 hr. After being washed with PBS, cells were placed in DMEM plus 50 mM HEPES, pH 7.2, with or without IBMX (0.4 mM) at 37°, and 10 min later, GRP or vehicle was added. After 30 min, cAMP was released from cells by the addition of 10% trichloroacetic acid. The aqueous sample containing the cAMP was extracted with ether to remove the acid and then lyophilized. The content of cAMP was determined with the cAMP enzyme immunoassay system (Amersham RPN 225) according to the manufacturer's instructions.

Formation of soluble IPs. Cells were grown onto six-well plates (Corning) until nearly confluent and then labeled with *myo*-[1,2-^{3}H]inositol (80–100 Ci/mmol) at a concentration of 5 μCi/ml for 24 hr in assay medium [DMEM/Waymouth (1:1) plus 1% ITS$^+$ and 1% glutamine]. Labeled cells were rinsed twice with cold PBS and then incubated for 30 min at 37° in DMEM plus 50 mM HEPES, pH 7.2, which contained LiCl (100 mM) in some cases. Cells were then treated with GRP or vehicle for the length of time indicated in figure legends before terminating the response by the addition of 0.5 ml of ice-cold 0.5 M $HClO_4$ containing 0.5 mM EDTA and 0.1 mM diethylenetriaminepentaacetic acid. The samples were then neutralized with 1.5 M KOH/60 mM HEPES and applied to Dowex AG1-X8 columns to separate IP species from labeled inositol (30). Columns were washed with water (10 ml) and 5 mM sodium borate plus 180 mM sodium formate (15 ml). Radioactively labeled IP species recovered in fractions eluted with 1.3 M ammonium formate/0.1 M formic

acid (determined to be predominantly IP_2 and IP_3) were measured by liquid scintillation counting.

Analysis of intracellular calcium concentration. Intracellular calcium was measured in cells loaded with the dye Fura-2 as described previously (27). Briefly, cells were loaded with Fura-2 acetoxymethyl ester (5 μM) for 45 min in RPMI 1640 plus 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 finally 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. Cellular fluorescence measurements were made at 37° with a PTI Deltascan 4000 fluorometer (Photon Technology, South Brunswick, NJ) equipped with dual excitation monochromators. From the fluorescence of Fura-2 in the presence of Triton X-100 we determined that the concentration of unchelated extracellular calcium under our experimental conditions ranged from 50 to 100 μM.

Results

The effect of GRP on the proliferation of Balb 3T3 cells transfected with the human GRP receptor. To study the mitogenic activity of the human GRP receptor in fibroblast cells, we stably transfected Balb 3T3 cells with the human GRP receptor. Nontransfected Balb 3T3 cells did not display detectable high affinity binding sites for ^{125}I-GRP or express mRNA encoding the GRP receptor or NMB receptor at levels detectable by Northern blot analysis (data not shown). A panel of clones expressing the GRP receptor was identified by screening transfectants for high affinity binding of ^{125}I-GRP. To further characterize the level of GRP receptor expression in transfected clones, we performed competitive-displacement ^{125}I-GRP binding studies. As shown in Table 1, our clones expressed levels of the GRP receptor ranging from $\sim 5 \times 10^4$ to 1.4×10^6 receptors/cell.

To assess whether GRP stimulates DNA synthesis by quiescent Balb 3T3 transfectants, we measured its effect on [^{3}H]thymidine incorporation by representative clones in our collection expressing a relatively low (GR1 cells) or high (GR9 cells) level of the GRP receptor. The incorporation of [^{3}H]thymidine was measured 16 hr after stimulation of cells with GRP in a defined, serum-free medium containing insulin, transferrin, and selenium. As shown in Fig. 1, GRP promoted a dose-dependent stimulation of [^{3}H]thymidine incorporation by GR1 cells, which expressed relatively low levels of receptor. The peptide displayed no effect on [^{3}H]thymidine incorporation by untransfected Balb 3T3 cells or vector-only transfectants (data not shown). These data demonstrate that GRP can trigger a mitogenic response in Balb 3T3 cells through the activation of ectopically expressed GRP receptors.

TABLE 1

Balb 3T3 cell lines ectopically expressing the human GRP receptor

The level of GRP receptor expression and the K_D for GRP were determined from [^{125}I]-GRP binding studies performed as described in Experimental Procedures. Values are mean ± standard error of three independent determinations.

Transfectant	GRP	K_D
	receptors/cell	*pM*
GR0.1	156,000 ± 5,300	20 ± 2.1
GR1	46,000 ± 2,200	10 ± 1.2
GR6	130,000 ± 2,313	27 ± 1.2
GR8	1,370,000 ± 150,000	25 ± 5.3
GR9	1,340,000 ± 130,000	67 ± 0.5
GR10	450,000 ± 40,000	21 ± 2.8
GR14	740,000 ± 96,000	36 ± 6.5

