Glycosylation of Bombesin Receptors: Characterization, Effect on Binding, and G-Protein Coupling

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ABSTRACT: In the present study, we investigated the nature and the importance of glycosylation of two mammalian bombesin receptors, the neuromedin B receptor (NMB-R) and the gastrin-releasing peptide receptor (GRP-R), using chemical cross-linking and enzymatic deglycosylation. [^{125}I]-(D-Tyr^0)NMB cross-linked to native NMB-R on rat C-6 glioblastoma cells or rat NMB-R transfected into BALB 3T3 cells revealed a single broad band, M_r = 63 000, on both cell types that was not altered by DTT. NMB inhibited cross-linking specifically and saturably with an IC_{50} of 4.8 and 6.1 nM for C-6 and NMB-R transfected cells, respectively, and there was a close correlation between its ability to inhibit binding and its ability to inhibit cross-linking. A single broad band of M_r = 82 000 was cross-linked with [^{125}I]GRP on mouse GRP-R transfected BALB 3T3 cells. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) digestion increased the mobility of the original band in C-6, NMB-R, and GRP-R transfected cell membranes. Endoglycosidase H (Endo-H) and endoglycosidase F_2 (Endo-F_2) digestion had no effect on both transfected cells. Neuraminidase digestion slightly increased the mobility of the original band in NMB-R transfected cell membranes; however, it had no effect on GRP-R transfected cell membranes. Endo-α-*N*-acetylglucosaminidase (*O*-glycanase) digestion subsequent to neuraminidase treatment showed no additional effect on either receptor. Serial partial deglycosylation of cross-linked NMB-Rs with PNGase F treatment for different incubation periods revealed one band of partially glycosylated receptor (53 kDa) besides the fully glycosylated and fully deglycosylated ones, showing that NMB-R has two oligosaccharide chains. Similarly, three partially deglycosylated species (72, 62, and 52 kDa) are seen with the GRP-R, indicating that the GRP-R has four oligosaccharide chains. Treatment of unlabeled membranes with PNGase F followed by affinity labeling resulted in fully deglycosylated NMB-R or 75% deglycosylated GRP-R. Deglycosylation of the NMB-R did not alter its affinity for NMB or alter G-protein coupling; however, 75% deglycosylation of the GRP-R both decreased its affinity for GRP and altered its ability to couple to G-proteins. The present results demonstrate that NMB-R on native and transfected cells is an N-linked sialoglycoprotein with two triantenary and/or tetraantenary complex oligosaccharide chains. The apparent M_r of this sialoglycoprotein is 63 000, and this protein does not contain disulfide-linked subunits or O-linked carbohydrates. GRP-R is a similar N-linked glycoprotein of an apparent M_r = 82 000, with four triantenary and/or tetraantenary complex oligosaccharide chains containing no sialic acid. In NMB-R, glycosylation is not required for full affinity or G-protein coupling, whereas in GRP-R it plays a significant role in maintaining full high affinity and G-protein coupling.

Bombesin-related peptides have a wide spectrum of diverse biological activities, including marked effects in the central nervous system (CNS), such as regulation of body temperature (Brown et al., 1988b), circadian rhythm (Albers et al., 1991), and thyrotropin release (Rettori et al., 1992), and in peripheral tissues, such as stimulation of the release of numerous gastrointestinal peptides (Ghatei et al., 1982; Kaneto et al., 1978), stimulation of pancreatic secretion (Jensen et al., 1988; Tache et al., 1988), smooth muscle contraction (Falconieri Erspamer et al., 1988), satiety (McCoy & Avery, 1990), and stimulation of chemotaxis (Ruff et al., 1985). Bombesin-related peptides also have potent growth effects in normal and tumorigenic tissues (Rozengurt, 1988; Bologna et al., 1989), and it has been shown that these peptides can function as autocrine growth factors in human small cell lung cancer cells (Cuttitta et al., 1985).

Two mammalian peptides, gastrin-releasing peptide (GRP) and neuromedin B (NMB), have been described and are found widely in both the CNS and peripheral tissues (Minamino et al., 1983; Spindel, 1986). Recent functional, binding, and cloning studies have now established that at least two receptors mediate the action of these different peptides, the GRP and the NMB receptors (von Schrenck et al., 1989, 1990; Ladenheim et al., 1990; Jensen & Coy, 1991; Battey et al., 1991; Spindel et al., 1990; Wada et al., 1991; Wang et al., 1992; Rozengurt, 1988). These receptors are closely related, as they share a 56% amino acid sequence homology and both activate phospholipase C; however, they differ in their pharmacology, distribution, and involvement in altering biological activities of different tissues.

For mammalian bombesin receptors, similar to receptors for most other gastrointestinal hormones, little is known about their receptor carbohydrate structure or its importance. In this regard, they differ from receptors for classical hormones, such as luteinizing hormone (LH) or thyroid-stimulating

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hormone (TSH) (Zhang et al., 1991; Liu et al., 1993; Petaja-Repo et al., 1991, 1993; Ji et al., 1990; Russo et al., 1991), or receptors for classical neurotransmitters, such as those for adrenergic agents (Rands et al., 1990) or cholinergic agents (Gehle & Sumikawa, 1991; Giovannelli et al., 1991; Ohara et al., 1990), which have been studied extensively. Only limited data are available from cross-linking or lectin binding studies for the GRP-R, which demonstrate that it is a glycoprotein that has a variable extent of N-glycosylation in different tissues (Feldman et al., 1990; Brown et al., 1988a; Sinnett-Smith et al., 1988; Huang et al., 1990; Kris et al., 1987), while no data are available for the NMB receptor. For both bombesin receptor subtypes, the nature of the glycosylation is unknown, as is whether all extracellular potential glycosylation sites are glycosylated or whether glycosylation is involved in mediating high-affinity binding or G-protein coupling. Recent studies demonstrate that receptor glycosylation is important in protein folding, maintaining receptor stability, and trafficking and targeting of receptors to cell membranes (Petaja-Repo et al., 1991, 1993; Russo et al., 1991; Kuwano et al., 1991; Giovannelli et al., 1991; Rands et al., 1990; West, 1986; Elbein, 1987; Rademacher et al., 1988). When receptors for the classical hormones such as LH/HCG and TSH, or receptors for neurotransmitters such as adrenergic agents or muscarinic cholinergic agents are examined, glycosylation has no effect on high-affinity receptor binding (Liu et al., 1993; Russo et al., 1991; Rands et al., 1990; Ohara et al., 1990). However, in recent studies glycosylation has been shown to be important in maintaining high-affinity binding for a number of peptidergic receptors, such as those for somatostatin (Rens-Domiano & Reisine, 1991), cholecystokinin (Santer et al., 1990), and VIP (Chochola et al., 1993), as well as receptors for PGE_2, PGD_2 (Morii & Watanabe, 1992), insulin (Bastian et al., 1993), EGF (Soderquist & Carpenter, 1984), and low-density lipoproteins (Kuwano et al., 1991). Furthermore, in recent studies receptor glycosylation has been shown to be important in maintaining receptor–signal transduction pathway coupling for the insulin receptor (Leconte et al., 1992) and β_2-adrenergic receptor (Rands et al., 1990).

Recently, we established that BALB-3T3 fibroblasts, which do not contain receptors for bombesin-related peptides when stably transfected with the coding sequence for the NMB receptor, function in a pharmacologically identical fashion to the cells natively expressing the receptor (Benya et al., 1992). In this study, we characterize the native NMB-R on rat C-6 glioblastoma cells and rat NMB-R stably transfected into BALB 3T3 cells. We demonstrate that the receptors on both cell types are structurally indistinguishable, but that the NMB-R differs from the GRP-R in the extent of glycosylation, the nature of the glycosylation, the availability of reactive amino and sulfhydryl groups near the binding pockets, and the importance of glycosylation in maintaining G-protein coupling. Deglycosylation did not alter receptor affinity or G-protein coupling of NMB-R; however, deglycosylation decreased GRP-R affinity and altered GRP-R G-protein coupling.

MATERIALS AND METHODS

Materials

Neuromedin B (NMB), porcine gastrin-releasing peptide (GRP), (Tyr^4)bombesin, cholecystokinin octapeptide (CCK-8), vasoactive intestinal peptide (VIP), and substance P were obtained from Peninsula Laboratories (Belmont, CA); phosphate-buffered saline (PBS) (pH 7.4) was from Biofluids (Rockville, MD); Dulbecco's modified essential medium (DMBM), fetal bovine serum, and the aminoglycoside G-418 were from Gibco (Waltham, MA); tris, glycine, and Coomassie Blue R-250 were from Schwarz/Mann Biotech (Cleveland, OH); ethylenediaminetetraacetic acid (EDTA), bacitracin, soybean trypsin inhibitor (SBTI), dithiothreitol (DTT), dimethyl sulfoxide (DMSO), and cacodylic acid were from Sigma (St. Louis, MO); phenylmethanesulfonyl fluoride (PMSF) and Nonidet P-40 (NP-40) from were Fluka (Ronkonkoma, NY); bovine serum albumin (BSA) fraction V was from Miles Inc. (Kankakee, IL); aprotinin and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) were from Boehringer Mannheim Biochemicals (Indianapolis, IN); *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (MBS), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS^3), ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES), dithiobis(succinimidyl propionate) (DTSP), 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, $NaH_2PO_4 \cdot H_2O$, and $Na_2HPO_4 \cdot 7H_2O$ were from Mallinckrodt Inc. (Paris, KY); Protogel (30% acrylamide and 0.8% bisacrylamide stock solution) was from National Diagnostics (Manville, NJ); peptide-N^4(*N*-acetyl-β-glucosaminyl) asparagine amidase (PNGase F), neuraminidase, endo-α-*N*-acetylgalactosaminidase (*O*-glycanase), endoglycosidase H (Endo-H), and endo-β-*N*-acetylglucosaminidase F_2 (Endo-F_2) were from Genzyme (Cambridge, MA); and $Na^{125}I$ was from Amersham (Arlington Heights, IL). Rat glioblastoma C-6 cells were obtained from the American Type Culture Collection (Rockville, MD).

