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Bombesin receptor from Swiss 3T3 cells

Affinity chromatography and reconstitution into phospholipid vesicles

Arnold Coffer, James Sinnett-Smith and Enrique Rozengurt

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

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Bombesin and its mammalian counterpart gastrin releasing peptide (GRP) are potent mitogens for Swiss 3T3 cells in which distinct high affinity receptors have been identified. We developed here a probe for specific ligand affinity chromatography by coupling biotin to [lys^3]bombesin. The resulting biotinylated [lys^3]bombesin (BLB) retained biological activity as judged by inhibition of [^{125}I]GRP binding to intact cells and membrane preparations and stimulation of rapid Ca^{2+} mobilization and DNA synthesis in intact cells. Using this ligand and magnetised beads coated with streptavidin, we extracted differentially a single protein from detergent-solubilized Swiss 3T3 membranes in a BLB-dependent manner. Visualization was achieved either after autoradiograph of metabolically labelled proteins with [^{35}S]methionine or by silver staining of larger preparations. In other experiments, elution of BLB-receptor complexes bound to streptavidin beads was carried out at neutral pH and the eluted fraction was reconstituted into phospholipid vesicles. This procedure revealed [^{125}I]GRP binding activity that exhibited saturability, specificity and a 1946-fold increase in specific activity.

Signal transduction; Growth control; Biotinylated bombesin

1. INTRODUCTION

Neuropeptides are increasingly implicated in the control of cell proliferation [1–3]. Bombesin and structurally related peptides including gastrin-releasing peptide (GRP) are potent mitogens for Swiss 3T3 cells [3,4] and may act as autocrine growth factors for small cell lung carcinoma [5,6]. Prior to stimulation of DNA synthesis in 3T3 cells, bombesin and GRP elicit a set of early molecular responses [1] including enhanced phosphoinositide metabolism, Ca^{2+} and Na^+ fluxes, activation of protein kinase C, enhancement of cAMP accumulation and induction of the cellular oncogenes c-*fos* and c-*myc* [reviewed in 8–10].

The characterization of bombesin receptors is an essential step in the elucidation of the molecular basis of the potent mitogenic response initiated by neuropeptides of the bombesin family in cultures of Swiss 3T3 cells. ^{125}I-labelled GRP ([^{125}I]GRP) binds to high-affinity receptors in intact Swiss 3T3 cells and can be cross-linked to a M_r 75 000–85 000 glycoprotein [5,11–14]. Subsequently, [^{125}I]GRP is internalized and degraded by intact Swiss 3T3 cells [15] and the number of receptors decreases after a lag period [16]. Membrane preparations have been used to determine the characteristics of the binding reaction, to identify the binding component(s) and to demonstrate modulation of ligand affinity by guanine nucleotides [16]. Recently, we described the solubilization of [^{125}I]GRP-receptor complexes from Swiss 3T3 cell membranes in a functional state, as judged by their sensitivity to guanine nucleotides [17,18].

The molecular and regulatory characterization of plasma membrane receptors requires a procedure for their purification. The interaction of streptavidin with biotinylated peptides has been extremely useful to differentially extract several membrane receptors [19]. In the present study we developed an affinity chromatographic procedure to isolate the bombesin receptor from Swiss 3T3 cell membranes using biotinylated [lys^3]bombesin (BLB) bound to the receptor prior to detergent solubilization.

2. MATERIALS AND METHODS

2.1. *Materials*

Bombesin, GRP, GRP(1–16), substance P, vasopressin, sodium taurodeoxycholate (TDOC), bovine serum albumin (BSA), aprotonin, bacitracin, soybean trypsin inhibitor, leupeptin, phenylmethylsulphonyl fluoride (PMSF) and polyethylenimine were purchased from Sigma. [^{125}I]GRP (1800–2200 Ci/mmol) and [^{35}S]methionine (1000 Ci/mmol) were from Amersham International. Sulphosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-Biotin), ethylene glycolbis (succinimidyl succinate) (EGS) and Extracti-Gel D were purchased from Pierce. L-α-lecithin was obtained from Avanti Polar Lipids Inc. Cultispher-GL macroporous gelatin microcarriers were purchased from Percell, Biolytica, Sweden. All other reagents were of the highest grade available.