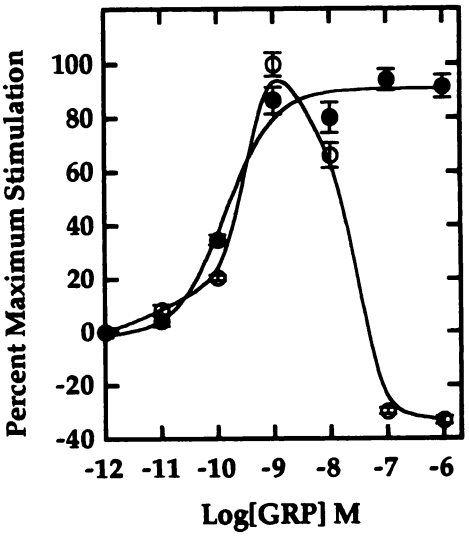

Fig. 1. Effect of GRP on [^{3}H]thymidine incorporation by quiescent GR1 and GR9 cells. GRP-stimulated [^{3}H]thymidine incorporation by quiescent GR1 cells (●) or GR9 cells (○) was determined as described in Experimental Procedures. Data were normalized to the maximal level of stimulation achieved (4.8-fold over basal for GR1 cells and 3.4-fold over basal for GR9 cells). Shown is the average ± standard error from triplicate determinations, and the results are representative of three independent experiments.

[^{3}H]Thymidine incorporation by quiescent GR9 cells, which express relatively high levels of the GRP receptor, was also stimulated by GRP but only at concentrations of ≤10 nM. At GRP concentrations of ≥100 nM, [^{3}H]thymidine incorporation was below the basal rate. GRP had a similar biphasic effect on DNA synthesis by other Balb 3T3 transfectants if they expressed the GRP receptor at a level of ≥~100,000 receptors/cell (data not shown). GRP also inhibited [^{3}H]thymidine incorporation stimulated by 3% fetal calf serum with an IC_{50} of 10 nM (data not shown). These data indicate that activation of too many GRP receptors can produce a dominant growth-inhibitory response.

Due to the instability of GRP in the assay, it is difficult to accurately derive the number of GRP receptors required to promote growth inhibition from the IC_{50} for GRP-mediated inhibition of [^{3}H]thymidine incorporation. Assuming no degradation of GRP, the pool of GRP receptors on GR9 cells would have been relatively saturated (~50–90%) at a concentration of only 1–3 nM GRP, based on the K_D of the receptor (70 pM, Table 1) and the relatively high number of receptors present on GR9 cells. Because the mitogenic effects of GRP require the continuous presence of the peptide for hours, however,[1] there was ample time for GRP degradation to occur.

We also tested whether GRP could inhibit the growth of subconfluent Balb 3T3 clones that were rapidly proliferating. GRP had no effect on the proliferating GR1 cells in a medium containing 10% fetal calf serum (Fig. 2A), which is consistent with its effect on Swiss 3T3 cells. In Swiss 3T3 cells, GRP failed to promote growth unless serum levels were reduced to 3.5% (17). In contrast, GRP strikingly inhibited the proliferation of clones expressing moderate to relatively high levels

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

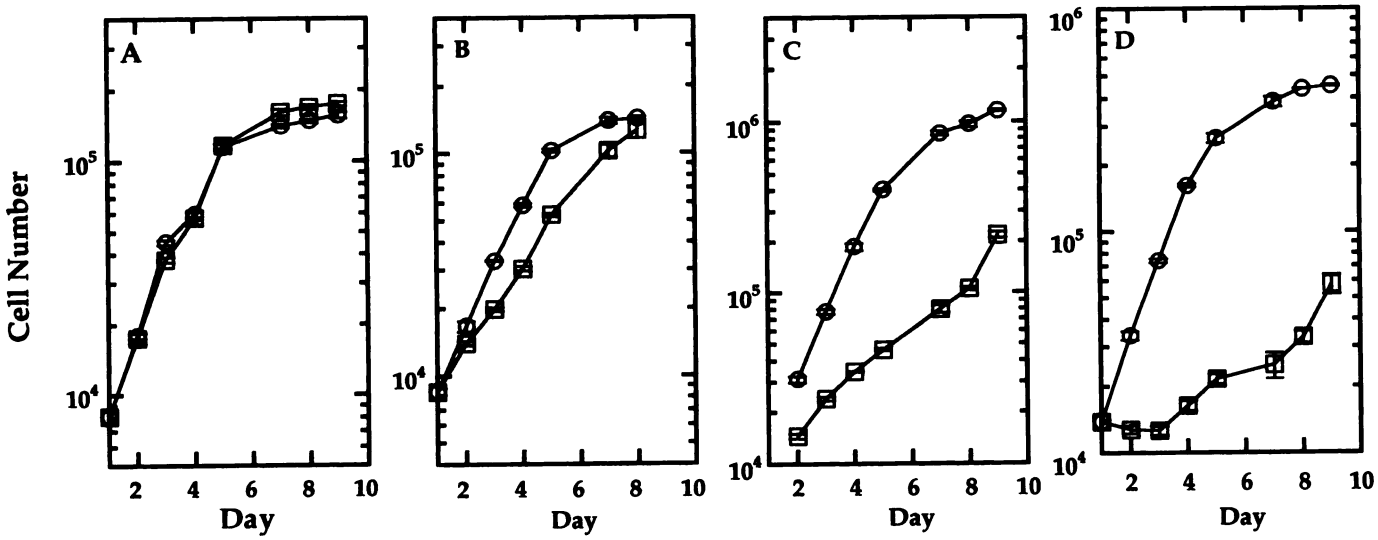

Fig. 2. Effect of GRP on growth curves of Balb 3T3 clones expressing different levels of the GRP receptor. A, GR1 cells were grown as described in Experimental Procedures in the presence (□) or absence (○) of 100 nM GRP. Cells were placed in fresh media and GRP on days 3, 5, and 7 after plating. Data are presented as the average ± standard error of three replicates. B, Same as in A but with GR6 cells. C, Same as in A but with GR14 cells. D, Same as in A but with GR8 cells.

