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# Characterization of a Bombesin Receptor on Swiss Mouse 3T3 Cells by Affinity Cross-Linking

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We have previously identified by chemical cross-linking a cell surface protein in Swiss 3T3 cells of apparent Mr 75,000-85,000, which may represent a major component of the receptor for peptides of the bombesin family in these cells [1]. Because bombesin-like peptides may interact with other cell surface molecules, it was important to establish the correlation between receptor binding and functions of this complex and further characterize the Mr 75,000-85,000 cross-linked protein. Detailed time courses carried out at different temperatures demonstrated that the Mr 75,000-85,000 affinity-labelled band was the earliest cross-linked complex detected in Swiss 3T3 cells incubated with $^{125}$I-labelled gastrin-releasing peptide ($^{125}$I-GRP). Furthermore, the ability of various nonradioactive bombesin agonists and antagonists to block the formation of the Mr 75,000-85,000 cross-linked complex correlated extremely well ($r = 0.994$) with the relative capacity of these peptides to inhibit $^{125}$I-GRP specific binding. Pretreatment with unlabelled GRP for up to 6 h caused only a slight decrease in both specific $^{125}$I-GRP binding and the affinity labelling of the Mr 75,000-85,000 protein. We also show that the cross-linked complex is a glycoprotein. First, solubilized affinity labelled Mr 75,000-85,000 complex applied to wheat germ lectin-sepharose columns was eluted by addition of 0.3 M N-acetyl-D-glucosamine. Second, treatment with endo-β-N-acetylglucosaminidase F reduced the apparent molecular weight of the affinity-labelled band from 75,000-85,000 to 43,000, indicating the presence of N-linked oligosaccharide groups.

**Key words: cell surface glycoprotein, peptide**

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 [reviewed in 1]. In particular, Rozengurt and Sinnett-

Abbreviations: SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; EGS, ethylene glycolbis (succinimidyl succinate); DSS, disuccinimidyl suberate; DSP, dithiobis (succinimidyl propionate); BSOCOES, Bis[2-(succinimidooxycarbonyloxy) ethyl] sulfone; GRP, gastrin releasing peptide; endo F, endo-β-N-acetylglucosaminidase F; GlcNAc, N-acetyl-D-glucosamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; EDTA, ethylene diaminetetraacetic acid; NP40, nonidet NP40; DMEM, Dulbecco's modified Eagle's medium; SP, substance P.

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Smith [2] found that the amphibian tetradecapetide bombesin [3] is a potent mitogen for Swiss 3T3 cells and acts synergistically with insulin and other growth-promoting factors at subnanomolar concentrations. Mammalian peptides structurally related to bombesin, including gastrin-releasing peptide (GRP) and the neuromedins [4-7], also stimulate DNA synthesis in these cells [8]. These peptides bind to high-affinity receptors in Swiss 3T3 cells, which are distinct from those of other mitogens for these cells [8]. Structure–activity relationships demonstrate that the stereospecific requirement for receptor binding [8,9] and elicitation of biological responses, including mitogenesis [8], is satisfied by the highly conserved COOH-terminal heptapeptide of this family of neuropeptides.

After binding, bombesin and structurally related peptides elicit a complex array of early biological responses [reviewed in 10] including enhanced phosphoinositide metabolism [11-13], mobilization of $Ca^{2+}$ from intracellular stores [12-14], activation of protein kinase C [9,15-17], inhibition of $^{125}$I-epidermal growth factor–binding [9,15,18] stimulation of $Na^+/H^+$ antiport activity (14), and induction of the cellular oncogenes c-fos and c-myc [19-22]. In addition, bombesin-like peptides are present in high concentrations in small-cell lung carcinoma [23-25], where they could act as autocrine growth factors [2]. In spite of the potential importance of peptides of the bombesin family as modulators of animal cell growth [1], little is known about the molecular properties of the receptor for these peptides.

Recently, we have identified by chemical cross-linking a cell surface protein in Swiss 3T3 cells of apparent Mr 75,000–85,000 that specifically recognizes $^{125}$I-GRP [28]. These findings suggest that this protein is a major component of the receptor for peptides of the bombesin family in these cells. Here we provide further evidence supporting this proposition and show that the affinity-labelled Mr 75,000–85,000 complex is a glycoprotein, as judged by chromatography on wheat germ lectin columns and by treatment with endo-β-N-acetylglucosaminidase (endo F), an enzyme that cleaves glycans from asparagine-linked glycoproteins [29].

## MATERIALS AND METHODS

### Cell Culture

Stock cultures of Swiss 3T3 cells [30] were maintained in Dulbecco-Vogt's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 10% $CO_2$:90% air at 37°C. For experimental purposes, $10^5$ cells were subcultured in 33 mm Nunc Petri dishes with 2 ml DMEM containing 10% fetal bovine serum and used at least 6 days after the last change of the medium. The cells were arrested in the $G_1/G_0$ phase of the cell cycle, as judged by cytofluorometric analysis and by the fact that less than 1% of the cells were autoradiographically labelled after a 40-h exposure to ($^3$H)-thymidine [31].