Correspondence address: E. Rozengurt, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

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2.2. Methods

2.2.1. Cell culture

Cultures of Swiss 3T3 cells [20] were maintained in 90 mm Nunc Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin 100 U/ml, and streptomycin (100 μg/ml) in humidified 10% CO_2 and 90% air at 37°C. For the preparation of membranes, 3×10^6 cells were subcultured into 1850 cm^2 Falcon roller bottles with 200 ml of the same culture medium and were grown to confluence without a change of medium for 6–7 days. The final cell density was 3×10^7 cells/flask.

Swiss 3T3 cells were also grown on cultispher-GL microcarriers when metabolic labelling with [^{35}S]methionine was required. Briefly, 10 g of cultispher were swollen in phosphate buffered saline (PBS) (0.15 M NaCl, 5 mM KCl, 0.02 M Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2), autoclaved, washed with DMEM and seeded with 2×10^8 Swiss 3T3 cells in 3 l of DMEM containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The mixture of cells, microcarriers and medium was gassed with 10% CO_2:90% air, maintained at 37°C and stirred intermittently (2 min on, 30 min off). On the following day, the volume of medium was increased to 5 l and the mixture re-gassed with 10% CO_2:90% air and stirred continuously. Labelling with [^{35}S]methionine and harvesting of the cells was carried out 4–6 days later. The cells were harvested as recommended by the manufacturers, briefly, the cultispher microcarriers were washed with 1 l PBS containing 0.2% EDTA, then the cultispher microcarriers were digested with dispase (1.5 mg/ml) dissolved in PBS containing 5 mM $MgCl_2$ and 1.8 mM $CaCl_2$ for 10 min at 37°C. The resulting cell suspension (4×10^9 cells) was centrifuged at 1000 × *g* for 10 min and the cell pellet used to prepare membranes as described later.

2.2.2. Binding assay, Ca^{2+} mobilization and DNA synthesis

For binding at 37°C, confluent and quiescent cultures of Swiss 3T3 cells in 33 mm dishes were washed twice with DMEM and incubated with 1 ml of binding medium, which consisted of 1:1 (vol/vol) DMEM and Waymouth medium supplemented with 1 mg of bovine serum albumin per ml, 50 mM 2-[bis(2-hydroxyethyl)-2-amino]-ethanesulfonic acid (BES) pH 7.0, and GRP labeled with ^{125}I at tyrosine-15 ([^{125}I]GRP) at 1 nM. After 30 min of incubation, cultures were washed rapidly 4 times with PBS supplemented with 0.1% bovine serum albumin at 4°C and extracted in 0.5 ml of 0.1 M NaOH containing 2% Na_2CO_3 and 1% $NaDodSO_4$. Total cell-associated radioactivity was determined in a γ counter. Affinity labelling of the bombesin receptor in intact Swiss 3T3 cells was carried out using the bifunctional cross-linking agent ethylene glycolbis (succinimidyl succinate) (EGS), exactly as previously described [11,13]. The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in 3T3 cells loaded with the fluorescent indicator fura-2 [15] and the incorporation of [^{3}H]thymidine into acid-precipitable material [4] were determined as previously described.

2.2.3. Membrane preparation

Membranes were prepared as described previously [16]. Briefly, cultures in roller bottles were washed twice with 150 ml PBS at room temperature. The cells were then harvested at 4°C by scraping into ice cold PBS containing 5 mM $MgCl_2$, 1 mM [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid (EGTA), 1 mg/ml bacitracin, 10 μg/ml aprotonin, 1 mg/ml soybean trypsin inhibitor and 50 μM PMSF. All subsequent steps were carried out at 4°C. The cells were pelleted by centrifugation at 750 × *g* for 10 min and resuspended at 5×10^6/ml in solution A containing 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 5 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml bacitracin, 10 μg/ml aprotonin, 1 mg/ml soybean trypsin inhibitor and 50 μM PMSF, adjusted to pH 7.4 with NaOH at 4°C. Cells were then disrupted using a Dounce homogeniser (A pestle; 75 strokes). The homogenate was centrifuged at 500 × *g* for 10 min to remove nuclear material and intact cells and the supernatant was centrifuged again at 30 000 × *g* for 30 min. The resulting pellet, representing a membrane-enriched preparation was resuspended at a protein concentration of 5–10 mg/ml in solution A and stored in liquid nitrogen. Protein concentration in the membrane preparations was measured by the method of Bradford [19]. BSA was used as the protein standard.