of the GRP receptor, i.e., GR6, GR14, GR8 (Fig. 2), and GR9 cells (not shown). The population-doubling time of the cell line that was most inhibited, GR8 cells, was increased ~5-fold by GRP. Furthermore, sensitivity of each clone to growth inhibition by GRP correlated with the number of GRP receptors expressed, i.e., GR8 (1.4×10^6 receptors/cell) > GR14 (7.4×10^5 receptors/cell) > GR6 (1.3×10^5 receptors/cell). These data demonstrate that GRP can inhibit the growth of Balb 3T3 transfectants in a medium containing 10% fetal calf serum that supports rapid, GRP-independent growth of cells.

The data also suggest that the sensitivity of cells to GRP-mediated growth inhibition depends on the number of GRP receptors expressed. To extend these observations, we examined the effect of GRP on the growth of a larger number of Balb 3T3 transfectants (Fig. 3). Consistent with the results described above, clones that were the most sensitive to growth inhibition by GRP after 2 days of treatment expressed the greatest number of receptors (Fig. 3A). GRP maximally inhibited the growth of such transfectants (i.e., GR10, GR14, GR9, and GR8 cells) by ≥80%. The IC_{50} for GRP-stimulated growth inhibition was ~1 nM (Fig. 3A), which corresponds to a calculated receptor occupancy of ~50%, based on a K_D for GRP of 70 pM and the relatively large number of receptors available for binding GRP. These data, therefore, provide evidence that growth inhibition is mediated through the GRP receptor. This was further substantiated by our finding that GRP had no effect on the growth of vector-only-transfected Balb 3T3 cells (Fig. 3A) or untransfected Balb 3T3 cells (not shown). GRP maximally inhibited the growth of two transfectants, GR0.1 and GR6 cells, by ~40%. The growth of one transfectant, GR1 cells, was not affected by GRP. In Fig. 3B, we plotted the GRP receptor expression level in Balb 3T3 transfectants versus the extent to which their growth was inhibited by 100 nM GRP. The data demonstrate that GRP-mediated growth inhibition clearly correlates with the number of GRP receptors expressed on host Balb 3T3 cells.

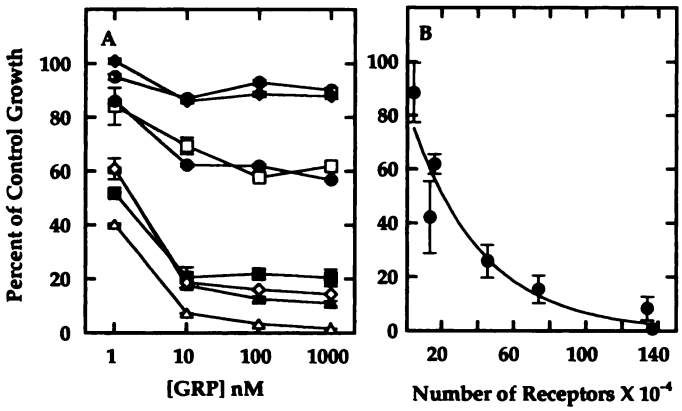

Fig. 3. Dose response for the inhibition of Balb 3T3 transfectants expressing the GRP receptor and the correlation of the maximal inhibitory response with the number of receptors expressed. A, Effect of various concentrations of GRP on the growth of GR1 (♦), GR6 (●), GR0.1 (□), GR10 (■), GR14 (◇), GR9 (▲), GR8 (△), and vector-only (pBBS70)-transfected Balb 3T3 cells (○). Results are calculated as the percentage decrease in population density relative to untreated cultures after 2 days as described in Experimental Procedures and represent the average ± standard error of three replicates. B, Correlation of GRP-mediated growth inhibition and GRP receptor expression level among different Balb 3T3 clones tested (see Table 1). Growth data represent the average ± range of two independent experiments.

GRP inhibits the growth of 184-B5 mammary epithelial cells ectopically expressing the GRP receptor. Although the consequences of GRP receptor activation have been extensively studied in fibroblast cells, much less is known about such responses in nontransformed epithelial cells. To address this, we stably transfected mammary epithelial 184-B5 cells with the human GRP receptor and determined the effects of GRP on their growth. ^{125}I-GRP binding studies indicated that the parental 184-B5 cells did not display detectable levels of the GRP receptor (data not shown). Furthermore, 184-B5 cells did not express GRP receptor mRNA at levels detectable by Northern blot analysis. We isolated two transfected 184-B5 clones: B5-GR4 cells, which expressed 42,900 ± 9,700 receptors/cell (K_D = 0.018 ± 0.002 nM) and B5-GR6 cells, which expressed 18,200 ± 520 receptors/cell (K_D = 0.022 ± 0.003 nM).

