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Glycosylation of the GLP-1 Receptor Is a Prerequisite for Regular Receptor Function

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GÖKE, R., R. JUST, B. LANKAT-BUTTGEREIT AND B. GÖKE. *Glycosylation of the GLP-1 receptor is a prerequisite for regular receptor function.* PEPTIDES **15**(4) 675–681, 1994.—The GLP-1 receptor on RINm5F cells is a glycoprotein with a M_r of 63,000. Treatment of the receptor with glycopeptidase F generated a protein with a M_r of 51,000, indicating that the GLP-1 receptor contains *N*-linked glycans. Tunicamycin pretreatment concentration-dependently decreased GLP-1 binding to RINm5F cells due to a decreased receptor number without change of receptor affinity. Tunicamycin exerted no effect on the GLP-1 receptor mRNA expression. The stimulation of cAMP production was decreased in tunicamycin-treated cells. Our data show that glycosylation of the GLP-1 receptor is a precondition for regular receptor function.

GLP-1 Glycoprotein Receptor Tunicamycin RINm5F cells

THE preproglucagon-derived glucagon-like peptide-1(7–36)-amide (GLP-1) is a member of the glucagon/VIP/secretin family of peptides. GLP-1 has been identified in pancreatic A-cells and in the L-cells of the intestine (4). After food ingestion, GLP-1 is released into the circulation, stimulating insulin release. Its important role as an incretin hormone in the entero-insular axis has been described in severals studies [for review see (11)].

Receptors for GLP-1 were demonstrated on different rat insulinoma cell lines (6,10), rat gastric glands (27), rat lung membranes (23), and in certain brain regions (28). Cross-linking studies with RINm5F cell membranes have revealed a molecular mass of the GLP-1 receptor of 63,000 Da and have shown that the receptor protein does not contain S-H bound subunits (9). The biological action of GLP-1 is mediated by an activation of the adenylate cyclase system and a rise of the intracellular cAMP level (12). Furthermore, GLP-1 was reported to potentiate glucose-stimulated insulin release by gating the voltage-dependent Ca^{2+} channels to increase intracellular [Ca^{2+}] (19). Recently, using a rat islet cDNA library, a cDNA for the GLP-1 receptor was isolated (26). The receptor protein contains seven transmembrane regions. The GLP-1 receptor belongs to the family of G-protein-coupled receptors and shows sequence homology to the secretin receptor (13), the parathyroid hormone receptor (15), the calcitonin receptor (18), and the glucagon receptor (14,25). The *N*-terminal region of the GLP-1 receptor contains a hydrophilic domain of about 120 amino acids that exhibits three *N*-linked glycosylation sites (26). To date, no information about GLP-1 receptor glycosylation and its possible meaning for receptor function is available. The present study shows that the GLP-1 receptor in RINm5F cells is a functional glycoprotein and that glycosylation is a prerequisite for normal receptor function.

Because of the limited availability of islet tissue, especially in amounts necessary for biochemical studies, in our experiments we used the RINm5F cell line. This cell line is derived from a radiation-induced, insulin-producing tumor (7). RINm5F cells show many of the differentiated functions characteristic for B-cells (29), and are, therefore, widely used as a model for studies on insulin secretory mechanisms.

METHOD

Reagents

Glucagon-like peptide-1(7–36)amide was purchased from Bissendorf Biochemicals (Hannover, Germany). Tunicamycin was from Calbiochem (Bad Soden, Germany). Stock solutions were made by dissolving tunicamycin in 0.01 N NaOH; they were stored frozen in aliquots. Disuccinimidyl suberate (DSS) was obtained from Pierce Europe B.V. (Oud-Beijerland, The Netherlands). [^{125}I]GLP-1(7–36)amide (specific activity approximately 2000 Ci/mmol) was prepared as described previously (10).

Cell Culture

RINm5F cells were grown in plastic culture bottles under conditions as described by Praz et al. (21). For experiments, cells at stationary growth were diluted with fresh medium and incubated at 37°C for 65 h with or without tunicamycin (0–1 μg/ml). Control cells received equivalent amounts of 0.01 N NaOH.