2.2.4. Binding of [^{125}I]GRP to membranes

Swiss 3T3 membranes (50 μg) were incubated at 37°C for 10 min in binding medium (100 μl) containing 0.5 nM [^{125}I]GRP, 50 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.25 M sucrose, 1 mg/ml bacitracin and 10 μg/ml aprotonin. The incubation was stopped by rapid vacuum filtration over GF/B filters, presoaked in polyethyleneimine (5%) at 4°C. These were then washed with 5 × 5 ml of PBS containing 1% BSA. Under these conditions specific binding was determined as the difference between the amount of [^{125}I]GRP bound in the absence (total binding) and the presence (non-saturable binding) of 10 μM bombesin.

2.2.5. Biotinylation of [Lys^3]bombesin

Sulphosuccinimidyl 6-(biotinamido)hexanoate (60 μmol, NHS-LC-Biotin) was added to a solution of [Lys^3]bombesin (12 μmol) in 10 ml of 100 mM Hepes, pH 7.4. The solution was stirred for 1 h after which time the reaction was terminated with 20 μl ethanolamine. The products were chromatographed on a Bio-gel P2 column (2.5 × 65 cm) equilibrated and eluted with 1% v/v acetic acid (0.16 M). Fractions of the main peak of UV absorption at 280 nm (fractions 33–37; 5 ml/fraction) were collected, lyophilised and dissolved in distilled H_2O. Further purification of the [lys^3]bombesin was achieved using fast protein liquid chromatography (FPLC) on a Pep RPC HR5/5 column (Pharmacia). The biotinylated [lys^3]bombesin (BLB) was eluted by a gradient of 0.1% trifluoroacetic acid in water (solvent system A) and 0.1% trifluoracetic acid in 99.9% acetronitrile (solvent system B 10–40%) for 33 min at a flow rate of 0.7 ml/min. The [lys^3]bombesin and BLB eluted at 28% and 31.5% of solvent B, respectively.

2.2.6. Affinity chromatography of the bombesin receptor

Membranes (40–400 mg) were incubated in 8–80 ml of binding medium in the presence of either 100 nM BLB or 100 nM bombesin for 15 min at 37°C. Following centrifugation at 16 000 × *g* for 15 min to remove unbound ligand, the pellet was resuspended at 4°C in 8–80 ml of solubilization buffer consisting of 30 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.25 M sucrose, 10% glycerol, 1 mg/ml bacitracin, 10 μg/ml aprotonin and 1% sodium taurodeoxycholate (TDOC). After 30 min at 4°C, the detergent concentration was reduced to 0.33% and the solubilized proteins were separated from non-extractable membrane material by centrifugation for 1 h at 100 000 × *g*. The supernatant was then incubated with 10–100 mg of streptavidin coated magnetic beads (Dynal beads) at 4°C for 1 h. Following this incubation the beads were separated from the supernatant with a magnet (MPC-1) and washed 5 × with solubilization buffer containing 0.33% TDOC at 4°C. The bound proteins, were eluted by one of the following methods; (A) total bound proteins were eluted with 100 μl 2 × sample buffer (0.2 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 6% (w/v) sodium dodecyl sulphate (SDS), 4% (w/v) mercaptoethanol and 2 mM EDTA); (B) acid-dissociable proteins were eluted by incubating the beads with 100 μl of 30 mM Hepes containing 0.1% TDOC, 5 mM $MgCl_2$, 1 mM leupeptin and 50 μM PMSF pH 5.0, for 30 min at 4°C; (C) dissociable proteins at increased temperature were eluted by incubating the beads in 1 ml 30 mM Hepes containing 0.33% TDOC 5 mM $MgCl_2$, 1 mM leupeptin and 50 μM PMSF, pH 7.4 at 28°C for 30 min. The eluted proteins from A and B were analysed by SDS-PAGE and the eluate from C was assayed for [^{125}I]GRP binding activity after reconstitution into phospholipid vesicles as described below.