Methods

Transfection and Maintenance of Cell Lines. As described previously (Benya et al., 1992), BALB 3T3 cells expressing a stably transfected rat NMB receptor were obtained using calcium phosphate precipitation of a full length NMB-preferring bombesin receptor clone generated from rat esophagus and subcloned into a modified version of the pCD_2 plasmid (Davis et al., (1986). BALB 3T3 cells expressing a stably transfected murine GRP receptor were produced similarly using a GRP receptor clone generated from Swiss 3T3 cells (Benya et al., 1993). Cells were maintained in DMEM containing 10% fetal bovine serum plus 300 μg/mL G-418 and passaged every 3–4 days at confluence, using 0.1% trypsin in 1 mM EDTA. Rat glioblastoma C-6 tumor cells were maintained in DMEM containing 10% fetal bovine serum and were passaged weekly at confluence. All cell lines were cultured at 37 °C in a 5% CO_2 atmosphere.

Preparation of Peptides. (D-Tyr^0)NMB, *N*-acetyl-GRP-(20–26)ethyl ester, and cyclo-SS-octa(D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2) were synthesized using solid phase methods, as described previously (Sasaki & Coy, 1987; Wang et al., 1990; Orbuch et al., 1993).

Preparation of [^{125}I]-(D-Tyr^0)NMB, [^{125}I]GRP, and [^{125}I]-(Tyr^4)Bombesin. [^{125}I]-(D-Tyr^0)NMB, [^{125}I]GRP, and [^{125}I]-(Tyr^4)bombesin (2200 Ci/mmol) were prepared using IODO-GEN as described previously (Wang et al., 1992; Benya et al., 1992; von Schrenck et al., 1989). 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 incubation for 6 min 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 column (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.

Preparation of Cell Membranes. Confluent cells were mechanically disaggregated, washed twice with PBS at 4 °C, and resuspended in 10 mL of homogenization buffer [20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.25 M sucrose, 1 mM EDTA, 1 mg/mL bacitracin, 0.1 mg/mL SBTI, and 1 mM PMSF] as described previously (Svoboda et al., 1988). 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 for 5 min at 500*g* at 4 °C. The supernatant was centrifuged at 30000*g* for 60 min at 4 °C. The pellet was resuspended in binding buffer [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5% (w/v) BSA, 0.5% (w/v) bacitracin, and 10 mg/mL aprotinin] at a 2.5 mg of protein/mL concentration and stored at −70 °C until use.

Binding of [^{125}I]-(D-Tyr^0)NMB and [^{125}I]GRP to Membranes. Membranes were thawed rapidly and diluted with binding buffer to a protein concentration of 0.25–1 mg/mL. In a typical experiment, a 500 μL aliquot was incubated with 0.5 nM [^{125}I]-(D-Tyr^0)NMB, [^{125}I]GRP, or [^{125}I]-(Tyr^4)-bombesin in the presence or absence of various agents at 25 °C for 15 min. Preliminary experiments demonstrated that binding became maximal by 15 min. Duplicate 50 μL samples were layered over 300 μL of PBS (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 PBS (4 °C). The tops of the tubes were cut, and the radioactivity associated with the membranes was measured with a gamma 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 ligand and was >80% of total binding in all experiments. At the end of an experiment, 10 μL of the incubation mixture was taken for determination of the total radioactivity added.

Cross-Linking of [^{125}I]-(D-Tyr^0)NMB, [^{125}I]GRP, and [^{125}I]-(Tyr^4)Bombesin to Membranes. Five hundred microliters of membranes (0.25–2 mg of protein/mL) was incubated with 0.5 nM [^{125}I]-(D-Tyr^0)NMB, [^{125}I]GRP, or [^{125}I]-(Tyr^4)-bombesin as described earlier 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 PBS (4 °C) and resuspended in 200 μL of cross-linking buffer [50 mM HEPES (pH 7.5) and 5 mM $MgCl_2$] containing a cross-linking agent. Preliminary studies showed that MBS was the most effective of the agents tested for cross-linking of [^{125}I]-(D-Tyr^0)NMB to the transfected cell membrane. Unless otherwise indicated, the cross-linking agent for cross-linking of [^{125}I]-(D-Tyr^0)NMB was 1 mM MBS. For cross-linking of [^{125}I]GRP to GRP-R transfected cell membranes, DSS was used in most experiments unless stated otherwise. Preliminary studies comparing cross-linking for 15, 30, and 60 min at 4, 25, and 37 °C demonstrated that a 25 °C incubation for 30 min gave the best cross-linking of [^{125}I]-(D-Tyr^0)NMB and [^{125}I]GRP. After cross-linking for 30 min at 25 °C, the reaction was stopped by adding 25 μL of stopping solution (1 M glycine and 1 M 2-mercaptoethanol for MBS; 1 M glycine for the other cross-linkers), unless otherwise indicated. After 10 min at 4 °C, 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× concentrated) and incubated at 25 °C for 1 h. The gel loading buffer (5× 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.

Cross-Linking of [^{125}I]-(D-Tyr^0)NMB to Intact Cells. Cells harvested from one 175 cm^2 flask (approximately 5 × 10^7 C-6 cells or 2 × 10^7 NMB-R transfected cells) were washed twice, resuspended in 4 mL of HEPES-buffered saline [50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 125 mM $NaCl_2$], and incubated with 100 pM [^{125}I]-(D-Tyr^0)NMB at 25 °C. After a 10 min incubation, the reaction mixture was centrifuged at 10000*g* for 30 s. The pellet was washed twice with 1 mL of PBS (4 °C) and resuspended in 1 mL of HEPES-buffered saline containing 1 mM MBS. Cross-linking was performed at 4 °C for 120 min and stopped by adding 100 μL of stopping solution. After 10 min on ice the sample was centrifuged at 10000*g* for 30 s.

Analysis of Cross-Linked Membrane Proteins. Solubilized membranes were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After the protein concentration was adjusted, samples (60–100 μg of protein/lane for C-6 cells and 10–20 μg of protein/lane for NMB-R transfected cells and GRP-R transfected cells) were applied to the gel. In most cases, 1.5 mm thick 3% (w/v) acrylamide/0.1% (w/v) SDS stacking gel over a 10% single percentage or a 4–15% linear gradient (w/v) acrylamide/0.1% (w/v) SDS separating gel with the Laemmli buffer system (Laemmli, 1970) was used. In some experiments involving PNGase F digestion of receptor proteins, a 6% acrylamide gel with the Weber and Osborn buffer system (Weber & Osborn, 1969) was used to avoid the anomalous results seen with PNGase F-digested cross-linked NMB-R transfected cell membranes analyzed by SDS–PAGE using the Laemmli buffer system. The electrophoresis solution contained 25 mM Tris, 0.2 M glycine, and 0.1% (w/v) SDS for the Laemmli buffer system and 100 mM sodium phosphate buffer (pH 7.0) and 0.1% SDS for the Weber and Osborn buffer system. The electrophoresis was carried out at 40–50 mA per gel in both cases. 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 an 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. To obtain better autoradiograms faster, dried gels were exposed to a storage phosphor screen for 1–7 days at room temperature instead of using conventional X-ray films. 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 obtained using the PhosphorImager.

Enzyme Treatment of Cross-Linked Membrane Proteins. (1) PNGase F Treatment. Cross-linked membrane proteins

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 μg of protein) of the above was added 10 μL of 7.5% NP-40. The mixture was incubated in a volume of 60 μL with various concentrations of PNGase F (0, 10, 30, or 100 units/mL) for 18 h at 37 °C. The reaction was stopped by 4% SDS, and samples were subsequently analyzed by SDS–PAGE.

PNGase F treatment of non-cross-linked membranes was performed in nondenaturing conditions using a buffer containing 50 mM Tris-HCl (pH 7.7), 1 mM EDTA, and 1 mM PMSF.

(2) Endo-H Treatment. Cross-linked membrane proteins 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 a 0, 2, or 5 units/mL final enzyme concentration in a volume of 20 μL for 18 h at 37 °C. The reaction was stopped by 4% SDS, and samples were subsequently analyzed by SDS–PAGE.

(3) Endo-F_2 Treatment. Cross-linked membrane proteins 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 a 0, 20, or 60 munits/mL final enzyme concentration in a volume of 20 μL for 18 h at 37 °C. The reaction was stopped by 4% SDS, and samples were subsequently analyzed by SDS–PAGE.

(4) Neuraminidase Treatment. Pellets of cross-linked membrane proteins were resuspended in 80 μL of 50 mM sodium cacodylate (pH 6.0) containing 10 mM calcium acetate. Twenty-five microliter aliquots of samples (50 μg of protein) were incubated in a volume of 50 μL with various concentrations of neuraminidase (0, 1, or 5 units/mL). After 60 min at 37 °C, the membranes were again pelleted (10000*g*, 3 min), resuspended in 50 μL of 120 mM Tris-HCl (pH 6.8), solubilized in gel loading buffer, and analyzed by SDS–PAGE.

(5) O-Glycanase Treatment. Cross-linked membrane proteins were treated with 1 unit/mL neuraminidase as above. The pellet was then resuspended in 50 μL of 50 mM sodium cacodylate buffer (pH 6.0) with 1% (v/v) NP-40, 0.1% (w/v) SDS, and 1% (v/v) 2-mercaptoethanol. *O*-Glycanase (0, 100, or 400 munits/mL final concentration) was added to the suspension and incubated for 18 h at 37 °C. At the end of the incubation, the samples were solubilized by adding gel loading buffer and analyzed by SDS–PAGE.