### Chemical Cross-Linking of $^{125}$I-GRP

Chemical cross-linking was performed as described previously [28]. Briefly, confluent and quiescent cultures of Swiss 3T3 cells were incubated at 37°C in 1 ml of binding medium (0.14M NaCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES at pH 7.0) containing $^{125}$I-GRP at 1 nM. After 10 min of incubation (unless otherwise indicated) the cultures were washed

and incubated in binding medium, pH 7.4, containing 6 mM ethylene glycolbis (succininimidyl succinate) (EGS) or 1 mM dithiobis (succinimidyl propionate) (DSP) at 15°C for 15 min. The cross-linking agents (EGS and DSP) were dissolved in dimethyl sulfoxide immediately prior to use and were added to binding medium to give a final solvent concentration of 1–2%. The cultures were then washed twice with phosphate buffered saline and solubilized in 0.1 ml of 2× sample buffer, which contained 0.2 M Tris-HCl pH 6.8, 10% (w/v) glycerol, 6% sodium dodecyl sulfate (SDS) (w/v), 4% β-mercaptoethanol (v/v) and 2 mM EDTA. Samples were immediately heated at 100°C for 3–5 min and analyzed by SDS-polyacrylamide gel electrophoresis.

### SDS-Polyacrylamide Gel Electrophoresis

Slab gel electrophoresis was performed with 7.5% acrylamide in the separating gel and 5% in the stacking gel, and 0.1% SDS [32]. After electrophoresis, gels were stained, destained, and dried down onto paper for autoradiography with Fuji x-ray film. Dried gels were exposed to film for 4–8 days. The Mr 75,000–85,000 band from autoradiograms was scanned with a Joyce-Loebl double-beam densitometer, and the areas under specific peaks were measured with a Hewlett-Packard digitizer.

## MATERIALS

EGS, DSS, DSP and BSOCOES were purchased from Pierce and Warriner (UK) Ltd. (Chester, England). Bombesin, 3-[(3-cholamidopropyl) dimethylammonia] 1-propanesulphonate (CHAPS), Triton X-100, deoxycholate, digitonin, and nonidet-NP40 (NP40) were obtained from Sigma chemical Co. (St Louis, MO). Wheat germ lectin sepharose 6 MB was obtained from Pharmacia (Uppsala, Sweden). Endo F was purchased from NEN Research Products (Boston, MA). $^{125}$I-GRP was obtained from Amersham (England). GRP, Neuromedin B, bombesin [8-14], [DArg$^{1}$, DPro$^{2}$, DTrp$^{7,9}$, Leu$^{11}$]substance P, and [DArg$^{1}$, DPhe$^{5}$, DTrp$^{7,9}$, Leu$^{11}$]substance P were purchased from Pensinsula Laboratories Europe Ltd. (St. Helens, England).

## RESULTS

### Formation of the Mr 75,000–85,000 Cross-Linked Complex: Time and Temperature Dependence

Analysis by SDS–PAGE of extracts of Swiss 3T3 cells that had been preincubated with $^{125}$I-GRP for various times at different temperatures and subsequently treated with the homobifunctional disuccinimidyl cross-linking agent EGS for 15 min at 15°C revealed the presence of a major broad band migrating with an apparent Mr 75,000–85,000. Figure 1 shows that the level of this band increased rapidly at 37°C and reached a maximum after 7.5 min of incubation with $^{125}$I-GRP. The rate of formation of the cross-linked complex decreased in a progressive fashion when the incubations with $^{125}$I-GRP were carried out at 24°C or at 15°C instead of at 37°C (Fig 1). In other experiments, cultures incubated with $^{125}$I-GRP for 5 min were washed and subsequently treated with EGS for various times at 37°C. Maximum cross-linking was obtained after 5 min of exposure to EGS. Longer incubations at 37°C with the cross-linking agent resulted in the formation of additional complexes of higher molecular weight (results not shown). These kinetic studies demonstrate

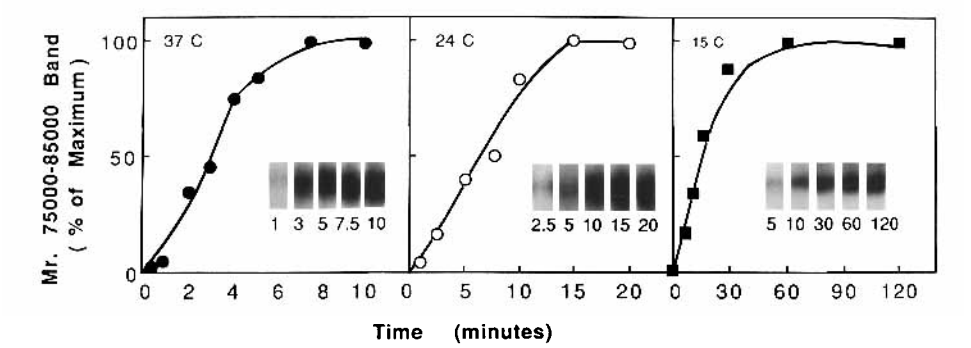


Fig. 1. Time course of the appearance of the Mr 75,000–85,000 affinity-labelled protein at various temperatures. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with $^{125}$I-GRP (1 nM) at either 37°C **(left-hand panel)**, 24°C **(centre panel)**, or 15°C **(right-hand panel)**. At the times indicated, chemical cross-linking was carried out and the level of Mr 75,000–85,000 quantified as described in Materials and Methods. The values shown are expressed as a percentage of the maximum level and are the mean of three independent experiments obtained at each temperature. The inserts show some representative autoradiograms.

that the Mr 75,000–85,000 affinity labelled band is the earliest cross-linked complex detected in Swiss 3T3 cells incubated with $^{125}$I-GRP.

