Characterization of ligand binding and processing by gastrin-releasing peptide receptors in a small-cell lung cancer cell line

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The ligand-binding properties of the gastrin-releasing peptide (GRP) receptor and the cellular processing of GRP have been studied in the small-cell lung cancer (SCLC) cell line COR-L42. Scatchard analysis of GRP receptor expression indicated a single class of high-affinity receptors (K_d 1.5 nM) and approx. 6700 receptors/cell. GRP bound to its receptor with a K_i of 2.4 nM. The bombesin-related peptides neuromedin B (NMB) and phyllolitorin also bound to GRP receptors with K_i values of 22.7 and 59.1 nM respectively. Binding of ^{125}I -GRP to COR-L42 cells increased rapidly at 37 °C, achieved a maximum at 10 min and declined rapidly thereafter. At 4 °C, maximum binding was achieved at 30 min and the subsequent decline in cell-associated radioactivity was slower than that seen at 37 °C. Acid/salt extraction, to separate surface-bound ligand from internalized GRP, indicated that after receptor binding ^{125}I -GRP was rapidly internalized. To determine the pathway of ^{125}I -GRP degradation, binding studies were carried out with the lysosomotropic agent chloroquine (5 mM), and with phosphoramidon (10 μM), an inhibitor of the membrane-bound enzyme (EC 3.4.24.11). Both agents markedly inhibited the degradation of GRP, indicating that this process involves a lysosomal pathway and a phosphoramidon-sensitive pathway, possibly involving the EC 3.4.24.11 enzyme. GRP receptor down-regulation was observed following a 10 min exposure to 100 nM-GRP. With longer pretreatment times the number of binding sites recovered to 80 % of control values. Treatment with 5 mM-chloroquine plus GRP or cycloheximide (10 $\mu\text{g}/\text{ml}$) plus GRP demonstrated that the majority of GRP receptors are recycled. NMB and phyllolitorin pretreatment did not influence the subsequent binding of ^{125}I -GRP, suggesting that these peptides do not down-regulate GRP receptors.

INTRODUCTION

Amphibian bombesin-like peptides are a family of structurally related peptides (Nakajima *et al.*, 1970; Anastasi *et al.*, 1971; Yasuhara *et al.*, 1983; Barra *et al.*, 1985) comprising the bombesin, ranatensin and phyllolitorin subfamilies. Only bombesin- and ranatensin-like peptides have mammalian counterparts, namely gastrin-releasing peptide (GRP) and neuromedin B (NMB) respectively (McDonald *et al.*, 1978; Minamino *et al.*, 1983, 1985). GRP is a potent mitogen for Swiss 3T3 cells (Rozengurt & Sinnett-Smith, 1983; Zachary & Rozengurt, 1985) and also has been shown to be an autocrine growth factor for small-cell lung cancer (SCLC) (Cuttitta *et al.*, 1985). SCLC cells synthesize (Moody *et al.*, 1981) and secrete (Korman *et al.*, 1986) GRP, express high-affinity GRP receptors (Moody *et al.*, 1985; Gaudino *et al.*, 1988; Layton *et al.*, 1988; Kado-Fong & Malfroy, 1989) and respond mitogenically to GRP stimulation (Carney *et al.*, 1987). In addition, they are growth-inhibited by antibodies to GRP, confirming an autocrine function for this peptide in SCLC (Cuttitta *et al.*, 1985).

Receptor binding and internalization of GRP in Swiss 3T3 and HIT pancreatic islet cells has been shown to be a rapid process which is followed by ligand internalization (Zachary & Rozengurt, 1987a; Swope & Schonbrunn, 1987; Brown *et al.*, 1988) and lysosomal degradation (Westendorf & Schonbrunn, 1983; Zachary & Rozengurt, 1987a; Swope & Schonbrunn, 1987; Brown *et al.*, 1988). Unlike the receptors for several growth factors (Carpenter & Cohen, 1976; Heldin *et al.*, 1982; Moscatelli, 1988; Galizzi *et al.*, 1989; Samanta *et al.*, 1990), GRP receptors appear not to undergo down-regulation in Swiss 3T3 cells (Zachary & Rozengurt, 1987a; Brown *et al.*, 1988). This

observation raises the possibility that extensive receptor down-regulation is not in every case an obligatory event for the initiation of cell proliferation. In contrast, GRP receptor down-regulation has been demonstrated in the HIT pancreatic islet cell line following the addition of 100 nM-GRP for 90 min at 37 °C (Swope & Schonbrunn, 1987). These observations indicate differences in GRP receptor regulation in different cell types.