(6) Protein Assay. Membrane protein concentration was determined using the Bio-Rad protein assay dye agent, using bovine serum albumin as a standard.

RESULTS

NMB caused half-maximal inhibition of binding at 3.7 ± 0.4 (mean ± SD) and 7.1 ± 1.8 nM for membranes from C-6 cells and those from NMB-R transfected BALB 3T3 cells (NMB-R transfected cells), respectively (Figure 1). GRP also inhibited the binding of [^{125}I]-(D-Tyr^0)NMB to both membranes, but was 200 times less potent than NMB (Figure 1). The dose–inhibition curves of NMB for the binding of [^{125}I]-(D-Tyr^0)NMB to membranes from both cell types were analyzed by a least-squares analysis curve-fitting program (LIGAND) (Munson & Rodbard, 1980). Membranes from C-6 cells and NMB-R transfected cells were best fit by a single high-affinity binding site model for NMB, and their K_d's were 2.2 ± 0.5 and 6.6 ± 1.2 nM, respectively. These values agree closely with those reported previously for binding

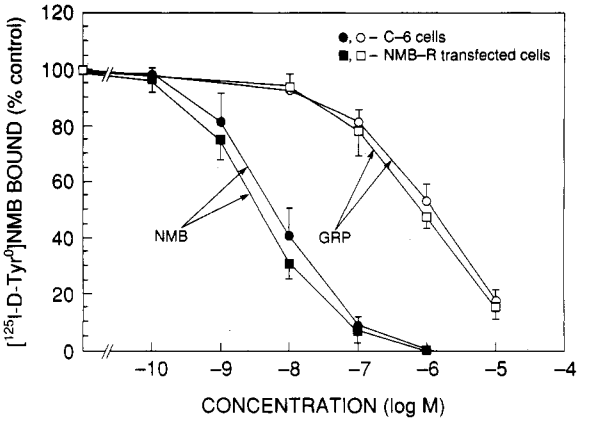

FIGURE 1: Comparison of the abilities of NMB and GRP to alter the binding of [^{125}I]-(D-Tyr^0)NMB to membranes from C-6 glioblastoma and NMB-R transfected BALB 3T3 cells. Membranes from rat C-6 glioblastoma cells (circles) or NMB-R transfected cells (squares) were incubated with 0.5 nM [^{125}I]-(D-Tyr^0)NMB alone or in the presence of the indicated concentration of NMB or GRP 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 and expressed as a percentage of the radioactivity saturably bound to the membrane with no additions. Each value was determined in duplicate, and results are means ± SEM from three separate experiments.

to intact cells (Benya et al., 1992), demonstrating that binding to cell membranes and to intact cells is similar. The binding capacity of NMB to C-6 cell membranes was 56 ± 8 fmol/mg of protein, and that for membranes from NMB-R transfected cells was 2200 ± 200 fmol/mg of protein.

When the initial incubation of both membranes from C-6 cells and those from NMB-R transfected cells (Figure 2, left panel, lane 6) with [^{125}I]-(D-Tyr^0)NMB was followed by incubation with the cross-linking agent, MBS, a single broad protein band of M_r = 63 000 was labeled with radioactivity. Addition of 1 μM NMB to the initial incubation (Figure 2, left panel, lane 5) prevented the cross-linking of NMB-R transfected and C-6 cells to both membranes. Addition of a reducing agent, DTT (100 mM), did not alter the pattern of cross-linking or the size of the single protein band, and without cross-linking agents, no cross-linking was seen (data not shown). The apparent bands of M_r > 200 000 seen in autoradiograms of 10% (w/v) acrylamide gels (Figure 2, left panel) represented an accumulation of precipitated, aggregated labeled receptor protein at the border of the stacking and separating gels because this was not seen in 4–15% gradient gels (data not shown). In preliminary studies with other cross-linkers (all at 1 mM), such as DSS, BSOCOES, BS_3, EGS, DTSP, and sulfo-EGS, only a single band of M_r = 63 000 was cross-linked on NMB-R transfected cell membranes, and in each case these cross-linkers were 10-fold less efficacious than MBS (data not shown).

In previous studies investigating the structure of the GRP receptor, which shares 56% homology with the NMB-R (Wada et al., 1991), the NH_2-reacting homofunctional cross-linking agent DSS was primarily used (Brown et al., 1988a; Huang et al., 1990; Kris et al., 1987). To compare the ability of DSS or MBS, a heterofunctional cross-linker reacting with free NH_2 and SH groups, to cross-link to GRP or NMB receptors, we compared the ability of each cross-linker to couple various GRP and NMB receptor ligands to NMB-R or GRP-R expressed on transfected cells. [^{125}I]GRP was cross-linked on GRP-R transfected cell membranes to a single broad band of M_r = 82 000 protein by either DSS or MBS (Figure 2, left panel, lanes 1–4), which is similar to that previously shown

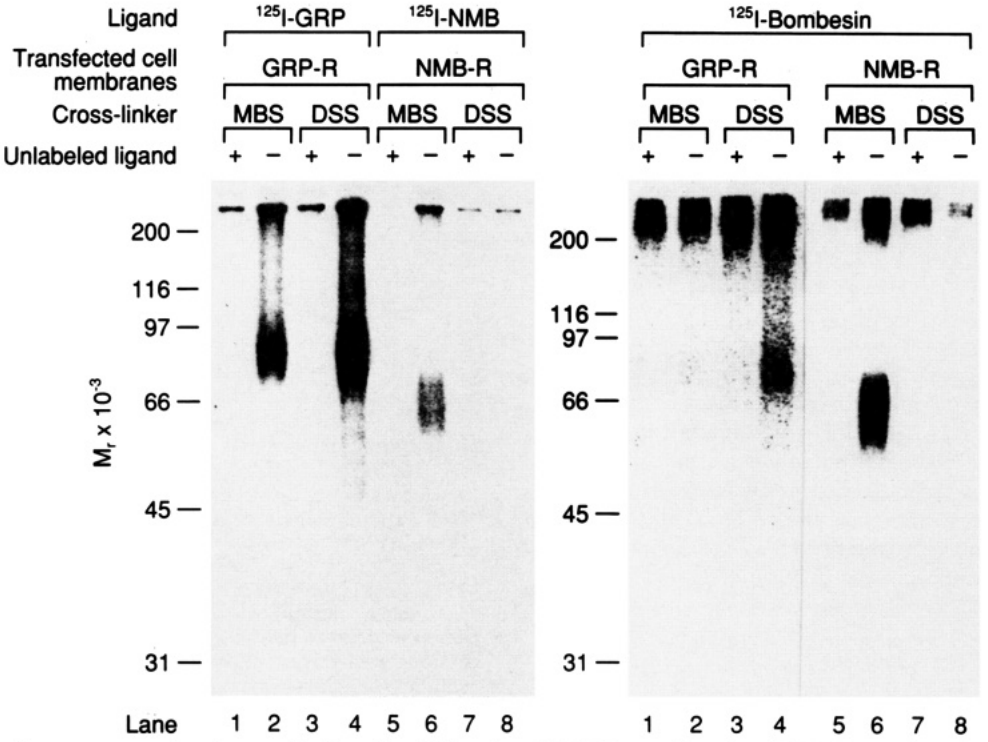

FIGURE 2: Autoradiograms comparing the cross-linking of radioligands to NMB-R transfected and GRP-R transfected BALB 3T3 cells. In the left panel, NMB-R transfected cell membranes and GRP-R transfected cell membranes were incubated with 0.5 nM [^{125}I]-(D-Tyr^0)NMB or 0.5 nM [^{125}I]GRP, respectively, at 25 °C for 15 min in the presence or absence of non-radiolabeled ligands. After washing, cross-linking was performed with 1 mM DSS or MBS as indicated. Samples were subjected to SDS–PAGE using 10% acrylamide gels and analyzed by autoradiography. The right panel shows an autoradiogram for the cross-linking of [^{125}I]-(Tyr^4)bombesin to membranes from either GRP-R or NMB-R transfected cells with 1 mM DSS or MBS. Each of these autoradiograms is representative of at least two others. The positions of molecular weight standards are shown on the left side of each panel.

on Swiss 3T3 cells (Sinnett-Smith et al., 1988; Brown et al., 1988a; Rozengurt, 1988). DSS was slightly more effective than MBS for the cross-linking of [^{125}I]GRP to GRP-R transfected cell membranes; however, MBS was much more effective than DSS for the cross-linking of [^{125}I]-(D-Tyr^0)-NMB to NMB-R transfected cell membranes (Figure 2, left panel, lanes 5–8). To determine whether this difference in the relative potencies of MBS and DSS for the two receptors was caused by a difference in the radiolabeled ligand used or a difference in the receptor protein structures, [^{125}I]-(Tyr^4)-bombesin, which binds to both receptors (Wang et al., 1993), was cross-linked to membranes using either MBS or DSS (Figure 2, right panel). Although the amount of radioactivity cross-linked was less than that seen with [^{125}I]-(D-Tyr^0)NMB or [^{125}I]GRP, the relative potencies of the cross-linkers were similar to those seen when [^{125}I]-(D-Tyr^0)NMB or [^{125}I]GRP was used as tracer, with the same M_r cross-linked proteins seen.