The effect of GRP on the growth curves of parental and

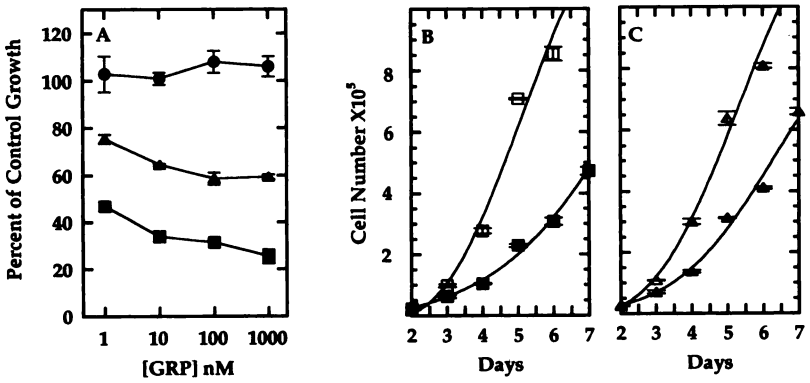

Fig. 4. Inhibitory effect of GRP on the growth of 184-B5 cells transfected with the human GRP receptor. A, Effect of various concentrations of GRP on the growth of parental B5 cells (●), B5-GR4 cells (▲), or B5-GR6 cells (■). Results are calculated as the percentage decrease of population density relative to untreated cultures after 2 days of treatment as described in Experimental Procedures. B, Growth curve of B5-GR6 cells in the presence (■) or absence (□) of 100 nM GRP. Data represent the average ± standard error of three replicates. C, Growth curve of B5-GR4 cells in the presence (▲) or absence (△) of 100 nM GRP. Data represent the average ± standard error of three replicates.

transfected 184-B5 cells in DFCI-1 media is shown in Fig. 4. GRP inhibited the proliferation of B5-GR4 and B5-GR6 transfectants by 40–70% but did not inhibit the growth of parental 184-B5 cells. Furthermore, GRP inhibited the growth of B5 transfectants at relatively low concentrations (IC_{50} = ~1 nM) (Fig. 4A), providing additional evidence that the response was mediated through the GRP receptor. These results demonstrate that the GRP receptor can couple to an inhibition of cell growth in a human epithelial cell line. Indeed, 184-B5 cells could be rendered sensitive to GRP-mediated growth inhibition by the expression of far fewer GRP receptors than that required by Balb 3T3 cells.

PGHS inhibitors block the inhibitory effects of GRP on DNA synthesis by Balb 3T3 cells transfected with the GRP receptor. In a number of cells, formation of arachidonic acid is thought to play a central role in either positive or negative effects of factors on growth. This led us to investigate the role of GRP-stimulated arachidonic acid formation and metabolism in GRP-mediated growth responses in Balb 3T3 transfectants. As shown in Fig. 5, GRP stimulated arachidonate release from both GR1 and GR9 cells. In GR1 cells, which express relatively low levels of the GRP receptor, GRP stimulated arachidonic acid release by ~1.5-fold. There was a larger, 3-fold response by GR9 cells, which express a greater number of GRP receptors and are sensitive to growth inhibition by GRP. As shown in Fig. 5, the dose response for GRP-stimulated arachidonic acid release by GR9 cells was monophasic (EC_{50} = 0.1 nM). These data demonstrate that the GRP receptor can couple to arachidonic acid formation in Balb 3T3 cells, as has been seen in other systems.

We then addressed whether the metabolic products of arachidonic acid played a role in the growth-inhibitory effects of GRP. PGHS is a rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. To determine whether the activity of this enzyme was important for growth-inhibitory effects of GRP, we examined the effect of the PGHS inhibitor ibuprofen on [^{3}H]thymidine incorporation by quiescent GR1 and GR9 cells measured 16 hr after stimulation of cells with various doses of GRP. As discussed above, GRP promotes DNA synthesis in GR9 cells with a characteristic bell-shaped dose response indicative of both positive and negative effects on growth. Treatment of GR9 cells with 10 μM ibuprofen markedly reduced the inhibitory arm of the GRP dose-response curve (Fig. 6B) but displayed no effects on GRP-stimulated DNA synthesis by quiescent GR1 cells (Fig. 6A). In four independent experiments, we found that GRP (1 μM) stimulated DNA synthesis by quiescent GR9 cells an average of 2.67 ± 0.61-fold in the presence 10 μM ibuprofen compared with 1.12 ± 0.22-fold in the absence of ibuprofen. Ibuprofen, at the concentration used, effectively blocks growth factor-stimulated production of prostaglandins (≥90%) in Balb 3T3 cells (31) and other systems (32, 33).

To further demonstrate the importance of prostaglandin production on GRP-mediated growth-inhibitory effects, we performed similar experiments with indomethacin (10 μM), which, like ibuprofen, inhibits PGHS. Indomethacin (10 μM) effectively blocked GRP-stimulated prostaglandin synthesis in Swiss 3T3 cells (34) and growth factor-stimulated prostaglandin synthesis in other cell types (33, 35). In three independent experiments, GRP (1 μM) promoted a 6.19 ± 0.78-fold induction of DNA synthesis by GR9 cells in the presence of 10 μM indomethacin compared with 0.49 ± 0.21-fold in the absence of indomethacin. Analogous results were observed in experiments where cells were labeled continuously for 24 hr with [^{3}H]thymidine after GRP treatment (data not shown). Taken together, our data show that PGHS activity plays a role in growth-inhibitory but not growth-stimulatory effects of GRP.