Although it has been shown that ^{125}I -GRP binds to specific cell surface receptors on SCLC cell lines (Moody *et al.*, 1985; Gaudino *et al.*, 1988; Layton *et al.*, 1988; Kado-Fong & Malfroy, 1989) and stimulates rapid and transient mobilization of Ca^{2+} (Heikkila *et al.*, 1987; Woll & Rozengurt, 1989; Bunn *et al.*, 1990), little is known about the cellular processing of GRP and its receptor in these cells. Given the acknowledged role of GRP as an autocrine growth factor for SCLC and its potential as a target for novel anti-cancer therapies, the present study investigates GRP receptor binding and internalization, GRP catabolic pathways and GRP receptor down-regulation in the recently-derived SCLC cell line COR-L42.

MATERIALS AND METHODS

Materials

^{125}I -GRP (2000 Ci/mmol) was purchased from Amersham. GRP (pig) was obtained from Cambridge Research Biochemicals, Cambridge, U.K., and phyllolitorin and [^{35}S]bombesin were from Peninsula Laboratories Europe, Ltd., St. Helens, Merseyside, U.K. Sodium selenite, transferrin, insulin, Bes, aprotinin, phosphoramidon, chloroquine, cycloheximide, GRP (human), GRP-10, GRP-(1–16), ranatensin, litorin, alytesin, NMB and substance P were all obtained from Sigma Chemical Co.

Abbreviations used are: GRP, gastrin-releasing peptide; GRP-10, C-terminal decapeptide fragment of GRP; NMB, neuromedin B; SCLC, small-cell lung cancer.

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Cell line

The derivation and characterization of the SCLC COR-L42 cell line has been described elsewhere (Baillie-Johnson *et al.*, 1985). Briefly, this cell line shows a deletion in the short arm of chromosome 3 (Ibson *et al.*, 1987), contains high levels of neuron-specific enolase and creatinine kinase BB, a low level of L-dopa decarboxylase activity (Baillie-Johnson *et al.*, 1985), and does not express the GRP gene (Cardona *et al.*, 1991). These properties are consistent with its classification as a variant SCLC (SCLC-V) cell line (Carney *et al.*, 1985). Cells were grown routinely in RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM-L-glutamine, 100 µg of penicillin/ml and 100 µg of streptomycin/ml (all from Gibco Ltd.).

Time course of ^{125}I -GRP binding

COR-L42 cells were harvested by centrifugation. Cells were then rinsed in RPMI-1640 medium supplemented with sodium selenite (30 nM), insulin (50 µg/ml) and transferrin (100 mg/ml) (RPMI/SIT medium) and resuspended in the same medium containing 0.25% BSA, 50 mM-Bes and aprotinin (100 kallikrein-inhibiting units/ml) at pH 6.5 (binding buffer). Cells were monodispersed by gentle syringing, transferred to silicone-coated Eppendorf tubes (4×10^6 cells/tube), and 0.5 nM- ^{125}I -GRP was added in the presence or absence of 1 µM unlabelled GRP. The total volume was 200 µl and each assay was performed in duplicate. Cells were rapidly washed after various incubation times with ice-cold washing solution containing 0.15 M-NaCl, 0.02 M- NaH_2PO_4 , 5 mM-KCl, 1.8 mM- KH_2PO_4 , 5 mM- MgCl_2 and 0.25% BSA, pH 6.7 (washing buffer). Cell-associated radioactivity was determined in a γ -radiation counter (Compugamma CS; LKB Wallace). Non-specific binding is defined as the cell-associated radioactivity not displaced in the presence of excess GRP (1 µM). Specific binding was calculated by subtracting the non-specific binding from the total cell-associated radioactivity.