In previous studies, [^{125}I]GRP has been shown to be cross-linked specifically to GRP-R using NH_2 homobifunctional cross-linkers such as DSS (Huang et al., 1990; Sinnett-Smith et al., 1988; Singh et al., 1990). To assess the specificity of cross-linking of [^{125}I]-(D-Tyr^0)NMB to NMB-R, we examined several agents that do not interact with NMB receptors to affect cross-linking. VIP (1 μM), substance P (1 μM), CCK-8 (1 μM), and N-acetyl-GRP(20–26)ethyl ester, a GRP-R specific receptor antagonist (Jensen & Coy, 1991), had no effect on the cross-linking of [^{125}I]-(D-Tyr^0)NMB to the M_r = 63 000 protein band of both types of NMB-R-containing cells (data not shown).

NMB inhibited the cross-linking of [^{125}I]-(D-Tyr^0)NMB to the C-6 and NMB-R transfected cell membrane M_r = 63 000 protein bands in a dose-dependent manner (Figure 3), with half-maximal inhibition of cross-linking occurring with 4.8 ± 0.4 nM for C-6 cell membranes and 6.1 ± 1.5 nM for NMB-R transfected cell membranes, respectively (Figure 3). GRP also inhibited cross-linking of [^{125}I]-(D-Tyr^0)NMB to both membranes; however, it was >200-fold less potent than NMB (Figure 3).

To provide additional evidence that the cross-linked protein seen on the membranes was the same as that seen on the intact cell, [^{125}I]-(D-Tyr^0)NMB was cross-linked directly to intact C-6 and NMB-R transfected cells (data not shown). The results with the intact cells were similar to those obtained with membrane preparations for both cells. A single M_r = 63 000 band was seen, and the cross-linking to it was completely inhibited by 1 μM unlabeled NMB.

To characterize the glycosylation of the NMB-R, digestion by various glycosidases was performed (Figures 4 and 5 and Table 1). PNGase F (30 units/mL) digestion of cross-linked membranes from C-6 cells increased the mobility of the original cross-linked band to a band of M_r = 43 000 (Figure 4, lane 11, and Table 1), and 100 units/mL PNGase F had no additional effect (not shown). Similarly, a 10 units/mL PNGase F (Figure 4, lane 8) digestion of cross-linked membranes from NMB-R transfected cells increased the mobility of the original band to a band of M_r = 43 000, and 30 units/mL PNGase F (Figure 4, lane 9) had no additional effect. Endo-H (2 or 5 units/mL) (Figure 4, lanes 1–3) and Endo-F_2 (20 or 60 munits/mL) (Figure 4, lanes 4–6) had no effect on the mobility of the original bands seen in NMB-R transfected cells. Neuraminidase (1 unit/mL) digestion slightly increased the mobility of the original cross-linked band to M_r = 60 000 in NMB-R transfected cell membranes (Figure 5, lanes 1 and 2, and Table 1). Higher concentrations of neuraminidase (5 units/mL) showed the same effect (Figure

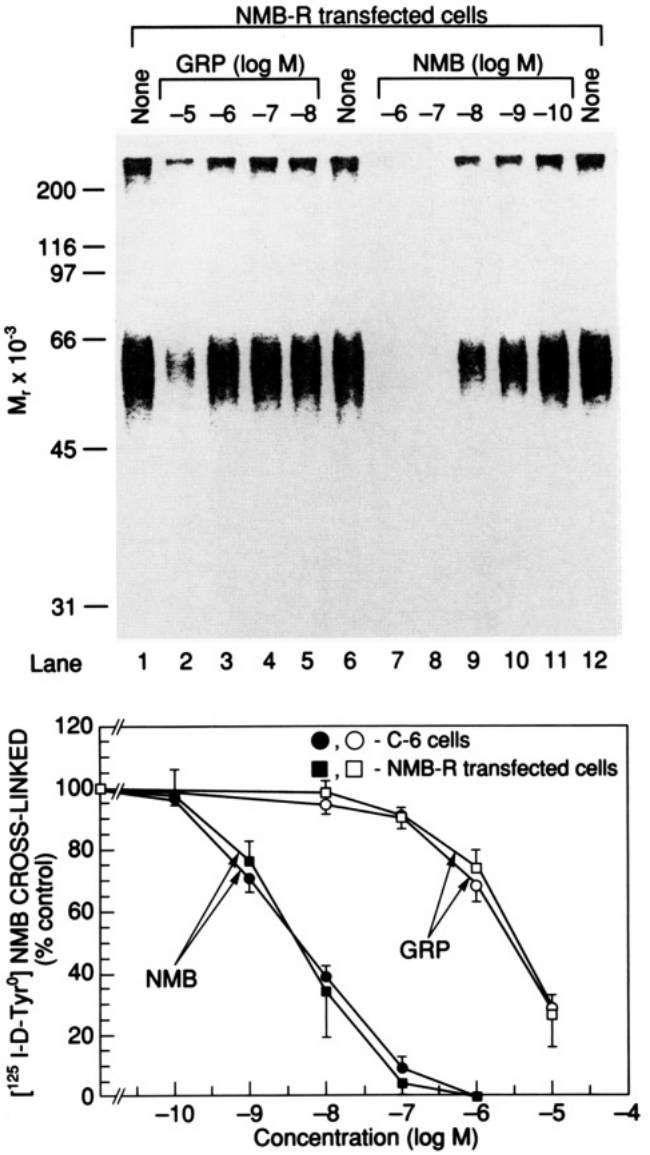

FIGURE 3: Ability of NMB and GRP to inhibit cross-linking of [^{125}I]-(D-Tyr^0)NMB to membranes from rat C-6 glioblastoma and NMB-R transfected cells. Membranes were incubated with 0.5 nM [^{125}I]-(D-Tyr^0)NMB for 15 min at 25 °C with no additions or with the indicated concentrations of peptides. Further processing was as described in the Figure 4 legend. The top panel shows an autoradiogram from NMB-R transfected cells, which is representative of two others. Each lane contains a sample incubated with the concentration of peptide indicated at the top of the figure expressed in log *M*. The positions of molecular weight standards are shown on the left side of the figure. The bottom panel shows the amount of radioactivity saturably cross-linked to the 63 000 protein band from C-6 glioblastoma and NMB-R transfected cells. Results are expressed as a percentage of the amount of radioactivity saturably cross-linked with no additions. Values are means ± SEM from three separate experiments.

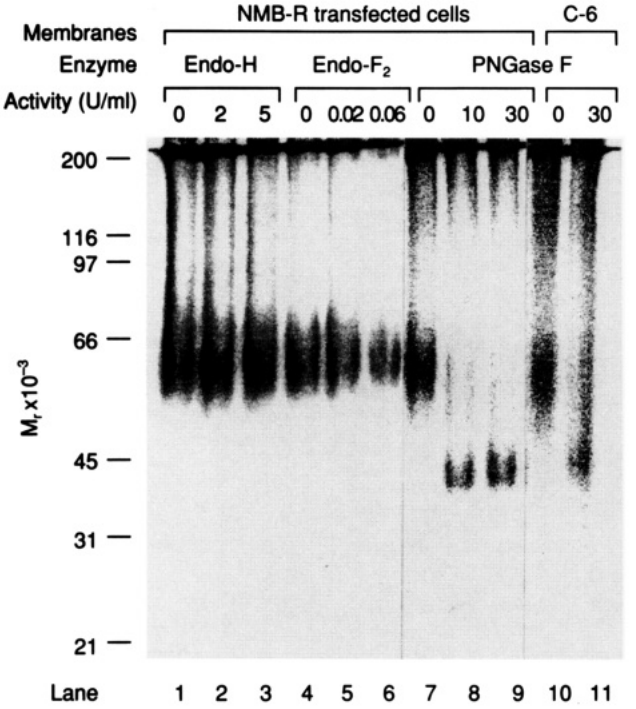

FIGURE 4: Effects of Endo-H, Endo-F_2, or PGNase F treatment on the migration of [^{125}I]-(D-Tyr^0)NMB cross-linked protein. [^{125}I]-(D-Tyr^0)NMB was cross-linked with 1 mM MBS for 30 min at 25 °C to either NMB-R transfected cell membranes (lanes 1–9) or C-6 glioblastoma cell membranes (lanes 10 and 11). Cross-linked membranes were incubated with the indicated concentrations of Endo-H, Endo-F_2, or PNGase F for 18 h at 37 °C. After digestion was stopped with 4% SDS, samples were subjected to SDS–PAGE using the Weber and Osborn buffer system and analyzed by autoradiography. Each of these results is representative of two others. The positions of molecular weight standards are shown on the left side of the figure.

5, lane 3). The addition of *O*-glycanase (up to 400 munits/mL) (Figure 5, lanes 4–7) had no additional effect after-neuraminidase treatment of the [^{125}I]-(D-Tyr^0)NMB cross-linked band.

To compare the results with the glycosidase treatment of the NMB-R to that of the structurally related GRP-R, similar studies were performed on GRP-R transfected cells (Figures 5 and 6 and Table 1). PGNase F (30 units/mL) (Figure 6, lane 8) increased the mobility of the cross-linked protein from 82 ± 1 to 43 ± 2 kDa. Neither Endo-H (Figure 6, lanes 1–3) nor Endo-F_2 (Figure 6, lanes 4–6) treatment of the GRP-R had any effect on mobility of the cross-linked band. In contrast to the NMB-R, neuraminidase treatment (Figure 5, lanes 8–10) had no effect on the mobility of the GRP-R cross-linked band (Figure 5 and Table 1), and the addition of *O*-glycanase (Figure 5, lanes 11–14) to neuraminidase had no effect.