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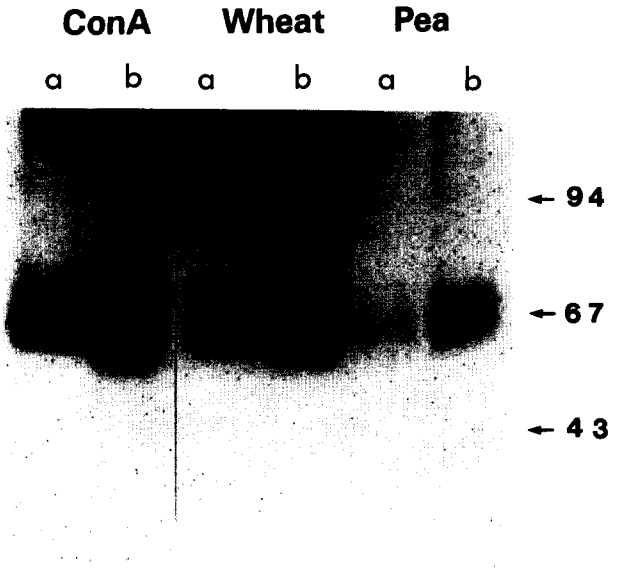

FIG. 1. Binding of radiolabeled GLP-1 receptor to different lectins. RINm5F cell plasma membranes were incubated with [^{125}I]GLP-1 for 30 min at 37°C and then treated with 0.1 mmol/l DSS. The labeled receptor was then applied to the lectin columns and recovered by washing the columns with an excess of the appropriate sugars.

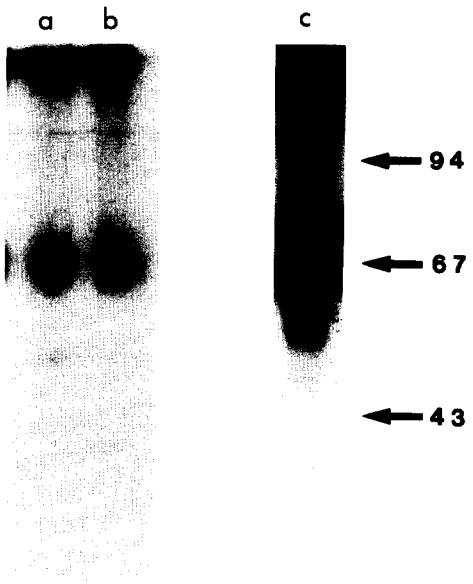

FIG. 2. Glycopeptidase F treatment of labeled GLP-1 receptor. Unpurified (a) and purified RINm5F cell plasma membranes were incubated with [^{125}I]GLP-1, treated with DSS, and then submitted to glycopeptidase F treatment as described in the Method section. Control samples (b) did not receive any enzymes. The labeled proteins were analyzed by SDS-PAGE. M_r standards are indicated on the right side by arrows.

The cells were detached from the surface of the bottles before experimentation using phosphate-buffered saline (NaCl, 136 mmol/l; KCl, 2.7 mmol/l; Na_2HPO_4, 8.1 mmol/l; KH_2PO_4, 1.5 mmol/l, pH 7.3) containing EDTA (0.7 mmol/l). Cell concentrations were determined by measurement of cellular DNA (2).

Binding Studies

Detached RINm5F cells were centrifuged (100 × *g*, 3 min) and resuspended in incubation buffer (Tris-HCl, 2.5 mmol/l; pH 7.4; NaCl, 120 mmol/l; $MgSO_4$, 1.2 mmol/l; KCl, 5 mmol/l; CH_3COONa, 15 mmol/l) containing 1% (w/v) human serum albumin, bacitracin (0.1%; w/v), and EDTA (1 mmol/l). Cells were incubated for 30 min at 37°C with [^{125}I]GLP-1 (approximately 20,000 cpm) in the absence or presence of a range of concentrations of unlabeled GLP-1. The reaction was stopped by centrifugation of aliquots of the cell suspension through an oil layer (10). The pellets were counted in a gamma-counter. Unspecific binding was defined as binding of tracer in the presence of 1 μmol/l unlabeled GLP-1.