2.2.7. Reconstitution of affinity chromatographed bombesin receptor into phospholipid vesicles

Phospholipid vesicles were prepared from α-lecithin (70 mg/ml) either by sonication for 30 min [22] or by freeze-thaw fracture and extrusion through 0.4 μm nylon filters [23]. The soluble bombesin receptors were reconstituted into the phospholipid vesicles by mixing equal

volumes (1 ml) of vesicles and affinity eluate together with 50 μg/ml BSA as carrier, followed by removal of the CHAPS using an extracti-gel D column (1 × 1 cm), equilibrated and eluted with 30 mM Hepes, 5 mM $MgCl_2$, pH 7.4.

2.2.8. [^{125}I]GRP binding to reconstituted bombesin receptor

Aliquots (100 μl) of reconstituted eluate containing 0.2% BSA, 1 mg/ml bacitracin and 10 μg/ml aprotonin were incubated with 1 nM [^{125}I]GRP at 22°C for 30 min. At the end of the incubation [^{125}I]GRP associated with macromolecular components was separated from free [^{125}I]GRP by applying the reaction mixture (50–100 μl) to 1 ml syringes packed with Sephadex G-100 equilibrated in 30 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1% BSA followed by centrifugation at 1000 × g for 1 min at 4°C. The columns were then washed by centrifugation (1000 × g, 1 min) with 200 μl 30 mM Hepes pH 7.4, 5 mM $MgCl_2$ (spin-column chromatography). The radioactivity in the total elution volume was then determined using a Beckman γ-counter.

2.2.9. SDS-Polyacrylamide Gel Electrophoresis

Slab gel electrophoresis was performed using 7.5% acrylamide in the separating gel and 3% in the stacking gel and 0.1% SDS [24]. After electrophoresis gels were fixed and soaked in an amplify solution (Amersham International) and dried down onto paper for autoradiography with Fuji X-ray film. Dried gels were exposed for 2–4 days at −70°C. Alternatively, the gels were fixed and the proteins were visualized by silver staining (Koch-light silver stain kit).

3. RESULTS AND DISCUSSION

Peptides structurally related to bombesin and GRP contain a highly conserved C-terminal heptapeptide which confers binding specificity and biological activity to this neuropeptide family [5,8–10]. In order to develop a probe for specific ligand-affinity chromatography, we used [lys^3]bombesin to couple biotin at the lysine residue which is separated from the binding domain (residues 8–14). The resulting biotinylated lys^3-bombesin (BLB) was purified by FPLC (see section 2) and its binding and biological properties were then examined. Fig. 1 shows that BLB, like [lys^3]bombesin, inhibited specific binding of [^{125}I]GRP to either intact cells or to membrane preparations in a concentration-dependent manner. Concentrations giving rise to half-maximal inhibitions (IC_{50}) for [lys^3]bombesin and BLB were 1 nM and 2 nM in intact cells and 1 nM and 8 nM in membrane preparations. BLB also inhibited the cross-linking of [^{125}I]GRP to the M_r 75 000–85 000