To investigate how many of the potential glycosylation sites (Battey et al., 1991; Spindel et al., 1990) in both classes of bombesin receptors are actually glycosylated, serial partial digestion of cross-linked membranes from NMB-R transfected and GRP-R transfected cells by PNGase F was performed (Figure 7). When cross-linked NMB-R transfected cell membranes were treated with 10 units/mL PNGase F for various periods of less than 18 h, radiolabeled receptors were observed to be digested in a stepwise fashion (Figure 7, left panel). Besides the original fully glycosylated (M_r = 63 000) and fully deglycosylated protein bands (M_r = 43 000), one additional band of M_r = 53 000 was seen (Figure 7, top left and bottom left panels). When GRP-R transfected cell membranes containing the fully glycosylated GRP-R (M_r = 82 000) were treated similarly with 2 units/mL PNGase F, three bands (M_r = 72 000, 62 000, and 52 000) in addition to the full, deglycosylated receptor (M_r = 43 000) were seen (Figure 7, top right and bottom right panels). These results suggest that the NMB-R has at least two and the GRP-R has four N-linked oligosaccharide chains.

To investigate the possible importance of glycosylation in high-affinity binding or G-protein coupling, treatment with glycosylation inhibitors such as tunicamycin has been used (el Battari et al., 1991; Kaushal et al., 1994). When GRP-R and NMB-R transfected cells were cultured for 48 h with

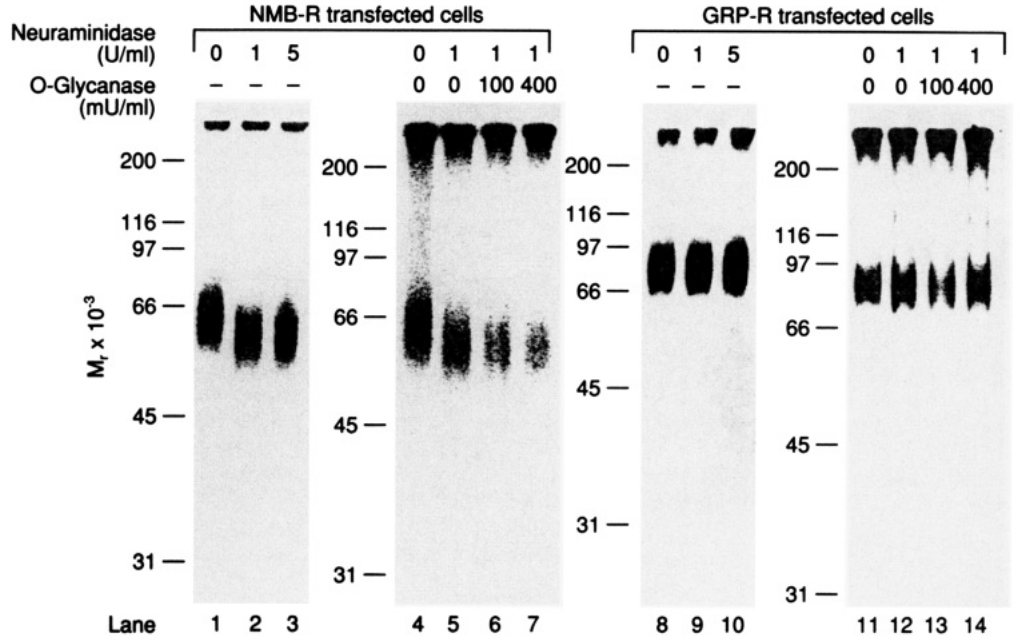

FIGURE 5: Effects of neuraminidase and neuraminidase plus *O*-glycanase digestion on the migration of the [^{125}I]-(D-Tyr^0)NMB cross-linked protein in NMB-R transfected BALB 3T3 cell membranes and the [^{125}I]GRP cross-linked protein in GRP-R transfected cell membranes. [^{125}I]-(D-Tyr^0)NMB was cross-linked to NMB-R transfected BALB 3T3 cell membranes with 1 mM MBS for 30 min at 25 °C. [^{125}I]GRP was cross-linked to GRP-R transfected cell membranes with 1 mM DSS for 30 min at 25 °C. Cross-linked membranes were incubated with the indicated concentrations of neuraminidase for 1 h at 37 °C. After neuraminidase digestion, membranes were solubilized in 4% SDS or digested further with the indicated concentration of *O*-glycanase for 18 h at 37 °C and solubilized. Samples were subjected to SDS–PAGE and analyzed by autoradiography. Each of these autoradiograms is representative of two others. The positions of molecular weight standards are shown on the left side of each panel.

Table 1: Effect of Enzymatic Digestions on the Mobility of ^{125}I-[D-Tyr^0]NMB or ^{125}I-GRP Cross-Linked Proteins[a]

	M_r of cross-linked protein ($\times 10^3$)		
enzyme added	C-6 cells	NMB-R transfected 3T3 cells	GRP-R transfected 3T3 cells
none	63 ± 1	63 ± 1	82 ± 2
PNGase F (30 units/mL)	43 ± 1	43 ± 1	43 ± 2
neuraminidase (1 unit/mL)	NT	60 ± 1	82 ± 1
neuraminidase (1 unit/mL) plus *O*-glycanase (400 munits/mL)	NT	60 ± 1	82 ± 1
Endo-H (5 units/mL)	NT	60 ± 1	82 ± 1
Endo-F_2 (5 units/mL)	NT	63 ± 1	82 ± 1

[a] The mobilities of cross-linked proteins after enzymatic digestions of membranes from C-6 and transfected BALB 3T3 cells were obtained from the autoradiograms shown in Figures 2–6. M_r was corrected for the relative molecular weight of the radiolabeled ligand used. Values are means ± SEM of at least three separate experiments. NT: not tested.

0.01–3 μg/mL tunicamycin present, concentrations of up to 0.1 μg/mL tunicamycin dose-dependently decreased the number of receptors, and only fully glycosylated receptors were seen. High doses (1 and 3 μg/mL) were toxic to the cells. Therefore, the approach of enzymatic deglycosylation of the receptors in the membrane was used to elucidate whether the N-linked oligosaccharide chains in NMB-R and the GRP-R are essential for maintaining high-affinity interactions with agonists. When NMB-R transfected cell membranes were pretreated with 30 units/mL PNGase F at 37 °C for 3 h and then cross-linked with [^{125}I]-(D-Tyr^0)NMB, only a fully deglycosylated species of M_r = 43 000 was radiolabeled (data not shown). Binding dose–inhibition results for deglycosylated and control membranes from NMB-R transfected cells were superimposable, with K_d values for binding of 4.1 ± 0.7 and 4.7 ± 0.5 nM and B_{max} values of 2.0 ± 0.1 and 2.0 ± 0.1 pmol/mg of protein for PNGase F-treated and untreated membranes, respectively (Figure 8, top panel). When GRP-R

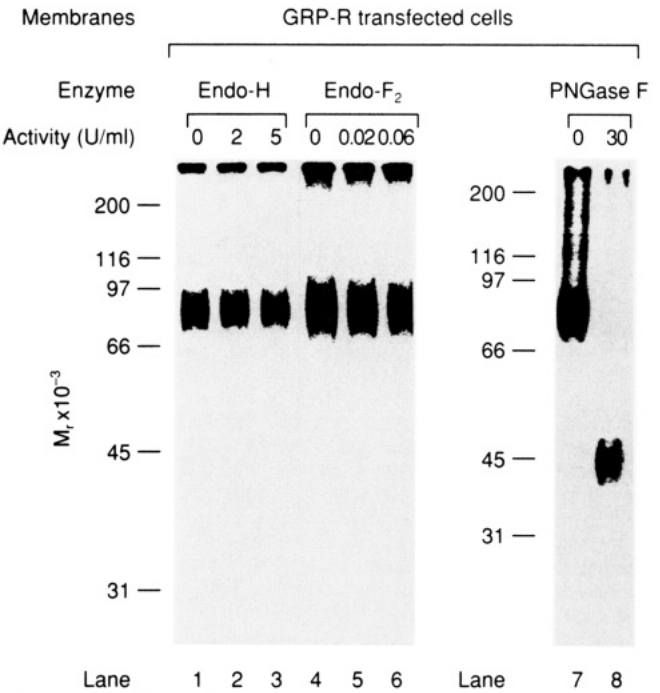

FIGURE 6: Effects of Endo-H, Endo-F_2, or PGNase F treatment on the migration of [^{125}I]GRP cross-linked protein in membranes from GRP-R transfected cells. [^{125}I]GRP was cross-linked with 1 mM DSS for 30 min at 25 °C. Cross-linked membranes were incubated with the indicated concentrations of Endo-H, Endo-F_2, or PNGase F for 18 h at 37 °C. After digestion was stopped with 4% SDS, samples were subjected to SDS–PAGE using either the Laemmli buffer system (lanes 1–6) or the Weber and Osborn buffer system (lanes 7 and 8) and analyzed by autoradiography. Each of these autoradiograms is representative of two others. The positions of molecular weight standards are shown on the left side of each panel.

transfected cell membranes were pretreated with 30 units/mL PNGase F at 37 °C for 3 h and then cross-linked with [^{125}I]GRP, a 75% deglycosylated protein of M_r = 52 000 was