Other than PGHS, cytochrome P450 and lipoxygenase can also metabolize arachidonic acid. Therefore, another poten-

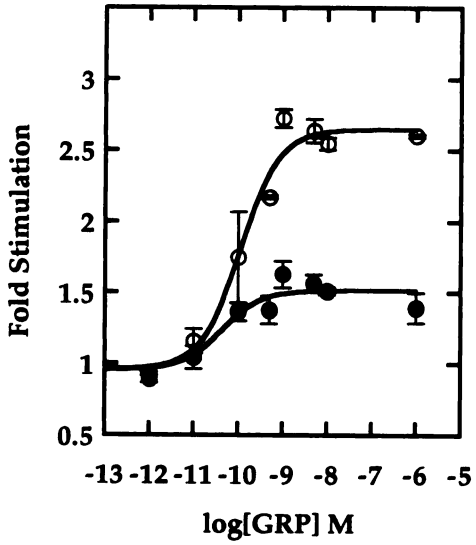

Fig. 5. Dose response for GRP-stimulated arachidonic acid release in GR9 and GR1 cells. Arachidonic acid release was determined as described in Experimental Procedures after treatment of GR1 (●) or GR9 (○) cells with the concentrations of GRP indicated. *Error bars*, range of duplicate determinations. The results are representative of three independent experiments.

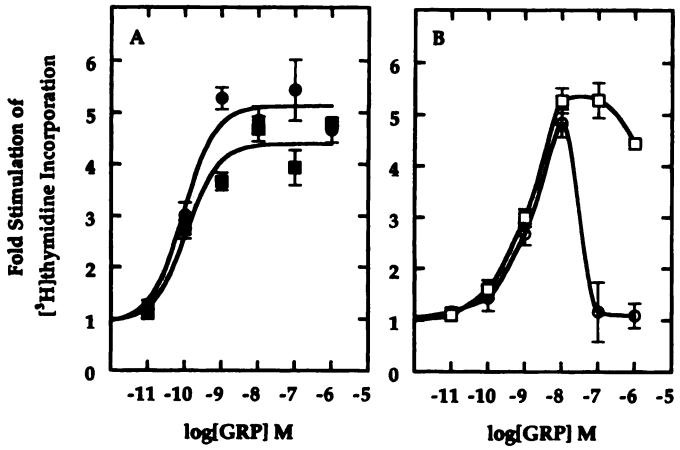

Fig. 6. Effect of ibuprofen on GRP-stimulated [^{3}H]thymidine incorporation by GR1 and GR9 cells. A, GRP dose response of [^{3}H]thymidine incorporation by GR1 cells performed in the presence (■) or absence (●) of 10 μM ibuprofen was measured as described in Experimental Procedures. Results are expressed as the average ± standard error stimulated/basal [^{3}H]thymidine incorporation from triplicate determinations. B, GRP dose response of [^{3}H]thymidine incorporation performed performed as in A, except with GR9 cells in the presence (□) or absence (○) of 10 μM ibuprofen.

tial effect of blocking PGHS was to increase the levels of arachidonic acid metabolites produced by these enzymes. To test this possibility, we examined the effects of the lipoxygenase inhibitor 5,8,11-eicosatrienoic acid (40 μM) and the cytochrome P450 inhibitor SKF 525A (Proadifen; 50 μM) on GRP-stimulated [^{3}H]thymidine incorporation by GR1 or GR9 cells. Neither inhibitor altered the effects of GRP on GR1 or GR9 cells, however, in either the presence or absence of ibuprofen (data not shown). Taken together, these results indicate that the production of a prostanoid through the metabolism of arachidonic acid by PGHS is an important event in the triggering of growth inhibition through the GRP receptor. In contrast, the metabolism of arachidonic acid through either PGHS-, lipoxygenase-, or cytochrome P450-dependent pathways did not seem to be important for the stimulatory effects of GRP on DNA synthesis by Balb 3T3 cells expressing the GRP receptor.

Potential role of other GRP receptor-mediated signal transduction pathways in GRP-mediated growth inhibition. To potentially identify other mediators of growth inhibition by GRP, we characterized the effects of GRP on cAMP formation, PLC activity, and calcium mobilization in GR1 and GR9 cells.

It was of particular interest to test whether GRP promoted cAMP formation in Balb 3T3 transfectants, because high levels of cAMP can inhibit the growth of fibroblast cells. As shown in Table 2, however, treatment of GR9 or GR1 cells with GRP (100 nM) for 30 min did not promote a significant increase in the level of cAMP. To more sensitively probe for the activation of adenylate cyclase activity, we also added an inhibitor of cAMP breakdown, IBMX. Under these conditions, GRP still had no effect on cAMP levels in GR9 cells and only slightly elevated cAMP levels in GR1 cells. There also was no significant stimulation of cAMP levels after 5 or 10 min of treatment (data not shown). As a positive control, we treated cells with forskolin, a direct activator of adenylate cyclase activity, which, as expected, greatly increased cAMP levels. Taken together, these results rule out the possibility

TABLE 2

Effect of GRP on cAMP levels in GR1 and GR9 cells

cAMP levels were determined after 30 min of treatment as described in Experimental Procedures. Values are mean ± standard error of varying numbers of independent experiments (shown in parentheses).

