Characterization of Gastrin-Releasing Peptide Receptor Expressed in Sf9 Insect Cells by Baculovirus

Takashi Kusui,[‡] Mark R. Hellmich,[§] Lu-Hua Wang,[||] Richard L. Evans,[⊥] Richard V. Benya,[‡] James F. Battey,[§] and Robert T. Jensen*,[‡]

Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Laboratory of Biological Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Laboratory of Cellular Metabolism, National Heart, Lung and Blood Institute, and Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892-1804

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ABSTRACT: Whereas baculovirus expression systems have been extensively used for high-level expression of steroid receptors and receptors coupled to adenylate cyclase, there are few studies on peptide receptors coupled to phospholipase C (PLC). In the present study we have expressed the murine gastrin-releasing peptide receptor (mGRP-R) in Sf9 cells using a recombinant baculovirus and characterized it structurally and functionally. mGRP-R was detectible 12 h post infection with recombinant baculovirus carrying mGRP-R cDNA and became maximal at 60 h post infection (B_{max} = 6 pmol/mg protein), which is a 4−60-fold greater density than is found in native tissues. The mGRP-R in Sf9 cells assessed by affinity labeling or immunoblotting was smaller than that in native tissues (M_r = 51 kD vs 82 kD), and the difference was due to the extent of glycosylation. In Sf9 cells the mGRP-R had at least two of the four potential extracellular glycosylation sites glycosylated, whereas in the native receptor all four were approximately equally glycosylated. In Sf9 cells the glycosylation was entirely biantennary complex, in contrast to the native mGRP-R, where it was entirely tri- and tetraantennary complex *N*-linked oligosaccharides. Affinity labeling studies revealed a band with an apparent molecular mass about 40 kDa higher than the 51-kDa mGRP-R band. The intensity of this band correlated with the extent of functional G protein coupling, suggesting that it may represent an mGRP-R−G protein complex. In binding studies the affinity of the mGRP-R in Sf9 cells for the agonists bombesin (Bn), GRP, and neuromedin B (NMB) varied differently with infection time: with Bn the affinity decreased 3-fold with longer infection times, with GRP it remained unchanged, and with NMB it decreased 10-fold. GPP(NH)p inhibited binding of either [^{125}I]Tyr^{4}Bn or [^{125}I]GRP at 24 h post infection, but not at 96 h post infection. Agonists activated PLC, increasing both [^{3}H]IP and $[Ca^{2+}]_i$; however, the efficacy of each agonist decreased with infection time. These results demonstrate that by the use of recombinant baculovirus infected Sf9 cells the PLC-linked receptor mGRP-R can be expressed in amounts significantly greater than those in native tissues. The mGRP-R expressed in these Sf9 cells is incompletely glycosylated and has less complex *N*-linked oligosaccharide chains, yet it is fully coupled to G proteins and activates phospholipase C, similar to the native receptor, if short infection times are used. The ability of this system to express mGRP-R in increased amounts should be useful for obtaining enough receptor for making antibodies, performing reconstitution studies, and studying GRP-R modulation by agonists, and this system also provides useful insights into the importance of both glycosylation and G protein coupling with various agonists.

Gastrin-releasing peptide (GRP) is the mammalian homologue of the amphibian tetradecapeptide bombesin and is thought to play a physiological or pathological role in numerous, diverse processes (Tache et al., 1988). These include effects on the central nervous system (thermoregulation, maintenance of circadian rhythms, increased satiety) (Brown et al., 1988b; Albers et al., 1991; McCoy & Avery, 1990), effects in the gastrointestinal tract (stimulation of pancreatic exocrine secretion, potent stimulation of the release of many gastrointestinal hormones, regulation of motility) (Ghatei et al., 1982; Severi et al., 1991; Jensen, 1994, potent developmental effects (stimulation of chondrocytes, lung maturation) (Hill & McDonald, 1992; Sunday et al., 1993), and effects on the immune system (stimulation of macrophages, antibody secretion, natural killer cell cytoxicity) (Ruff et al., 1985; DelaFuente et al., 1993). GRP and related peptides also have potent growth effects on both normal tissues and tumors (Tache et al., 1988; Rozengurt, 1988), as well as functioning as autocrine growth factors in some small cell lung cancer cells (Cuttitta et al., 1985). GRP mediates its effects by interacting with a specific high-affinity receptor, which is a member of the seven transmembrane G protein linked receptor superfamily, activation of which results in stimulation of phospholipase C in all cells examined (Jensen, 1994; Rozengurt, 1988; Tache et al., 1988; Battey et al., 1991; Spindel et al., 1990).

* Address correspondence to this author: NIH/NIDDK/DDB, 10/9C103, 10 Center Dr. MSC 1804, Bethesda, MD 20892-1804. Tel: (301) 496-4201. FAX: (301) 402−0600.
[‡] National Institute of Diabetes and Digestive and Kidney Diseases.
[§] National Cancer Institute.
[||] National Heart, Lung and Blood Institute.
[⊥] National Institute of Dental Research.
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Like most peptide hormone receptors, the GRP receptor is present in very small quantities in tissues, which can limit the ability to carry out certain studies (Jensen, 1994; Moody et al., 1983). It is difficult to purify the receptor in sufficient quantity to carry out reconstitution studies with various G proteins or to generate antibodies. Furthermore, studies of receptor modulation by phosphorylation may be difficult because of the low signals.

Recombinant baculovirus infected insect cell systems have been used for the large-scale expression of a number of mammalian proteins including various receptors (Luckow, 1990). This system has been used extensively for steroid receptors and more recently for the G protein linked receptors interacting with protonated amines (adrenergic, muscarinic, and dopaminergic receptors) (Richardson & Hosey, 1992; Richardson et al., 1993; Kleymann et al., 1993; Ravet et al., 1993; Mills et al., 1993). Experience in using this system with receptors for various peptide hormones is more limited, although it has been reported to be successfully used for neurokinin type 2 (NK-2), prolactin, FSH, and chemotaxic receptors (Aharony et al., 1993; Cahoreau et al., 1992; Christophe et al., 1993; Quehenberger et al., 1992). Particularly limited is the use of this system for expressing peptide hormone receptors coupled to phospholipase C. Unlike the bacterial gene expression system, the baculovirus system often allows posttranslational modification (Luckow, 1990). Because most G protein coupled receptors undergo glycosylation, palmitoylation, phosphorylation, or other modifications that effect their functional expression, the baculovirus system has proved suitable for large-scale expression of mammalian G protein coupled receptors. This increased level of receptor expression in a baculovirus system has been useful not only for purification of the receptor; with some receptors such as the M_2 muscarinic cholinergic receptor, which is appropriately coupled to G proteins in baculovirus, this system has allowed detailed studies of agonist alterations of receptor function in the baculovirus (Richardson & Hosey, 1992; Richardson et al., 1993). Recent studies suggest that such posttranslational modifications as glycosylation may be particularly important for function of a number of such peptide hormone receptors. For receptors for classical hormones such as those for luteinizing hormone and thyroid-stimulating hormone (Zhang et al., 1991; Liu et al., 1993; Petaja-Repo et al., 1993; Ji et al., 1990) and for receptors for classical neurotransmitters such as those for adrenergic agents (Rands et al., 1990) or cholingeric agents (Gehle & Sumikawa, 1991; Giovannelli et al., 1991; Ohara et al., 1990), receptor glycosylation has no effect on high-affinity receptor binding or G protein coupling. However, with GRP receptors (Kusui et al., 1994), like a number of other peptide hormone receptors such as those for somatostatin (Rens-Domiano & Reisine, 1991), cholecystokinin (Santer et al., 1990), and VIP (Chochola et al., 1993), glycosylation is essential to maintain high-affinity binding and G protein coupling. In most previous studies (Luckow, 1990; Reilander et al., 1991; Richardson et al., 1993; Parker et al., 1991; Davidson & Castellino, 1991), the baculovirus expression system did not fully glycosylate G protein coupled receptors; therefore, whether peptide receptors such as those for the above hormones will be fully functional and fully coupled when expressed in this system is unknown.

In the present study we report the successful expression of the mouse GRP (mGRP) receptor (mGRP-R) in a recombinant baculovirus system and characterize the expressed receptors both functionally and structurally. We demonstrate that mGRP-R is expressed in increased amounts over that seen in native tissues but that it differs from the native receptor in type and extent of glycosylation; however, it remains coupled to G proteins in the Sf9 insect cell, with receptor activation resulting in phospholipase C activation acting to alter changes in inositol phosphates and cellular calcium. Additionally, these studies demonstrate that changing receptor agonist affinities with varying baculovirus infection times provides support for the proposal that the different agonist's receptor interactions are differentially regulated by receptor coupling to different guanine nucleotide binding proteins.

MATERIALS AND METHODS

Bombesin, [Tyr^4]bombesin, porcine gastrin-releasing peptide (GRP), and neuromedin B (NMB) were obtained from Peninsula Laboratories, Belmont, CA. Phosphate-buffered saline (PBS), pH 7.4, was from Biofluids, Rockville, MD. Sf900 insect cell media, Dulbecco's modified essential media (DMBM), and fetal bovine serum were from Gibco, Waltham, MA. Tris, glycine, and Coomassie blue R-250 were from Schwarz/Mann Biotech, Cleveland, OH. Ethylenediaminetetraacetic acid (EDTA), octopamine, bacitracin, soybean trypsin inhibitor (SBTI), dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) were from Sigma, St. Louis, MO. Phenylmethanesulfonyl fluoride (PMSF) and Nonidet P 40 (NP-40) were from Fluka, Ronkonkoma, NY. Bovine serum albumin (BSA) fraction V was from Miles Inc., Kankakee, IL. Aprotinin and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) was from Boehringer Mannheim Biochemicals, Indianapolis, IN. Disuccinimidyl suberate (DSS) and IODO-GEN were from Pierce, Rockford, IL. Sodium dodecyl sulfate (SDS), bromophenol blue, molecular weight standards, 2-mercaptoethanol, and protein assay dye reagent were from Bio-Rad, Richmond, CA. Glycerol was from Mallinckrodt Inc., Paris, KY. Protogel (30% acrylamide and 0.8% bis(acrylamide) stock solution) was from National Diagnostics, Manville, NJ. Peptide-N^4-[*N*-acetyl-β-glucosaminyl]asparagine amidase (PNGase F), endoglycosidase H (Endo-H), and endo-β-*N*-acetylglucosaminidase F_2 (Endo-F_2) were from Genzyme, Cambridge, MA. Na^{125}I was from Amersham, Arlington Heights, IL. *Spodoptera frugiperda* cells (Sf9 cells) and Swiss 3T3 murine fibroblast cells were obtained from the American Type Culture Collection, Rockville, MD. Modified baculovirus AcNPV DNA (*Autographa californica* nuclear polyhedrosis virus:BaculoGold DNA) and the transfer vector pVL1393 were obtained from PharMingen, San Diego, CA (Gruenwald & Heitz, 1993).

DNA Construction. A cDNA fragment encoding the open reading frame of the mGRP receptor (mGRP-R) flanked by *Eco*RI sites immediately preceding the initiation codon and following the termination codon was generated by polymerase chain reaction using a mouse GRP-R cDNA (Battey et al., 1991) as a template. The sense primer was 5′-CTGGTTCCGGCGAGAATTCATGGCTCCAAATAATTGTTC-3′, and the antisense primer was 5′-CTCGCTCCGGCGAGAATTCCTAGACATACCCCTCATGAC-3′. After digestion of the PCR product with *Eco*RI, the fragment

was purified by agarose gel electrophoresis and ligated into an *Eco*RI-cut alkaline phosphatase-treated transfer vector, pVL1393 (Gruenwald & Heitz, 1993). A recombinant pVL1393 plasmid containing a single copy of the mGRP-R cDNA fragment in the correct orientation was identified by restriction endonuclease mapping and designated pVL1393/mGRP-R. The nucleotide sequence of the entire coding region was confirmed by DNA sequencing to eliminate the possibility of changes introduced during the polymerase chain reaction.