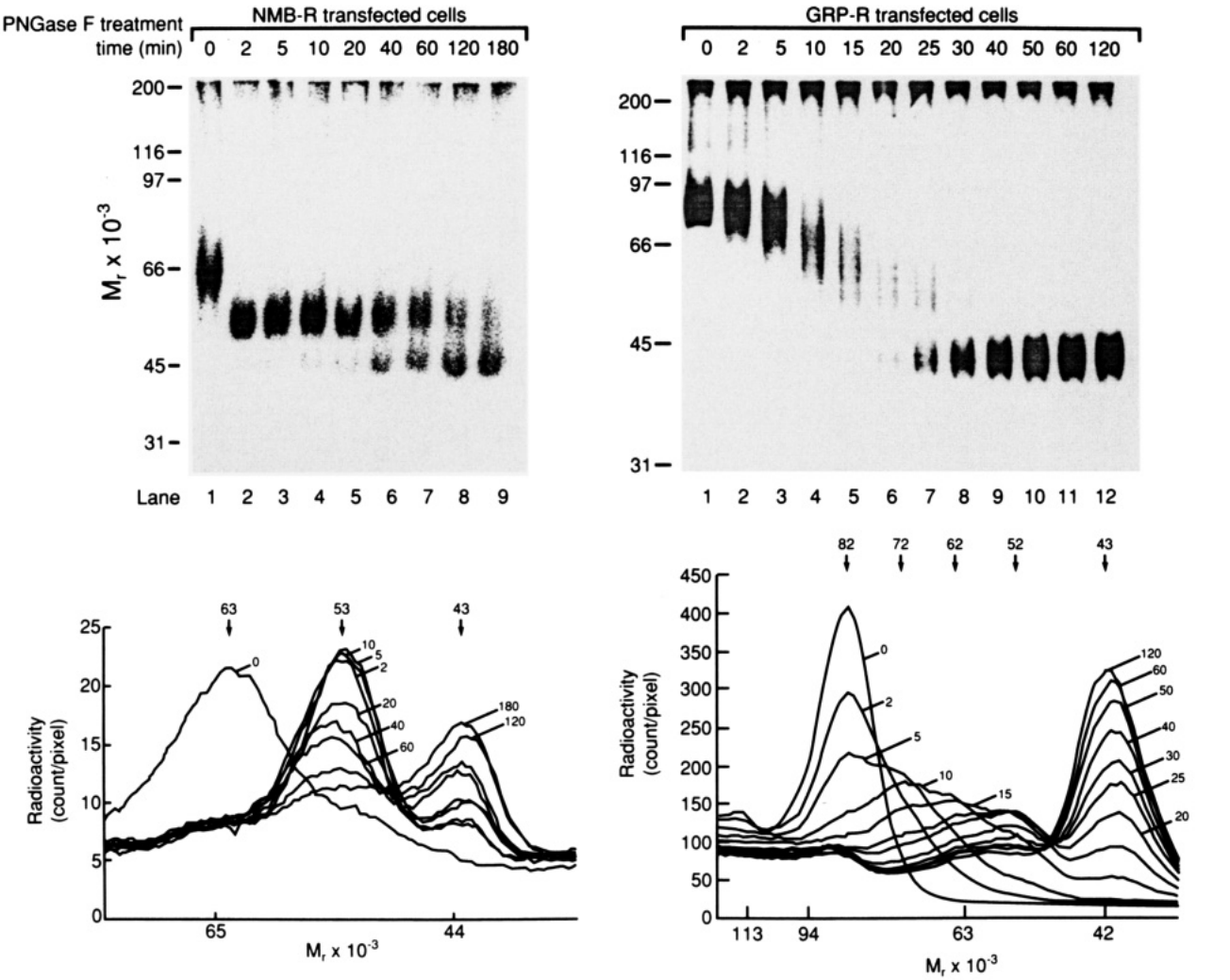

FIGURE 7: Serial partial deglycosylation of [^{125}I]-(D-Tyr^0)NMB cross-linked NMB-R transfected cell membranes and [^{125}I]GRP cross-linked GRP-R transfected cell membranes. [^{125}I]-(D-Tyr^0)NMB was cross-linked with 1 mM MBS to NMB-R transfected cell membranes (top left panel). [^{125}I]GRP was cross-linked with 1 mM DSS to GRP-R transfected cell membranes (top right panel). Cross-linked membranes were incubated with PNGase F (10 units/mL for NMB-R transfected cell membranes and 2 units/mL for GRP-R transfected cell membranes) at 37 °C for the various times indicated at the top of each lane. The digestion was stopped by denaturing the reaction mixture in 4% SDS. Samples were analyzed by SDS–PAGE using either the Weber and Osborn buffer system (NMB-R transfected cell membranes) or the Laemmli buffer system (GRP-R transfected cell membranes) followed 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 bottom left panel shows the spatial distribution of radioactivity in each lane shown in the top left panel. The bottom right panel shows the spatial distribution of radioactivity in each lane shown in the top right panel. The numbers at the top of the right and left bottom panels are the molecular weights of each radioactive peak.

radiolabeled (data not shown). The presence of increasing concentrations of GRP in binding buffer inhibited cross-linking of [^{125}I]GRP to the M_r = 52 000 band and binding to the membranes in a dose-dependent manner, similar to that observed with cross-linking of [^{125}I]GRP to the M_r = 82 000 band in untreated membranes (Figure 8, bottom panel). PNGase F pretreatment significantly decreased the affinity of the GRP-R for GRP compared to control cell membranes (p = 0.032, n = 5) (Figure 8, bottom panel) without a significant effect on receptor capacity (B_{max}). The calculated K_d values were 3.7 ± 0.7 and 2.2 ± 0.3 nM and the calculated B_{max} values were 1.1 ± 0.2 and 1.1 ± 0.1 pmol/mg of protein for PNGase F-pretreated and untreated GRP-R transfected cell membranes, respectively. For both cell types, their specific ligands inhibited cross-linking to the deglycosylated receptor band in a manner similar to the binding inhibition results.

To investigate the role of oligosaccharide chains in the G-protein coupling of bombesin receptors, we tested the effect of PNGase F treatment on the ability of Gpp(NH)p to cause a decrease in binding of radiolabeled ligands to NMB-R and GRP-R transfected cell membranes induced (Figure 9). Gpp(NH)p decreased the binding of [^{125}I]-(D-Tyr^0)NMB to intact NMB-R transfected cell membranes by 62%, with a half-maximal effect occurring at 4.5 nM. Treatment of the membranes with 30 units/mL PNGase F at 37 °C for 3 h, which results in total deglycosylation of the NMB-R, did not alter the effect of Gpp(NH)p on the binding of [^{125}I]-(D-Tyr^0)NMB (64% decrease in maximal binding, with a half-maximal effect occurring at 6.3 nM) (Figure 9, top panel). This indicates that the oligosaccharide chains on the NMB-R are not essential to mediating receptor–G-protein coupling. Gpp(NH)p decreased the binding of [^{125}I]GRP to intact GRP-R transfected cell membranes by 45%, with a half-maximal effect occurring at 24 nM (Figure 9, bottom panel). In contrast to NMB-R transfected cell membranes, Gpp(NH)p showed a minimal effect (10% decrease) after treatment of the GRP-R transfected cell membranes with 30 units/mL PNGase F at 37 °C for 3 h, which results in 75% deglycosylation of the GRP-R (Figure 9, bottom panel).

To be certain that each of the glycosidases had activity in our system, they were tested for their ability to alter various glycoproteins (data not shown). PGNase F digestion increased

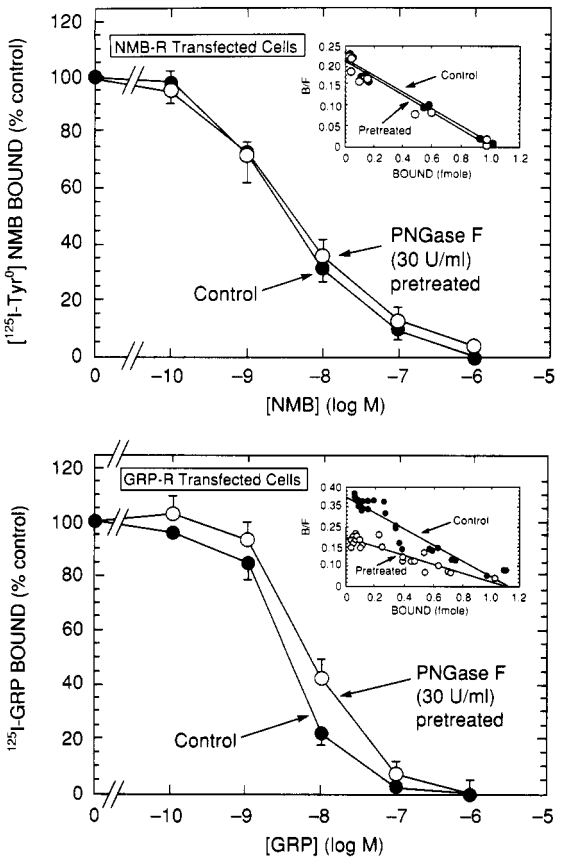

FIGURE 8: Effect of NMB or GRP on the binding of radiolabeled ligands to PNGase F-pretreated membranes from NMB-R transfected and GRP-R transfected cells. The top panel shows the results with NMB-R transfected cell membranes. NMB-R transfected cell membranes were incubated in the presence or absence of 30 units/mL PNGase F at 37 °C for 3 h. After washing, membranes were incubated with 0.5 nM [^{125}I]-(D-Tyr^0)NMB with or without the addition of various concentrations of unlabeled NMB indicated. Radioactivity saturably bound was determined as described in Materials and Methods and expressed as a percentage of radioactivity saturably bound to the membrane with no additions, which was 2680 ± 60 and 2540 ± 120 cpm per 50 μL sample (12.5 μg of protein) for control and PNGase F-treated samples, respectively. The bottom panel shows the results with similarly processed GRP-R transfected cell membranes. Saturably bound radioactivity was 1480 ± 90 and 950 ± 50 cpm per 50 μL sample (12.5 μg of protein) for GRP-R control and PNGase F-treated samples, respectively. The inset in each figure shows the Scatchard plots obtained from experiments shown in the main panels. Each value was determined in duplicate, and results are means ± SEM from three separate experiments.