Cells	Additions	cAMP produced
		fmol/μg protein
GR1	None	2.1 ± 0.09 (3)
	GRP (100 nM)	1.3 ± 0.18 (3)
	Forskolin (25 μM)	40.0 ± 12 (3)
	IBMX	13 ± 2.6 (2)
	IBMX + GRP (500 nM)	21 ± 4.1 (2)
	IBMX + forskolin (25 μM)	203 (1)
GR9	None	4.0 ± 1.1 (4)
	GRP (100 nM)	3.7 ± 1.1 (4)
	Forskolin (25 μM)	13.0 ± 1.7 (4)
	IBMX	17 ± 2.9 (2)
	IBMX + GRP (500 nM)	14.0 ± 1.1 (2)
	IBMX + forskolin (25 μM)	230 (1)

that GRP directly activates adenylate cyclase, thereby stimulating levels of cAMP that are growth inhibitory in transfectants expressing a relatively high level of the GRP receptor, such as GR9 cells.

We also compared GRP-stimulated PLC activation in GR1 and GR9 cells. In Swiss 3T3 cells, PLC can be activated by G proteins coupled to the GRP receptor. The enzyme hydrolyzes phosphatidylinositol bisphosphate to diacylglycerol and inositol[1,4,5]trisphosphate. This latter product promotes the release of calcium from intracellular stores. As shown in Fig. 7A, PLC activity is also promoted through activation of the GRP receptor in Balb 3T3 fibroblasts. The maximal level of IP formation promoted by GRP was approximately three times greater in GR9 cells than in GR1 cells if the response was normalized to basal levels. This larger response correlates with the presence of higher levels of the GRP receptor in GR9 cells. The dose response of GRP-stimulated IP formation in GR1 and GR9 cells was similar (EC_{50} = 0.5 nM).

Another significant effect of the higher receptor expression in GR9 cells was to prolong the activation of PLC by GRP. As

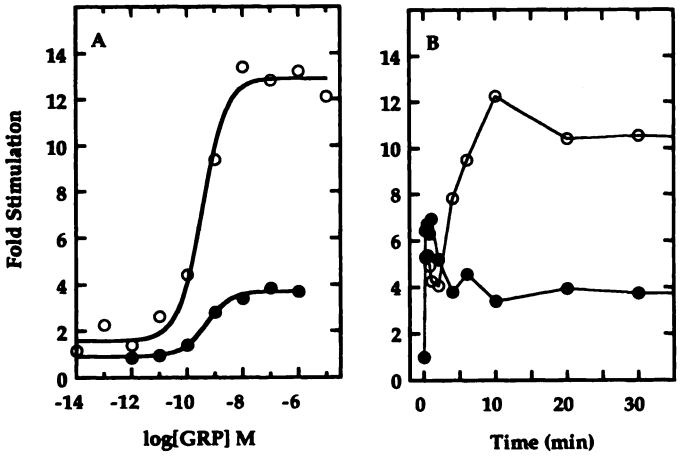

Fig. 7. Dose response and time course of GRP-stimulated production of IPs in GR1 and GR9 cells. A, Production of IPs by GR1 (●) or GR9 (○) cells was determined as described in Experimental Procedures after treatment for 10 min with GRP at the indicated concentrations. Cells were also treated with 100 mM LiCl to reduce IP degradation. Data are representative of three independent experiments. B, Time course of soluble IP formation stimulated by 1 μM GRP. Soluble IP formation was measured as described in A, except that no LiCl was present. Data are representative of three independent experiments.

shown in Fig. 7B, IP formation promoted by GRP (1 μM) in GR1 cells declined rapidly after ~1 minute of treatment, whereas in GR9 cells, an initial peak of IP was formed after 1 min, followed by a larger and more stable peak at ~10 min of treatment. These results demonstrate that GRP stimulates a greater and longer-lived activation of PLC in Balb 3T3 transfectants, such as GR9 cells, expressing a higher level of the GRP receptor.

These data raised the possibility that GRP inhibits the growth of GR9 cells by promoting a prolonged, and possibly toxic, elevation of intracellular calcium. To test this hypothesis, we compared GRP-stimulated calcium mobilization in GR9 and GR1 cells. As shown in Fig. 8, calcium mobilization stimulated by GRP was, in fact, somewhat greater in GR1 cells than in GR9 cells. The responses were similar with respect to the kinetics of intracellular calcium increase and dissipation. The addition of EGTA to the medium had no effect on the response (not shown), demonstrating that calcium was released from intracellular stores. These results indicate that toxic or persistently elevated levels of intracellular calcium cannot account for the growth-inhibitory effects of GRP on Balb 3T3 cells transfected with the GRP receptor.

Discussion

We found that GRP exhibits pleiotropic effects on the growth of nontransformed fibroblast and epithelial cells ectopically expressing the GRP receptor. Although our study shows that activation of the GRP receptor in fibroblast cells can produce a mitogenic response, consistent with previous studies (17), it also demonstrates that the activation of a relatively large number of receptors leads to growth inhibition. To understand the mechanism of GRP-mediated growth inhibition, we compared GRP-mediated responses of two Balb 3T3 clones that were either sensitive or insensitive to growth inhibition by GRP by virtue of their expression of different levels of the GRP receptor. An important conclusion of these experiments was that GRP-mediated growth inhibition depends, at least in part, on the activity of PGHS.

We also demonstrated that the growth-inhibitory effects of GRP are not restricted to Balb 3T3 cells. In fact, normal mammary epithelial 184-B5 cells transfected with the GRP receptor were rendered sensitive to growth inhibition by GRP by the expression of fewer receptors (1.8×10^4 receptors/cell) than that required by Balb 3T3 cells. This result indicates that the efficiency of receptor coupling to intracellular signaling mediators of growth inhibition may vary greatly among different cell types. GRP has been shown to transmodulate the EGF receptor in Swiss 3T3 cells. Because the growth of 184-B5 epithelial cells is dependent on EGF, it was possible that the activation of the GRP receptors in these cells inhibited growth by blocking the EGF receptor. This was not the case, however, as the binding of ^{125}I-EGF to B5-GR4 cells was not affected by GRP treatment (data not shown).