Formation of Recombinant Baculovirus. Transfer of the full-length mGRP-R from pVL1393/mGRP-R to the AcNPV genome was achieved by homologous recombination into the polyhedrin gene locus (replaced by a *Lac*Z gene) after calcium phosphate co-transfection of Sf9 cells with modified wild-type linearized AcNPV DNA (BaculoGold DNA) and pVL1393/mGRP-R using the BaculoGold transfection kit (PharMingen, San Diego, CA, Cat. No. 21100K) (Gruenwald & Heitz, 1993). The transfection procedure was as follows: 3×10^6 Sf9 cells were seeded in a 25-cm^2 tissue culture flask. After the cells were incubated at 27 °C for 15 min to allow them to attach firmly to the flask, the culture medium was removed, and 1 mL of transfection buffer A [Grace's medium supplemented with 10% (v/v) fetal bovine serum] was added. After 5 min at room temperature, 1 mL of transfection solution [0.5 μg of linearized virus DNA (BaculoGold DNA) and 2 μg of the recombinant plasmid DNA, pVL 1393/mGRP-R, in 1 mL (Gruenwald & Heitz, 1993) of buffer B [125 mM HEPES/NaOH (pH 7.1)], 125 mM $CaCl_2$, and 140 mM NaCl from the kit] was added dropwise and mixed gently. After a 4-h incubation at 27 °C, the transfection solution was removed and 3 mL of insect cell culture media [Sf900 insect cell media supplemented with 10% (v/v) fetal bovine serum] was added. After an incubation at 27 °C for 4 days, the culture medium was collected and centrifuged at 1000*g* for 10 min, and the supernatant was used to infect more cells for amplification. Amplification of the recombinant virus was repeated three times, and a large amount of supernatant containing the recombinant baculovirus was obtained, which was stored in the dark at 4 °C and used for standard infections as outlined below.

Sf9 Insect Cell Culture and Viral Infection. Sf9 cells were cultured in insect cell culture media at 27 °C in room air. A standard infection was carried out by adding 1 mL of virus stock to 1×10^7 Sf9 cells in 15 mL of insect cell culture media per a 75 cm^2 tissue culture flask. After the indicated incubation time, cells were collected by mechanical disaggregation and pelleted at 1000*g* for 10 min and resuspended in the appropriate buffers for various experiments.

Transfection and Maintenance of Mammalian Cell Lines. BALB 3T3 cells stably expressing mGRP-R was generated as described previously (Benya et al., 1994b). Briefly, the receptor cDNA obtained from Swiss 3T3 fibroblasts was subcloned into a modified version of the pCD2 plasmid, and transfected using calcium phosphate precipitation. Stable transfectants were isolated in the presence of 800 μg/ml aminoglycoside G-418. Transfected cells and Swiss 3T3 cells were maintained identically by culturing in DMEM containing 10% fetal bovine serum (plus 300 μg/ml G-418 for stable transfectants), at 37 °C in a 5% CO_2 atmosphere. Cells were passaged every 3−4 days at confluence using 0.1% trypsin in 1 mM EDTA.

Preparation of Mouse Pancreatic Acini. Dispersed mouse pancreatic acini which contained native mGRP-R were prepared as described previously (Huang et al., 1990). Briefly, pancreata were injected with collagenase in standard buffer containing 98 mM NaCl, 6 mM KCl, 25 mM HEPES/NaOH (pH 7.4), 5 mM pyruvate, 5 mM fumarate, 5 mM glutamate, 0.01% soybean trypsin inhibitor, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.2 mM KH_2PO_4, 2 mM glutamine, 11 mM glucose, and 0.2% (w/v) BSA. After a 21-min incubation at 37 °C, the pancreata were shaken and processed as described previously (Huang et al., 1990; Jensen et al., 1982).

Measurement of Inositol Phosphates. Total inositol phosphates were determined in either recombinant baculovirus infected Sf9 cells or mouse pancreatic acini as described previously (Rowley et al., 1990; Benya et al., 1994b). Cells or acini were washed and resuspended in standard buffer and then loaded with 100 μCi/mL of *myo*-[2-^{3}H]inositol in the same buffer for 60 min either at 37 °C (mouse pancreatic acini) or at 25 °C (Sf9 cells). Cells were washed and incubated in IP buffer (standard buffer additionally contained 10 mM LiCl, 2 mM $CaCl_2$, 2% BSA, and 1.2 mM $MgSO_4$) for 15 min and then for 30 min at the same temperature with agonists at various concentrations. Reactions were terminated using ice-cold 1% (v/v) HCl in methanol containing 33% (v/v) chloroform. The inositol phosphates were isolated using a Dowex anion-exchange column as described previously (Qian et al., 1993; Berridge et al., 1983). Briefly, free [^{3}H]inositol was removed by washing with 10 vol of water; [^{3}H]glycerophosphorylinositol was removed by washing with 4 vol of 5 mM disodium tetraborate in 60 mM sodium formate; and total [^{3}H]inositol phosphates were eluted using 100 mM formic acid in 1.0 M ammonium formate. Eluates were then assayed for their radioactivity after the addition of Hydro-Fluor scintillation fluid.

Measurement of Changes in Cytosolic Calcium ($[Ca^{2+}]_i$). Cells or acini were suspended in standard buffer at a concentration of 2×10^6 cells/mL containing 2 μM fura-2 AM and incubated at 25 °C for 45 min. After fura-2 loading, cells were washed 3 times in standard buffer. For measurement of $[Ca^{2+}]_i$, 2.0-mL samples were placed in quartz cuvettes in a Delta PTI Scan 1 spectrophotometer (PTI Instruments, Gaithersburg, MD). This instrument was modified so as to be able to maintain a constant incubation temperature while continuously mixing the cuvette contents by means of a magnetic stirrer. Fluorescence was measured at 500 nm after excitation at 340 nm (F_{340}) and at 380 nm (F_{380}). Experiments with Sf9 cells were performed at 25 °C, and those with mouse pancreatic acini were performed at 37 °C. Autofluorescence of the unloaded cells was subtracted from all measurements. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. (Grynkiewicz et al., 1985), using the formula $[Ca^{2+}]_i = K_d \times (R - R_{min})-(R_{max} - R)S_f/S_b$, where K_d is the affinity of fura-2 for Ca^{+2}, $R = F_{340}/F_{380}$, or the ratio of the fluorescence of the two excitation wavelengths, R_{max} = the ratio of F_{340}/F_{380} in a saturated Ca^{2+} environment after the addition of 0.1% Triton X-100, R_{min} = the fluorescence ratio at virtually zero calcium after the addition of 25 mM EGTA, S_f is the F_{380} at zero calcium, and S_b is the F_{380} at saturated calcium.

Preparation of Cell Membranes. Sf9 cells and mouse pancreatic acini were washed twice with PBS at 4 °C and resuspended in 10 mL of TMEE buffer (50 mM Tris/HCl,

pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 3 mM benzamidine, 0.1 mg/mL SBTI, and 1 mM PMSF). Cells were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) for 30 s with power level 6 at 4 °C. The homogenate was centrifuged at 1000*g* for 5 min at 4 °C. The supernatant was centrifuged at 30000*g* for 60 min at 4 °C. The pellet was resuspended in TMEE-BB buffer [TMEE buffer additionally containing 0.1% (w/v) BSA and 0.1% (w/v) bacitracin] at a 2.5 mg of protein/mL concentration and used immediately or stored at −70 °C until used.

Preparation of [^{125}I-Tyr^4]Bombesin and [^{125}I]GRP. [^{125}I-Tyr^4]Bombesin and [^{125}I]GRP (2200 Ci/mmol) were prepared using the modification recently described (Wang et al., 1992) of the procedure described previously (Jensen et al., 1978). Briefly, 0.4 μg of IODO-GEN was added to 8 μg of peptide and 2 mCi of Na^{125}I in 20 μL of 0.5 M potassium phosphate buffer (pH 7.4). After a 6-min incubation at 22 °C, 300 μL of 1.5 M dithiothreitol was added and the reaction mixture was incubated at 80 °C for 60 min. Free ^{125}I was separated by a Sep-Pak (Waters Associates, Milford, PA), and radiolabeled peptide was separated from unlabeled peptide by reverse-phase high-performance liquid chromatography (Waters Associates, Model 204, with a Rheodyne injector) using a 0.46 × 25 cm μBondapak column as described previously (Wang et al., 1992).

Binding of [^{125}I-Tyr^4]Bombesin and [^{125}I]GRP to Membranes. Membranes were diluted with TMEE-BB buffer to a protein concentration of 0.1−0.25 mg/mL. In a typical experiment, a 200-μL aliquot was incubated with 0.5 nM [^{125}I-Tyr^4]bombesin or [^{125}I]GRP in the presence or absence of various agents at 25 °C for 15 min. Duplicate 50 μL samples were layered over 300 μL of binding buffer (4 °C) in 500-μL polypropylene tubes and centrifuged at 10000*g* for 3 min (Beckman Model B microfuge). After aspiration of the supernatant, the pellets were washed once with 300 μL of binding buffer (4 °C). The tops of the tubes were cut, and the radioactivity associated with the membranes was measured with a γ counter. Nonsaturable binding of the radiolabeled ligand was measured as the amount of radioactivity associated with the membranes when the incubation was performed in the presence of 1 μM non-radioactive bombesin and was less than 15% of the total binding in every experiment. At the end of an experiment, 10 μL of incubation mixture was taken for determination of total radioactivity added.

Cross-Linking of [^{125}I]GRP to Membranes. Cross-linking of [^{125}I]GRP to the mGRP-R was performed as described recently (Kusui et al., 1994). Briefly, 500 μL of membranes (0.25−1 mg of protein/mL) were incubated with 0.5 nM [^{125}I]GRP in TMEE-BB buffer at 25 °C in the presence or absence of various agents in a 1.6-mL polypropylene tube. After a 15-min incubation, the reaction mixture was centrifuged at 10000*g* for 3 min. The pellet was washed twice with 1 mL of ice-cold cross-linking buffer (50 mM HEPES, pH 7.5, and 5 mM $MgCl_2$) and resuspended in 200 μL of the same buffer containing 1 mM DSS as a cross-linking agent. After 30 min of cross-linking at 25 °C, the reaction was stopped by adding 25 μL of 1 M glycine. After 10 min on ice, the sample was centrifuged at 10000*g* for 3 min. The supernatant was aspirated, and the pellet was resuspended in 100 μL of 120 mM Tris/HCl, pH 6.8. A 6-μL aliquot of the mixture was taken and used to measure protein concentration. Cross-linked membranes were solubilized by adding 25 μL of gel-loading buffer (5 times concentrated) and incubated at 25 °C for 1 h. The gel loading buffer (5 times concentrated) contained 0.4 M Tris/HCl, pH 6.8, 20% SDS (w/v), 50% glycerol (v/v), and 0.05% bromophenol blue (w/v) plus 0.5 M DTT, unless otherwise indicated.

