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Internalization and degradation of peptides of the bombesin family in Swiss 3T3 cells occurs without ligand-induced receptor down-regulation

Ian Zachary and Enrique Rozengurt

Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London, WC2A 3PX, UK

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The binding of [^{125}I]gastrin releasing peptide ([^{125}I]GRP) to Swiss 3T3 cells at 37°C increases rapidly, reaching a maximum after 30 min and decreasing afterwards. The decrease in cell-associated radioactivity at this temperature is accompanied by extensive degradation of the labelled peptide. At 4°C equilibrium binding is achieved after 6 h and [^{125}I]GRP degradation is markedly inhibited. Extraction of surface-bound ligand at low pH demonstrates that the iodinated peptide is internalized within minutes after addition to 3T3 cells at 37°C. The rate of internalization is strikingly temperature-dependent and is virtually abolished at 4°C. In addition, lysomotropic agents including chloroquine increase the cell-associated radioactivity in cells incubated with [^{125}I]GRP. The binding of [^{125}I]GRP to Swiss 3T3 cells was not affected by pretreatment for up to 24 h with either GRP or bombesin at mitogenic concentrations. Furthermore, pretreatment with GRP did not reduce the affinity labelling of a M_r 75 000–85 000 surface protein recently identified as a putative receptor for bombesin-like peptides. These results demonstrate that while peptides of the bombesin family are rapidly internalized and degraded by Swiss 3T3 cells, the cell surface receptors for these molecules are not down-regulated.

Key words: gastrin releasing peptide/lysomotropic agent/bombesin

Introduction

The binding of several peptide growth factors to their specific surface sites is followed by the rapid internalization of the ligand–receptor complexes and the subsequent degradation of both the growth factor and the receptor in lysosomes (Carpenter and Cohen, 1979; King and Cuatrecasas, 1981; Pastan and Willingham, 1981; James and Bradshaw, 1984; Wileman *et al.*, 1985; Goldstein *et al.*, 1985; Bergeron *et al.*, 1985; Stahl and Schwartz, 1986). These processes result in a marked decrease in the number of available receptors at the cell surface (down-regulation). In addition to the possible role of receptor down-regulation in the control of target-cell responsiveness, it has been proposed that the internalization and degradative processing of the receptor may be important for the propagation of the mitogenic signal into the cell, but this issue remains unresolved (King and Cuatrecasas, 1981; James and Bradshaw, 1984; Bergeron *et al.*, 1985; Wakshull and Wharton, 1985).

Regulatory peptides which act as local hormones or neurotransmitters in an autocrine or paracrine fashion on adjacent cells are increasingly implicated in the control of cell proliferation (Rozengurt *et al.*, 1979; Rozengurt and Sinnett-Smith, 1983; Nilsson *et al.*, 1985; Payan, 1985; Singh *et al.*, 1986). The amphibian tetradecapeptide bombesin (Anastasi *et al.*, 1971) and structurally related mammalian peptides including gastrin-releasing peptide (GRP) and the neuromedins (Wharton *et al.*, 1978; McDonald *et al.*, 1979; Moody and Pert, 1979; Minamino *et al.*, 1983, 1984, 1985) are potent mitogens for Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983; Zachary and Rozengurt, 1985a). These peptides bind to high-affinity cell-surface receptors in Swiss 3T3 cells (Zachary and Rozengurt, 1985a) and elicit a complex array of early biological responses (Rozengurt, 1986) including enhanced phosphoinositide metabolism and mobilization of Ca^{2+} from intracellular stores (Heslop *et al.*, 1986; Mendoza *et al.*, 1986; Takuwa *et al.*, 1987), stimulation of Na^+/H^+ antiport activity (Mendoza *et al.*, 1986), activation of protein kinase C (Zachary and Rozengurt, 1985b; Zachary *et al.*, 1986; Isacke *et al.*, 1986), inhibition of [^{125}I]epidermal growth factor ([^{125}I]EGF) binding (Brown *et al.*, 1984; Zachary and Rozengurt, 1985b; Zachary *et al.*, 1986), and induction of the cellular oncogenes *c-fos* and *c-myc* (Letterio *et al.*, 1986; Palumbo *et al.*, 1986; Rozengurt and Sinnett-Smith, 1987). Recently, Zachary and Rozengurt (1987) identified a surface protein in Swiss 3T3 cells with apparent Mr 75 000–85 000 as a putative component for the bombesin/GRP receptor. In addition, bombesin-like peptides are present in high concentrations in small cell lung carcinoma (Moody *et al.*, 1981; Wood *et al.*, 1981; Erisman *et al.*, 1982; Roth *et al.*, 1983) where they could act as auctocrine growth factors (Cuttita *et al.*, 1985). Thus, peptides of the bombesin family may provide a novel and valuable model for the elucidation of the mechanism(s) underlying cellular proliferation.

The experiments presented in this study were designed to examine the fate of [^{125}I]GRP and its receptor following the binding of this mitogen to Swiss 3T3 cells. Our results indicate that [^{125}I]GRP is internalized and extensively degraded by these cells. However, it is shown by a number of criteria that peptides of the bombesin family, in contrast to other growth factors, do not cause down-regulation of their specific cell-surface receptors.