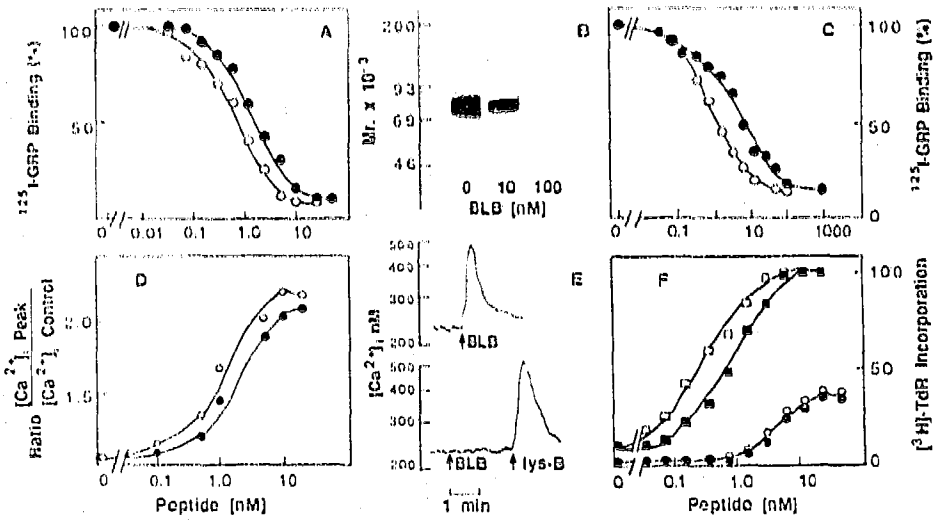

Fig. 1. Comparison of the biological activity between [lys^3]bombesin and biotinylated [lys^3]bombesin (BLB). (A) Competition of [^{125}I]GRP binding to Swiss 3T3 cells. Confluent and quiescent cultures of Swiss 3T3 cells were incubated at 37°C for 30 min with 1 nM [^{125}I]GRP in the presence of either [lys^3]bombesin (○) or BLB (●) at the concentrations indicated. The results are expressed as a percentage of the control value, 225 ± 20 fmol/10^6 cells (mean ± SEM, $n=6$). (B) Effect of BLB on the affinity labelling of the M_r 75 000–85 000 protein. Swiss 3T3 cells were incubated with 1 nM [^{125}I]GRP at 37°C for 10 min in the presence of BLB at the indicated concentrations. Chemical cross-linking was then performed with 6 mM EGS as described in section 2. (C) Competition of [^{125}I]GRP binding to Swiss 3T3 membranes. Membrane fractions (50 μg) were incubated in 100 μl of binding medium containing 0.5 nM [^{125}I]GRP for 10 min at 37°C in the presence of either [lys^3]bombesin (○) or BLB (●) at the indicated concentrations. The results are the composite of 3 independent experiments and are expressed as a percentage of the control value in each case. The mean control value of [^{125}I]GRP binding was 260 ± 22 fmol/mg of protein (mean ± SEM, $n=12$). (D) Dose-dependent effects of [lys^3]bombesin and BLB on $[Ca^{2+}]_i$ in Swiss 3T3 cells. Confluent and quiescent cultures of Swiss 3T3 cells in 90 mm dishes were washed twice with DMEM and incubated for 10 min in 5 ml DMEM containing 1 μM fura-2 AME. The cultures were then washed and suspended in 2 ml of electrolyte solution [25] in a quartz cuvette and $[Ca^{2+}]_i$ monitored by fluorescence as described in section 2 following addition of [lys^3]bombesin (○) or BLB (●) at the indicated concentrations. The Ca^{2+} response was calculated as the ratio between peak $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ just prior to addition of the peptides. (E) Incubation of BLB with streptavidin coated magnetic beads leads to loss of $[Ca^{2+}]_i$ response. Cultures of Swiss 3T3 cells were loaded with fura-2 AME and $[Ca^{2+}]_i$ monitored as described in 1D. Upper: effect of BLB (10 nM) on $[Ca^{2+}]_i$. Lower: BLB (10 nM) and [lys^3]bombesin (10 nM lys-B) were incubated with streptavidin coated magnetic beads for 1 h at 22°C. Following the removal of the magnetic beads the supernatants were sequentially assayed for the ability to mobilize Ca^{2+} in the same cell preparation as indicated. (F) [Lys^3]bombesin and BLB-induced DNA synthesis in Swiss 3T3 cells. Quiescent cultures of Swiss 3T3 cells were incubated in DMEM: Waymouth medium (1:1, v/v) containing [^{3}H]thymidine (1 μCi/ml) and various concentrations of [lys^3]bombesin (open symbols) or BLB (closed symbols) either in the absence (circles) or presence (squares) of insulin. The incorporation of radioactivity into acid-precipitable material was determined after 40 h of incubation. The results, the average of 3 experiments, are expressed as a percentage of the maximum values (10% FBS, 6.5 ± 0.5 × 10^5, $n=6$) in each case.

glycoprotein, identified as the bombesin receptor (Fig. 1B).

In order to determine whether BLB retains biological activity we tested its ability to increase the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in fura-2 loaded 3T3 cells [25–27] and to stimulate DNA synthesis in the absence or presence of insulin [4]. Fig. 1D shows that BLB caused a dose-dependent increase in $[Ca^{2+}]_i$; the peak $[Ca^{2+}]_i$ values and the transient kinetics of this response were similar to those induced by [lys^3]bombesin (Fig. 1E). BLB stimulated initiation of DNA synthesis as effectively as [lys^3]bombesin. The mitogenic response induced by both ligands was markedly potentiated by insulin (Fig. 1F). We verified that binding competition and stimulation of Ca^{2+} mobilization and DNA synthesis by BLB were abolished by binding the BLB preparation to streptavidin conjugated to beads (Fig. 1 and results not shown). Hence, receptor binding, biological activity and the association of the biotin moiety to streptavidin were maintained after biotinylation of [lys^3]bombesin.