SDS−PAGE. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using the Laemmli buffer system as described previously (Laemmli, 1970). For cross-linking study, 10−30 μg of protein/lane samples were applied to a 1.5 mm thick 3% (w/v) acrylamide, 0.1% (w/v) SDS stacking gel over a 10% (w/v) acrylamide, 0.1% (w/v) SDS separating gel. The electrophoresis solution contained 25 mM Tris, 0.2 M glycine, and 0.1% (w/v) SDS. The electrophoresis was carried out at 40 mA/gel. Gels were stained with 0.1% (w/v) Coomassie blue R-250 in 40% (v/v) ethanol and 10% (v/v) acetic acid and destained with 10% (v/v) ethanol and 7.5% (v/v) acetic acid. After overnight destain, gels were equilibrated in 45% (v/v) ethanol and 5% (v/v) glycerol for 30 min and dried in a gel-slab drier (Hoefer Scientific Instruments, Model SE 540, San Francisco, CA). Molecular weight standards (myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin) were applied to each gel before electrophoresis. Dried gels were exposed to storage phosphor screen for 1−7 days at room temperature. A phosphorimager (Molecular Dynamics, Sunnyvale, CA) was used to analyze the exposed storage phosphor screen, and the results were printed by a LaserJet III (Hewlett Packard, Boise, ID). The relative densities of the radioactive bands were also obtained using the PhosphorImager. For the immunoblotting study, a 1.0 mm thick minisized 7.5% acrylamide gel and SeeBlue prestained molecular weight marker (Novex, San Diego, CA) were used.

Enzyme Treatment of Cross-Linked Membrane Proteins. Deglycosylation with PNGase F, Endo-H, and Endo-F2 were performed as described previously (Kusui et al., 1994) and are briefly summarized below.

PNGase F Treatment. Membrane preparations were denatured by a 3-min incubation at 95 °C in 80 μL of 50 mM Tris/HCl, pH 7.7, containing 50 mM EDTA, 50 mM 2-mercaptoethanol and 0.5% (w/v) SDS. To a 20 μL aliquot (40−160 μg of protein) of the above, 10 μL of 7.5% NP-40 was added. The mixture was incubated in a volume of 60 μL with the indicated concentrations of PNGase F for 3 h at 37 °C. Fifteen microliters of gel loading buffer was then added, and samples were subsequently analyzed by SDS−PAGE.

Endo-H Treatment. Cross-linked membrane preparations were denatured by a 3-min incubation at 95 °C in 80 μL of 50 mM sodium phosphate, pH 6.0, containing 1 mM PMSF and 0.5% (w/v) SDS. Eight-microliter aliquots of samples (16 μg of protein) were incubated with 5 units/mL Endo-H in a volume of 20 μL for 18 h at 37 °C. Samples were then solubilized by adding gel loading buffer and analyzed by SDS−PAGE.

Endo-F_2 Treatment. Cross-linked membrane preparations were denatured by a 3-min incubation at 95 °C in 80 μL of 0.5 M sodium acetate, pH 4.75, containing 1% (w/v) SDS. To a 4-μL aliquot (8 μg of protein) was added 2 μL of 10% NP-40, and digestion was performed with 60 munits/mL Endo-F_2 in a volume of 20 μL for 18 h at 37 °C. Samples

were then solubilized by adding gel loading buffer and analyzed by SDS−PAGE.

Immunoblotting of the GRP-R. Membrane preparations (50 μg of protein/lane) were separated by SDS−PAGE as described in the SDS-PAGE section above. Separated proteins were then electrotransferred to a nitrocellulose membrane in transfer buffer [25 mM Tris, 192 mM glycine, and 20% (v/v) methanol] at 4 °C and 25 mA overnight. The membrane was blocked with blocking buffer [50 mM Tris/HCl (pH 8.0), 80 mM NaCl, 2 mM $CaCl_2$, 0.05% TWEEN20, 0.02% NaN_3, and 5% (w/v) non-fat dry milk] at 25 °C for 2 h. Blocked membrane was washed 10 min × 2 with blocking buffer and was incubated in blocking buffer at 25 °C for 2 h with polyclonal antibody obtained from a rabbit immunized with synthetic peptide corresponding to the third intracellular loop of the mGRP-R (CVEGNIHVKKQIES-RKR) coupled to Keyhole limpet hemocyanin through the amino-terminal cysteine (Kroog et al., 1995). After two 10-min washes with blocking buffer, the membrane was incubated with goat anti-rabbit antibody in blocking buffer at 25 °C for 1 h, washed 10 min × 2 with blocking buffer, and probed with [^{125}I]protein A in blocking buffer (0.2 μCi/mL) at 25 °C for 45 min. Membranes were then rinsed twice, washed twice for 10 min with blocking buffer, rinsed twice, washed twice for 10 min with blocking buffer without non-fat dry milk, and exposed to a storage phosphor screen for 1−2 days at room temperature.

Western Blotting: Analysis of Sf9 Cell Membranes Infected with Either GRP-R or $G\alpha_q$ Containing Recombinant Baculovirus. Sf9 cells were maintained as suspension cultures in Sf-900II serum-free media (GibcoBRL, Gaithersburg, MD) at 27 °C. Log-phase Sf9 cell cultures (density = 1−2 × 10^6 cells/mL) were infected with either a recombinant baculovirus containing a mouse GRP-R cDNA or a mouse $G\alpha_q$ cDNA, at a multiplicity of infection (MOI) of approximately 2, and incubated for various lengths of time. Cells were harvested by centrifugation (1000*g* for 10 min). Cell membranes were prepared as described under Preparation of Cell Membranes, except that 10 μg/mL aprotinin was added to the other protease inhibitors. Cross-linking and SDS−PAGE were performed as described above except that cell membranes were incubated with or without 0.5 nM GRP at 25 °C for 15 min. Samples were resolved by SDS−PAGE and electrophoretically transferred to a nitrocellulose membrane as described above. The nitrocellulose membrane was blocked in blotto (50 mM Tris pH 8, 2 mM $CaCl_2$, 80 mM NaCl, 5% (w/v) non-fat milk, 0.2% (v/v) NP-40, and 0.02% Na-azide) plus 20% fetal calf serum for 1 h at room temperature and then probed with an anti-$G\alpha_q$ rabbit antiserum (1:500 dilution in blotto) overnight at 4 °C. The next day, the membrane was washed (2 × 15 min) with blotto, probed with an HRP-conjugated donkey anti-rabbit antibody (1:1000 dilution in blotto) for 1 h at room temperature, and washed 2 × 15 min with blotto and 2 × 15 with buffer A (50 mM Tris, pH 8, 2 mM $CaCl_2$, and 80 mM NaCl). Specific immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL, Amersham, MA).

Protein Assay. Membrane protein concentration was determined using the Bio-Rad protein assay dye agent with BSA as a standard.

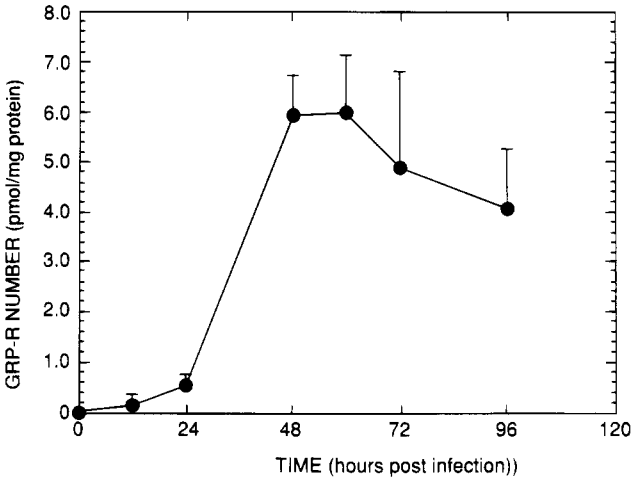


FIGURE 1: Time course of mGRP-R expression in recombinant baculovirus infected Sf9 cells. Sf9 cells were infected with recombinant baculovirus carrying mGRP-R cDNA as described in Materials and Methods. At various times after infection, Sf9 cells were collected by mechanical disaggregation and membrane preparations were prepared. Membranes were incubated with [^{125}I-Tyr^4]Bn in the presence or absence of various amounts of non-radioactive Bn. Radioactivities associated with membranes were measured, and the receptor number (B_{max}) was calculated by using a nonlinear, least-squares curve-fitting program (LIGAND). Each value was determined in duplicate, and results are means ± SEM from three separate experiments.

RESULTS

There was no saturable binding of [^{125}I-Tyr^4]Bn to noninfected Sf9 cells, demonstrating that these cells do not possess GRP receptors. When Sf9 cells were infected by recombinant baculovirus carrying mGRP-R cDNA, specific and saturable binding of [^{125}I-Tyr^4]Bn to membrane preparation was first detectable at 12 h post infection (Figure 1). It reached a maximum with a B_{max} of 6 pmol/mg of protein (0.5 pmol/10^6 cells, or 300 000 receptors/cell) at 60 h post infection (Figure 1). Incubations longer than 60 h resulted in a decrease in the number of mGRP-R (Figure 1). When Sf9 cells were infected with wild-type AcNPV or a recombinant AcNPV containing exogeneous cDNA encoding a protein other than the mGRP-R (i.e., a phosphodiesterase), no detectable binding of [^{125}I-Tyr^4]Bn was seen (data not shown).

To determine the affinities of the mGRP-R expressed by Sf9 cells for various GRP-related peptides, a dose-inhibition study was performed at various times post infection (Figures 2 and 3). At all time points after infection, Bn, GRP, and NMB each inhibited binding of [^{125}I-Tyr^4]Bn (Figure 2). However, the affinities of those agonists showed different time-dependencies (Figure 3). Specifically, at 24 h post infection the K_i for Bn was 3.0 nM, which is similar to the K_i seen with the native mGRP-R on pancreatic acini, but by 96 h post infection it demonstrated a 3-fold decrease in affinity to 9.0 nM (Figure 3, left panel). The mGRP-R demonstrated a 10-fold decrease in affinity for NMB from 24 to 96 h post infection (Figure 3, middle panel). The K_i value of this receptor for NMB was similar to that of the native mGRP-R on pancreatic acini at 96 h post infection instead of 24 h as was the case with Bn. In contrast to NMB and Bn, the mGRP-R did not change its affinity for GRP with infection time (Figure 3, right panel). At both 24 and 96 h post infection, the affinity of the mGRP-R on bacu-

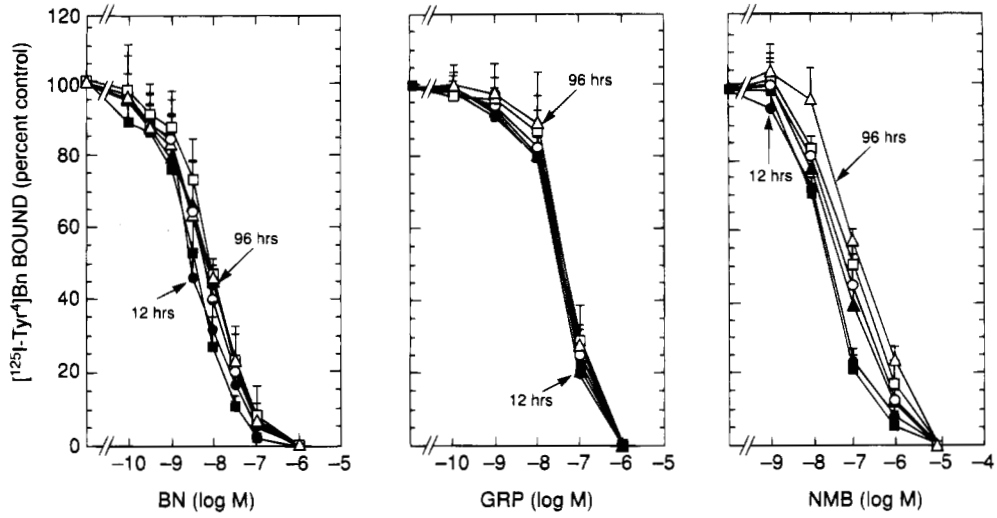


FIGURE 2: Ability of Bn (left panel), GRP (middle panel), and NMB (right panel) to alter binding of [^{125}I-Tyr^4]Bn to recombinant baculovirus infected Sf9 cell membranes at different infection times. Membranes from Sf9 cells expressing mGRP-R were prepared as described in the Caption for Figure 1 at 12 (●), 24 (■), 48 (▲), 60 (○), 72 (□) and 96 h (△) post infection with baculovirus. Membranes were incubated with [^{125}I-Tyr^4]Bn in the presence or absence of various amounts of non-radioactive Bn, GRP, or NMB at 25 °C for 15 min. Fifty-microliter aliquots of the incubation mixture were taken, and radioactivity saturably bound was determined as described in Materials and Methods. Results are expressed as the percentage of radioactivity saturably bound to the membranes with no additions. Each value was determined in duplicate, and results are means ± SEM from three separate experiments.