Results

Binding and degradation of [^{125}I]GRP: influence of temperature and lysomotropic agents

Figure 1A shows the time-course of [^{125}I]GRP binding to confluent cultures of Swiss 3T3 cells at 37°C. Cell-associated radioactivity increased rapidly after addition of the peptide reaching a maximum after 30 min and declining afterwards. At longer time points than those shown, binding of the radiolabelled ligand was virtually undetectable. The marked decrease in specific binding after 30 min suggested that labelled peptide was being extensively degraded either intracellularly via a lysosomal pathway or extracellularly by an ectopeptidase. To test this possibility, cultures were incubated with [^{125}I]GRP and after various times the medium was removed and analysed by chromatographic separation on Sep Pak C18 cartridges as described under Materials and methods. Using this procedure we found that there was a time-dependent decrease in the level of the in-

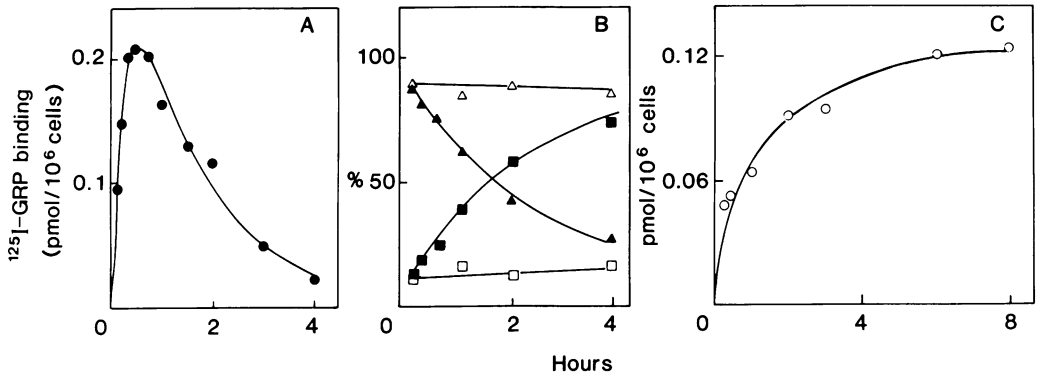

Fig. 1. A. Time-course of [^{125}I]GRP binding to Swiss 3T3 cells at 37°C. Confluent and quiescent cultures of 3T3 cells were washed and incubated with [^{125}I]GRP (1 nM). After various times cells were washed, extracted and cell-associated radioactivity was determined as described under Materials and methods. Each point represents the mean of duplicate determinations. **B.** Degradation of [^{125}I]GRP by Swiss 3T3 cells. Cultures were incubated with 1 nM [^{125}I]GRP either at 37°C (closed symbols) or at 4°C (open symbols). After various times the medium was removed and the relative amounts of [^{125}I]GRP (▲,△) and [^{125}I]tyrosine (■,□) were measured by chromatography on Sep-Pak C18 cartridges as described under Materials and methods. Each point represents a composite of three experiments. **C.** Time-course of [^{125}I]GRP binding to Swiss 3T3 cells at 4°C. Confluent cultures were incubated with 1 nM [^{125}I]GRP at 4°C for various times. Other experimental details were as described in Materials and methods. Each point represents the mean of two determinations.

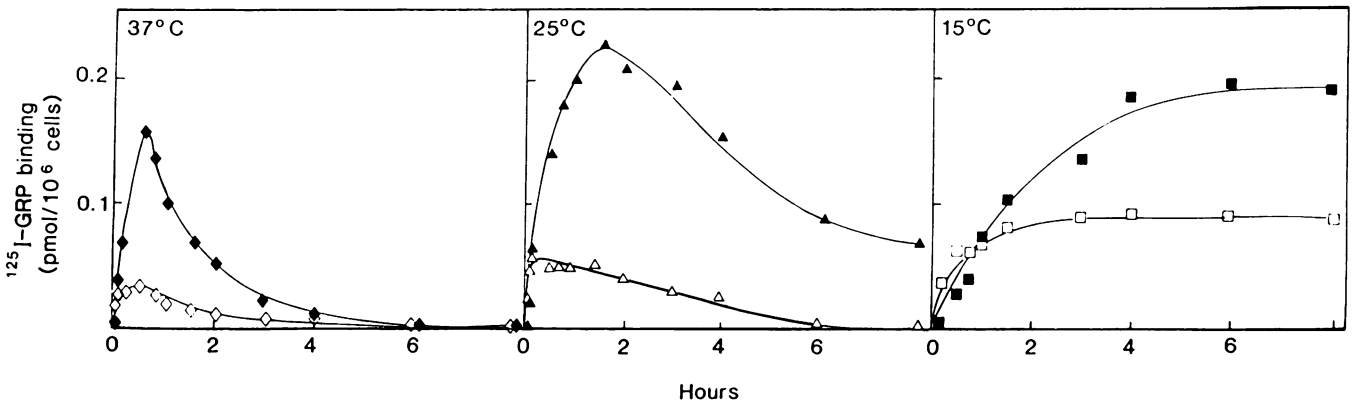

Fig. 2. Effect of temperature on the internalization of [^{125}I]GRP by Swiss 3T3 cells. Parallel confluent cultures of Swiss 3T3 cells were incubated with 1 nM [^{125}I]GRP at the temperature indicated. After various times cells were washed at 4°C and surface-bound (open symbols) and intracellular (closed symbols) cell-associated radioactivity were determined as described in Materials and methods. Each point represents the mean of duplicate determinations.

tact peptide at 37°C and a concomitant increase in the level of iodotyrosine in the medium. Furthermore, ligand breakdown correlated with a decrease in binding. Thus, when medium was removed from cells preincubated with [^{125}I]GRP and then added to fresh cultures for 30 min at 37°C a progressive decline in specific binding was observed with increasing length of preincubation (not shown). At 4°C peptide degradation was almost completely inhibited (Figure 1B, open symbols) and the specific binding of the labelled ligand increased slowly reaching equilibrium after about 6 h (Figure 1C).