We have recently shown that [^{125}I]GRP-receptor complexes were solubilized from Swiss 3T3 cell membranes by using the detergents taurodeoxycholate (TDOC) or deoxycholate [17]. These detergents extracted the ligand-receptor complex formed in intact membranes prior to solubilization. In addition, only partial dissociation of the solubilized [^{125}I]GRP-receptor complex occurred during gel filtration in G-100 or G-200 columns at 4°C [17,18]. Since BLB also binds to the receptor in membranes (Fig. 1), we reasoned that BLB-receptor complexes solubilized by TDOC could be retrieved from detergent-solubilized extracts by exploiting the affinity of biotin for streptavidin conjugated to beads. To test this approach cultures of Swiss 3T3 cells were metabolically labelled with [^{35}S]methionine and radioactively labelled membrane preparations were incubated with BLB. The specificity of this procedure was assessed by a parallel incubation of radioactive membranes with bombesin instead of BLB. Ligand-receptor complexes were solubilized in a solution containing 1% TDOC for 30 min at 4°C and separated from the non-extractable material by centrifugation for 60 min at 100 000 × *g* at 4°C. Then, the solubilized materials were incubated with magnetised beads coated with streptavidin. The beads were separated, washed and treated with an SDS-containing buffer to dissolve retained proteins (see section 2). The resulting samples were analyzed by SDS-PAGE followed by autoradiography. Although several bands were retained in a non-specific fashion, i.e. these were bands of identical intensity regardless of whether the membranes were incubated with either BLB or bombesin, a single additional band migrating with an apparent molecular mass of 82 kDa was bound to streptavidin beads in extracts from membranes treated with BLB (Fig. 2).

In order to assess whether the BLB-dependent isolation of the 82 kDa band can be scaled up, Swiss 3T3 membranes (400 mg of membrane protein) were incubated in parallel with either BLB or bombesin. All subsequent procedures up to the elution of the streptavidin-bound proteins were similar to those described above for the [^{35}S]methionine labelled membranes. Since [^{125}I]GRP bound to either intact cells or membrane preparations (unpublished results) dissociates very rapidly at acidic pH values [14], the elution was performed by incubating the streptavidin-conjugated beads with a solution adjusted at pH 5.0. The samples were analyzed by SDS-PAGE and the proteins revealed by silver staining. As shown in Fig. 2 (right), a single extra band migrating with an apparent molecular mass of 82 kDa was extracted from membranes treated with BLB. This protein migrated with the expected molecular mass of the bombesin receptor, as judged by affinity cross-linking of either intact 3T3 cells [11–13] or membrane preparations of these cells [16].

Although specific binding of [^{125}I]GRP to detergent solubilized bombesin receptors was poor [17], specific binding was markedly increased by reconstitution of the solubilized receptor into phospholipid vesicles (unpublished results). If the protein differentially extracted by BLB is in fact the bombesin receptor, it should be possible to demonstrate [^{125}I]GRP binding activity to