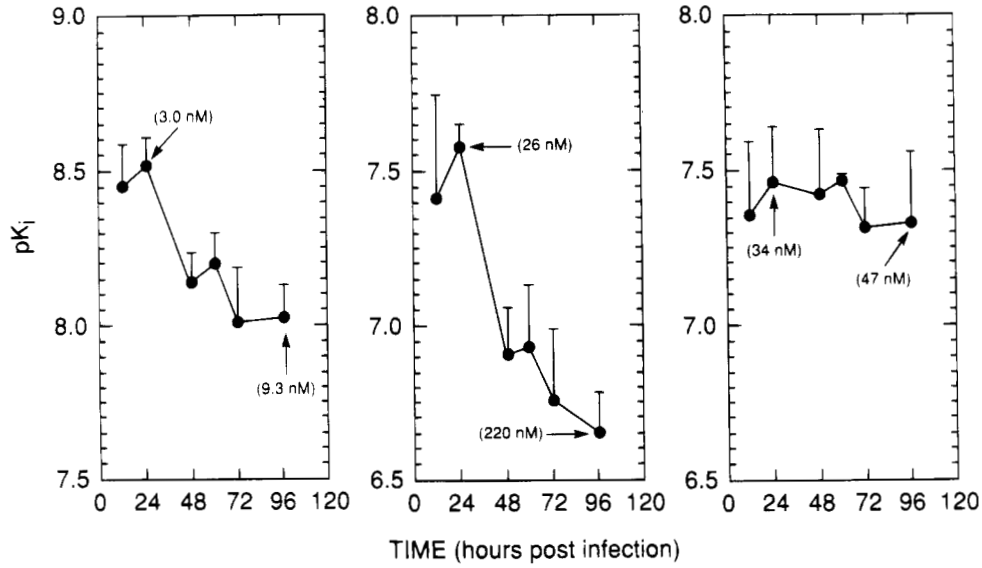


FIGURE 3: Effect of infection time with the recombinant baculovirus on the affinity of the mGRP-R expressed in Sf9 cells. Binding data shown in Figure 2 were analyzed using the least-squares curve-fitting program LIGAND. K_i values were expressed as $pK_i = -\log(K_i)$ with K_i expressed in molar units. Data in the left panel are for Bn; middle panel, NMB; and right panel, GRP. Each result is the mean ± SEM from three separate experiments. The K_i values at 24 and 96 h post infection are shown in the parentheses in nanomolar units.

lovirus for GRP was 10-fold lower than that of the native mGRP-R on pancreatic acini.

To examine whether the mGRP-R in the Sf9 cells was coupled to G proteins and whether this coupling changed with time, we examined the effect of Gpp[NH]p on the binding of [^{125}I-Tyr^4]Bn and [^{125}I]GRP to GRP-Rs in mouse pancreatic acini and the infected Sf9 cells at 24 and 96 h post infection. Gpp[NH]p caused a 45% decrease in binding of [^{125}I-Tyr^4]Bn or [^{125}I]GRP to native mGRP-R in mouse pancreatic acinar cell membranes with IC_{50} values of 14 ± 3 and 6 ± 2 nM for [^{125}I-Tyr^4]Bn and [^{125}I]GRP, respectively (Figure 4, left panel). The effect of Gpp[NH]p on agonist binding to mGRP-R expressed in Sf9 cells at 24 h post infection was similar to that observed with the native receptor in the mouse pancreas. Specifically, at 24 h post infection a maximal effective concentration of Gpp[NH]p caused a 40% decrease in binding of [^{125}I-Tyr^4]Bn and a 45% decrease in binding of [^{125}I]GRP with IC_{50} values of 13 ± 3 and 4 ± 2 nM for [^{125}I-Tyr^4]Bn and [^{125}I]GRP, respectively (Figure 4, middle panel). In contrast, at 96 h post infection the effect of Gpp[NH]p on the binding of [^{125}I-Tyr^4]Bn or [^{125}I]GRP to GRP-R in recombinant baculovirus infected Sf9 cells was minimal (Figure 4, right panel). These results indicate that the mGRP-R in recombinant baculovirus infected Sf9 cell is well coupled to G proteins in the early phase of infection, but is not well coupled with longer infection times.

To investigate the cellular coupling of the mGRP-R in Sf9 cells further, we measured agonist-induced inositol phosphate production (Figure 5). With *myo*-[2-^{3}H]inositol preloaded mouse pancreatic acini, Bn, GRP, and NMB caused a 600% increase in [^{3}H]inositol phosphates, and the increase was caused in a dose-dependent manner (Figure 5, left panel).

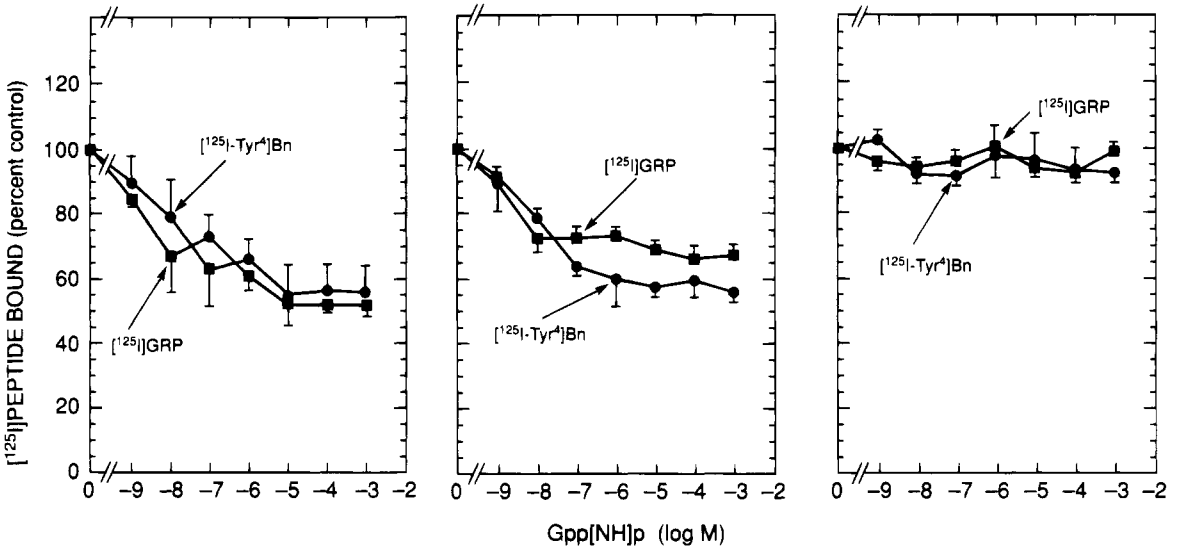


FIGURE 4: Comparison of the effect of Gpp[NH]p on agonist binding to the native mGRP-R in mouse pancreatic acinar cells (left) and the mGRP-R in recombinant baculovirus infected Sf9 cells at 24 (middle) and 96 h (right) post infection. Membranes from the mouse pancreatic acinar cells and mGRP-R expressing Sf9 cells at 24 and 96 h post infection were incubated with 0.1 nM [^{125}I-Tyr^4]Bn or 0.1 nM [^{125}I]GRP in the presence or absence of various concentrations of Gpp[NH]p for 15 min at 25 °C with or without 1 μM bombesin. Results are expressed as the percentage of radioactivity saturably bound to the membrane with no additions. Each value was determined in duplicate, and results are means ± SEM from three separate experiments.

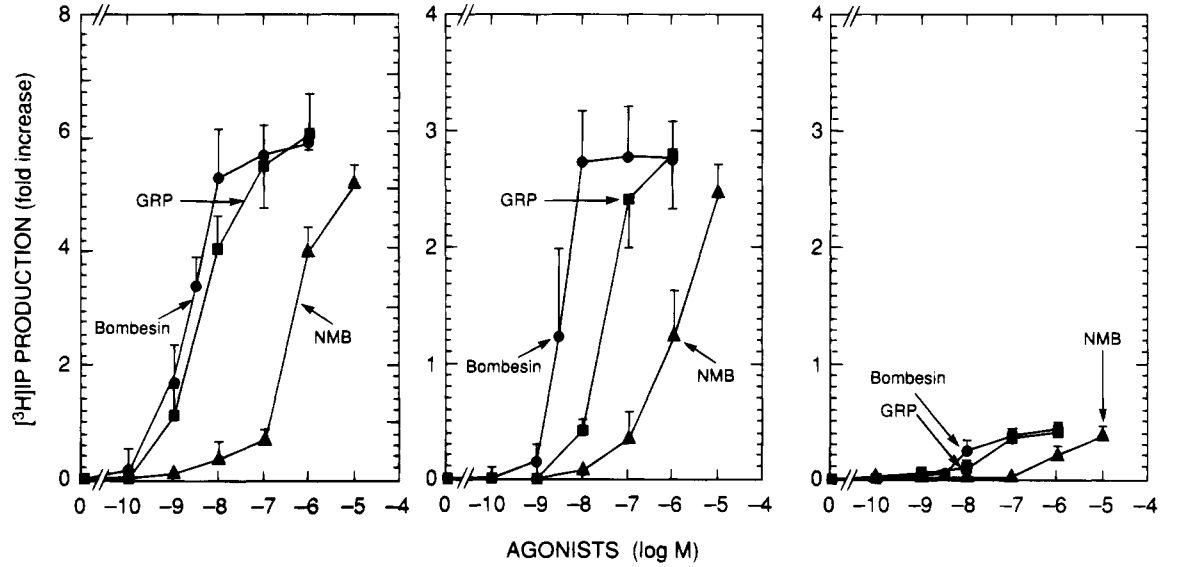


FIGURE 5: Comparison of the ability of GRP and related peptides to stimulate an increase in [^{3}H]IP in mouse pancreatic acini (left) and recombinant baculovirus infected Sf9 cells at 24 (middle) and 96 h (right) post infection. Dispersed mouse pancreatic acini and baculovirus-infected Sf9 cells were suspended in standard buffer and incubated with *myo*-[2-^{3}H]inositol for 1 h at 37 °C (pancreatic acini) or at 25 °C (Sf-9 cells). After a wash with standard buffer, acini or cells were incubated in IP buffer for 15 min. Acini and cells were stimulated with various concentrations of Bn, GRP, or NMB for 30 min at the same temperature used for *myo*-[2-^{3}H]inositol loading. Total tritiated inositol phosphate ([^{3}H]IP) was measured as described in Materials and Methods. Results are expressed as the fold increase over basal levels. Each value was determined in duplicate, and results are means ± SEM from three separate experiments.