## Covalent Nature of the Mr 75,000–85,000 Complex

Previous studies demonstrated that not only EGS but also the homobifunctional cross-linking agents DSS, DSP, and BSOCOES were effective in promoting covalent cross-linking of $^{125}$I-GRP to Swiss 3T3 cells, resulting in the formation of a major band of Mr 75,000–85,000 [28]. In the present studies we confirmed these observations, using these cross-linking agents at 37°C instead of at 15°C. That the Mr 75,000–85,000 constitutes genuine covalent linking of $^{125}$I-GRP to the putative bombesin receptor was verified with the thiol-cleavable cross-linking agent DSP. As shown in Figure 2, DSP promoted the cross-linking of $^{125}$I-GRP to the Mr 75,000–85,000 protein. Reduction of the disulfide bond of DSP by incubating the extracts with various concentrations of 2-mercaptoethanol resulted in dose-dependent disappearance of the Mr 75,000-85,000 band.

## Effect of Bombesin Agonists and Antagonists on the Formation of the Mr 75,000–85,000 Complex

The possibility that the Mr 75,000–85,000 band is a surface component of Swiss 3T3 cells closely related to the receptor for peptides of the bombesin family was substantiated by cross-linking $^{125}$I-GRP to cultures incubated in the presence of different concentrations of unlabelled bombesin agonists and antagonists. Figure 3 shows that addition of bombesin, GRP, neuromedin B or the 8–14 amino acid fragment of bombesin (bombesin 8-14) decreased the level of the Mr 75,000–85,000 complex in a concentration-dependent fashion. The concentrations of bombesin, GRP, neuromedin B, and bombesin 8-14 required to produce 50% inhibition of cross-linking ($IC_{50}$, cross-linking) were 0.7 nM, 8 nM, 34 nM, and 900 nM, respectively. In contrast, the $NH_2$-terminal fragment of GRP (GRP, 1–16) did not prevent the formation of the cross-linked complex even at 10 $\mu$M.

The neuropeptide substance P (SP) neither inhibits the binding of $^{125}$I-GRP to 3T3 cells [8] nor prevents the formation of the Mr 75,000–85,000 cross-linked

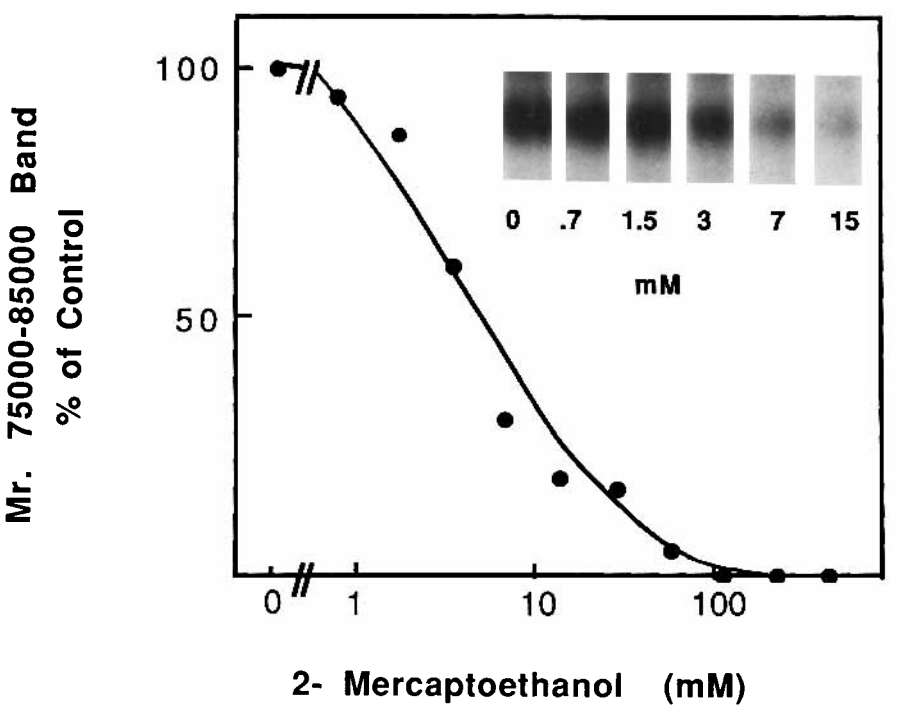


Fig. 2. Effect of 2-mercaptoethanol on DSP-mediated cross-linking of $^{125}$I-GRP to the Mr 75,000–85,000 protein. Confluent and quiescent cultures of 3T3 cells were incubated with $^{125}$I-GRP (1 nM) at 37°C for 10 min. Chemical cross-linking using DSP was performed at 15°C for 15 min, as described in Materials and Methods. The cell monolayers were then dissolved with 2× sample buffer containing varying concentrations of 2-mercaptoethanol, boiled for 5 min, and analysed by SDS–PAGE. The areas under the individual peak for the Mr 75,000–85,000 protein are shown as a function of the concentration of 2-mercaptoethanol and are expressed as a percentage of the control value. The inset shows the autoradiogram obtained at the lower concentrations of reducing agent.

complex [28]. SP also fails to stimulate DNA synthesis in quiescent cells [2]. However, SP antagonists are potent bombesin antagonists in 3T3 cells [8,33]. Figure 3 also includes the effect of various concentrations of [DArg$^1$, DPro$^2$, DTrp$^{7,9}$, Leu$^{11}$]SP and [DArg$^1$, DPhe$^5$, DTrp$^{7,9}$, Leu$^{11}$]SP [33] on the formation of the Mr 75,000–85,000 band. These antagonists decreased the level of the Mr 75,000–85,000 band in a concentration-dependent fashion. The concentrations of [DArg$^1$, DPhe$^5$, DTrp$^{7,9}$, Leu$^{11}$]SP and [DArg$^1$, DPro$^2$, DTrp$^{7,9}$, Leu$^{11}$]SP that exhibit a 50% inhibition of cross-linking ($IC_{50}$, cross-linking) were 3,700 nM and 17,000 nM, respectively.