Consistent with our finding that GRP inhibits cells that ectopically express the GRP receptor, GRP has been found to inhibit the growth of nontransfected cells, providing additional evidence that this activity has physiological relevance. For example, it has been shown that the proliferation of small cell lung cancer cell lines in a defined medium was dependent on BN concentrations of <50 nM but was inhibited by higher levels of the peptide. Because this occurred in cell lines expressing very low levels of receptor (12), SCLC cells, like 184-B5 cells, seem to be particularly sensitive to growth

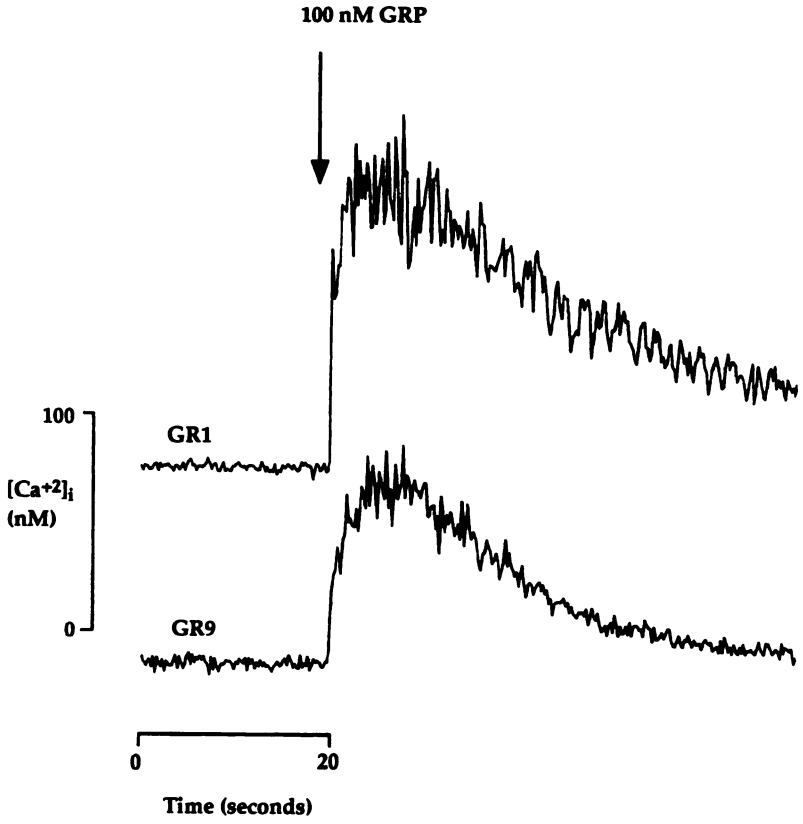

Fig. 8. Mobilization of Ca^{2+} by GRP in GR1 and GR9 cells. Effects of 100 nM GRP on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in GR1 (*top*) and GR9 (*bottom*) were monitored with the use of Fura 2 fluorescence as described in Experimental Procedures. For GR1 cells, the basal calcium concentration was 19 nM and the peak GRP-stimulated concentration was 155 nM. For GR9 cells, the basal calcium concentration was 29 nM and the peak GRP-stimulated concentration was 119 nM.

inhibition through activation of the GRP receptor. As suggested previously (36), the growth-inhibitory effects of GRP could cause a powerful *in vivo* selection, accounting for the relatively low level of receptor expression found in SCLC tumor cells (12).

It has also been observed that GRP inhibited the growth of SKI and H2T human ductal pancreatic adenocarcinoma xenografts in nude mice (10, 11), showing that GRP can also promote growth inhibition *in vivo*. Furthermore, GRP inhibited the formation of preneoplastic lesions derived from the ductal cell types in the pancreas of rats and hamsters treated with azaserine (37). In contrast, a number of studies indicated that BLPs exhibit a growth-promoting effect on pancreatic cells (1, 10, 38). This dichotomy may be explained by differences in the number of GRP receptors expressed in these tissues, which we found to be an important determinant for whether GRP promotes growth stimulation or inhibition. Because GRP can promote the secretion of other factors, its effect on the growth of pancreatic cells *in vivo* could also be indirect.

A significant finding of our study was that PGHS plays a role in GRP-mediated growth inhibition. PGHS is a rate-limiting enzyme in the conversion of arachidonate to a number of prostaglandins that have been found to have either growth-stimulatory or -inhibitory effects on cells (34, 35, 39–45). The products of arachidonic acid metabolism that promote GRP-mediated growth inhibition remain to be identified, however. Furthermore, it is not clear how the GRP receptor regulates the level of such compounds. It may be that, in addition to controlling the production of arachidonic acid, the GRP receptor differentially regulates the production of different prostaglandins. In the Balb 3T3 cell, such regulation could require the activation of a relatively large number of receptors, but other cells, such as B5 epithelial cells, may require the activation of far fewer receptors.