EC_{50} values for Bn, GRP, and NMB were 2 ± 1, 4 ± 1, and 470 ± 50 nM, respectively, and were closely related to corresponding K_i values from binding studies. In mGRP-R recombinant baculovirus infected Sf9 cells at 24 h post infection, Bn and GRP caused a 300% increase in [^{3}H]-inositol phosphates, and the increase occurred in a dose-dependent manner over dose ranges where binding inhibition occurred (compare Figure 5, middle and Figure 2). In contrast to the fact that NMB and GRP showed similar affinities for the mGRP-R in infected Sf9 cells at 24 h post infection (Figure 3), NMB was 50-fold less potent than GRP at stimulating an increase in [^{3}H]inositol phosphates at this infection time (Figure 5, middle panel). Specifically, at 24 h post infection the EC_{50} values for stimulating increases in [^{3}H]inositol phosphates of Bn, GRP, and NMB were 4 ± 1, 30 ± 7 and 1200 ± 200 nM, respectively (Figure 5, middle panel). At 96 h post infection, the efficacy of these agonists were markedly reduced (Figure 5, right panel). Only a 40% increase in [^{3}H]inositol phosphates was seen, and the increase occurred over the dose ranges where binding inhibition occurred.

We next compared the ability of Bn to increase $[Ca^{2+}]_i$ in mouse pancreatic acini and in recombinant baculovirus infected Sf9 cells. When 1 μM Bn was added to Fura-2 preloaded mouse pancreatic acini, $[Ca^{2+}]_i$ increased rapidly from a basal level of 100 ± 10 nM, peaked at 1 s with

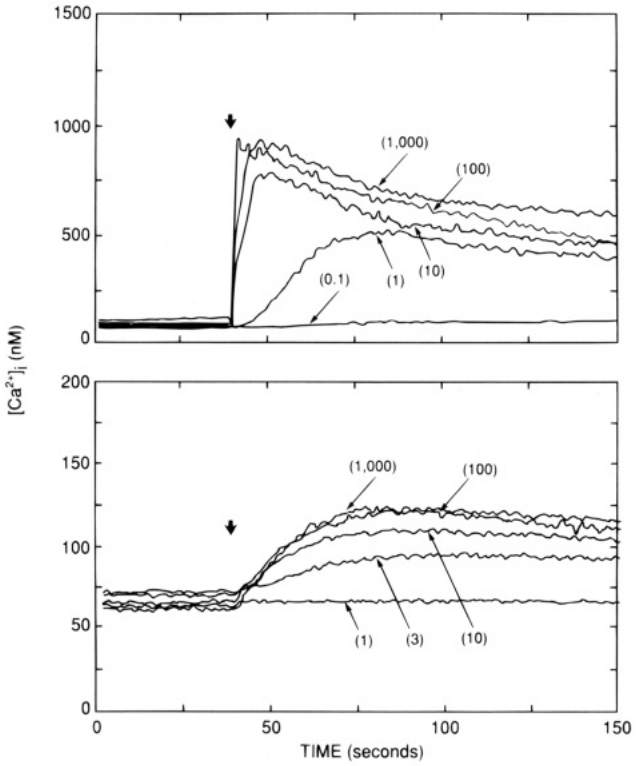


FIGURE 6: Ability of bombesin to increase $[Ca^{2+}]_i$ in mouse pancreatic acini (top) and recombinant baculovirus infected Sf9 cells (bottom). Fura-2 was loaded in pancreatic acini or Sf9 cells by a 45-min incubation of acini or cells with 2 μM fura-2 AM. After being washed with standard buffer, 2.0-mL samples were incubated in quartz cuvettes in a Delta PTI Scan 1 spectrophotometer either at 37 °C (pancreatic acini) or at 25 °C (Sf9 cells). Fluorescence was measured at 500 nm after excitation at 340 nm (F_{340}) and at 380 nm (F_{380}). Autofluorescence of the unloaded cells was subtracted from all measurements. $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. The numbers in parentheses are the nanomolar concentration of Bn added in each case. Each tracing is representative of two others.

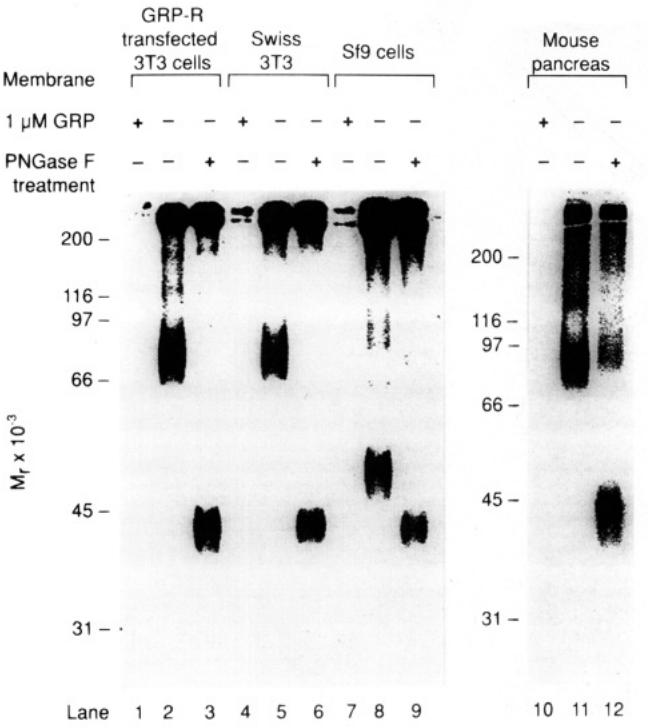


FIGURE 7: Cross-linking of [^{125}I]GRP to the mGRP-R expressed in various cells and the effects of PGNase F treatment. Membranes from mouse pancreatic acinar cells, Swiss 3T3 fibroblasts, BALB 3T3 cells transfected with mGRP-R, and Sf9 cells expressing mGRP-R were incubated with 0.5 nM [^{125}I]GRP at 25 °C for 15 min in the presence or absence of non-radiolabeled GRP. After washing, cross-linking was performed by a 30-min incubation with 1 mM DSS at 25 °C. Some of the samples were treated with PNGase F to remove N-linked oligosaccharide chains as described in Materials and Methods (lanes 3, 6, 9, and 12). Samples were subjected to SDS−PAGE and analyzed by autoradiography. Each of these autoradiograms is representative of at least two others. The positions of molecular mass standards are shown on the left side of each panel.

maximal level of 960 ± 60 nM, and then declined (Figure 6, top panel). The ability of Bn to increase $[Ca^{2+}]_i$ in pancreatic acini was dose-dependent with an EC_{50} value of 1.2 ± 0.5 nM (Figure 6, top panel). Bn also increased $[Ca^{2+}]_i$ from a basal level of 66 ± 6 nM in recombinant baculovirus infected Sf9 cells at 24 h post infection (Figure 6, bottom panel). However, the increase was slower and smaller than that seen in mouse pancreatic acinar cells. $[Ca^{2+}]_i$ increased to a maximal value 130 ± 10 nM at 30 s after addition of Bn. Similar to what was observed in pancreatic acini, the ability of Bn to increase $[Ca^{2+}]_i$ in transfected Sf9 cell was dose-dependent with an EC_{50} of 3.2 ± 0.8 nM (Figure 6, bottom panel). With recombinant baculovirus infected Sf9 cells at 96 h post infection, Bn failed to increase $[Ca^{2+}]_i$ (data not shown). At both 24 and 48 h post infection the increase in $[Ca^{2+}]_i$ in recombinant baculovirus infected Sf9 cells caused by 1 μM Bn was examined in both 0.5 Ca^{2+}-containing standard incubation buffer and a Ca^{2+}-free buffer (data not shown). At both infection times the increase in $[Ca^{2+}]_i$ was not altered by the presence or absence of extracellular Ca^{2+}; however, the increase was sustained in $[Ca^{2+}]_i$-containing buffer, whereas it decreased to baseline by 2 min in Ca^{2+}-free buffer.

To assess the size and the extent of glycosylation of the mGRP-R in recombinant baculovirus infected Sf9 cells, cross-linking of [^{125}I]GRP to the expressed GRP-R was performed using the homo-bifunctional cross-linking agent DSS, and the results were compared with those for native mGRP-R in mouse pancreatic acinar cells and in Swiss 3T3 cells, as well as for mGRP-R in mGRP-R-transfected BALB 3T3 cells. SDS−PAGE and autoradiogram analysis of cross-linked membranes from mGRP-R transfected BALB 3T3 cells, Swiss 3T3 cells, and mouse pancreatic acini showed a broad main band of 82 kDa (Figure 7, lanes 2, 5, and 11). Cross-linking was inhibited by 1 μM non-radiolabeled GRP in each of these three cell types (Figure 7, lanes 1, 4, and 10). In contrast, at any time of recombinant baculovirus infection, Sf9 cell membranes showed a main band of 51 kDa, labeling of which was also inhibited by 1 μM non-radiolabeled GRP (Figure 7, lanes 7 and 8: the result for 48 h post infection is shown). PNGase F-deglycosylation of cross-linked membranes from all four cell types resulted in a decrease in the size of the main bands to the same 43 kDa (Figure 7, lanes 3, 6, 9, and 12), indicating that the difference in the molecular weights of the mGRP-Rs could be attributed to the difference in the extent of glycosylation and is not caused by different degradation or processing of the polypeptide core in each cell type. Besides the main bands mentioned above, faint bands which were 45−50 kDa larger than the main bands were seen. Specifically, faint bands of approximately 130 kDa were frequently seen with membranes from the mouse pancreatic acini, mGRP-R-transfected BALB 3T3 cells, and Swiss 3T3 cells (see Figure 7, lanes 2