Specificity of GRP receptors

Competition for GRP-binding sites was studied by incubating cells in the presence of 0.5 nM- ^{125}I -GRP and 0.1–10 000 nM unlabelled peptides. After 10 min at 37 °C the cells were rapidly washed in ice-cold washing buffer and cell-associated radioactivity was determined.

Cellular distribution of ^{125}I -GRP

Surface-bound and internalized ligand were separated using an acid/salt extraction procedure previously used to dissociate

GRP from GRP receptors expressed on Swiss 3T3 cells (Zachary & Rosengurt, 1987a), based on the method of Haigler *et al.* (1980). Briefly, after various incubation times in the presence of 0.5 nM- ^{125}I -GRP at either 4 °C or 37 °C, cells were washed rapidly three times with ice-cold washing buffer and then treated for 6 min with 1 ml of 0.2 M-acetic acid/0.5 M-NaCl, pH 2.5, at 4 °C. Cells were centrifuged at 13 400 g for 10 s and were washed once with 0.5 ml of phosphate-buffered saline. Washings and cell pellets were counted for radioactivity in a γ -radiation counter to determine both the surface-bound cell-associated radioactivity and the intracellular cell-associated radioactivity.

Determination of ^{125}I -GRP degradation

To determine the optimum concentrations of chloroquine and phosphoramidon to inhibit degradation of GRP, cells were incubated with 0.5 nM- ^{125}I -GRP in the presence of 5–5000 µM-chloroquine or 10–10 000 nM-phosphoramidon for 30 min. Cells were washed in ice-cold buffer and cell-associated radioactivity was determined. Control cultures received no inhibitor pre-treatment and were incubated with 0.5 nM- ^{125}I -GRP only. In subsequent studies, cells were incubated in the absence or the presence of 5 mM-chloroquine and/or 10 µM-phosphoramidon. Cells were washed and counted for radioactivity as previously described. Cell viability, as determined by Trypan Blue exclusion, was not affected by these agents.

Effect of pretreatment with unlabelled GRP, cycloheximide and chloroquine on the subsequent binding of ^{125}I -GRP

Cells were pretreated in the presence of 5 mM-chloroquine at 37 °C for 30 min. These and parallel untreated control cells were then incubated with 100 nM-GRP in the presence or the absence of 10 µg of cycloheximide/ml or 5 mM-chloroquine at 37 °C. After various incubation times, cells were washed twice with binding buffer at 4 °C and incubated in fresh binding buffer with 0.5 nM- ^{125}I -GRP at 37 °C for 10 min. Cells were then washed and counted for radioactivity as previously described. Cell viability, as determined by Trypan Blue exclusion, was not affected by the action of these agents.

Effect of pretreatment with various unlabelled peptides on the subsequent binding of ^{125}I -GRP

In order to examine the ability of unlabelled peptides to down-regulate GRP receptors, cells were incubated either in the presence or in the absence of alytesin (100 nM), NMB (100 nM), phyllolitorin (100 nM) or substance P (1 µM). Following incubation at 37 °C for 1 h, cells were washed twice with ice-cold

Fig. 1. ^{125}I -GRP binding to COR-L42 cells

(a) Concentration-dependence of ^{125}I -GRP binding at 37 °C to COR-L42 cells. ●, Specific binding; ○, non-specific binding. (b) Scatchard plot of GRP binding.

binding buffer and incubated in fresh binding buffer with 0.5 nM-¹²⁵I-GRP at 37 °C for 10 min. Cells were then washed and counted for radioactivity as previously described.

RESULTS

Scatchard analysis of binding to GRP receptors

A representative binding curve and Scatchard analysis of ¹²⁵I-GRP receptor binding in COR-L42 cells is shown in Figs. 1(a) and 1(b) respectively. Non-specific binding was approx. 20 % of total binding. Scatchard analysis indicated approx. 6700 high-affinity sites/cell (*K_d* 1.5 nM).