This hypothesis is supported by the finding that EGF and arachidonic acid, when combined, but not separately, resulted in the inhibition of human fibroblast proliferation (46). Growth inhibition by EGF and arachidonic acid could be abrogated by indomethacin (46). One explanation for this is that EGF stimulates key enzymes in the synthesis of growth-inhibitory compounds derived from arachidonic acid, such as prostaglandins, but cannot, by itself, generate the levels of arachidonic acid needed to synthesize growth-inhibitory levels of these compounds. On the other hand, EGF alone can inhibit the proliferation of cells, such as human epidermoid carcinoma A431 cells, that express a large number of EGF receptors (47, 48). Although the involvement of arachidonic acid or its metabolism in such cases is speculation, it may be that both of these elements are promoted through the activation of the EGF receptor, if it is present at a sufficiently high level.

It has also been shown that surface receptors can regulate the formation of arachidonic acid metabolites through the induction of key metabolic enzymes. For example, the expression of a rate-limiting form of PGHS, PGHS-2, is stimulated by serum and a number of mitogens in Swiss 3T3 cells (49). Furthermore, expression of another enzyme involved in arachidonate metabolism, arachidonate-12-lipoxygenase, is promoted by EGF in A431 cells (50). These results suggest that cell surface receptors may regulate many enzymes that are important in the synthesis of prostaglandins and other metabolites derived from arachidonic acid.

Bradykinin and acetylcholine receptors have also been found to promote growth inhibition. Like BLP receptors, these receptors are members of the superfamily of G protein-coupled receptors with seven transmembrane spanning domains. Activation of B_1 bradykinin receptors inhibits DNA synthesis by human breast stromal fibroblasts. The effect could be partially reversed with the prostaglandin synthase inhibitor indomethacin (51). In contrast, the inhibitory effects of carbachol on [^{3}H]thymidine incorporation by A9L cells transfected with either m1 or m3 acetylcholine receptor subtypes were largely insensitive to indomethacin (52). Further work is needed to understand whether arachidonic acid metabolites play a role in the growth-inhibitory effects of other factors.

Several studies have correlated GRP-stimulated arachidonic acid formation with the ability of GRP to stimulate mitogenesis in Swiss 3T3 cells under different conditions (20, 53). Indomethacin (1 μM) was found to inhibit BN-stimulated DNA synthesis by quiescent Swiss 3T3 cells by ~30% (34). Our results showing that the mitogenic effects of GRP on GR1 cells were not blocked by indomethacin (10 μM) suggest that in Balb 3T3 cells, and possibly other cells, the production of prostaglandins is not required for the growth-promoting effects of GRP. The role of arachidonic acid metabolism in the mitogenic responses of other growth factors on Balb 3T3 cells is mixed. PDGF stimulates DNA synthesis by Balb 3T3 fibroblasts, which was not affected by indomethacin or nordihydroguaiaretic acid, a lipoxygenase inhibitor (45). On the other hand, EGF-stimulated DNA synthesis by Balb 3T3 fibroblasts can be blocked by indomethacin (31, 45). Furthermore, the mitogenic activity of EGF in the presence of indomethacin could be restored by the addition of prostaglandin G_2 (45), demonstrating that the metabolism of arachidonic acid to prostaglandins is required for the mitogenic activity of EGF.

Because production of cAMP can have a negative impact on the growth of fibroblast cells, we examined the effect of GRP on cAMP levels in Balb 3T3 transfectants expressing different levels of the GRP receptor. GRP couples to pertussis toxin- and cholera toxin-insensitive G proteins in Swiss 3T3 cells, which are thought to be in the G_q family (54, 55) and do not directly increase cAMP levels in these cells. Nevertheless, receptor coupling could be less specific in cells, such as GR9 cells, that express a relatively high level of the receptor. However, GRP failed to increase the basal levels of cAMP in either GR9 cells or GR1 cells within 30 min of treatment. This result indicates that GRP-induced growth inhibition of Balb 3T3 clones did not result from the promiscuous coupling of the GRP receptor to G proteins that regulate cAMP levels. Another possibility that has not been ruled out by our data is that GRP-induced growth inhibition resulted from cAMP that was generated as a later event during the G_1 phase of the cell cycle through a prostaglandin-dependent pathway.

Consistent with our study, it has been observed that NMB inhibits DNA synthesis by Balb 3T3 transfectants ectopically expressing a relatively high level of the NMB receptor (8×10^5 receptors/cell) (36). It was suggested that this effect involved the generation of toxic levels of intracellular calcium. Our data suggest otherwise, however, because GRP actually promoted a slightly greater increase in intracellular calcium

levels in GR1 cells, which are not sensitive to growth inhibition by GRP, than in GR9 cells. This result may be explained by our observation that during the first several minutes of GRP treatment, during which the calcium mobilization response occurs, GRP stimulated slightly less IP formation in GR9 cells than in GR1. IP formation is upstream in the receptor signaling cascade leading to calcium mobilization. At later times, however, GRP promoted formation of significantly higher IP in GR9 cells, which apparently did not significantly affect calcium mobilization.

In summary, our results demonstrate that GRP receptor activation can mediate a growth-inhibitory response in Balb 3T3 fibroblasts and 184B-5 mammary epithelial cells transfected with the GRP receptor. The data also indicate that PGHS activity plays a significant role in the growth-inhibitory effects of GRP in transfected Balb 3T3 cells and possibly in other cells. Further investigation of Balb 3T3 and 184-B5 transfectants will provide additional insight into the signaling pathways that promote growth stimulation or inhibition.

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