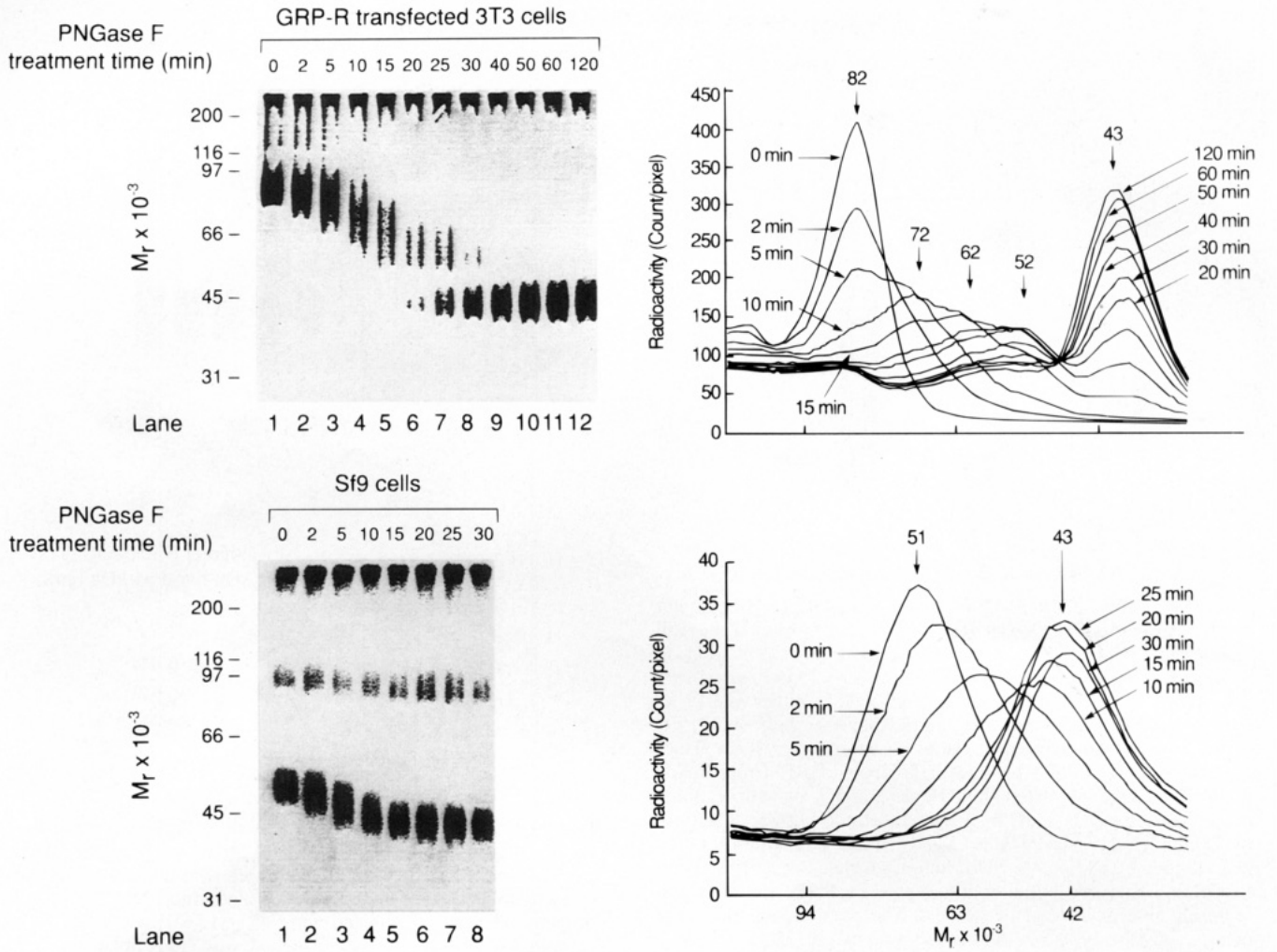


FIGURE 8: Serial partial deglycosylation of [^{125}I]GRP cross-linked to the mGRP-R in transfected BALB 3T3 cells (top) and recombinant baculovirus infected Sf9 cells (bottom). [^{125}I]GRP was cross-linked with 1 mM DSS to membranes from transfected BALB 3T3 cells (top left panel) or membranes from Sf9 cells expressing mGRP-R (bottom left panel). Cross-linked membranes were incubated with PNGase F (2 units/mL for transfected BALB 3T3 cell membranes; 0.2 unit/mL for mGRP-R-transfected Sf9 cell membranes) at 37 °C for the various times indicated at the top of each lane. The digestion was stopped by denaturing the enzyme in 4% SDS. Samples were subjected to SDS−PAGE and analyzed by autoradiography. Each autoradiogram is representative of at least two others. The positions of molecular weight standards are shown on the left side of each panel. The top right panel shows the spacial distribution of radioactivity in each lane shown in the top left panel analyzed using a PhosphorImager. The bottom right panel shows the spacial distribution of radioactivity in each lane shown in the bottom left panel. In each of the right panels, the times in minutes indicate the time of the PNGase F treatment, and numbers with short arrows indicate the molecular weights of the peak bands of radioactivity.

and 11, for examples with GRP-R-transfected 3T3 cells and mouse pancreas), and with Sf9 cell membranes a faint band at 97 kDa was seen (Figure 7, lane 8). A faint 90-kDa band was seen with deglycosylated membranes from all four cell types (best seen in mouse pancreas, Figure 7, lane 12).

To investigate further the nature of the difference in glycosylation between native mGRP-R and those expressed in recombinant baculovirus infected Sf9 cells, a serial partial deglycosylation study was performed on recombinant baculovirus infected Sf9 cells and mGRP-R transfected BALB 3T3 cells which have glycosylation identical to that of the native mGRP-R on pancreatic acini or Swiss 3T3 cells (Kusui et al., 1994) (Figure 8). When cross-linked mGRP-R-transfected BALB 3T3 cell membranes were incubated with 2 units/mL PNGase F for various times, the radiolabeled receptor was deglycosylated in a stepwise manner (Figure 8, top left panel). Analysis of these bands using a PhosphorImager demonstrated that there were three bands of partially deglycosylated receptors (72, 62, and 52 kDa) besides the original fully glycosylated and fully deglycosylated ones (Figure 8, top right panel). This observation suggests that the transfected mGRP-R has four oligosaccharide chains with each oligosaccharide chain being approximately 10 kDa in size. Serial partial deglycosylation of the mGRP-R in recombinant baculovirus infected Sf9 cells with 0.2 unit/mL PNGase F showed at least one additional band between the original band, which has a size (51 kDa) similar to that of the smallest partially deglycosylated species obtained from digestion of the native mGRP receptor, and the fully deglycosylated band (Figure 8, bottom panels). Because of the rapidity of the digestion and small amount of glycosylation on the mGRP-R in Sf9 cells, it was not possible to resolve clearly whether three intermediate bands could be seen in these cells as was seen in the mGRP-R-transfected BALB 3T3 cells. These results suggest that the mGRP-R expressed in recombinant baculovirus infected Sf9 cells has only 25% of the glycosylation of the native mGRP-R because it has smaller oligosaccharide chains on at least two of the four potential N-linked glycosylation sites instead of the possibility that it has one fully glycosylated

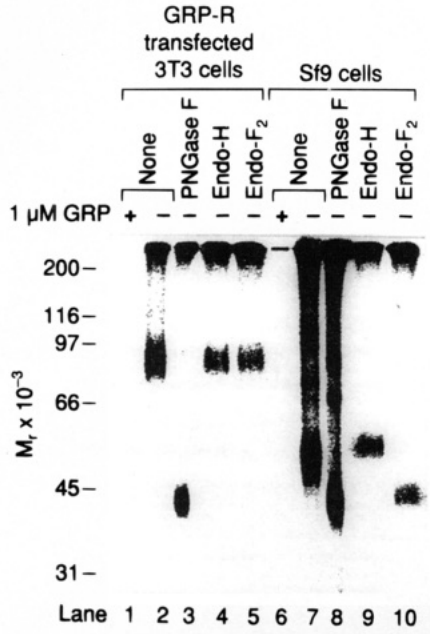
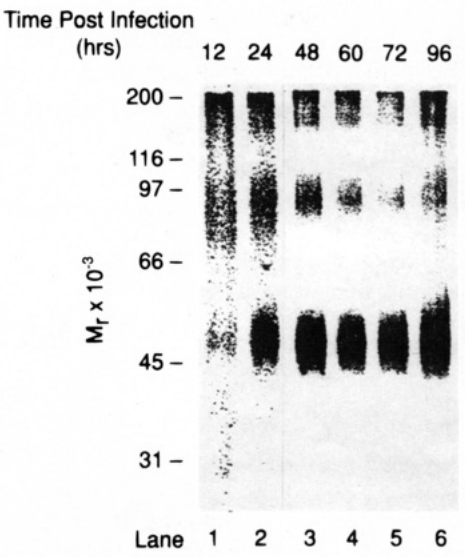


FIGURE 9: Effect of Endo-H, Endo-F_2, and PGNase F treatment on the migration of [^{125}I]GRP cross-linked proteins in cell membranes of mGRP-R-transfected BALB 3T3 cells and in recombinant baculovirus infected Sf9 cells. [^{125}I]GRP was cross-linked with 1 mM DSS for 30 min at 25 °C to either mGRP-R-transfected BALB 3T3 cell membranes (lanes 1−5) or mGRP-R-expressing Sf9 cell membranes (lanes 6−10) in the presence (lanes 1 and 6) or absence of 1 μM non-radiolabeled GRP. Cross-linked membranes were incubated alone (lanes 2 and 7) or with 10 units/mL PNGase F (lanes 3 and 8), 5 units/mL Endo-H (lanes 4 and 9), or 60 munits/mL Endo-F_2 (lanes 5 and 10) for 18 h. After digestion was stopped by adding 4% SDS, samples were subjected to SDS−PAGE and analyzed by autoradiography. Each of these results is representative of at least two others. The positions of molecular mass standards are shown on the left side of the figure.

oligosaccharide chain instead of four as is seen with the native mGRP receptor.

To test the possibility that there is also a difference in the nature of the oligosaccharide chains between mGRP-R expressed in Sf9 cells and mGRP-R in transfected BALB 3T3 cells, digestion of the cross-linked membranes by various glycosidases was performed (Figure 9). The mGRP-R expressed in transfected BALB 3T3 cells was resistant to digestion by Endo-H and Endo-F_2, indicating that it contains only tri- and/or tetraantennary complex type N-linked oligosaccharide chains (Figure 9, lanes 4 and 5). In contrast, the mGRP-R expressed in baculovirus-infected Sf9 cells was deglycosylated by Endo-F_2 (Figure 9, lane 9) to a similar extent as it was deglycosylated by PNGase F (Figure 9, lane 8), although it was not altered by Endo-H digestion (Figure 9, lane 10). These observations indicate that the N-linked oligosaccharide chains in the mGRP-R expressed in Sf9 cells consist of a biantennary complex type. Control experiments demonstrated that the lack of effect with Endo-H was not due to inactive enzyme because Endo-H decreased the molecular weight of ovalbumin (from 45 to 43 kDa) and of RNase B (from 19.0 to 16.7 kDa) in parallel incubations (data not shown).

To determine whether there was a change in the amount of glycosylation with varying infection times, cross-linking studies were performed after different infection times (Figure 10). With increasing time of infection, there was a progressive decrease in the proportion of the radioactivity in the high molecular weight band of 97 kDa (Figure 10).



Lane	Time (hrs)	Ratio of Radioactivity 97 kDa band/51 kDa band
1	12	1.99
2	24	0.41
3	48	0.27
4	60	0.25
5	72	0.19
6	96	0.12

FIGURE 10: Effect of infection time with recombinant baculovirus on the migration of [^{125}I]GRP cross-linked proteins in Sf9 cell membranes. Sf9 cells were infected with recombinant baculovirus carrying mGRP-R cDNA for the indicated times. Membranes were prepared and were incubated with 0.5 nM [^{125}I]GRP at 25 °C for 15 min. After washing, bound ligand was cross-linked by a 30-min incubation with 1 mM DSS at 25 °C. Samples were subjected to SDS−PAGE and analyzed by autoradiography. The top panel shows an autoradiogram which is representative of two others. The positions of molecular mass standards are shown on the left side of each panel. The bottom panel analyzes the ratio of radioactivity associated with the 97-kDa band to that associated with the main 51-kDa band. Values were calculated from the gel shown in the top panel.