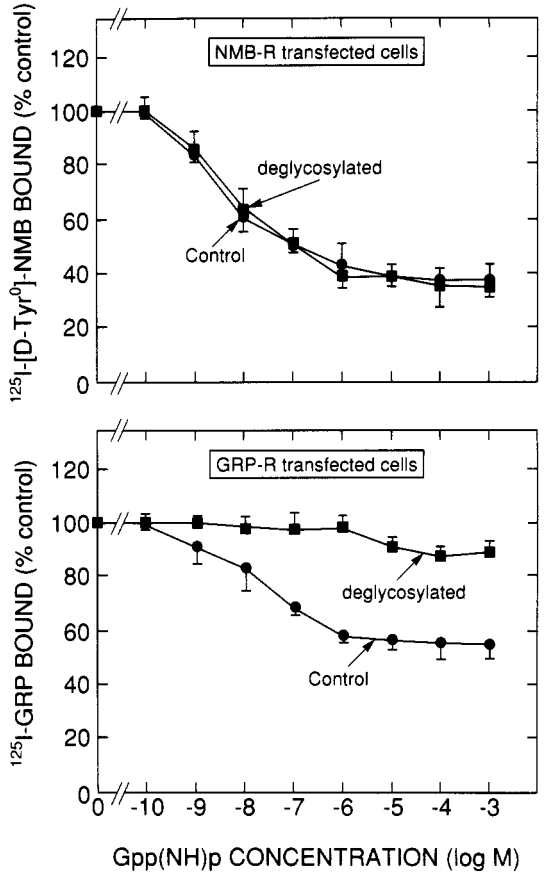

FIGURE 9: Effects of PNGase F treatment on G-protein coupling in NMB-R and GRP-R transfected cell membranes. The top panel shows the results with NMB-R transfected cell membranes. NMB-R transfected cell membranes were incubated in the presence or absence of 30 units/mL PNGase F at 37 °C for 3 h. After washing, membranes were incubated with 0.5 nM [^{125}I]-(D-Tyr^0)NMB with or without the addition of various concentrations of Gpp(NH)p. Radioactivity saturably bound was determined as described in Materials and Methods and expressed as a percentage of radioactivity saturably bound to the membrane with no additions. The bottom panel shows the results with similarly processed GRP-R transfected cell membranes. Each value was determined in duplicate, and results are means ± SEM from three separate experiments.

the mobility of each of the glycoproteins examined: fetuin, from M_r = 65 000 to 52 000; ovalbumin, from M_r = 45 000 to 43 000; and ribonuclease B, from M_r = 19 100 to 16 700. Endo-H increased the mobility of ovalbumin from M_r = 45 000 to 43 000. It also increased the mobility of RNase B from M_r = 19 100 to 16 700. Endo-F_2 increased the mobility of RNase B to an extent similar to that of Endo-H; however, it had little effect on the mobility of fetuin and it did not affect the mobility of ovalbumin, demonstrating that Endo-F_2 was free of contaminating PNGase activity. Neuraminidase increased the mobility of the original band of the fetuin (from M_r = 65 000 to 57 000). *O*-Glycanase digestion after neuraminidase treatment caused an additional increase in the mobility of fetuin from M_r = 65 000 to M_r 54 000.

DISCUSSION

In contrast to receptors for neurotransmitters such as adrenergic agents (Rands et al., 1990) and cholinergic agents (Giovannelli et al., 1991; Gehle & Sumikawa, 1991; Ohara et al., 1990) and classical hormones such as the glycoprotein hormones LH and TSH (Zhang et al., 1991; Liu et al., 1993; Petaja-Repo et al., 1991, 1993; Ji et al., 1990; Russo et al., 1991), but similar to other receptors for most gastrointestinal neurotransmitters and hormones, very little is known about the glycosylation of GRP or NMB receptors. Although the protein structure of both bombesin receptors is known from recent cloning studies, only limited information is available regarding GRP receptor glycosylation from cross-linking and lectin binding studies of the GRP receptor (Battey et al., 1991; Spindel et al., 1990; Wada et al., 1991; Kris et al., 1987). From cross-linking studies of the GRP receptor in murine Swiss 3T3 cells, mouse pancreatic acinar cells, human SCLC cells, human U-118 glioblastoma cells, and the rat pancreatic acinar tumor cell line AR42J cells (Huang et al., 1990; Kris et al., 1987; Singh et al., 1990; Sinnett-Smith et al., 1988; Brown et al., 1988a; Feldman et al., 1990; Staley et al., 1993), the GRP-R is known to be a glycoprotein varying in the extent glycosylation from 30% in human U-118 glioblastoma cells to 50% in mouse Swiss 3T3 cells. It is known from cloning studies that the mouse GRP receptor possesses four potential extracellular N-glycosylation sites (Asn-X-Ser/Thr, where

X is not Pro) (Battey et al., 1991; Spindel et al., 1990). From cross-linking and glycosylation studies, it is known that the GRP receptor contains N-linked glycosylation (Huang et al., 1990; Kris et al., 1987; Feldman et al., 1990; Sinnett-Smith et al., 1988); the nature of the N-linked glycosylation is unclear, as is whether O-linked glycosylation exists, whether all four potential glycosylation sites are glycosylated, and whether glycosylation affects receptor affinity, coupling, or function. From cloning studies, it is known that the rat NMB receptor has two possible extracellular glycosylation sites (Wada et al., 1991), but no information is available from cross-linking or solubilization studies and, thus, no information existed until the present study about the importance of glycosylation for this receptor.

Recent studies have demonstrated the importance of the carbohydrate moiety of receptors for maintaining receptor stability, intracellular trafficking, proper cell surface expression, and protein folding (Petaja-Repo et al., 1991, 1993; Russo et al., 1991; Kuwano et al., 1991; Giovannelli et al., 1991; Rands et al., 1990; West, 1986; Elbein, 1987; Rademacher et al., 1988). Furthermore, in some receptors the carbohydrate moieties are needed for high-affinity receptor interaction or proper intracellular signaling (Bastian et al., 1993; Soderquist & Carpenter, 1984; Feige & Baird, 1988; Chochola et al., 1993; Rens-Domiano & Reisine, 1991). Numerous studies demonstrate that N-glycosylation is not required for high-affinity interactions of muscarinic cholinergic receptors, adrenergic receptors, or the receptors for the glycoprotein hormones, FSH or LH (Giovannelli et al., 1991; Rands et al., 1990; Dattatreyamurty & Reichert, 1992; Liu et al., 1993). However, with some growth factor receptors, such as those for insulin (Leconte et al., 1992), epidermal growth factor, or basic fibroblast growth factor (Soderquist & Carpenter, 1984; Feige & Baird, 1988), as well as a number of peptidergic receptors such as those for the gastrointestinal hormone receptors, such as vasoactive intestinal peptide (Chochola et al., 1993), cholecystokinin (Santer et al., 1990), or somatostatins (Rens-Domiano & Reisine, 1991), N-linked glycosylation is required for high-affinity receptor binding and/or transduction.

With receptors for other peptides and neurotransmitters, cross-linking studies have been used alone or in combination with various glycosidase enzymatic digestions to provide insights into receptor structure and receptor subtype differences (Sinnett-Smith et al., 1988; Chochola et al., 1993; Jarvie et al., 1989; Honda et al., 1993; Pearson et al., 1987; Maley et al., 1989; Petaja-Repo et al., 1991, 1993). In the present study, we have used this methodology to investigate the glycosylation of two receptors mediating the actions of bombesin-related peptides. NMB receptors were investigated using this methodology in both the rat glioblastoma C-6 cells and the NMB-R from rat esophageal muscularis mucosa transfected into BALB-3T3 cells (Benya et al., 1992). These NMB receptor cell types were selected because recent studies demonstrate that each is pharmacologically similar to NMB receptors on esophageal mucosa and that activation of each alters cell function in a similar manner, including increasing $[Ca^{2+}]_i$ and inositol phosphates and causing receptor down-regulation, internalization, and desensitization (Benya et al., 1992, 1994). These results were compared with those for the mouse GRP receptor, which was also transfected into these cells and is glycosylated to an extent identical to that in native mouse 3T3 cells and which has been shown to behave in a pharmacologically similar fashion to the native murine GRP receptor in Swiss 3T3 cells (Benya et al., 1994). The present studies show that the NMB-R ligand, [^{125}I]-(D-Tyr^0)NMB, is covalently linked to a single polypeptide, M_r = 63 000 (when corrected for the relative molecular weight of [^{125}I]-(D-Tyr^0)-NMB), in membranes from both C-6 and NMB-R transfected cells. This establishes that the NMB receptor is also a glycoprotein.

Recent studies (Benya et al., 1992) show that the NMB receptors on transfected BALB 3T3 cells and those on native C-6 cells behave in a functionally and pharmacologically similar manner, and several results in this study demonstrate that the NMB receptor on the NMB-R transfected cells and those on C-6 glioblastoma cells are indistinguishable. First, cross-linking of [^{125}I]-(D-Tyr^0)NMB to NMB-R transfected cells or their membranes and to C-6 cells or their membranes showed identical results, with only a single band of M_r = 63 000 seen. Second, the electrophoretic motility patterns after PGNase F digestion of the cross-linked membranes from both cell types were similar, with both showing an M_r = 43 000 band. Third, the abilities of various agents such as NMB, GRP, cyclo-SS-octa, and *N*-acetyl-GRP(20–26)ethyl ester to inhibit binding and cross-linking of [^{125}I]-(D-Tyr^0)NMB to the membranes were similar for both cell types. These observations support the usefulness of the NMB-R transfected cells in investigating either the structure of the NMB receptor or the ability of its activation to alter cell function.