Membrane Preparation

RINm5F plasma membranes were prepared as described previously (9). Briefly, RINm5F cells were suspended in ice-cold homogenization buffer (Tris-HCl, 10 mmol/l, pH 7.5; NaCl, 30 mmol/l; dithiothreitol, 1 mmol/l; phenylmethylsulphonyl fluoride 5 μmol/l) and disrupted in a glass-glass potter. The suspension was layered over a 41% (w/v) sucrose solution and centrifuged at 100,000 × *g* for 60 min at 4°C. The band of membranes at the interface of the layers was collected and diluted fourfold with homogenization buffer. Membranes were collected by centrifugation at 40,000 × *g* for 30 min at 4°C. The pellets were stored in liquid nitrogen. Protein was measured as described by Bradford (1).

Covalent Cross-Linking Experiments

[^{125}I]GLP-1 was cross-linked to RINm5F plasma membranes as described previously (9). In brief, plasma membranes (1 mg protein/ml) were incubated with [^{125}I]GLP-1 (approximately 9 × 10^5 cpm) with or without unlabeled GLP-1 (1 μmol/l) in HEPES buffer (50 mmol/l, pH 7.5) containing 0.02% (w/v) human serum albumin for 30 min at 37°C. After centrifugation for 5 min at 10,000 × *g* and 4°C, pellets were resuspended in HEPES buffer (pH 9.0). A solution of DSS dissolved in dimethyl sulfoxide was added to give a final concentration of 0.1 mmol/

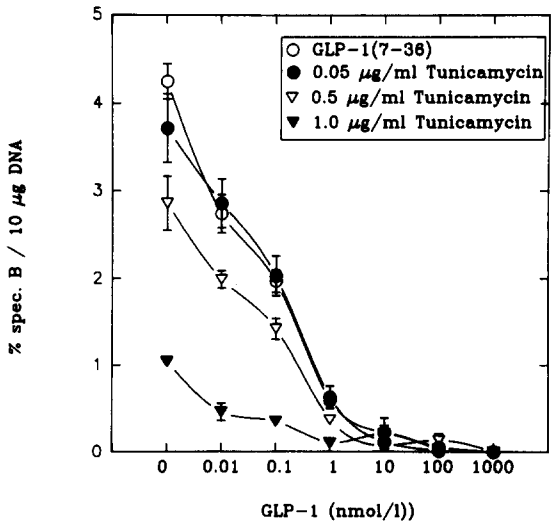

FIG. 3. Effect of tunicamycin on GLP-1 binding. RINm5F cells were incubated for 65 h at 37°C with a range of tunicamycin concentrations. Then cells were incubated for 30 min at 37°C with [^{125}I]GLP-1 in the presence or absence of unlabeled GLP-1 in the concentrations indicated. Data points show means ± SEM of six experiments.