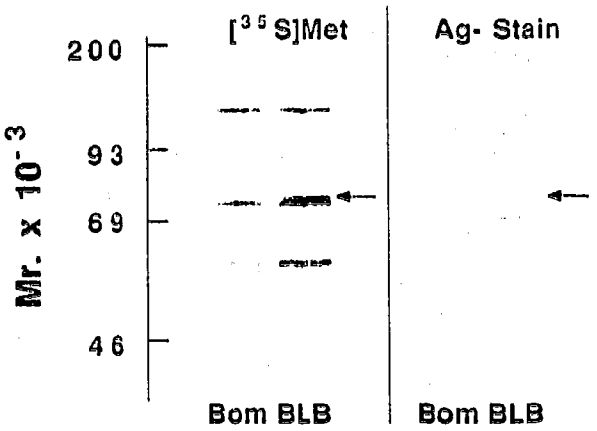

Fig. 2. Differential extraction of a 82 kDa protein (arrowed) in a BLB-dependent manner. **Left:** Swiss 3T3 cells, cultured on cultisphere-GL microcarriers for 4–5 d, were washed 3× with DMEM without methionine and incubated in the same medium containing [^{35}S]methionine (1.5 μCi/ml), unlabelled methionine (1.5 mg/l) and 10% dialysed FBS. After 18 h, the cells were harvested and membranes prepared as described in section 2. The membranes were then incubated in the presence of bombesin (100 nM; B) or BLB (100 nM) for 15 min at 37°C and solubilised. Affinity chromatography, using streptavidin-coated magnetic beads, was performed on the supernatant as described in section 2. The autoradiograms shown represent the SDS-PAGE analysis of total bound proteins eluted with 2× sample buffer. **Right:** membranes (400 mg) were incubated in the presence of either bombesin (100 nM) or BLB (100 nM) at 37°C for 15 min. Following solubilisation and affinity chromatography with streptavidin-coated magnetic beads, proteins eluted at pH 5.0 were analysed by SDS-PAGE and silver staining as described in section 2.

reconstituted phospholipid vesicles provided that the receptor is not denatured during the elution. It is known that the rate of dissociation of [^{125}I]GRP from either membranes or receptor solubilized preparations is strikingly enhanced by an increase in the temperature (e.g. from 4°C to 30°C) at neutral pH [16,17]. Consequently, BLB-receptor complexes solubilized with TDOC and bound to streptavidin-coated beads were incubated at 28°C for 30 min in the presence of 0.33% TDOC to dissociate bombesin receptors from the immobilized complexes. The results depicted in Figs 3 and 4 show that [^{125}I]GRP binding activity was clearly demonstrated when the eluted fraction was reconstituted into phospholipid vesicles prior to the binding measurements. Several lines of evidence (Fig. 3) indicate the specificity of [^{125}I]GRP binding to reconstituted receptors: (a) no specific binding was obtained to either phospholipid vesicles reconstituted without protein or to vesicles reconstituted with detergent-solubilized extracts of Swiss 3T3 membranes initially incubated with [lys^3]bombesin instead of BLB, (b) specific [^{125}I]GRP binding could not be demonstrated when membranes from either BALB/c 3T3 or 3T6 cells, which lack bombesin receptors [4,5,16] were treated with BLB exactly as those from Swiss 3T3 cells, and (c) the binding of [^{125}I]GRP to the reconstituted receptor was displaced by either unlabelled bombesin or GRP but it was virtually unaffected by identical concentrations of substance P, vasopressin or GRP(1–16), the NH_2-terminal fragment of GRP which neither binds to the receptor in membranes or intact cells [5,16] nor elicits any biological response in Swiss 3T3 cells [10].

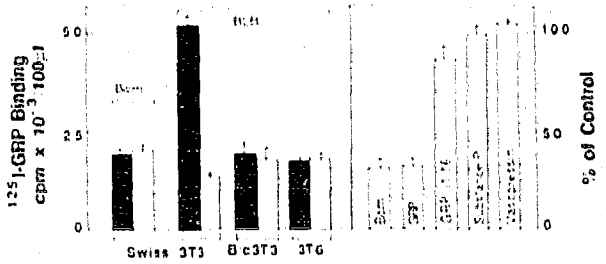

Fig. 3. Specificity of [^{125}I]GRP binding to affinity purified receptors reconstituted into phospholipid vesicles. **Left:** membranes (40 mg) prepared from Swiss 3T3, BALB/c 3T3 and 3T6 cells were incubated with BLB (100 nM) at 37°C for 15 min. Also, an identical aliquot of Swiss 3T3 membranes was incubated in the presence of bombesin (100 nM; BOM). The membranes were solubilised with 1% TDOC and the supernatants incubated with streptavidin-coated magnetic beads. The beads were then washed and temperature dissociable proteins were eluted with 1 ml of 30 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1 mM leupeptin and 50 µM PMSF as described in section 2. Eluted proteins were reconstituted into phospholipid vesicles and [^{125}I]GRP binding activity determined in 100 µl aliquots as described in section 2, either in the absence (closed bars), or presence (open bars) of 10 µM bombesin. The results represent the actual cpm eluted from applying the 100 µl aliquots of reconstituted proteins to Sephadex G-100 spin columns. **Right:** Swiss 3T3 membranes (4 × 40 mg aliquots) were incubated with BLB (100 nM) at 37°C for 15 min, then solubilised and affinity chromatographed with streptavidin-coated beads as described in section 2. Each aliquot was then eluted with 1 ml of 30 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1 mM leupeptin and 50 µM PMSF, for 30 min at 28°C. The eluted proteins (4 × 0.8 ml) were then reconstituted into phospholipid vesicles (4 × 0.8 ml) and pooled (final volume 5.0 ml). Aliquots (120 µl) were then incubated with 1 nM [^{125}I]GRP in the presence of bombesin (BOM), GRP, GRP(1–16), substance P and vasopressin all at 10 µM. Bound [^{125}I]GRP was separated from free [^{125}I]GRP by applying 100 µl of the reaction mixture to 1 ml G-100 spin column (see section 2). The results shown represent the mean values ± SEM, (n = 9) of 3 independent experiments expressed as a percentage of the level obtained in the absence of additions.