Since the endocytotic uptake and degradation of environmental ligands is markedly diminished at 4°C (King and Cuatrecasas, 1981; Pastan and Willingham, 1981), the above results suggested that [^{125}I]GRP was being rapidly internalized by Swiss 3T3 cells. Indeed internalization of the related ligand [^{125}I]Tyr^4 bombesin has been demonstrated in rat pituitary cells (Westendorf and Schonbrunn, 1983). To investigate this possibility an acid−salt extraction procedure (Haigler *et al.*, 1980) was used to remove surface-bound ligand. The cellular distribution of specifically bound peptide at 37°C, 25°C and 15°C is shown in Figure 2. At 37°C [^{125}I]GRP was rapidly transformed from an acid-extractable to an acid-resistant form; after only 5 min approximately 50% of specific cell-associated radioactivity was internalized, and at 30 min only 20% remained in an acid-extractable form (Figure 2A). The rate of ligand internalization was markedly decreased at lower temperatures. Internalized [^{125}I]GRP represented 34% and 52% of total cell-associated radioactivity respectively after 5 min at 25°C and 1 h at 15°C (Figure 2B and C). In another experiment performed at 4°C internalization of [^{125}I]GRP virtually ceased; 86, 85 and 86% of cell-associated radioactivity remained surface-bound after 2, 4 and 6 h of incubation respectively (results not shown).

Our results suggested that [^{125}I]GRP was being internalized and subsequently degraded, but did not rule out the possibility that the labelled peptide was being removed from the medium extracellularly. Indeed, recent reports suggest that a number of small regulatory peptides, including substance P and cholecystokinin, are degraded by an ecto-endopeptidase (Matsas *et al.*, 1983, 1984; Connelly *et al.*, 1985). To distinguish between these possibilities cells were incubated for 1 h with [^{125}I]GRP to allow internalization to take place, washed and then further incubated in medium lacking the labelled ligand. After various times, the medium was removed and analysed by chromatographic separation on Sep Pak C18 cartridges. Figure 3 shows that there was a time-dependent increase in the level of radioactivity in the medium of the preincubated and washed cultures which occur-

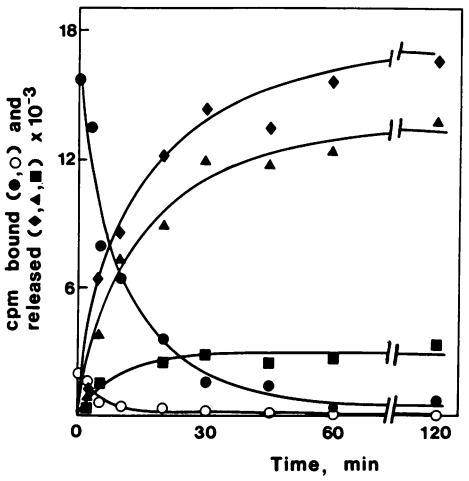

Fig. 3. Confluent cultures of Swiss 3T3 cells were incubated with [^{125}I]GRP (1 nM) for 1 h at 37°C, washed 3 × with DME medium containing 0.1% DBSA, and then incubated in medium without the radiolabelled ligand. After various times the medium was removed and the relative amounts of [^{125}I]GRP (■) and [^{125}I]tyrosine (▲) measured as described under Materials and methods. ◆, total specific cell-associated radioactivity released into the medium. The cells were rapidly chilled to 4°C in 0.5 M NaCl, 0.2 M acetic acid, pH 2.5 and incubated at 4°C for 6 min to determine specific surface binding (open circles). This medium was removed for counting, and the remaining specific intracellular cell-associated radioactivity (closed circles) was extracted as described in Materials and methods. Each point represents a composite of two experiments.

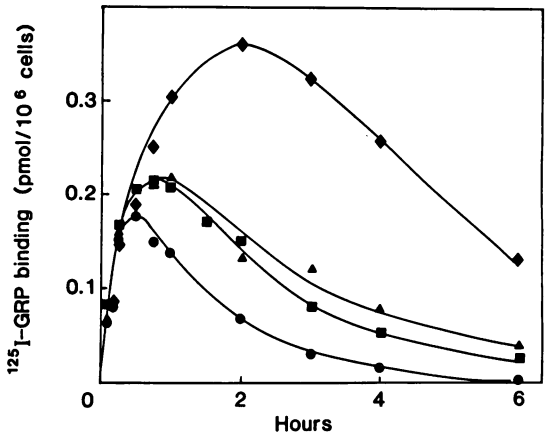

Fig. 4. Time-course of [^{125}I]GRP binding to Swiss 3T3 cells in the presence or absence of inhibitors of degradation. Confluent cultures of cells were pretreated at 37°C for 2.5 h in the presence of 2 μM colchicine or in the presence of either 500 μM chloroquine or 10 mM NH_4Cl for 30 min. These and parallel unpretreated control cultures were then incubated for various times with 1 nM [^{125}I]GRP either in the absence (●) or presence of 2 μM colchicine (▲), 10 mM NH_4Cl (■) or 500 μM chloroquine (◆). Other experimental details were as described in Materials and methods. Each point represents the mean of duplicate determinations.

red concomitantly with a decrease in intracellular cell-associated radioactivity. Most (>60%) of the labelled material was in the form of iodotyrosine, and was therefore derived from the degradation of internalized [^{125}I]GRP. Intact [^{125}I]GRP accounted for a significant fraction (>20%) of the cell-associated radioactivity released into the medium. This was most likely due either to the dissociation of residual surface-bound ligand or, alternatively, to internalized and undegraded [^{125}I]GRP which has been returned to the plasma membrane.