The concentrations of agonist or antagonists required to produce 50% inhibition of the level of the Mr 75,000–85,000 complex ($IC_{50}$, cross-linking) were derived from Figure 3, whereas the concentrations of these peptides that cause 50% inhibition of $^{125}$I-GRP specific binding to Swiss 3T3 cells were derived from previous published curves [8,33]. Figure 4 shows that the $IC_{50}$ for cross-linking correlated extremely well with the values of $IC_{50}$ for binding ($r = 0.994$). The striking parallelism between the ability of the peptides to inhibit binding and cross-linking considerably strengthens our conclusion that the Mr 75,000–85,000 complex is a major component of the receptor for peptides of the bombesin family.

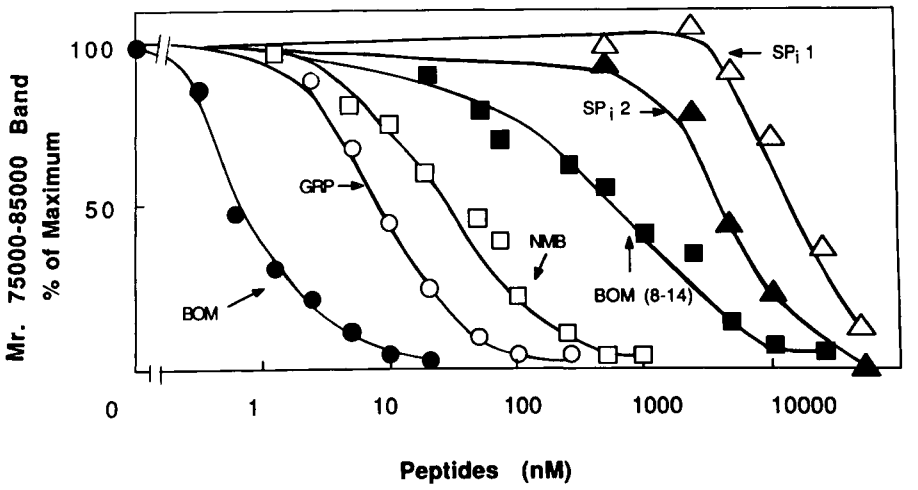


Fig. 3. Effect of various bombesin agonists and antagonists on the affinity labelling of the Mr 75,000–85,000 protein. Confluent and quiescent cultures of 3T3 cells were incubated at 37°C for 10 min with 1 nM $^{125}$I-GRP either in the presence or absence of the following agonists and antagonists at the concentrations indicated: bombesin (●, Bom): GRP (○); neuromedin B (□, NMB); bombesin 8-14 (■, Bomb 8–14); [DArg$^{1}$, DPro$^{2}$, DTrp$^{7,9}$, Leu$^{11}$]substance P (△, SPi 1); and [DArg$^{1}$, DPhe$^{5}$, DTrp$^{7,9}$, Leu$^{11}$]substance P (▲, SPi 2). Chemical cross-linking was then performed with 6 mM EGS, as described in Materials and Methods. The results are the composite of three independent experiments and are expressed in each case as a percentage of the control value, obtained from scanning densitometry of the autoradiograms.

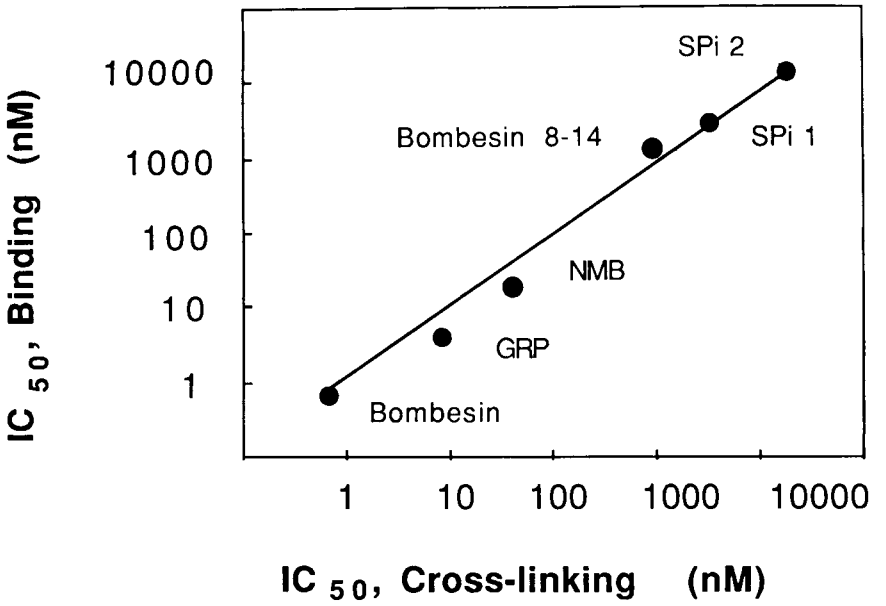


Fig. 4. The ability of various bombesin agonists and antagonists to inhibit $^{125}$I-GRP binding correlates with the capacity of these peptides to block the formation of the Mr 75,000–85,000 affinity-labelled complex. The concentrations of peptides that gave 50% inhibition in the binding of $^{125}$I-GRP to 3T3 cells ($IC_{50}$ binding) from previously published data [8,33] were plotted against the concentrations of the same peptides that gave a 50% inhibition of chemical cross-linking ($IC_{50}$, cross-linking) obtained from Figure 3.