Specificity of ¹²⁵I-GRP binding to COR-L42 cells

Fig. 2 shows that [Lys³]bombesin, alytesin, GRP-10, human and pig GRP, ranatensin, litorin, NMB and phyllolitorin competed with ¹²⁵I-GRP for GRP-binding sites on COR-L42 cells in a concentration-dependent manner. Substance P and GRP-(1-16), the *N*-terminal amino acid sequence of GRP which is known to have no receptor-binding properties (Zachary & Rozengurt, 1987b), failed to compete for GRP-binding sites. Table 1 shows that for human GRP the concentration of unlabelled peptide required to inhibit 50 % of the specific binding

Fig. 2. Specificity of GRP binding to COR-L42 cells

Cells were incubated for 10 min at 37 °C with 0.5 nM-¹²⁵I-GRP and different concentrations of [Lys³]bombesin (●), alytesin (○), GRP-10 (■), human GRP (□), pig GRP (▲), ranatensin (△), litorin (◆), NMB (◇), phyllolitorin (■), GRP-(1-16) (□) and substance P (×). Data are expressed as percentages of ¹²⁵I-GRP binding in the absence of unlabelled peptide.

Table 1. Ability of various bombesin-related peptides to inhibit the binding of ¹²⁵I-GRP to COR-L42 cells

K_i values were determined from the IC₅₀ value (concn. causing 50 % inhibition), as calculated by the Cheng & Prusoff (1973) method.

Peptide	<i>K_i</i> (nM)
[Lys ³]Bombesin	0.40
Alytesin	0.40
GRP-10	0.49
GRP (human)	0.91
GRP (pig)	2.42
Ranatensin	6.06
Litorin	13.63
NMB	22.72
Phyllolitorin	59.09
GRP-(1-16)	> 10000
Substance P	> 10000

of ¹²⁵I-GRP was 0.91 nM. Ranatensin, litorin, NMB and phyllolitorin competed less potently for GRP-binding sites, having *K_i* values of 6.03, 13.63, 22.72 and 59.09 nM respectively.

Binding and cellular distribution of ¹²⁵I-GRP

Fig. 3(a) shows the time course of ¹²⁵I-GRP binding at 37 °C and 4 °C in COR-L42 cells. Maximum binding was achieved after 10 min at 37 °C, followed by a decline in cell-associated radioactivity at longer incubation times. At 4 °C maximum binding was achieved after 30 min, and the decline in specific binding at longer incubation times was markedly slower than that seen at 37 °C.

To investigate GRP receptor internalization, surface-bound and internalized ligand were separated using an acid/salt extraction procedure. Internalized versus surface-bound cell-

Fig. 3. Effect of temperature on binding and internalization of ¹²⁵I-GRP

(a) Time course of specific ¹²⁵I-GRP binding to COR-L42 cells at 37 °C (●) and 4 °C (○). Also shown are time courses of ¹²⁵I-GRP binding and internalization in COR-L42 cells at 37 °C (b) and 4 °C (c): ■, internalized ¹²⁵I-GRP; □, surface-bound ¹²⁵I-GRP. Each point represents the mean of two experiments.

associated radioactivity at 37 °C in COR-L42 cells can be seen in Fig. 3(b). Approx. 70 % of bound ^{125}I -GRP was internalized after 10 min. Cell-surface and internalized ^{125}I -GRP rapidly declined thereafter. Internalized ^{125}I -GRP remained at approx. 70 % of total cell-associated radioactivity throughout the time course. Fig. 3(c) shows that at 4 °C approx. 80 % of cell-associated radioactivity remained surface-bound throughout the time course.

Degradation of ^{125}I -GRP

To determine the pathway of ^{125}I -GRP degradation, the effects of the lysosomotropic agent chloroquine (5–50000 μM) and of phosphoramidon (10–10000 nM) on cell-associated radioactivity were studied. Fig. 4 shows that both chloroquine and phosphoramidon increased total binding of ^{125}I -GRP to COR-L42 cells in a dose-dependent manner. For subsequent experiments 5 mM and 10 μM concentrations of chloroquine and phosphoramidon respectively were used.

Fig. 4. Concentration-dependence of the effects of chloroquine and phosphoramidon on the binding of ^{125}I -GRP to COR-L42 cells

Cells were pretreated at 37 °C for 30 min in the presence of these inhibitors of degradation. These and parallel untreated control cultures were then incubated at 37 °C for 60 min with 0.5 nM- ^{125}I -GRP either in the absence or the presence of chloroquine (○) or phosphoramidon (●).