The results presented in Figures 1–3 supported the conclusion that [^{125}I]GRP was rapidly internalized and subsequently degraded by Swiss 3T3 cells at 37°C. It is generally recognised that the lysosomes are the principal site of degradation for extracellular ligands and their receptors, and pharmacological agents which interfere with lysosomal function have been widely used to support this conclusion (King and Cuatrecasas, 1981; Pastan and Willingham, 1981; Wileman *et al.*, 1985; Massagué and Kelly, 1986). To determine the pathway of [^{125}I]GRP degradation, we investigated the effect of a variety of lysomotropic agents on the time-course of peptide binding. Figure 4 shows that treatment of Swiss 3T3 cells with 500 μM chloroquine caused a striking increase in cell-associated [^{125}I]GRP. Chloroquine had no effect upon the rate of [^{125}I]GRP internalization but markedly inhibited the degradation of [^{125}I]GRP at 37°C (not shown). Ammonium chloride, methylamine and leupeptin also significantly enhanced the binding of [^{125}I]GRP to 3T3 cells (Figure 4 and results not shown). In addition, the microtubule-disrupting agent colchicine which acts synergistically with bombesin in the stimulation of DNA synthesis (Rozengurt and Sinnett-Smith, 1983) and inhibits the degradation of [^{125}I]EGF (Brown *et al.*, 1980), also enhanced the association of [^{125}I]GRP with Swiss 3T3 cells (Figure 4). None of the agents tested significantly affected the initial rate of binding (up to 30 min) of the labelled peptide (Figure 4), suggesting that the enhancement of cell-associated radioactivity was due to the inhibition of ligand degradation.

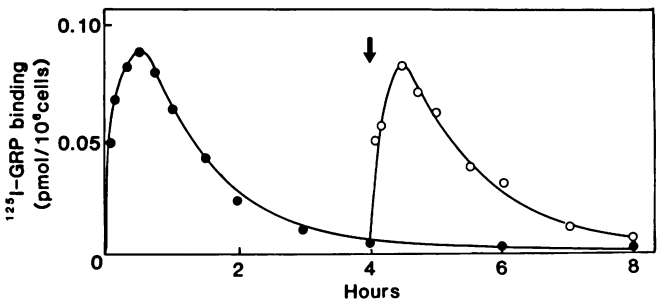

Fig. 5. Time-course of [^{125}I]GRP binding to Swiss 3T3 cells preincubated with [^{125}I]GRP. Confluent cultures of 3T3 cells were incubated at 37°C with 1 nM [^{125}I]GRP in the presence or absence of 360 nM unlabelled GRP, and cell-associated radioactivity was measured after various times (●). At the time point indicated by the arrow (4 h) some cells were rapidly washed at 37°C and incubated in fresh medium containing 1 nM [^{125}I]GRP either in the presence or absence of excess unlabelled peptide. Cell-associated radioactivity was then determined at the times indicated (○). Each point represents the mean of duplicate determinations.

Effect of exposure to [^{125}I]GRP, GRP and bombesin on the subsequent binding of [^{125}I]GRP

The striking decline in the specific binding of [^{125}I]GRP to Swiss 3T3 cells at 37°C (Figures 1A and 2A) could be due either to: (i) the removal of the labelled ligand from the medium by degradation; (ii) to the down-regulation of available receptors on the cell surface, or (iii) to both. The first possibility predicts that after incubating cells with [^{125}I]GRP for 4 h when binding has declined markedly, readdition of labelled peptide should initiate another cycle of binding and degradation. In contrast, the second possibility predicts a decrease in binding of newly added [^{125}I]GRP. To distinguish between these possibilities Swiss 3T3 cells were incubated with 1 nM [^{125}I]GRP at 37°C for 4 h; the cultures were then washed and fresh ligand added for various times. Figure 5 shows that neither the maximum binding nor the initial rate of association of [^{125}I]GRP were altered by a 4 h pretreatment with the labelled ligand. Furthermore, similar results

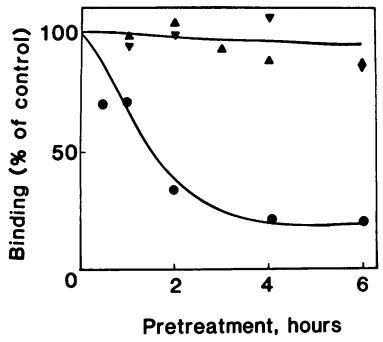

Fig. 6. Effect of pretreatment with GRP on the binding of [^{125}I]GRP to Swiss 3T3 cells. Confluent cultures of 3T3 cells were incubated at 37°C either in the presence or absence of 3.6 nM GRP (▲), 36 nM GRP (▼) or 3.3 nM EGF (●). After the times indicated, pretreated and control cultures were extensively washed and incubated at 37°C with either 5 nM [^{125}I]GRP for 30 min (▲,▼) or 8.3 nM [^{125}I]EGF for 1 h (●). Values are expressed as a percentage of the binding to control, unpretreated cultures and represent the mean of duplicate determinations.