To provide additional evidence that the expressed protein in Sf9 cells is mGRP-R, immunoblotting of the mGRP-R expressed in mGRP-R-transfected BALB 3T3 cells and recombinant baculovirus transfected cells was performed (Figure 11) using a polyclonal antibody raised against a portion of the third intracellular loop of the mGRP-R. With mGRP-R-transfected BALB 3T3 cells, a broad band of approximately 85 kDa was labeled without PNGase F treatment (Figure 11, lane 5). With PNGase treatment a single relatively sharp band of 43 kDa was seen (Figure 11, lane 4), suggesting that the difference in molecular weight of the two bands seen in lane 5 represents differences in glycosylation. These results were similar to those obtained in the cross-linking studies (compare to Figure 7). With Sf9 cells infected for 48 h by recombinant baculovirus, two separate bands were labeled without PNGase F treatment (Figure 11, lane 2). One was a broad band of approximately 50 kDa and was similar to the main band seen in the affinity-labeling study (compare to Figure 7). The other was a relatively sharp band of 43 kDa. After PNGase F treatment, only a 43-kDa band was labeled (Figure 11, lane 1). These data suggest that there was a detectable amount of ungly-

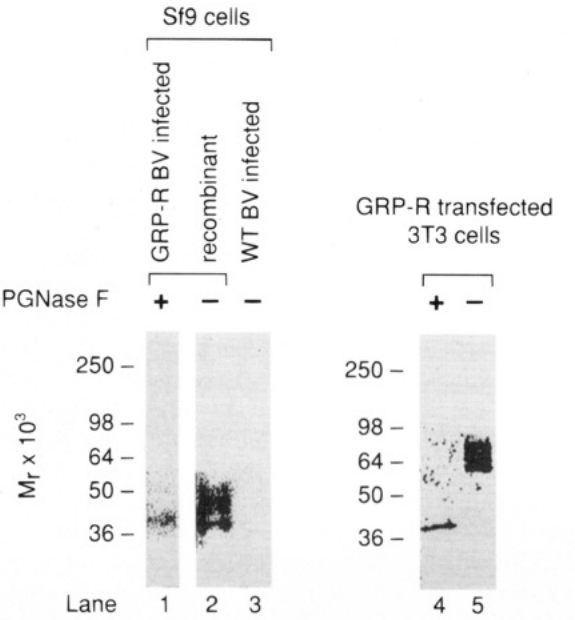


FIGURE 11: Immunoblotting of the mGRP-R expressed in transfected BALB 3T3 cells and recombinant baculovirus infected Sf9 cells. Membrane preparations (50 μg of protein/lane) were separated by SDS−PAGE. Samples applied to lanes 1 and 4 were pretreated with 10 units/mL PNGase F at 37 °C for 3 h as described in Materials and Methods. Separated proteins were electrotransferred to a nitrocellulose membrane. The blot was analyzed for mGRP-R using rabbit polyclonal antibody against synthetic peptide corresponding to a portion of the third intracellular loop of mGRP-R, and immunoreactivity was determined with [^{125}I]protein A and autoradiography as described in Materials and Methods. The positions of molecular mass standards are shown on the left side of each panel. Each of these results is representative of two others.

cosylated mGRP-R in Sf9 cells at 48 h post infection. With both cell types no bands corresponding to the faint bands seen in the cross-linking study, which were 45−50 kDa larger than the main bands, were seen. With Sf9 cells infected for 48 h with wild-type AcNPV, no bands were labeled (Figure 11, lane 3).

To attempt to assess whether a G protein might be being cross-linked with the GRP-R and solubilized and could account for the 97-kDa cross-linked band, a Western blot was performed with anti-α_q rabbit antiserum (which also interacts with α_{11}) (data not shown). Sf9 cells infected with the recombinant baculovirus containing mGRP-R or with a m$G\alpha_q$ cDNA were compared. After incubation with or without 0.5 nM GRP at 25 °C for 15 min, cross-linking with 1 mM DSS as described above, and transfer to nitrocellulose membranes after SDS−PAGE, the presence of α_q was detected using anti-α_q antibody (rabbit), and specific immunoreactive bands were visualized using enhanced chemiluminescence. Autoradiography revealed a single band of approximately 43 kDa in both GRP-R- and α_q-infected Sf9 cell membranes. There were no additional higher molecular weight bands seen in the GRP-R-transfected Sf9 cells (data not shown).

DISCUSSION

The purposes of the present study were to express the mGRP receptor (mGRP-R) using a baculovirus system to determine whether increased amounts of receptor could be obtained and to functionally and structurally characterize this receptor. Increased mGRP-R quantities would facilitate the ability to purify the mGRP-R for use in various studies of receptor function. As has been extensively done with adrenergic and muscarinic receptors (Kleymann et al., 1993; Ravet et al., 1993; Reilander et al., 1991; Richardson & Hosey, 1992; Parker et al., 1991), the purified receptor could then be used in reconstitution studies, for receptor antibody formation, and for investigations of G protein-coupling studies and receptor modulation by second messenger-independent and -dependent kinases. Furthermore, the increased expression of the mGRP-R in the baculovirus might provide a useful system to investigate receptor phosphorylation and modulation of function in these cells as has been done with muscarinic cholinergic receptors (Richardson & Hosey, 1992). To accomplish these future aims, it is necessary to structurally characterize the mGRP-R expressed by the baculovirus system, because recent studies demonstrate that the extent of glycosylation affects mGRP-R affinity and G protein coupling (Kusui et al., 1994), and the baculovirus system is known to usually not fully glycosylate G protein receptors (Luckow, 1990; Reilander et al., 1991; Kleymann et al., 1993; Parker et al., 1991; Davidson & Castellino, 1991). Furthermore, to accomplish studies of receptor modulation in the future, it is important to establish whether the mGRP-R in Sf9 cells is appropriately coupled to relevant effectors.

Several results demonstrate that we have successfully expressed the mGRP-R in Sf9 cells in increased amounts compared to normal tissues. First, prior to infection of the Sf9 cells with the mGRP-R baculovirus construct, no binding with mGRP-R ligands was seen, nor was any mGRP-R demonstrated by Western analysis using an mGRP-R antibody. Second, infection of Sf9 cells with baculovirus containing a gene distinct from the mGRP-R (a phosphodiesterase) did not demonstrate binding with mGRP-R ligands or demonstrate mGRP-R by Western analysis using an mGRP-R antibody. Third, after infection with the mGRP-R-containing baculovirus, the mGRP-R binding appeared in a time-dependent fashion, with the expressed mGRP-R demonstrating a high affinity for GRP and bombesin and a lower affinity for NMB with infection times $\geq$48 h. This pharmacology is therefore similar to that of the native receptor. Fourth, although the molecular weight of the expressed mGRP-R on Sf9 cells was lower than the mGRP-R in mouse pancreatic acini, Swiss 3T3 cells, or mGRP-R stably transfected BALB 3T3 cells, the size of the deglycosylated receptor was the same in each case and close to that predicted from sequence data. Fifth, the expressed receptor was detected immunologically by an antibody which recognizes the mGRP-R, and the molecular weight before and after deglycosylation was the same as that obtained from [^{125}I]GRP cross-linking studies. The mGRP-R was expressed in increased amounts compared to normal tissues with a maximal receptor capacity of 6 pmol/mg of protein (300 000 receptors/Sf9 cell). This is 4-fold higher than mouse pancreatic acinar membranes, which have a GRP receptor density of 1.5 pmol/mg of protein, 5-fold higher than rat pancreatic acini (Jensen, 1994; Sekar et al., 1991), 8-fold higher than human pancreatic acinar cell membranes (Scemama et al., 1986), and 60 times higher than GRP-R numbers in human small cell lung cancer cells (Moody et al., 1985). The mGRP-R number on Sf9 cells of 6 pmol/mg of protein, or 300 000 receptors/cell, is comparable to the value of 1−20 pmol/mg of protein reported with a number of other G

protein-linked receptors such as those for the β-adrenergic (Reilander et al., 1991; Kleymann et al., 1993; Ravet et al., 1993), M_2 muscarinic cholinergic (Richardson & Hosey, 1992), and human neurokinin type 2 (Aharony et al., 1993) receptors; but this number is considerably less than that seen with the nuclear receptor superfamily of ligand-inducible transcriptions factors (steroid, vitamin D) where levels of >100 pmol/mg of protein can be obtained (Janne et al., 1993; Collingwood et al., 1991; Ross et al., 1991; MacDonald et al., 1991)

Recent studies have demonstrated that the mGRP-R in native tissues or expressed in BALB 3T3 cells is heavily glycosylated (Kusui et al., 1994; Sinnett-Smith et al., 1988; Brown et al., 1988) and that this glycosylation is important in determining high-affinity binding and G protein interaction (Kusui et al., 1994). Glycosidase digestions show that the native mGRP-R glycosylation is entirely N-linked and that all four potential extracellular N-linked glycosylation sites are each approximately equally glycosylated with only tri- and/or tetraantennary complex oligosaccharide chains (Kusui et al., 1994). These results are confirmed in the present study because analysis of the radiolabeled proteins during serial deglycosylation of the mGRP-R stably expressed in BALB 3T3 cells was consistent with this receptor possessing four N-linked glycosylations. In contrast, the mGRP-R on baculovirus-infected Sf9 cells had a M_r of 51 kDa either by cross-linking or by immunoblotting instead of the 82 kDa seen with the native receptor. Because the fully deglycosylated mGRP-R has the same molecular mass when expressed natively or by Sf9 cells (i.e., 43 kDa), the difference in the molecular weights of mGRP-R between native and Sf9 cells is unlikely to be due to degradation or different processing of the polypeptide core and can be attributed to the extent of glycosylation. Few studies have investigated in detail the glycosylation of G protein-coupled receptors for peptides or hormones expressed by baculovirus in insect cells. However, extensive studies on β-adrenergic and muscarinic receptors and various proteins (Reilander et al., 1991; Kleymann et al., 1993; Parker et al., 1991) expressed in insect cells using baculovirus demonstrate that the glycosylation in insect cells usually does not progress beyond the high-mannose type. However, mixtures of the biantennary complex class also have been occasionally reported (Davidson & Castellino, 1991). To determine the basis for the decreased extent of mGRP-R glycosylation expressed in Sf9 cells, both a serial partial deglycosylation study with PNGase F and studies using different selective glycosidases were performed. Such experiments permitted us to compare the mGRP-R expressed in Sf9 cells with the mGRP-R expressed by stably transfected BALB 3T3 cells, which has already been shown to be identical to the native receptor (Kusui et al., 1994). The mGRP-R expressed on transfected BALB 3T3 cells had three partially deglycosylated bands of 72, 62, and 52 kDa with serial deglycosylation with PNGase F. Since PNGase F removes oligosaccharide chains *en bloc*, these data suggest that the native mGRP-R has four oligosaccharide chains of approximately uniform size (i.e., ≈10 kDa). With the mGRP-R on Sf9 cells, in addition to a 51-kDa and a 43-kDa deglycosylated band, at least one intermediate molecular mass band was seen. This result could possibly be due either to almost normal glycosylation (i.e., 8 kDa) of one of the four mGRP-R glycosylation sites or to partial glycosylation of a number of sites. Recent cross-linking studies in BALB 3T3 cells transfected with mGRP-R in which the glycosylation consensus sequences are selectively mutated suggests that the serial deglycosylation pattern seen in Sf9 cells cannot be explained by only one potential glycosylation site possessing full glycosylation and the other three possessing no glycosylation. Specifically, in a similar serial deglycosylation study (unpublished data) of a mutant mGRP-R possessing but one extracellular glycosylation consensus sequence, serial deglycosylation resulted in only two peaks, the glycosylated and unglycosylated mGRP-Rs, with no intermediate peaks being observed. These data suggest that the mGRP-R expressed in Sf9 cells has at least two N-linked glycosylated sites. We are unable to establish whether all four possible mGRP-R N-linked glycosylation sites are glycosylated when expressed by Sf9 cells, as they are when expressed by native tissues (Kusui et al., 1994), because the small differences that would be predicted to exist between each partially deglycosylated species cannot be resolved clearly on the gel. The studies with the different glycosidases demonstrate that the nature of the glycosylation differs between the native mGRP-R receptor and that expressed by Sf9 cells. Similar to that reported recently (Kusui et al., 1994) with native mGRP-R or when mGRP-R is stably transfected into BALB 3T3 cells, Endo-H, which cleaves N-linked high-mannose and hybrid type oligosaccharides (Trimble & Tarentino, 1991), and Endo-F_2, which cleaves N-linked high-mannose hybrid and biantennary complex type oligosaccharides with a preference for biantennary structures (Trimble & Tarentino, 1991), had no effect. However, in the Sf9 cells the mGRP-R was deglycosylated by Endo-F_2 to the same extent as PNGase F, whereas Endo-H had no effect. These observations indicate that in Sf9 cells mGRP-R glycosylation is of a biantennery complex type instead of the tri- and tetraantennary complex type seen in the native receptor. Furthermore, these data demonstrate that glycosylation of the mGRP-R in Sf9 cells differs from that reported with β-adrenergic receptors in this system, which is reported to primarily have only high-mannose-type oligosaccharides (Reilander et al., 1991). Receptor glycosylation has been shown to be important for maintaining receptor stability, intracellular trafficking, proper cell surface expression, and folding (Petaja-Repo et al., 1991; Rademacher et al., 1988; Russo et al., 1991; Rands et al., 1990). The fact that the 51-kDa mGRP-R in Sf9 cells, which has only 20% of the glycosylation of the native mGRP-R, was expressed on the cell surface demonstrates that full glycosylation is not needed for mGRP-R expression. Furthermore, glycosylation of the mGRP-R does not have to be of the tri- and tetraantennary type for cell surface expression. The fact that in the Sf9 cells an unglycosylated band of 43-kDa was seen by immunoblotting, whereas no cross-linked receptor was seen at this molecular mass, supports the conclusion that some glycosylation is necessary for cell surface expression and/or folding of the GRP-R that allows the receptor to interact with high affinity with ligands.