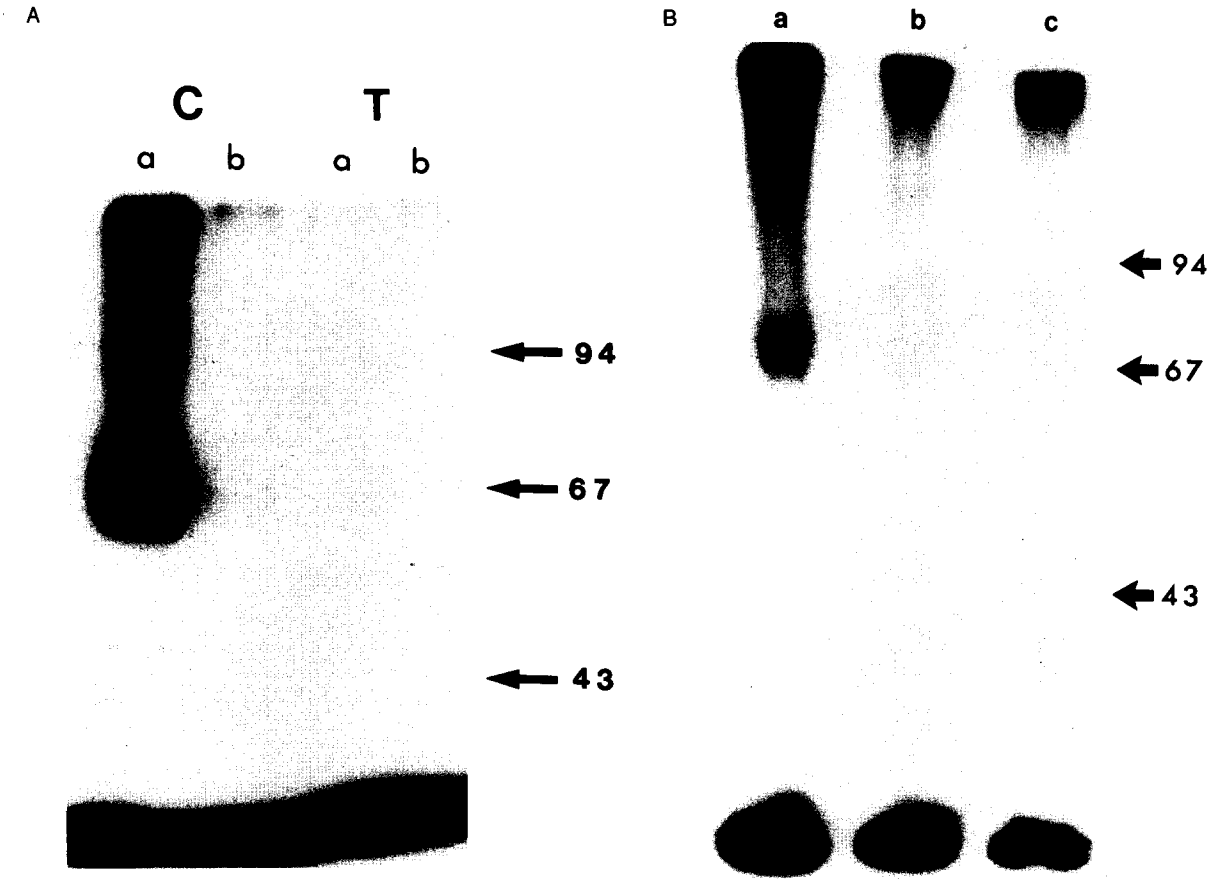

FIG. 4. (A) Cross-linking of [^{125}I]GLP-1 to plasma membranes of untreated or tunicamycin-treated RINm5F cells. Cells were incubated for 65 h at 37°C without (C) or with (T) tunicamycin (1.0 μg/ml). After incubation with [^{125}I]GLP-1 in the absence (a) or presence (b) of 1 μmol/l unlabeled GLP-1 for 30 min at 37°C cells were treated with DSS. The labeled proteins were analyzed by SDS-PAGE. M_r standards are indicated on the right side by arrows. (B) Cross-linking of [^{125}I]GLP-1 to plasma membranes of untreated and tunicamycin-treated RINm5F cells. Cells were incubated for 65 h at 37°C without (a) or with 0.5 μg/ml (b) or 1.0 μg/ml (c) tunicamycin. After incubation with [^{125}I]GLP-1 for 30 min at 37°C, cells were treated with DSS. The labeled proteins were analyzed by SDS-PAGE. M_r standards are indicated on the right side by arrows.

l. After incubation for 10 min at 2°C, the reaction was stopped by addition of ammonium acetate (final concentration 10 mmol/l). Membranes were centrifuged at 10,000 × *g* and 4°C for 5 min and washed twice with ice-cold HEPES buffer (pH 7.5). Finally, pellets were resuspended in sodium phosphate buffer (10 mmol/l, pH 7.5) containing 2% sodium dodecylsulphate (SDS). Samples were boiled for 5 min. Electrophoresis was carried out as described by Laemmli (16). After drying gels were exposed to Kodak type X-Omat AR film for up to 2 weeks at −80°C using an image-intensifying screen.

Binding to Lectin-Linked Sepharose

Before cross-linking with labeled GLP-1(7–36)amide, aliquots of the solubilized receptor were loaded onto lectin columns (8) (first column, 0.5 ml concanavalin A, 10–15 mg lectin/ml gel attached to Sepharose 4B; second column, 0.5 ml wheat germ, 5 mg lectin/ml gel insolubilized on Sepharose 6MB; and third column, 0.5 ml peanut agglutinin, 10 mg lectin/ml gel attached to Sepharose 4B, samples were loaded with/without previous sialidase treatment to expose putative binding dissacharide). After washing the columns with 50 m*M* HEPES buffer (pH 7.4) containing 150 m*M* NaCl, 10 m*M* $MgSO_4$, and 0.1% (w/v) digitonin, the receptors were eluted with the aforementioned HEPES buffer containing the appropriate sugar [con A, 0.3 *M* α-D-mannopyranoside; wheat germ, 0.3 *M* *N*-acetyl-β-D-glucosamin; and pea, 0.3 *M* galactose (β1–3)-galNac]. Samples were further analyzed by gel electrophoresis followed by autoradiography as described.