Fig. 5. Time course of ^{125}I -GRP binding to COR-L42 cells in the presence or the absence of inhibitors of degradation

Cells were pretreated at 37 °C for 30 min in the presence of either 5 mM-chloroquine or 10 μM -phosphoramidon. These and parallel untreated control cultures were then incubated for various times with 0.5 nM- ^{125}I -GRP either in the absence (●) or the presence of 5 mM-chloroquine (■), 10 μM -phosphoramidon (○) or both inhibitors at these concentrations (□). Each point represents the mean of two experiments.

Time courses of ^{125}I -GRP binding to COR-L42 in the presence or the absence of phosphoramidon, chloroquine or both can be seen in Fig. 5. The rate of GRP degradation was markedly decreased in the presence of phosphoramidon, since after an incubation time of 60 min total cell-associated radioactivity was approx. 3 times that of untreated cells at this time. Chloroquine also effectively inhibited the degradation of ^{125}I -GRP, since there was only a small decline in cell-associated radioactivity over a 4 h period compared with that seen in untreated controls. A combination of both inhibitors yielded a greater increase in cell-associated radioactivity compared with that obtained for each agent alone. No apparent changes in the association rates were seen in the presence of chloroquine and phosphoramidon.

Effect of exposure to GRP on the subsequent binding of ^{125}I -GRP

To investigate GRP receptor down-regulation COR-L42 cells were preincubated for various times with 100 nM-GRP prior to ^{125}I -GRP exposure. Fig. 6 shows that after a pretreatment time of 10 min, specific binding of ^{125}I -GRP was decreased to 40 % of that seen in cells receiving no GRP pretreatment. Longer GRP pretreatment times led to a progressive increase in ^{125}I -GRP binding which reached a plateau at 80 % of the ^{125}I -GRP binding seen in control cultures.

To determine whether this increase in ^{125}I -GRP binding was due to receptor recycling or to *de novo* synthesis of GRP receptors, cells were treated with the protein synthesis inhibitor cycloheximide (10 $\mu\text{g}/\text{ml}$) or with 5 mM-chloroquine to inhibit receptor recycling. Following addition of 10 μg of cycloheximide/ml, cell-associated radioactivity decreased to 75 % of that observed in cells receiving GRP pretreatment and no cycloheximide treatment. Treatment with 100 μg of cycloheximide/ml resulted in a further slight decrease in cell-associated radioactivity (results not shown). Treatment with 5 mM-chloroquine completely inhibited the increase in cell-associated radioactivity seen after 10 min in cultures pretreated with GRP only.

Fig. 6. Effect of pretreatment with GRP either in the presence or the absence of cycloheximide (10 $\mu\text{g}/\text{ml}$) or chloroquine (5 mM) on the binding of ^{125}I -GRP to COR-L42 cells

Cells were incubated at 37 °C either in the presence (●) or the absence (control) of 100 nM-GRP. Parallel cultured cells were incubated at 37 °C in the presence of cycloheximide (10 $\mu\text{g}/\text{ml}$), cycloheximide plus 100 nM-GRP (○), 5 mM-chloroquine, or 5 mM-chloroquine plus 100 nM GRP (■). After various times cultures were washed and incubated at 37 °C with 0.5 nM- ^{125}I -GRP for 10 min. Values are expressed as a percentage of the binding to their respective controls (i.e. no cycloheximide or chloroquine) and represent the means of two experiments.

Table 2. Effect of various peptides on the subsequent binding of ^{125}I -GRP in COR-L42 cells

Cells were incubated at 37 °C for 1 h in the presence or the absence (control) of pig GRP (100 nM), alytesin (100 nM), NMB (100 nM), phyllolitorin (100 nM) or substance P (1 μM). Values are expressed as a percentage of the binding to controls, and represent the means $\pm$ S.E.M. of two experiments.

Peptide	Binding (% of control)
None	100
GRP	53.3 $\pm$ 3.4
Alytesin	36.0 $\pm$ 2.8
NMB	102.3 $\pm$ 12.2
Phyllolitorin	95.6 $\pm$ 5.8
Substance P	98.6 $\pm$ 3.0

Effect of exposure to other peptides on the subsequent binding of ^{125}I -GRP

A comparison of the subsequent binding of ^{125}I -GRP to COR-L42 cells following pretreatment with GRP (100 nM), alytesin (100 nM), NMB (100 nM), phyllolitorin (100 nM) or substance P (1 μM) is shown in Table 2. It can be seen that pretreatment with alytesin significantly decreased the subsequent binding of ^{125}I -GRP. Pretreatment of cells with NMB, phyllolitorin or substance P failed to influence significantly the subsequent binding of ^{125}I -GRP.