G protein coupling of G protein-coupled receptors transfected by baculovirus into insect cells, and the ability of agonists to activate these receptors and other second messenger pathways, has been extensively studied with a number of receptors coupled to adenylate cyclase (Reilander et al., 1991; Kleymann et al., 1993; Ravet et al., 1993; Richardson & Hosey, 1992; Parker et al., 1991). However, much less

is known about the behavior of receptors coupled to phospholipase C in this expression system. M_5 muscarinic cholinergic, human thrombin, and bradykinin receptors (Hu et al., 1994), when expressed in Sf9 cells, couple to phospholipase C. With the M_5 muscarinic cholinergic receptor, the changes in cystolic calcium induced by agonists in these cells were similar to those seen in mammalian cells (Hu et al., 1994). Similarly, human neurokinin-2 receptors, which are coupled to phospholipase C, when expressed in Sf9 cells using baculovirus, remained coupled to G proteins in a similar manner as seen with natively expressed receptor (Aharony et al., 1993). A number of results in our study demonstrate that the transfected mGRP-R is coupled both to G proteins and to the phospholipase C-activated second messenger cascade, but that the efficiency of this coupling varies with baculovirus infection time. First, the nonhydrolyzable guanine nucleotide, GPP(NH)p, increased the dissociation of bound [^{125}I]GRP after 24 h of infection in a manner identical to that seen in mammalian cells. However, with prolonged infection times (i.e., 96 h), GPP(NH)p had no effect. Second, GRP receptor agonists stimulated increases in inositol phosphates and $[Ca^{2+}]_i$ in a temporal pattern similar to the coupling to G proteins. Specifically, with shorter baculovirus infection times, agonist-stimulated dose−response curves for changes in IP or $[Ca^{2+}]_i$ were similar to those seen with GRP-R in mammalian cells, and with longer baculovirus infection times minimal agonist-stimulated increases in either IP or $[Ca^{2+}]_i$ were seen. Therefore, with increasing baculovirus infection time mGRP-R becomes progressively uncoupled, probably secondary to the ability of the powerful polyhedrin promoter in the baculovirus construct to almost totally overwhelm the synthesis of other proteins. Third, the cross-linking studies could be interpreted as providing indirect evidence that the mGRP-R in Sf9 cells is coupled to G proteins and becomes progressively uncoupled with infection time. In mGRP-R-transfected BALB 3T3 cells, in mouse pancreatic acini containing native mGRP-R, and in mGRP-R baculovirus infected Sf9 cells, cross-linking using [^{125}I]GRP results in a faint protein band approximately 45 kDa greater than that of the principal cross-linked receptor protein. With deglycosylation both cross-linked proteins decrease a similar amount, suggesting both are equally glycosylated. With the GRP-R baculovirus infected Sf9 cells the principal protein band at short infection times is the higher molecular weight band (i.e., 97 kDa) and with increasing time of baculovirus infection it almost disappears, paralleling a time course similar to that seen with the loss of GPP(NH)p responsiveness and the ability of agonists to activate phospholipase C. This similarity in time courses suggests that the higher molecular mass protein could represent [^{125}I]GRP cross-linked to the mGRP-R, which in turn is cross-linked to a G protein which is lost at longer infection times because coupling is lost. A similar finding was reported previously in cross-linking studies of the rat GRP receptor with radiolabeled ligand in pancreatic acini (Lewin et al., 1990) and attributed to solubilization of a GRP-R−G protein complex which had also been cross-linked. In at least one study (Offermanns et al., 1994) mGRP-R has been shown in Swiss 3T3 cells to be coupled to Gq and G_{11}, whereas in another study it was also coupled to G_i and small GTP binding proteins (Profrock et al., 1992). In the present study Sf9 cells were found to possess an α_q- or α_{11}-like protein. However, on a Western blot no α_q or α_{11} could be detected in the position of the 97-kDa cross-linked band. This could be due to the fact that this band does not represent GRP-R cross-linked to the α_q or α_{11}, that the assay is insufficiently sensitive to detect the amount of α_q or α_{11} present, or that the GRP-R is cross-linked to some other Gq subunit or $\beta\gamma$ subunit or another protein. At present, without the ability to efficiently immunoprecipitate the GRP-R, these differences cannot be resolved.

Recent studies report that, with short-term incubations with agonists, the mGRP-R undergoes phosphorylation (Kroog et al., 1995) as well as desensitization (Walsh et al., 1993). With longer incubations (i.e., 24 h) the mGRP-R also undergoes desensitization and downregulation (Benya et al., 1994b). Recent studies suggest that phospholipase C activation is needed to mediate the downregulation and desensitization seen with these longer incubations (Benya et al., 1994a). These results suggest that in the future, if studies of mGRP-R phosphorylation or mGRP-R modulation are performed using mGRP-R-transfected Sf9 cells similar to those performed with muscarinic cholinergic receptors (Richardson & Hosey, 1992), it will be important to use Sf9 cells infected for shorter time periods with mGRP-R baculovirus to ensure full G protein coupling.

A recent study (Kusui et al., 1994) demonstrates that the extent of mGRP-R glycosylation affects the G protein coupling and receptor affinity. In that study (Kusui et al., 1994), when the mGRP-R transfected into BALB 3T3 cells was 80% deglycosylated by PNGase F (molecular mass decreased from 82 to 51 kDa), the mGRP-R receptor affinity for agonists was decreased and G protein coupling was lost, whereas with the closely related neuromedin B receptor treated in an identical fashion, no change in affinity or coupling was seen. The mGRP-R expressed in Sf9 cells was only partially glycosylated, similar to that reported for other G protein-coupled receptors expressed in these cells using baculovirus (Reilander et al., 1991; Kleymann et al., 1993; Ravet et al., 1993; Quehenberger et al., 1992; Parker et al., 1991). Specifically, the mGRP-R in Sf9 cells was glycosylated to only 20% of the extent of the wild type (i.e., molecular mass, 51 kDa). The fact that G protein coupling was seen with the mGRP-R expressed in Sf9 cells despite having its the same percentage glycosylation as the uncoupled wild type mGRP-R that was 80% deglycosylated with PNGase F treatment, supports the conclusion that the differences in either the distribution or the nature of the glycosylation account for these differences and further supports the importance of glycosylation in determining full G protein coupling with the GRP receptor.

A recent study of the GRP-R in rat pancreatic acini (Sekar et al., 1991) proposes that even though the agonists bombesin, GRP, and NMB all interact with only the GRP receptor in this tissue, they differ in their ability to couple the receptor to various G proteins. Our data with changes in receptor affinity with various baculovirus infection times are consistent with this hypothesis. With varying infection times of the mGRP-R-containing baculovirus there was a very different pattern of change in the affinity constants for the agonists bombesin, GRP, and NMB. Numerous previous studies demonstrate that the mGRP-R receptor affinity is regulated by coupling to G proteins (Mantey et al., 1993; Brown et al., 1988b; Sinnett-Smith et al., 1990; Benya et al., 1994b), and these data support the hypothesis (Sekar et al., 1991) that various full agonists at this receptor can

activate coupling to different G proteins. There are numerous examples of partial agonists for the GRP-R as well as other G protein-coupled receptors causing differential activation of various intracellular processes, presumably by differential coupling to different G proteins (Wang et al., 1990; Matozaki et al., 1990; Rowley et al., 1990). However, as far as we are aware, except for the study of Sekar et al. (Sekar et al., 1991), there are no studies that suggest differential G protein coupling of full agonists at the same receptor; therefore, whether this concept generally occurs with other G protein-coupled receptors is unclear. Although some glycosylation appears necessary for high-affinity receptor−ligand interaction (Kusui et al., 1994), it is unlikely that differing glycosylation accounts for this differential change in affinity for different agonists with time. Between 24 and 96 h post infection with mGRP-R baculovirus, there was a marked change in the affinity for different agonists (Figure 3); however, the molecular mass of the principal glycosylated protein band seen on cross-linking studies remained unchanged (Figure 10), suggesting that the extent of glycosylation did not account for this change. Preliminary experiments with Endo H, Endo-F_2, and PNGase F also suggested no time-dependent change in the nature of the glycosylation with infection time.

In conclusion, in the present study we have successfully expressed mGRP-R in Sf9 cells using an mGRP-R baculovirus construct at higher numbers than occur naturally. Structural characterization of the expressed receptor demonstrates that it is only 20% as glycosylated as native mGRP-R and that the glycosylation is less complex, consisting of biantennary complex oligosaccharides rather than the tri- and tetraantennary oligosaccharides present in the native mGRP-R. At least two of the four glycosylation sites in mGRP-R are glycosylated in Sf9 cells, demonstrating that neither full glycosylation nor complex tri- and tetraantennary oligosaccharide chains are needed for cell surface expression or G protein coupling of mGRP-R. Functionally, the mGRP-R in Sf9 cells is coupled to G proteins and activates phospholipase C; however, the stoichiometric relationships with various agonists differ with differing baculovirus infection times, suggesting differential coupling of the mGRP-R to G proteins with different agonists. These results demonstrate that the baculovirus system will be useful for expressing large amounts of mGRP-R to use for antibody studies and reconstitution studies. Furthermore, the full functional coupling with short baculovirus infection times should allow this system to be used to explore mechanisms of mGRP-R modulation, as has been previously done with muscarinic cholinergic receptors (Richardson & Hosey, 1992) expressed in this system.

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