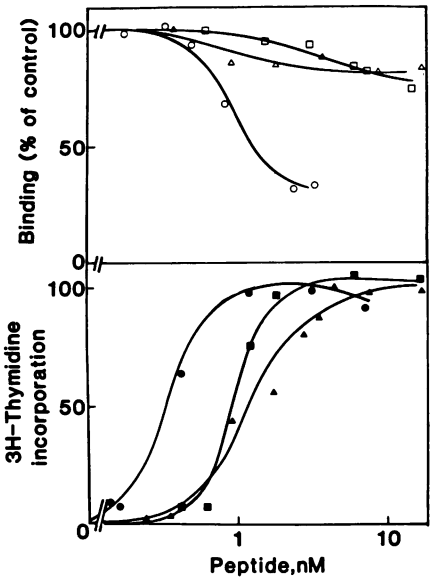

Fig. 7. Concentration-dependence of the effect of GRP and bombesin on [^{125}I]GRP binding and DNA synthesis in parallel cultures of Swiss 3T3 cells. **Upper panel.** Confluent and quiescent cultures of Swiss 3T3 cells were treated at 37°C either in the presence or absence of various concentrations of either GRP (Δ), bombesin (□) or EGF (○). Afer 3 h cultures were extensively washed and then incubated at 37°C with either 5 nM [^{125}I]GRP for 30 min (Δ,□) or 8.3 nM [^{125}I]EGF (○) for 1 h. Values are expressed as the percentage of binding to unpretreated cultures and represent the mean of duplicate determinations. **Lower panel.** Stimulation of DNA synthesis in confluent and quiescent cultures of Swiss 3T3 cells by various concentrations of EGF (●), bombesin (■) or GRP (▲). The assay for DNA synthesis was performed in the presence of 1 μg/ml insulin. Values are expressed as a percentage of the effect obtained in the presence of saturating concentrations of either EGF (1.2 nM), bombesin (6.2 nM) or GRP (5 nM). Each point represents the mean of duplicate determinations.

were obtained when fresh [^{125}I]GRP was directly added to cultures preincubated with the labelled peptide for 4 h without first removing the medium and washing the pretreated cells.

Since, it is known that exposure of Swiss 3T3 cells to EGF causes a marked loss of surface binding sites for this mitogen which occurs as a result of the internalization and degradation of its receptor (Pastan and Willingham, 1981; James and Bradshaw, 1984; Beguinot *et al.*, 1984; Stoscheck and Carpenter, 1984), we compared the effects on ligand binding of exposure of 3T3 cells to EGF and GRP. When cultures were pretreated with EGF at a concentration of 3.3 nM for various times, extensively washed to remove residual surface-bound ligand and then incubated with a saturating concentration of [^{125}I]EGF for 1 h a progressive and marked decrease in binding was observed (Figure 6). After 4 h of pretreatment specific binding was reduced to 20% of control level. In contrast exposure of cells to GRP at a concentration of either 3.6 or 36 nM for up to 6 h caused only a slight decrease in the level of specific binding (Figure 6). Furthermore, exposure of Swiss 3T3 cells to GRP at a concentration of 36 nM for 24 h did not significantly decrease binding of the labelled peptide. Figure 7 shows the effect of various concentrations of GRP, bombesin and EGF on both mitogenesis (lower panel) and ligand binding (upper panel). There was a close parallel between the down-regulation of EGF receptors and the mitogenicity of the polypeptide. In contrast, at concentrations of GRP and bombesin which produce a maximum stimulation of DNA synthesis the binding of [^{125}I]GRP was unaffected.

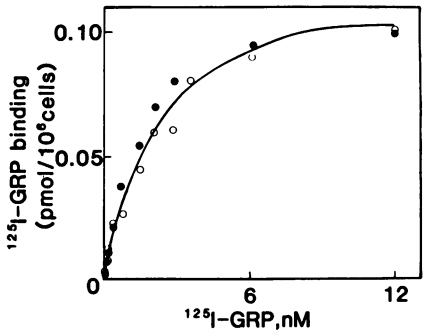

Fig. 8. Effect of pretreatment with GRP on the concentration-dependence of [^{125}I]GRP binding to Swiss 3T3 cells. Confluent cultures of 3T3 cells were incubated at 37°C either in the presence (○) or absence (●) of 18 nM GRP. After 3 h control and pretreated cultures were extensively washed and then incubated for 30 min with various concentrations of [^{125}I]GRP in either the presence or absence of 360 nm unlabelled GRP. Each point represents the mean of duplicate determinations.

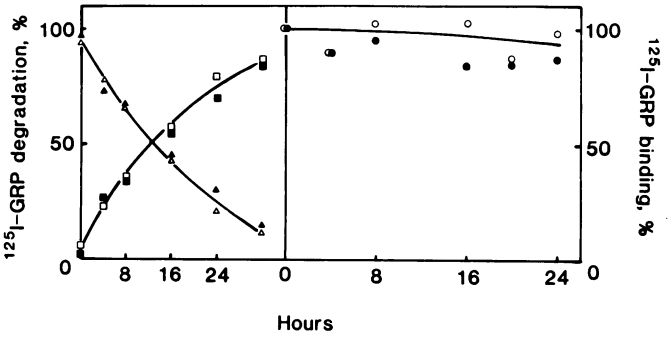

Fig. 9. Effect of cycloheximide on the degradation (**left panel**) and binding (**right panel**) of [^{125}I]GRP in Swiss 3T3 cells. **Left:** confluent and quiescent cultures were washed twice with DME medium at 37°C and incubated at 37°C with [^{125}I]GRP (5 nM) either in the absence (closed symbols) or presence (open symbols) of 250 ng/ml cycloheximide. After various times, the medium was removed and the relative amounts of [^{125}I]GRP (▲,Δ) and [^{125}I]tyrosine (■,□) were measured as described under Materials and methods. **Right:** cultures were washed and incubated at 37°C in the presence of either 250 ng/ml cycloheximide (●) or 250 ng/ml cycloheximide and 18 nM GRP (○). After various times cultures were extensively washed and incubated at 37°C with 5 nM [^{125}I]GRP for 30 min. Values are expressed as a percentage of the binding to parallel unpretreated cultures and represent the mean of duplicate determinations.