DISCUSSION

The mechanism by which a cell may be driven into mitogenesis requires the interaction between a growth factor and its receptor to form a ligand-receptor complex (Carpenter & Cohen, 1976; Heldin *et al.*, 1982; Walker & Burgess, 1987; Furlanetto, 1988; Galizzi *et al.*, 1989). Previous studies have shown that ^{125}I -GRP binds to a single class of high-affinity receptors on SCLC cells (Moody *et al.*, 1985; Layton *et al.*, 1988; Kado-Fong & Malfroy, 1989), and stimulates mobilization of intracellular Ca^{2+} in these cells (Heikkila *et al.*, 1987; Woll & Rozengurt, 1989; Bunn *et al.*, 1990). The present study shows that in the SCLC cell line COR-L42, ^{125}I -GRP binds to a single class of high-affinity receptors and is rapidly internalized and subsequently degraded. At 37 °C, approx. 75 % of radiolabelled GRP was internalized by COR-L42 cells, this energy-dependent process having a markedly lower rate at 4 °C, as indicated by retention of ^{125}I -GRP at the cell surface. These findings suggest that, like other peptide growth factors (Carpenter & Cohen, 1976; Heldin *et al.*, 1982; Walker & Burgess, 1987; Furlanetto, 1988; Galizzi *et al.*, 1989), GRP is internalized by receptor-mediated endocytosis in COR-L42 cells. Although similar findings have been reported in Swiss 3T3 fibroblasts (Zachary & Rozengurt, 1987a) and HIT (pancreatic islet) cells (Swope & Schonbrunn, 1987), an earlier study of ^{125}I -[Tyr⁴]bombesin binding to the SCLC cell line NCI-H446 reported that all binding of [Tyr⁴]bombesin was reversible and that little if any of the peptide was internalized by these cells (Moody *et al.*, 1985). The non-concordance between this finding and those of the present study may reflect differences in the ability of SCLC cell lines to internalize bombesin/GRP, a contention supported by the finding that no decrease in cell-associated radioactivity was observed in NCI-H446 cells after maximum binding had been reached (Moody *et al.*, 1985).

Competitive binding studies with bombesin-related peptides indicated that [Lys³]bombesin, alytesin, GRP-10 and GRP (human and pig) potentially bound to GRP receptors. Ranatensin-

like peptides also competed with ^{125}I -GRP, but less potently than the bombesin peptides. This finding is particularly noteworthy, given that lung tumour cell lines have been shown recently to express the NMB gene and to synthesize this peptide (Cardona *et al.*, 1991). Since NMB is a mitogen for SCLC (Bepler *et al.*, 1988), NMB may function as an autocrine growth factor in SCLC cells exerting its effects at least in part through interaction with GRP receptors. Interestingly, phyllolitorin, the third member of the bombesin-like peptide subfamilies, also bound to GRP receptors with a K_i of 59.1 nM.

After internalization of the ligand-receptor complex and the formation of endosomes, ligands and their receptors are dissociated and segregated (Brown *et al.*, 1983; Geuze *et al.*, 1983; Presky & Schonbrunn, 1986). Numerous reports have demonstrated degradation of ligands and ligand-receptor complexes by the action of several enzymes (King *et al.*, 1980; Heldin *et al.*, 1982; Walker & Burgess, 1987). The present study indicates that in COR-L42 cells, ^{125}I -GRP is degraded by endo/exopeptidases. Chloroquine is a weak base which acts by increasing intralysosomal pH, thus inhibiting further processing of ligand-receptor complexes (Wibo & Poole, 1974; Seglen *et al.*, 1979; Marshall & Olefsky, 1979; Poole & Ohkuma, 1981; Maxfield, 1982). Binding studies carried out in the presence of chloroquine showed that after ^{125}I -GRP binding and internalization, cell-associated radioactivity did not decline, indicating that the subsequent degradation of ^{125}I -GRP was inhibited by this agent. Similarly, phosphoramidon, which is an inhibitor of the EC 3.4.24.11 enzyme responsible for the degradation of GRP (Bunnett *et al.*, 1985), delayed the decrease in cell-associated radioactivity in COR-L42 cells. These findings indicate that in COR-L42 cells GRP is not only degraded by a lysosomal pathway as previously described for Swiss 3T3 cells (Zachary & Rozengurt, 1987a; Brown *et al.*, 1988), HIT cells (Swope & Schonbrunn, 1987) and pituitary cells (Westendorf & Schonbrunn, 1983), but is also degraded by a phosphoramidon-sensitive extracellular pathway possibly involving the EC 3.4.24.11 enzyme, recently shown to be the common acute lymphoblastic leukaemia antigen (CALLA) (Shipp *et al.*, 1989).