## Pretreatment With GRP Does Not Alter the Level of the Mr 75,000–85,000 Cross-Linked Complex

Zachary and Rozengurt [34] reported that exposure of Swiss 3T3 cells to either bombesin or GRP causes only a slight decrease in the number of cell-surface binding sites for these peptides. Furthermore, pretreatment with GRP did not reduce the level of the Mr 75,000–85,000 affinity-labelled band. These findings led to the conclusion that peptides of the bombesin family, in contrast to other mitogens, do not cause down regulation of their specific high affinity receptors in Swiss 3T3 cells. Recently Kris et al. [35] reported that treatment for 30 min with 1 nM GRP markedly reduced the level of the Mr 75,000–85,000 affinity-labelled band; they concluded that bombesin strongly down-regulates its receptor. In view of this discrepancy, we decided to reassess whether pretreatment with GRP alters the specific binding of $^{125}$I-GRP and/or the level of the affinity-labelled Mr 75,000–85,000 protein. Figure 5 shows that treatment of Swiss 3T3 cells with either 3.6 or 36 nM GRP for up to 6 h, followed by extensive washing at 37°C to remove surface-bound ligand, caused only a slight decrease in the level of specific binding. As a control, other cultures were pretreated with EGF at 3.3 nM for various times and then incubated with $^{125}$I-EGF. Pretreatment with EGF caused the expected down regulation of surface receptors (Fig. 5, left). To determine whether exposure of the cells to GRP changed the level of the Mr 75,000–85,000 protein, parallel cultures were treated with GRP for various times, washed extensively, incubated with $^{125}$I-GRP, and then treated with EGS. As shown in Figure 5 (right), pretreatment with 3.6 or 36 nM GRP did not cause any significant alteration of the Mr 75,000–85,000 protein. In other experiments we pretreated the cultures with 3.6 nM GRP and washed them at 4°C, as reported by Kris et al. [35]. We found

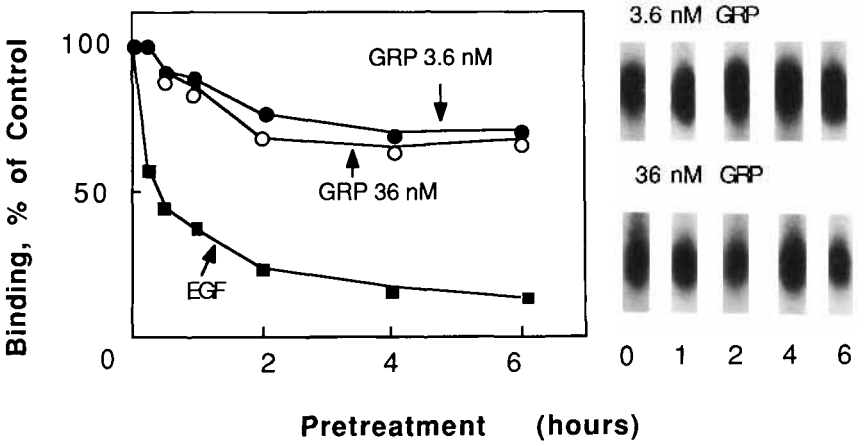


Fig. 5. Effect of pretreatment with GRP on the binding of $^{125}$I-GRP to Swiss 3T3 cells and on the $^{125}$I-GRP affinity labelling of the Mr 75,000-85,000 protein in Swiss 3T3 cells. **Left panel:** Confluent cultures of 3T3 cells were incubated at 37°C in either the absence or presence of 3.6 nM GRP (●), 36 nM GRP (○) or 3.3 nM EGF (■). After the times indicated, all cultures were washed 5× at 37°C, with a 15-min incubation after the third wash with DMEM, and incubated at 37°C with either 1 nM $^{125}$I-GRP for 30 min (○,●) or 2 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. **Right panel:** Parallel cultures of 3T3 cells were incubated with 1 nM $^{125}$I-GRP for 5 min at 37°C, and chemical cross-linking was carried out as described in Materials and Methods. The autoradiograms obtained from the chemical cross-linking are shown in the right-hand panel.

a marked decrease in $^{125}$I-GRP binding and cross-linking, suggesting that the apparent decrease in the level of the Mr 75,000–85,000 affinity-labelled band noted by Kris et al. [35] may be the result of incomplete removal of nonradioactive surface-bound ligand rather than genuine down regulation.

## Characterization of the Mr 75,000–85,000 Cross-Linked Complex as a Glycoprotein

Treatment with the nonionic detergents NP40 and Triton X-100 for 2 h at 4°C promoted effective solubilization of the Mr 75,000–85,000 affinity labelled band, whereas other detergents, including deoxycholate, digitonin, and CHAPS, were less effective (Fig. 6). Solubilization by NP40 depended on the amount of detergent; maximum solubilization by NP40 was achieved at a concentration of 1.25% (Fig. 6). The Mr 75,000–85,000 affinity-labelled band solubilized by NP40 or Triton X-100 was recovered in the supernatant after centrifugation at 100,000*g* for 1 h, as revealed by SDS–PAGE followed by autoradiography of the supernatant and pellet after treatment with each detergent.