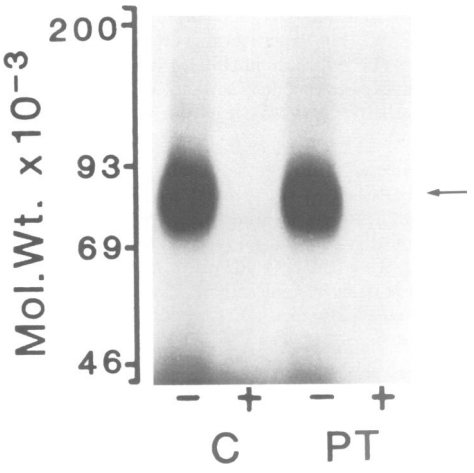

Fig. 10. Effect of GRP pretreatment on the [^{125}I]GRP affinity labelling of a Mr 75 000–85 000 protein in Swiss 3T3 cells. Confluent cultures were preincubated at 37°C in either the presence (PT) or absence (C) of 18 nM GRP. After 3 h cells were washed and then incubated at 37°C with 1.25 nM [^{125}I]GRP in either the presence (+) or absence (−) of a 500-fold excess of unlabelled peptide. After 30 min cultures were rapidly washed at 15°C and incubated at this temperature for 15 min wih 6 mM EGS. Cells were then extracted and samples were analysed by one-dimensional SDS–PAGE. Other experimental details were as described in Materials and methods. The arrow indicates the position of the Mr 75 000–85 000 protein.

In addition, we examined the effect of GRP pretreatment on the concentration-dependence of peptide binding. Quiescent cultures of 3T3 cells were incubated with GRP for 3 h, washed extensively and incubated a further 30 min with various concentrations of the labelled peptide. The pretreatment had no significant effect on either the affinity or the number of receptors (Figure 8). When this experiment was repeated using cultures only 4 days after plating instead of 6, identical results were obtained.

The preceding results (Figures 5–8) show that exposure of cells to GRP did not result in a significant decrease in the number of surface binding sites for the peptide and suggest that the receptors are recycled after internalization, rather than degraded. It remained plausible, however, that a significant fraction of the receptors were being degraded and subsequently replaced either by synthesis of new receptors or from a pool of receptors within the cytoplasm. This possibility predicts that prolonged treatment of cells with cycloheximide together with a saturating concentration of GRP should cause a progressive depletion of receptors from the cells. This would have two consequences: (a) inhibition of [^{125}I]GRP degradation, and (b) a decrease in the number of available receptors at the cell surface. To assess the effect of inhibiting protein synthesis on the degradation of [^{125}I]GRP Swiss 3T3 cells were pretreated for various times either in the presence or absence of cycloheximide at 250 ng/ml, a concentration which causes a 70–80% reduction in protein synthesis (Lopez-Rivas *et al.*, 1982). Figure 9 (left panel) shows that treatment with cycloheximide for up to 32 h did not inhibit degradation of [^{125}I]GRP (5 nM), as measured by the progressive increase of [^{125}I]tyrosine and concomitant decrease of intact labelled peptide in the medium. A similar conclusion was obtained when cells were incubated for 5 h with 5 nM [^{125}I]GRP following different times of exposure to cycloheximide (results not shown). Cycloheximide at a concentration of 10 μg/ml had no effect on the binding of [^{125}I]GRP in either control cultures or cells pretreated for 4 h with 18 nM GRP. Furthermore, continuous exposure of cells for up to 24 h with cycloheximide at a concentration of 250 ng/ml and a saturating concentration of GRP did not decrease the subsequent binding of the labelled peptide compared with control unpretreated cultures (Figure 9, right panel). The ability of Swiss 3T3 cells to continuously degrade [^{125}I]GRP in the presence of cycloheximide, and the inability of a prolonged pretreatment with cycloheximide and a saturating concentration of GRP to decrease the binding of [^{125}I]GRP to these cells strongly suggests that the receptors for peptides of the bombesin family are re-utilised, i.e. recycled to the cell surface after internalization.

Pretreatment with GRP does not alter the level of a putative receptor

Recently we identified a Mr 75 000–85 000 cell-surface protein in Swiss 3T3 cells which is affinity-labelled by [^{125}I]GRP. Several lines of evidence support the conclusion that this protein is a component of the receptor for peptides of the bombesin family in these cells (Zachary and Rozengurt, 1987). The Mr 75 000–85 000 protein was not found in other cell lines which do not exhibit receptors for bombesin-like peptides. Unlabelled GRP competed for affinity-labelling of this protein in a concentration-dependent manner and other peptides structurally related to GRP also inhibited the cross-linking of [^{125}I]GRP to this component. In contrast, a variety of other peptide hormones and mitogens, including EGF, had no effect. Finally, affinity-labelling of the Mr 75 000–85 000 protein was dependent on the concentration of [^{125}I]GRP and the increase in the level of the cross-linked band closely paralleled the ability of the peptide to stimulate DNA synthesis and a variety of early biological responses in quiescent Swiss 3T3 cells (Zachary and Rozengurt, 1987). To determine whether chronic exposure of Swiss 3T3 cells to bombesin-like peptides resulted in a decrease in the level of the Mr 75 000–85 000 protein parallel cultures were pretreated for 3 h in the presence or absence of GRP at 18 nM. After washing, cultures were incubated for a further 30 min with 1.25 nM [^{125}I]GRP and then treated with the homobifunctional cross-linking agent EGS. As shown in Figure 10, pretreatment with the peptide did not cause any significant alteration in the level of the Mr 75 000–85 000 protein. In addition, prolonged (24 h) pretreatment of cells with GRP also failed to reduce the level of the band relative to the control.