Glycopeptidase F (PNGase F) Treatment

After cross-linking, labeled receptors were solubilized in PNGase F buffer as has been detailed before (5). PNGase F (20 units/ml reaction mixture) was added and the mixture was incubated overnight at 37°C. Controls did not receive any enzyme. Samples were then analyzed by gel electrophoresis and autoradiography.

Preparation of RINm5F Cytosol

RINm5F cell cytosol was prepared after harvesting the cells and sonification of the cell pellets (10 s) in HEPES buffer with 0.1 m*M* PMSF and 1 m*M* benzamidine followed by ultracentrifugation (4°C, 40,000 rpm). The supernatant was then saved and subjected to the cross-linking procedure.

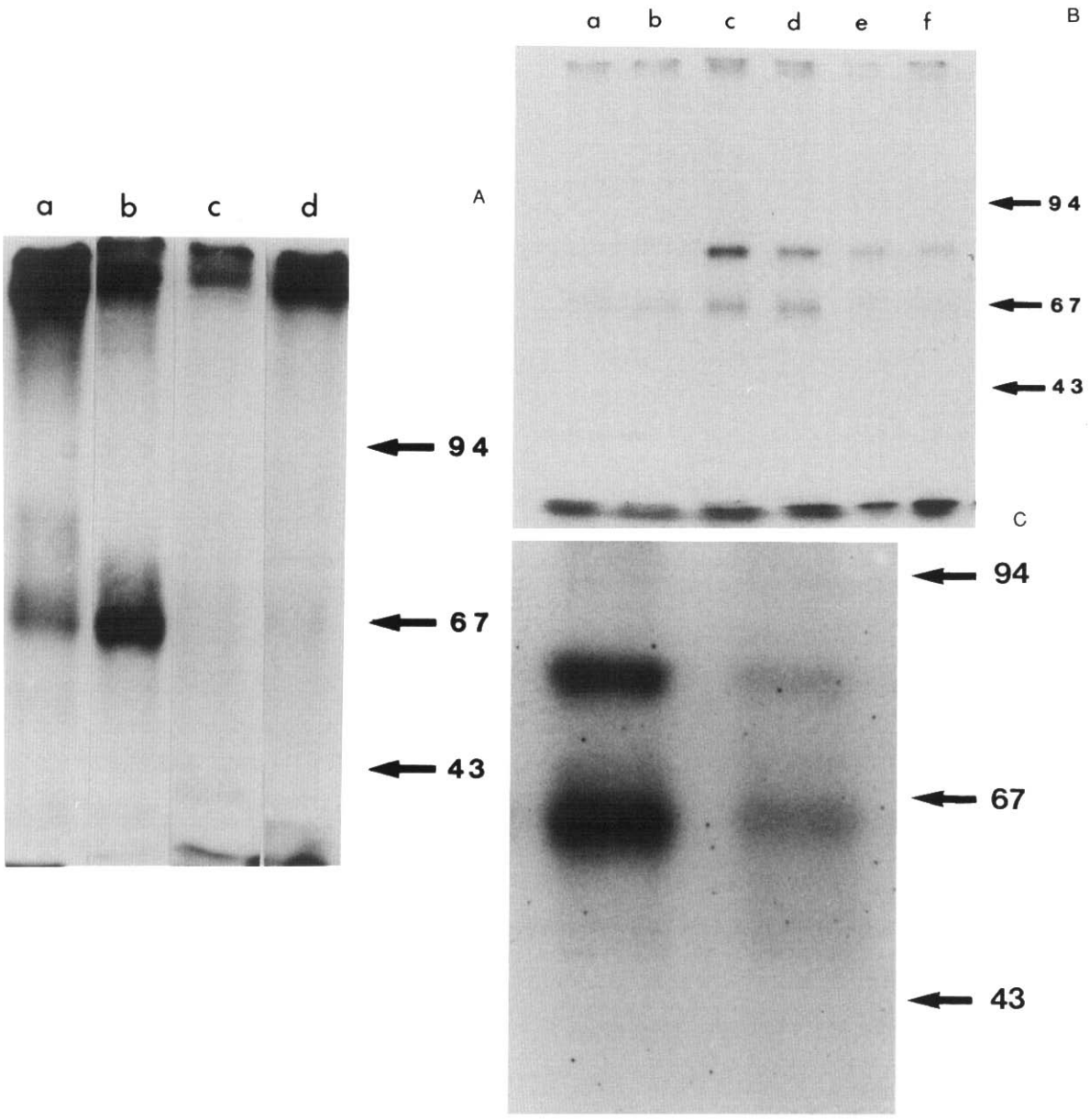

FIG. 5. (A) Cross-linking of [^{125}I]GLP-1 to plasma membranes and cytosol of RINm5F cells. Plasma membranes (a) and cytosol (b,c) of RINm5F cells were incubated for 30 min at 37°C with [^{125}I]GLP-1 without (a,b) or with (c) 1 μmol/l unlabeled GLP-1 and then treated with DSS. As a control, cytosol of receptor-negative cells was incubated for 30 min at 37°C with [^{125}I]GLP-1 before treatment with DSS. The labeled proteins were analyzed by SDS-PAGE. M_r standards are indicated on the right side by arrows. (B) Cross-linking of [^{125}I]GLP-1 to receptors in cytosol from untreated or tunicamycin-treated RINm5F cells. Cytosol from untreated (a,b) or tunicamycin-treated [0.5 μg/ml (c,d); 1.0 μg/ml (e,f)] cells was incubated with [^{125}I]GLP-1 for 30 min at 37°C