To determine whether the affinity-labelled Mr 75,000-85,000 complex is a glycoprotein, solubilized preparations were subjected to affinity chromatography in lectin columns [36]. After incubation with $^{125}$I-GRP and cross-linking with EGS, 3T3 cells were treated with 1% NP40. The solubilized material was chromatographed on a wheat germ lectin sepharose 6MB column. After extensive washing with the equilibrating buffer and subsequently with this buffer containing 0.4 M NaCl, bound

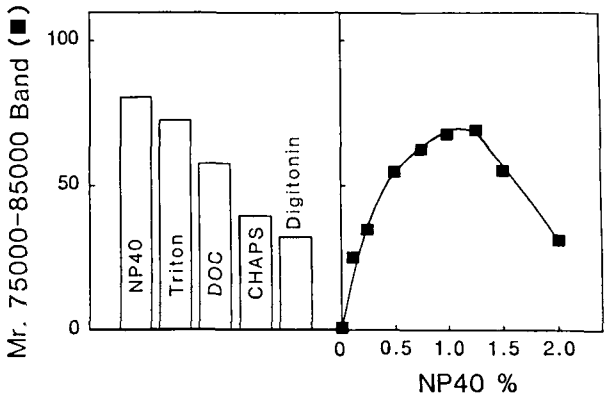


Fig. 6. Solubilization of the 75,000–85,000 affinity-labelled protein. Confluent cultures of 3T3 cells were incubated with $^{125}$I-GRP (1 nM) at 37°C for 10 min and chemically cross-linked with EGS as described in Materials and Methods. The individual cultures were solubilized and scraped at 4°C into a solubilization buffer consisting of 50 mM Hepes, 100 mM NaCl, 5 mM EDTA, phenylmethylsulfonyl fluoride (50 μM), aprotonin (3.5 μg/ml), pepstatin A (1 μg/ml), leupeptin (1 μg/ml), and bacitracin (1 μg/ml), plus the following detergents: 1% NP40, 1% Triton X-100 (TRITON), 5 mM deoxycholate (DOC), 6 mM CHAPS, and 2% digitonin (**left panel**). Other cultures were treated with solubilization buffer containing varying percentages of NP40 (**right panel**). In all cases, the cell suspensions were incubated at 4°C with gentle shaking for 2 h, and then passed through a 23-g needle before centrifugation at 100,000*g* for 1 h. The supernatants obtained were analyzed by SDS–PAGE. As a control, parallel cultures chemically cross-linked to $^{125}$I-GRP were scraped into the solubilization buffer in the absence of any detergent. Following centrifugation at 100,000*g* the pellet was solubilized with 2× sample buffer and analyzed by SDS–PAGE. The values shown are expressed as a percentage of the level of Mr 75,000–85,000 protein obtained from the pellet. The supernatant showed a complete absence of the Mr 75,000–85,000 protein.

glycoproteins were eluted from the column with buffer containing 0.3 M N-acetyl-N-glucosamine (GlcNAc). Individual fractions were examined by SDS-PAGE. As shown in Figure 7, the Mr 75,000–85,000 band was tightly bound to the lectin column and it was eluted by GlcNAc. Similar results were obtained when 3T3 cells were solubilized with 1% Triton X-100 instead of NP4O. In contrast, little retardation was observed when the solubilized material was applied to a lentil sepharose 4B column. These findings suggest that the Mr 75,000–85,000 contains carbohydrate chains with GlcNAc residues.

In order to substantiate the conclusion that the Mr 75,000–85,000 band is a glycoprotein, we examined the effect of endo-β-N-acetyl-glucosaminidase F (endo F) treatment on this complex. This enzyme cleaves N-linked oligosaccharides from glycoproteins and is therefore a useful probe for the further elucidation of the nature of putative cell-surface glycoproteins [29]. The affinity-labelled Mr 75,00–85,000 protein was located on dried, unfixed polyacrylamide gels by autoradiography and excised. Individual gel slices were then rehydrated and incubated with different amounts of endo F. As shown in Figure 8, treatment with endo F decreased the apparent molecular weight of the affinity-labelled band from 75,000–85,000 to 43,000. The appearance of this band was not due to a nonspecific proteolytic degradation of the Mr 75,000–85,000 protein, because neither the molecular weight nor the broad, fuzzy appearance of the higher-molecular-weight band was affected when gel slices

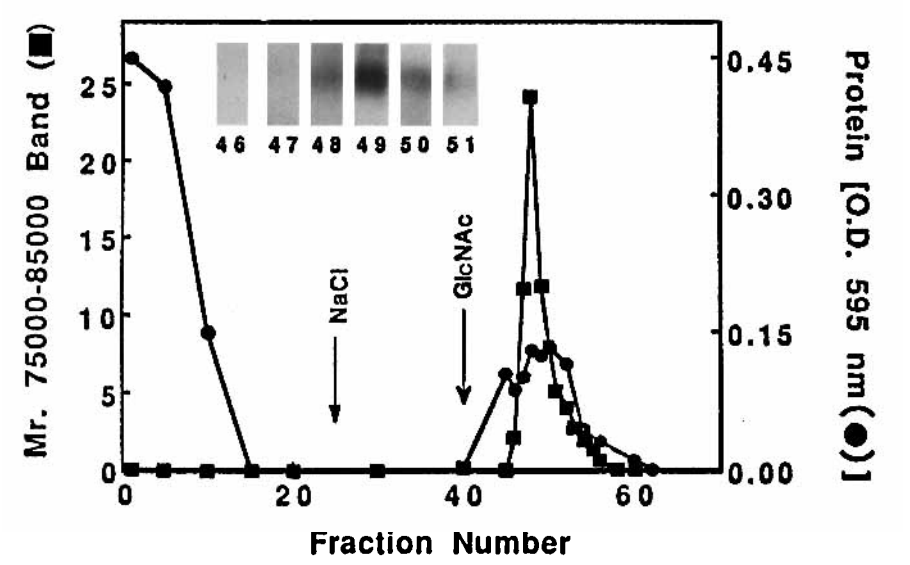


Fig. 7. Chromatography of the affinity-labelled Mr 75,000–85,000 protein on wheat germ lectin sepharose. $^{125}$I-GRP (1 nM) was cross-linked to confluent Swiss 3T3 cells ($10^7$) and the cultures were then solubilized with 1% NP40 as described in Figure 1. The supernatant from the 100,000*g* spin was diluted with solubilization buffer to give a final concentration of 0.2 % NP40, and loaded onto a 1-ml wheat germ lectin-sepharose 6MB column. The column was washed sequentially with solubilization buffer containing 0.1% NP40 then with solubilization buffer containing 0.1% NP40 and 0.4 M NaCl. Bound glycoproteins were eluted with the same buffer containing 0.1% NP40, 0.4 M NaCl, and 0.3 M GlcNAc. Aliquots (0.1 ml) of the fractions (0.3 ml) were analyzed by SDS-PAGE. The results represent the integration values in arbitrary units obtained from the autoradiogram shown in the inset. Proteins were measured by the method of Bradford with Coomassie blue G-250.