Discussion

The findings presented here demonstrate that [^{125}I]GRP is rapidly internalized and subsequently degraded by intact Swiss 3T3 cells at 37°C. The rates of both uptake and breakdown of [^{125}I]GRP are markedly reduced at lower temperatures suggesting that the internalization of [^{125}I]GRP, like other peptide growth factors, occurs by receptor-mediated endocytosis (Pastan and Willingham, 1981; James and Bradshaw, 1984). In addition, a variety of lysomotropic agents were found to enhance the association of [^{125}I]GRP to Swiss 3T3 cells and inhibit degradation of the peptide at 37°C. These data suggest that the pathway of [^{125}I]GRP processing in Swiss 3T3 cells is at least in part lysosomal.

The binding of polypeptide growth factors such as EGF and platelet-derived growth factor (PDGF) to their receptors is followed by rapid internalization and intracellular degradation of the ligand as well as the receptor. This process results in a marked reduction in the number of surface binding sites in the target cell (down-regulation). Receptor down-regulation was originally demonstrated by ligand binding studies with [^{125}I]EGF (Carpenter and Cohen, 1976; Das and Fox, 1978; Knipp *et al.*, 1982) and [^{125}I]PDGF (Bowen-Pope and Ross, 1982; Heldin *et*

al., 1982; Pike *et al.*, 1983), and subsequently verified for the EGF receptor using specific antibodies directed against the receptor molecule (Stoscheck and Carpenter, 1984; Beguinot *et al.*, 1984). Pandol *et al.* (1982) have reported that in pancreatic acinar cells exposure to bombesin also causes an apparent decrease in the number of its cell surface receptors. A salient feature of the results presented here is that exposure of Swiss 3T3 cells to mitogenic concentrations of bombesin or GRP for different times up to 24 h did not cause any significant change in the number of cell-surface binding sites for these peptides. Furthermore, pretreatment with GRP did not cause either an alteration in the concentration-dependence of binding of the labelled peptide or a reduction in the level of an M_r 75 000–85 000 surface protein recently identified as a putative component of the receptor for bombesin-like peptides in Swiss 3T3 cells. Replenishment of surface receptors during these experiments either by *de novo* receptor synthesis or from a putative intracellular pool of receptors is unlikely because: (i) Swiss 3T3 cells continue to degrade [^{125}I]GRP for prolonged periods in the presence of cycloheximide, and (ii) prolonged exposure of cells to cycloheximide together with GRP has no effect on [^{125}I]GRP binding. These findings demonstrate that mitogenic peptides of the bombesin family do not cause down-regulation of their specific high-affinity receptors in Swiss 3T3 cells.

The absence of ligand-induced down-regulation taken together with the rapid internalization and degradation of [^{125}I]GRP shown here, raises the possibility that receptors for peptides of the bombesin family in Swiss 3T3 cells return functionally intact to the plasma membrane. Recent findings have shown that several receptors involved in nutrient uptake and protein clearance systems move to intracellular locations and recycle in the absence of ligand. Typical examples are the receptors for low-density lipoprotein (Brown *et al.*, 1983; Goldstein *et al.*, 1985), asialoglycoprotein (Schwartz *et al.*, 1984; Stahl and Schwartz, 1985) and α_2-macroglobulin (Kaplan and Keogh, 1981). Recently, constitutive recycling was also demonstrated for the insulin-like growth factor type II receptor (Oka and Czech, 1986). The findings presented here suggest that receptors for peptides of the bombesin family may follow a similar pathway, and thus provide an example of a receptor for a potent mitogen which is recycled rather than extensively down-regulated. Further studies using additional reagents, such as specific antibodies, will be necessary to test this hypothesis directly.

It has been proposed that the internalization and degradation of growth factor receptors and/or the ligands may have a signalling function in mitogenesis (King and Cuatrecasas, 1981; James and Bradshaw, 1984; Bergeron *et al.*, 1985; Wakshull and Wharton, 1985). Specifically, down-regulation can be regarded as a necessary consequence of the informational content of the mitogenic receptor molecule; the proteolytic fragments generated by receptor processing would serve to propagate the biological response into the cell. The fact that peptides of the bombesin family are able to stimulate DNA synthesis and trigger a wide variety of signalling processes without reducing the number of their surface receptors strongly suggests that extensive receptor down-regulation can be dissociated from mitogenesis, and therefore may not represent in every case an obligatory event in the initiation of a proliferative response.

Materials and methods

Cell culture

Stock cultures of Swiss 3T3 cells (Todaro and Green, 1963) were maintained in Dulbecco's modified Eagle's (DME) medium containing 10% fetal calf serum (FCS) penicillin (100 units/ml), and streptomycin (100 μg/ml) in humidified 10% CO_2/90% air at 37°C. For experimental purposes cells were subcultured to 33-mm Nunc dishes (10^5 cells/dish) in DME medium containing 10% FSC. After 5–7 days, the cultures were confluent and quiescent as shown by autoradiography (<1% labelled nuclei) after a 40 h exposure to [^{3}H]thymidien (Dicker and Rozengurt, 1980).