and then treated with DSS. The labeled proteins were analyzed by SDS. M_r standards are indicated on the right side by arrows. (C) Cross-linking of [^{125}I]GLP-1 to receptors in cytosol from tunicamycin-treated RINm5F cells in the absence (left lane) or presence of an excess of unlabeled GLP-1 (1 μM; right lane).

Preparation of GLP-1 Receptor mRNA From RINm5F Cells

Total RNA was isolated from cells according to Chomczynski and Sacchi utilizing RNA isolation kits (3).

Northern Blot

A 1500 bp Eco RI fragment of the cDNA coding for rat GLP-1(7–36)amide receptor (gift of Dr. Thorens, Lausanne) (26) was radioactively labeled with [α^{32}P]dCTP, utilizing a random priming procedure (Amersham) according to the supplier's protocol and was purified with Nuctrap Columns. RNA was fractionated on 1% agarose gels containing 2.2 *M* formaldehyde (28), transfered to Hybond N membranes, and immobilized by UV cross-linking. Hybridizations were performed in 50% formamide, 5 × Denhardt's, 5 × SSC, and 0.1 mg/ml sonicated herring sperm DNA at 42°C using 2–3 × 10^6 cpm of the labeled

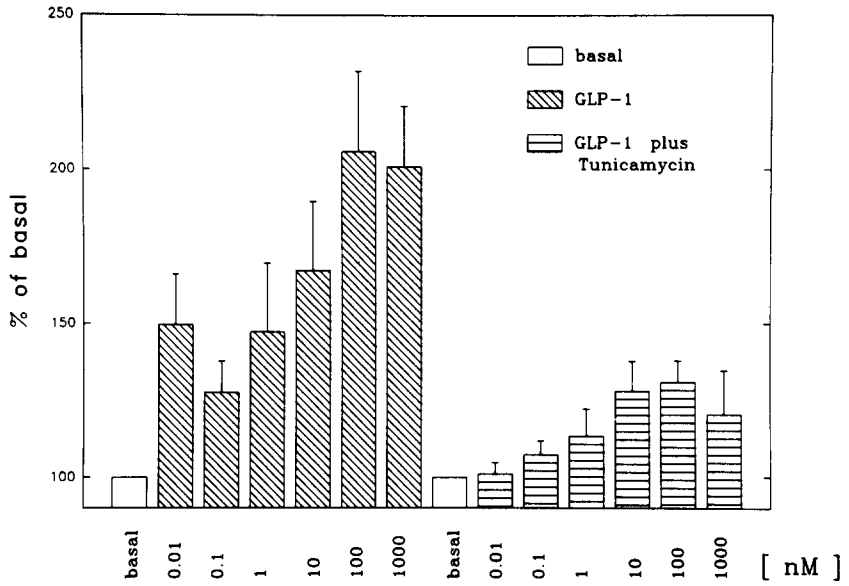

FIG. 6. Effect of GLP-1 on the cAMP generation in untreated or tunicamycin-treated RINm5F cells. Cells were treated without or with 1.0 μg/ml tunicamycin for 65 h at 37°C. Then cells were incubated with a range of GLP-1 concentrations for 3 min at 37°C. cAMP levels were determined as described in the Method section. Data shown are means ± SEM of six experiments.