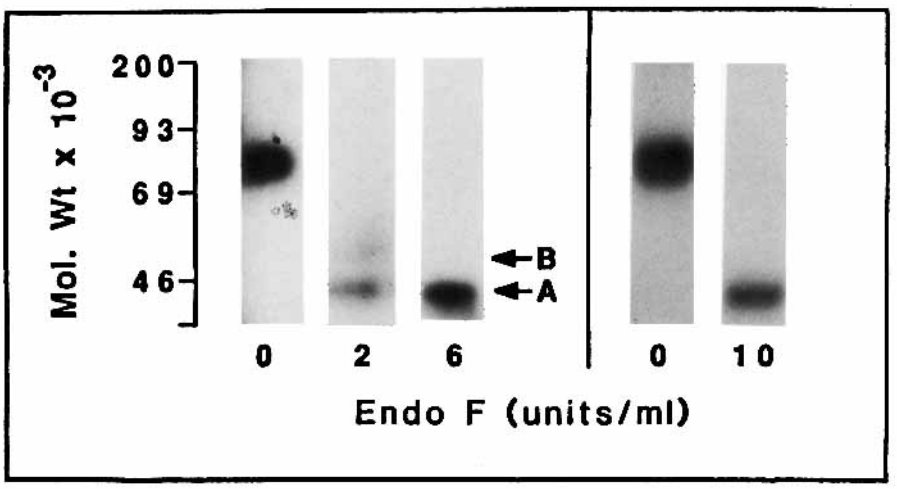


Fig. 8. Endo F treatment of the affinity-labelled Mr 75,000–85,000 protein. **Left:** Following chemical cross-linking of $^{125}$I-GRP to Swiss 3T3 cells, the affinity-labelled protein was resolved on a 10% acrylamide gel under reducing conditions, located by autoradiography of the dried unfixed gels, and excised. Each gel slice was placed in an individual tube and rehydrated in 0.3 ml of endo F digestion buffer (0.1 M Na phosphate, pH 6.1, 50 mM EDTA, 1% NP40, 0.1% SDS, and 1% β-mercaptoethanol). The accessibility of enzyme was then increased by sequentially freeze-thawing the tubes through three cycles of alternate incubations in dry ice and 37°C for 10 min each. Endo F was then added to each tube at the indicated concentrations for 24 h at 37°C. **Right:** Fractions containing the Mr 75,000–85,000 protein were pooled, and endo F (10 units/ml) was added directly to a 100-μl aliquot and incubated at 37°C for 24 h. The reactions were terminated by the addition of 10× sample buffer and heating for 5 min at 100°C. The gel slices and incubation mixtures were resolved on a second 10% SDS-PAGE.

were treated under identical conditions in the absence of the enzyme (Fig. 8). The effect of endo F on the molecular weight of the Mr 75,000–85,000 band was concentration-dependent, and a discrete intermediate band could be observed (Fig. 8), suggesting the presence of at least two N-linked oligosaccharide groups. Incubation with endo-β-acetylglucosaminidase H (1 μg/ml), which cleaves specifically high-mannose oligosaccharides [37], did not alter the level of the Mr 75,000–85,000 band or cause the appearance of any lower-molecular-weight species. In other experiments, fractions eluted from wheat germ lectin sepharose columns containing the Mr 75,000–85,000 affinity-labelled protein were treated with endo F. In agreement with the results shown in Figure 8 (left), the molecular weight of the complex decreased to 43,000 (Fig. 8, right).

## DISCUSSION

To gain information concerning some of the molecular properties of the bombesin/GRP receptor, we used affinity-labelling methodology to identify surface components of Swiss 3T3 cells that specifically recognize $^{125}$I-GRP. Homobifunctional disuccinimidyl cross-linking agents have been successfully employed to identify receptors for other peptide mitogens, including those for EGF and PDGF. Analysis of extracts of cells that had been preincubated with radiolabelled GRP and subsequently treated with a cross-linking reagent revealed the presence of a major band migrating with apparent Mr 75,000–85,000. Several lines of evidence support the