Receptor-mediated endocytosis results, in many cases, in a marked decrease in the number of receptors, a phenomenon known as receptor down-regulation. Indeed, it has been shown extensively that binding of growth factors and polypeptides to their receptors often leads to receptor down-regulation (Carpenter & Cohen, 1976; Heldin *et al.*, 1982; Moscatelli, 1988; Galizzi *et al.*, 1989; Samanta *et al.*, 1990). Furthermore, receptor down-regulation has been implicated in the initiation of mitogenesis (Rozengurt, 1986; Glenney *et al.*, 1988). In the present study evidence for GRP receptor down-regulation in COR-L42 cells was obtained, in that following GRP pretreatment ^{125}I -GRP binding was significantly decreased compared with control levels. It is unlikely that this decrease is due to the occupation of GRP receptors by unlabelled GRP, since acid/salt extraction studies indicate that much of the unlabelled GRP would have been internalized during the pretreatment period. Furthermore, both NMB and phyllolitorin are able to bind to GRP receptors and would similarly occupy surface GRP receptors. However, no decrease in the subsequent binding of ^{125}I -GRP was observed following pretreatment with these peptides. As expected, pretreatment with the amphibian peptide alytesin, which is also a member of the bombesin subfamily and has a C-terminal decapeptide sequence with only a single amino acid difference compared to that of GRP, markedly inhibited the subsequent binding of GRP to its receptor. Interestingly, GRP receptor down-regulation has also been demonstrated in a pancreatic islet cell line (Swope & Schonbrunn, 1987). In contrast, GRP receptors appear not to be down-regulated in the Swiss 3T3 cell line

(Zachary & Rozengurt, 1987a; Brown *et al.*, 1988). These findings indicate differences in GRP receptor regulation in Swiss 3T3 and SCLC cells. In this context, it is interesting to note that basic fibroblast growth factor receptors appear to be down-regulated in baby hamster kidney cells, in mouse lung capillary endothelial cells and in bovine capillary endothelial cells, but not in Swiss 3T3 cells (Neufeld & Gospodarowicz, 1985; Olwin & Hauschka, 1986; Friesel *et al.*, 1986; Moscatelli, 1988).

Following internalization and dissociation of the ligand-receptor complex, receptors and ligands can either be inactivated by the action of specific hydrolases or return intact to the cell surface to be re-utilized (Marshall, 1985). In the present study a substantial recovery in the number of GRP-binding sites was observed following receptor down-regulation. A similar re-appearance of down-regulated receptors has been observed for asialoglycoprotein, macroglobulin and low-density lipoprotein receptors (Brown *et al.*, 1983; Berg *et al.*, 1983; Dunn & Hubbard, 1984), cytokine receptors (Samanta *et al.*, 1990; Galizzi *et al.*, 1989; Murthy *et al.*, 1989) and calcitonin receptors (Schneider *et al.*, 1988). Pretreatment of COR-L42 cells with cycloheximide, to inhibit the synthesis of new GRP receptors, and with chloroquine, an agent which blocks receptor recycling (Marshall *et al.*, 1981; Knutson *et al.*, 1983), resulted in a decrease in ¹²⁵I-GRP binding to 60% and 30% respectively of control levels. These findings indicate that although some GRP receptors are synthesized *de novo*, the majority of GRP receptors are re-utilized.

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