Although receptors for NMB and GRP share considerable homology (Wada et al., 1991) and similar pharmacological properties, including high-affinity binding for bombesin (Wang et al., 1993) and being coupled to phospholipase C (Benya et al., 1992, 1994), the results in the present study disclose several structural differences between these two bombesin receptor types related to glycosylation. Both classes of receptors showed distinct relative molecular weights after cross-linking on SDS–PAGE (M_r = 63 000 and M_r = 82 000 for NMB-R and GRP-R transfected cells, respectively). In the present study, the core polypeptide size of both receptors after PNGase F digestion (M_r = 43 000) was similar and identical to that predicted from the amino acid structure. Thus, the difference in apparent molecular weights is attributed to the difference in the extent of glycosylation. Serial partial deglycosylation of [^{125}I]-(D-Tyr^0)NMB cross-linked NMB-R transfected cell membranes revealed one band of partially deglycosylated protein. Because PNGase F removes each oligosaccharide chain en bloc and does not modify oligosaccharide chains on the protein (Tarentino et al., 1985), the NMB receptor has two oligosaccharide chains of approximately the same size ($M_r \approx$ 10 000 each). Similarly, the fact that serial partial deglycosylation of [^{125}I]GRP cross-linked GRP-R transfected cell membrane revealed three bands of partially deglycosylated protein suggests that the GRP receptor has four oligosaccharide chains of approximately the same size ($M_r \approx$ 10 000 each). These results suggest that both of the extracellular potential N-linked glycosylation sites on the NMB-R and all four of the extracellular potential N-linked glycosylation sites on the GRP-R likely are glycosylated. The results with the GRP-R differ from those reported previously with the murine GRP-R, which suggests that two distinct N-linked oligosaccharide chains are present (Sinnett-Smith et al., 1988). This difference can be explained by the difference in methods in the two studies, in that only a single long incubation with enzyme was used in the previous study (Sinnett-Smith et al., 1988), whereas the present study demonstrated that the multiple glycosylation sites were only clearly seen with short incubation.

The fact that neither Endo-H, which can release N-linked high mannose and some kinds of hybrid type oligosaccharides (Tarentino & Maley, 1974), nor Endo-F_2, which releases N-linked high mannose, hybrid type, and biantenary oligosaccharides with a preference for biantenary structures (Trimble & Tarentino, 1991), changed the mobility of the original radiolabeled band in membranes from NMB-R or GRP-R transfected cells suggests that neither receptor contains these types of N-linkage. The lack of effectiveness of Endo-H and Endo-F_2 was not due to a lack of activity because these enzymes gave the expected results with RNase B and ovalbumin. The specificity of PNGase F for various N-linked oligosaccharides is much broader than that encountered with Endo-H and Endo-F_2, since complex oligosaccharides with bi-, tri-, and tetraantenary arms, as well as high mannose and hybrid types, are substrates for PNGase F, in addition to polysialyl complex oligosaccharides and those containing sulfate residues (Tarentino et al., 1985). In both cells, PNGase F digestion increased the mobility of the original band. The resultant protein band had an M_r = 43 000, which is identical to that predicted from cloning studies (Wada et al., 1991). These data indicate that the NMB and GRP receptors have N-linked complex oligosaccharides with tri- and/or tetraantenary arms. In these studies, neuraminidase slightly increased the mobility of the original cross-linked protein band in membranes from NMB-R transfected BALB 3T3 cells, but had no effect on the GRP-R. Since neuraminidase hydrolyzes sialic acid residues (Kiyohara et al., 1974), these results demonstrate that only the NMB receptor possesses terminal sialic acid residues; the GRP-R does not. The NMB-R resembles the receptors for cholecystokinin, calcitonin gene-related peptide, vasoactive intestinal peptide, and somatostatin in possessing terminal sialic acids (Honda et al., 1993; Rens-Domiano & Reisine, 1991; Ghatei et al., 1982; Iwamoto & Williams, 1980), while the GRP-R resembles the ileal endothelin receptor, interleukin-1 receptor type 1, and the rat and human neutrophil *N*-formylpeptide chemotaxic receptors in not possessing terminal sialic acids (Bousso-Mittler et al., 1991; Mancilla et al., 1992; Remes et al., 1991).

To look for O-linked oligosaccharide chains in both NMB receptors, *O*-glycanase digestion was performed (Umemoto et al., 1977). Because O-linked oligosaccharides may terminate in sialic acid, which interferes with the activity of *O*-glycanase, neuraminidase pretreatment was also performed (Pearson et al., 1987). In membranes from NMB-R and GRP-R transfected cells, *O*-glycanase digestion after neuraminidase treatment showed no further effects on the mobility of the band. These results demonstrate that both the NMB and GRP receptors do not contain O-linked oligosaccharide chains. These data demonstrate that the NMB and GRP receptors resemble receptors for CCK (Pearson et al., 1987) in rat pancreas, LH receptors (Petaja-Repo et al., 1993) in rat, and dopamine D_1 receptors in brain and parathyroid tissues (Jarvie et al., 1989), with each having N-linked but not O-linked oligosaccharide chains. These results differ from those for receptors for low-density lipoproteins (Kuwano et al., 1991) and prostaglandin E_2 and D_2 (Morii & Watanabe, 1992), which contain O-linked and N-linked oligosaccharide chains.

To explore the importance of the N-linked oligosaccharide chains for high-affinity ligand–receptor interactions, the effect of unlabeled ligand on binding and cross-linking of radiolabeled ligands to PNGase F-pretreated NMB-R transfected and GRP-R transfected cell membranes was determined. NMB interacted with the fully deglycosylated receptor with the same affinity that NMB interacted with the fully glycosylated NMB receptor. In contrast with the GRP-R, cross-linking of [^{125}I]-GRP to PNGase F-pretreated GRP-R transfected BALB 3T3 cell membranes resulted in 75% deglycosylation with labeling of an M_r = 52 000 protein band, which likely has one of the four oligosaccharide chains left on its polypeptide core. Deglycosylation of the GRP-R by 75% caused a significant decrease in the GRP-R affinity for GRP. These results suggest that full glycosylation of the GRP-R is necessary to maintain high affinity. These results demonstrate that the NMB-R resembles receptors for β-adrenergic agents (Rands et al., 1990) LH and FSH (Liu et al., 1993), where the extent of glycosylation does not affect binding affinity. In contrast, the GRP-R resembles receptors for VIP (Chochola et al., 1993), muscarinic cholinergic agents (Ohara et al., 1990), substance P (NK_1 agonists) (Fong et al., 1992), somatostatin (Rens-Domiano & Reisine, 1991), vasopressin (Jans et al., 1992), PGE_2, or PGD_2 (Morii & Watanabe, 1992), where the extent of glycosylation affects binding affinity.

Previous studies demonstrate that the GRP-R and NMB-R are G-protein-coupled receptors (Wang et al., 1992; Rozengurt, 1988; Benya et al., 1992; Profrock et al., 1992; Mantey et al., 1993; Fischer & Schonbrunn, 1988). G-protein coupling was not altered in the NMB-R transfected cells, even though the NMB-R was completely deglycosylated; however, 75% deglycosylation of the GRP-R markedly decreased the effect of Gpp(NH)p on binding. It is likely that this altered G-protein coupling is responsible for the decreased binding affinity because previous studies have shown that Gpp(NH)p alters GRP-R binding by altering affinity, not the receptor number (Mantey et al., 1993; Wang et al., 1993). While these results suggest that the extent of glycosylation of the GRP-R is important in affecting the ability of the receptor to effectively couple with G-proteins, it cannot be excluded that the PNGase F digestion itself is responsible for this alteration by altering membrane integrity or the G-protein directly in some manner. Yet this seems unlikely because NMB-R and GRP-R were transfected into the same BALB 3T3 cells, and the identical digestion technique did not alter the NMB-R G-protein coupling. These results suggest that glycosylation of the GRP-R appears to have importance for effective coupling and transduction similar to that shown recently for the insulin receptor (Leconte et al., 1992) and β-adrenergic receptor (β-AR) (Rands et al., 1990). With the β-AR, prevention of N-glycosylation by site-directed mutagenesis (Rands et al., 1990) resulted in decreased coupling to G_s. With the insulin receptor, prevention of N-glycosylation (Leconte et al., 1992) of the β-subunit by site-directed mutagenesis failed to alter binding, but decreased coupling to intracellular processes, decreasing the ability of insulin to cause tyrosine phosphorylation, glucose transport, glycogen synthesis, and DNA synthesis.

In conclusion, because of similar cross-linking patterns and glycosidase digestion patterns, results in this study indicate that the glycosylation of NMB receptors on C-6 cells and NMB-R transfected BALB 3T3 cells is the same. These NMB receptors are N-linked sialoglycoproteins with two N-linked tri- and/or tetraantenary complex oligosaccharide chains, demonstrating that both of the extracellular potential glycosylation sites are glycosylated. Their apparent M_r is 63 000, with a deglycosylated core peptide of M_r = 43 000, and these polypeptides do not contain disulfide-linked subunits or O-linked oligosaccharide chains. Despite a 56% protein homology with the NMB receptors, the glycosylation of the GRP-R differs from that of NMB-R in that it is a nonsialylated

N-linked glycoprotein with an apparent M_r of 82 000, consisting of a deglycosylated core peptide of M_r = 43 000 and at least four tri- and/or tetraantenary complex oligosaccharide chains. All four extracellular potential N-linked glycosylation sites are glycosylated to a similar extent. With the NMB-R, glycosylation was not an essential requirement for high-affinity binding to agonist, whereas with the GRP-R, 75% deglycosylation resulted in a decrease in affinity for GRP. Deglycosylation of the GRP-R also altered effective G-protein coupling, whereas it had no effect on NMB-R G-protein coupling. Finally, in addition to differences in glycosylation and pharmacology, the two mammalian bombesin receptors differ in the relationship between the free amino terminus of the ligand in its binding pocket and the availability of reactive NH_2 and SH groups in the receptor.

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