conclusion that this protein is the receptor or a major component of the receptor for peptides of the bombesin family in these cells: (a) The formation of the Mr 75,000–85,000 affinity labelled complex is promoted by a variety of disuccinimidyl cross-linking agents, including EGS, DSS, BSOCOES, and the thiol-clearable agent DSP [28] (Fig. 2). (b) The inhibition of $^{125}$I-GRP affinity-labelling of this band with unlabelled GRP corresponds closely with the ability of GRP to inhibit the binding of the labelled ligand in parallel cultures [28]. Indeed, we have demonstrated that the ability of various nonradioactive peptide agonists and antagonists to inhibit the formation of the Mr 75,000–85,000 affinity labelled complex (Fig. 3) correlates extremely well ($r = 0.994$) with the relative capacity of these peptide to inhibit $^{125}$I-GRP specific binding (Fig. 4). (c) Time courses carried out at different temperatures show that the Mr 75,000–85,000 band is the earliest cross-linked complex detected in cells incubated with $^{125}$I-GRP (Fig. 1). (d) $^{125}$I-GRP affinity-labelling of the Mr 75,000–85,000 band is specific, as shown by the lack of effect of a panel of other mitogens and neuropeptides on cross-linking [28]. (e) The Mr 75,000–85,000 protein was not found in other cell lines that do not exhibit receptors for bombesin-like peptides [8,28]. (f) the dependence of affinity labelling of the Mr 75,000–85,000 protein on the concentration of $^{125}$I-GRP closely paralleled the ability of the unlabelled peptide to stimulate DNA synthesis and a variety of other biological responses in Swiss 3T3 cells [8,9,14,15,21]. All these findings taken together strongly suggest that an Mr 75,000–85,000 surface protein is the receptor or a major component of the receptor for peptides of the bombesin family in Swiss 3T3 cells.

It is well known that 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 [38-40] and $^{125}$I-PDGF [41-43] and subsequently verified with specific antibodies directed against the receptor molecule [44,45]. We reported that exposure of Swiss 3T3 to mitogenic concentrations of bombesin or GRP caused only a small decrease in $^{125}$I-GRP binding and no significant alteration in the level of the Mr 75,000–85,000 cross-linked complex [34]. These findings indicated that mitogenic peptides of the bombesin family do not cause down regulation of their receptors in Swiss 3T3 cells. In contrast, Kris et al. [35] reported that treatment with 1 nM GRP for 30 min was sufficient to completely down-regulate the Mr 75,000–85,000 complex. In view of this discrepancy, here we have reassessed the effect of GRP pretreatment on the binding and cross-linking of $^{125}$I-GRP to Swiss 3T3 cells. As shown in Figure 5, pretreatment with 3.6 or 36 nM nonradioactive GRP for up to 6 h caused only a slight decrease both in specific binding and in the level of the Mr 75,000–85,000, in accord with our previous results. The abolition of $^{125}$I-GRP cross-linking by prior exposure to GRP reported by Kris et al. [35] may represent incomplete removal of surface-bound GRP before the incubation with radiolabelled GRP. The absence of ligand-induced down regulation [34] (Fig. 5) taken together with the rapid internalization and degradation of $^{125}$I-GRP shown previously [34] raises the possibility that receptors for bombesin-like peptides in Swiss 3T3 cells return functionally intact to the plasma membrane, i.e., they recycle in the same fashion as receptors involved in nutrient uptake [46]. Further studies using additional reagents, such as specific antibodies, will be necessary to test this hypothesis directly.

Regardless of the precise mechanism, the results shown in Figure 5 provide an additional example of the tight correlation between specific $^{125}$I-GRP binding and formation of the Mr 75,000-85,000 cross-linked complex.

The broad, diffuse, appearance of the Mr 75,000–85,000 affinity-labelled band raised the possibility that the cross-linked complex is a cell surface glycoprotein. Various detergents, particularly NP40 and Triton X-100, effectively solubilize the affinity labelled Mr 75,000–85,000 complex. Solubilized complex binds to wheat germ lectin-sepharose columns and can be eluted with N-acetyl-D-glucosamine (Fig. 7). This indicates that the complex contains carbohydrate chains with GlcNAc residues [36]. Furthermore, treatment of the affinity-labelled band with endo F, an enzyme that cleaves N-linked oligosaccharides from glycoproteins [29], reduced the apparent molecular weight of the labelled cross-linked complex to 43,000. The effect of endo F on the apparent molecular weight of the Mr 75,000–85,000 complex was concentration dependent, and a discrete intermediate band could be detected, suggesting the presence of at least two N-linked oligosaccharide chains. The striking reduction in apparent molecular weight seen after endo-F treatment suggests that the cross-linked complex is heavily glycosylated and/or that it migrates anomalously in SDS gels. Kris et al. [35] also found that treatment of the Mr 75,000–85,000 cross-linked complex with N-glycanase caused a marked reduction in its apparent molecular weight. Recently, Masu et al. [47] reported cDNA cloning of a receptor for the tachykinin neuropeptide substance K using an oocyte expression system. It is noteworthy that the deduced molecular weight of the core polypeptide is 43,066 and that it possesses two potential N-glycosylation sites [47]. Thus, the small size of the core polypeptide noted here is not unique for a bombesin-family peptide receptor; it is shared with other neuropeptide receptors.

Addition of bombesin-like peptides to quiescent Swiss 3T3 cells stimulates a complex array of early and late molecular events [1,2,9-22]. The mechanism by which a small-size receptor (in comparison with EGF or PDGF receptors) can initiate such an array of responses remains an important and challenging question. A single protein or various G proteins [48] may be implicated in the signal transduction process [19,49]. It has also been reported that an Mr 115,000 protein in Swiss 3T3 cells was phosphorylated in tyrosine in response to bombesin [49], an observation that remains controversial [16]. Further experimental work is warranted to determine the relationship, if any, between the Mr 75,000–85,000 affinity-labelled glycoprotein and bombesin-stimulated tyrosine kinase activity.

In conclusion, we have identified further by chemical cross-linking a cell-surface protein in Swiss 3T3 cells of apparent Mr 75,000–85,000 that may represent the receptor or a major component of the receptor for bombesin-like peptides. The molecule is a glycoprotein with a core polypeptide component of Mr 43,000, which contains at least two N-linked oligosaccharide side chains. This knowledge should prove useful in future studies directed at the purification and molecular characterization of the bombesin receptor.

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