cDNA fragment. Final washings were performed in 0.1 × SSC at 42°C. RNA quantity and integrity was verified by reversibly staining the Hybond N membranes with methylene blue prior to hybridization. Migration positions of the signals were calculated as compared to RNA markers.

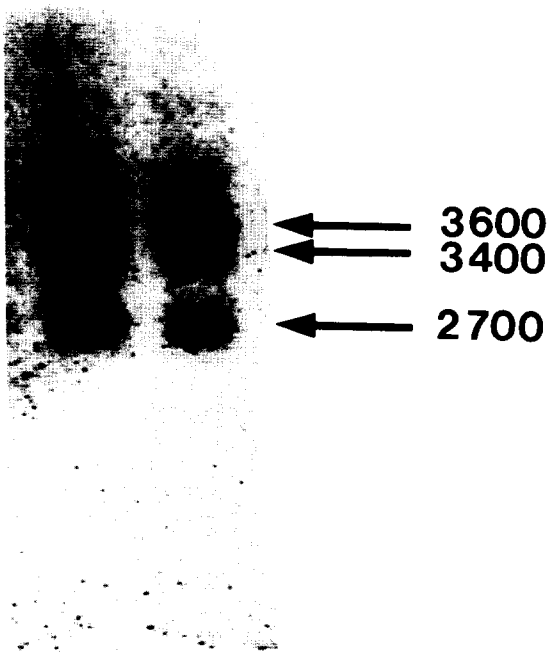

FIG. 7. Northern blot analysis of GLP-1 receptor expression in RINm5F cells with (right lane) or without (left lane) tunicamycin pretreatment (1 μg/ml). A 1500 bp Eco RI cDNA fragment coding for the rat GLP-1 receptor was used as probe.

Assay for cAMP

Cells in 500 μl buffer (HEPES, 10 mmol/l, pH 7.4; NaCl, 113 mmol/l; KCl, 4.7 mmol/l; KH_2PO_4, 1.2 mmol/l; $CaCl_2$, 2.5 mmol/l; $MgSO_4$, 1.2 mmol/l) containing 1% (w/v) human serum albumin were incubated for 3 min at 37°C with or without peptide. The reaction was stopped by addition of 200 μl ice-cold trichloroacetic acid (12%; w/v), sonication for 15 s at 50-W power (Labsonic 2000, Braun, Melsungen, Germany), and centrifugation for 5 min at 11,500 × *g* at 4°C. After extraction with diethyl ether, samples were freeze dried and redissolved in 200 μl sodium acetate buffer (50 mmol/l, pH 5.8). cAMP concentrations were determined using a test kit (Amersham–Buchler, Braunschweig, Germany) according to the manufacturer's instructions.

RESULTS

To determine whether the GLP-1 receptor is a glycoprotein, we initially investigated the ability of the cross-linked receptor protein to bind to immobilized WGA, Con A, or Pea. For this, the cross-linked receptor was solubilized and applied to the corresponding lectin. Approximately 5% of the radiolabeled GLP-1–receptor complex passed unbound WGA and approximately 25% Con A (Fig. 1). The remainder was bound to WGA and Con A and was readily eluted by an excess of *N*-acetylglucosamine and α-methylmannoside, respectively. Only a small amount of the peptide–receptor complex bound to Pea, irrespective of whether the receptors were pretreated with sialidase or not.

To examine whether the receptor protein contains *N*-linked carbohydrates, plasma membranes were cross-linked with [^{125}I]GLP-1 and then treated with glycopeptidase F. As shown in Fig. 2, treatment with glycopeptidase F resulted in an altered electrophoretic mobility of the peptide–receptor complex. The major complex with a molecular mass of 63,000 ± 1000 Da was partially converted to a smaller complex with a molecular mass

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