In conslusion, we describe a novel procedure to partially purify bombesin receptors which exploits the properties of biotinylated [lys^3]bombesin, a ligand that retains specific receptor binding and biological activity. Using this derivatized ligand, we extracted differentially a single protein from Swiss 3T3 membranes in a BLB-

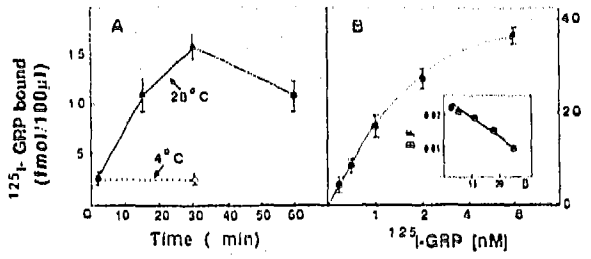

Fig. 4. Time-course of dissociation of bombesin receptor from streptavidin-coated beads (A) and analysis of binding as a function of [^{125}I]GRP concentration (B). Swiss 3T3 membranes (6 × 40 mg aliquots) were incubated with BLB (100 nM) at 37°C for 15 min, solubilised and affinity chromatographed as described in section 2. (A) Time-course of dissociation. Proteins were eluted either at 28°C for the times indicated or at 4°C for 30 min and reconstituted into phospholipid vesicles. Aliquots (100 µl) were incubated with 1 nM [^{125}I]GRP for 30 min at 22°C in the absence and presence of 10 µM bombesin. Specific binding of [^{125}I]GRP (fmol/100 µl) represent the mean ± SEM, n = 3. (B) Concentration dependence. Proteins eluted at 28°C for 30 min were reconstituted into phospholipid vesicles (see section 2) and aliquots were incubated in the presence of various concentrations of [^{125}I]GRP at 22°C. Specific binding was determined after 30 min as described in section 2. Non-specific binding was measured by the addition of at least 1000-fold excess unlabelled bombesin or of 1 µM bombesin for concentrations of [^{125}I]GRP below 1 nM. Inset: Scatchard analysis. Bound [^{125}I]GRP is expressed as fmol/100 µl of reconstituted receptor, free [^{125}I]GRP is expressed in pM.

Table I

Purification of the bombesin receptor from Swiss 3T3 membranes

	Protein (mg/ml)	Volume (ml)	Specific[a] activity (fmol/mg)	Total activity (pmol)	Yield	Fold purification
Solubilized extract	1.46	8	67	0.78	100	1
Effluent	0.67	16	"	"	"	"
Eluate	0.006	1.0	98300	0.59	83	1946

[a][^{125}I]GRP binding to solubilized extract, effluent and eluate was performed after reconstitution into phospholipid vesicles as described in section 2.

dependent manner. Visualization was achieved either by autoradiography of metabolically labelled proteins with [^{35}S]methionine or by silver staining in larger preparations. Crucially, when these preparations were eluted from streptavidin under mild conditions and reconstituted into phospholipid vesicles, we demonstrated [^{125}I]GRP binding activity that exhibited cellular and ligand specificity and saturability. The procedure described here should be useful to purify bombesin receptor from a variety of sources and also provides a strategy to isolate membrane receptors which require bound ligand prior to detergent solubilization. Finally, the reconstitution of the affinity purified bombesin receptor into phospholipid vesicles may provide an approach to elucidate the molecular nature of the G protein(s) that participate in the transduction of the mitogenic signal.

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