Assay of DNA synthesis

Determinations of DNA synthesis were performed as described before (Dicker and Rozengurt, 1980). Briefly, cultures were washed twice with DME medium at 37°C, and incubated in 2 ml of a 1:1 (vol/vol) mixture of DME and Waymouth media containing [^{3}H]thymidine (1 μCi/ml; 1 μM) and various additions as indicated. The incorporation of radioactivity into acid-precipitable material was measured as previously described (Dicker and Rozengurt, 1980).

Measurement of [^{125}I]GRP binding

Confluent and quiescent cultures of Swiss 3T3 cells were incubated with 1 ml of medium composed of 1:1 (vol/vol) DME and Waymouth media containing 0.1% bovine serum albumin (BSA), 50 mM 2-[bis(2-hydroxyethyl)-2-amino]ethane sulphonic acid (pH 7.0) and [^{125}I]GRP at the concentrations indicated. After various times cultures were rapidly washed with cold (4°C) phosphate-buffered saline (PBS) containing 0.1% BSA (wash solution), extracted with 1 ml 0.1 M NaOH containing 2% Na_2CO_3 and 1% sodium dodecyl sulphate (SDS), and total cell-associated radioactivity was determined in 10 ml Pico-Fluor in a Beckman counter. Non-specific binding, defined as the cell-associated radioactivity not displaced in the presence of 360 nM GRP represented 5–10% of total binding. Binding assays at 4°C were performed in 0.14 M NaCl, 5 mM KCl, 0.01 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (binding medium) pH 7.0 containing 0.1% BSA. The cellular distribution of [^{125}I]GRP was determined by the method of Haigler *et al.* (1980). After incubation with [^{125}I]GRP cultures of Swiss 3T3 cells were washed rapidly at 4°C with wash solution, and then treated for 6 min at 4°C with 1 ml of 0.2 M acetic acid, 0.5 M NaCl, pH 2.5 to remove surface-bound [^{125}I]GRP. This medium was then removed for counting and the remaining intracellular cell-associated radioactivity was extracted. Surface-bound and intracellular cell-associated radioactivity were determined as described above.

Chromatographic separation of [^{125}I]tyrosine and [^{125}I]GRP

Samples were applied to Sep-Pak C18 cartridges pre-washed with 5 ml methanol and 10 ml distilled water. Na^{125}I was eluted with two 5-ml aliquots of 0.1% trifluoroacetic acid, [^{125}I]tyrosine was eluted with two 5-ml aliquots of 0.1% trifluoroacetic acid in 25% methanol and intact peptide was eluted with two 5-ml aliquots of 0.1% trifluoroacetic acid in 25% methanol and intact peptide was eluted with two 5-ml aliquots of 0.1% trifluoroacetic acid in 80% methanol.

Chemical cross-linking of [^{125}I]GRP to receptors

Confluent and quiescent cultures of Swiss 3T3 cells were incubated for 30 min at 37°C in 1 ml of binding medium, pH 7.0, containing the appropriate concentration of [^{125}I]GRP in the presence or absence of a 500-fold excess of unlabelled GRP. Chemical cross-linking of [^{125}I]GRP to receptors was then carried out as described (Zachary and Rozengurt, 1987). Briefly, cells in 33-mm Nunc Petri dishes were washed three times at 15°C with PBS and then incubated for 15 min at 15°C in 1 ml binding medium, pH 7.4 in the presence of 6 mM ethyleneglycol *bis*(succinimidylsuccinate) (EGS). EGS was dissolved in dimethyl sulphoxide immediately prior to use and was added to medium to give a final concentration of dimethylsulphoxide of 2%. The cultures were rapidly rinsed twice with PBS at 4°C and solubilized in 0.1 ml of 2 × sample buffer 0.2 M Tris–HCl, pH 6.8, 10% (w/v) glycerol, 6% SDS (w/v) and 2 mM ethylenediaminetetraacetic acid. Samples were immediately heated at 100°C for 3–5 min and analysed by one-dimensional SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

SDS–PAGE

Slab gel electrophoresis was performed using 7.5% acrylamide in the separating gel and 5% in the stacking gel, and 0.1% SDS (Laemmli, 1970). After electrophoresis gels were stained, destained and dried down onto paper for autoradiography with Fuji X-ray film (Fuji Photo Film Co. Ltd, Japan). Dried gels were exposed to film for 4–8 days.

Materials

Bombesin was obtained from Sigma and GRP was purchased from Bachem Fine Chemicals (Saffron Walden, UK). Epidermal growth factor (EGF) was obtained from Collaborative Research. EGS was purchased from Pierce Chemical Company. [^{125}I]EGF was prepared as described (Rozengurt *et al.*, 1981, 1982). [^{125}I]GRP (2000 Ci/mmol; 1 Ci = 37 GBq) was obtained from the Radiochemical Centre (Amersham, UK) or was prepared by radiolabelling GRP with ^{125}I using the soluble lactoperoxidase method (Rozengurt *et al.*, 1981, 1982). The labelled peptide was separated from unreacted Na^{125}I as described (Wharton *et al.*, 1978). [^{125}I]GRP exhibited mitogenic activity within a similar concentration range to that observed with the unlabelled peptide. All other reagents used were of the highest grade available.

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