0026-895X/87/050565-07$02.00/0

MOLECULAR PHARMACOLOGY, 32:565–571

ACCELERATED COMMUNICATION

Glycosylation of the Mammalian α_1-Adrenergic Receptor by Complex Type *N*-Linked Oligosaccharides

DAVID G. SAWUTZ, STEPHEN M. LANIER, CHRISTOPHER D. WARREN, and ROBERT M. GRAHAM†

Cellular and Molecular Research Laboratory, Cardiac Unit (D.G.S., S.M.L., R.M.G.), and the Laboratory for Carbohydrate Research (C.D.W.), Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

Received June 2, 1987; Accepted August 4, 1987

SUMMARY

The binding subunit of the α_1-adrenergic receptor has been identified as an M_r = 80,000 peptide in several tissues. Adsorption of the α_1-adrenergic receptor to a wheat germ agglutinin lectin-agarose resin suggests that the receptor protein is glycosylated. In this study, we investigated the nature of the carbohydrate chains linked to the α_1-adrenergic receptor peptide. The α_1-adrenergic receptor from DDT_2 MF-2 smooth muscle cell and rat brain membranes was photolabeled with ^{125}I-azido-prazosin ([^{125}I]CP65,526) and then treated with exoglycohydrolases prior to SDS-PAGE and autoradiography. Removal of terminal sialic acid residues by neuraminidase decreased the receptor M_r by 6,000; however, α-mannosidase was without effect, indicating complex type glycosylation of the receptor-protein. Similar results were observed for the rat hepatic membrane α_1-adrenergic receptor. Removal of N-linked carbohydrates at asparagine residues by peptide-N^4[*N*-acetyl-β-glucosaminyl]asparagine amidase (from *Flavobacterium meningosepticum*) resulted in a specifically labeled peptide at M_r = 50,000–55,000 in DDT_1 MF-2 membrane and solubilized receptor preparations. Treatment of DDT_1 MF-2 cells with swainsonine or (+)-1-deoxymannojirimycin, inhibitors of complex type carbohydrate chain biosynthesis, caused a reduction in the apparent molecular weight of the receptor (M_r = 60,000) but did not alter the number of α_1-adrenergic receptors per cell or their affinity for the radioligand [^{3}H]prazosin. These findings indicate that the α_1-adrenergic receptor is heavily glycosylated, the major oligosaccharide moiety being of the complex type, *N*-linked to asparagine residues. The peptide backbone of the receptor has an $M_r \leq 55{,}000$, consistent with the predicted molecular mass of other membrane neurotransmitter receptors based on sequence analysis of isolated cDNA clones.

The α_1-adrenergic receptor has been purified to a homogeneity and the hormone binding subunit identified as an M_r = 80,000 peptide by photoaffinity labeling (for review see Ref. 1). Insel and coworkers (2) have demonstrated that the α_1-adrenergic receptor can be adsorbed to lectin resins, a finding that has been confirmed by Lomasney *et al.* (3). This suggests that the α_1-adrenergic receptor, like several other membrane receptors, is likely to be a glycoprotein (4–8). Carbohydrate chains associated with the receptor may be either *N*-linked to asparagine residues or *O*-linked to serine or threonine residues in the peptide backbone (9, 10). The specific functions of these carbohydrate moieties are not clearly understood, but may be important in the processing of the glycoprotein for insertion into the plasma membrane or in receptor metabolism.

As an initial approach to understanding the role of the carbohydrate moieties in α_1-adrenergic receptor receptor function, we have attempted to characterize the type of oligosaccharide chains linked to the receptor-protein and to determine the molecular weight of the receptor's peptide backbone. In the present study, we have photolabeled the α_1-adrenergic receptor in DDT_1 MF-2 smooth muscle cell and rat brain membranes using [^{125}I]CP65,526, an arylazide analog of prazosin (11) and then treated the photolabeled receptor preparations with various exo- and endoglycohydrolases. Based on our results, we conclude that the α_1-adrenergic receptor is a glycoprotein that has complex type carbohydrate chains N-linked to asparagine residues. The molecular weight of the deglycosylated receptor protein is no greater than 50,000–55,000 following enzymatic removal of the *N*-linked carbohydrate chains.

These studies were supported in part by National Institutes of Health (NIH) Grant NS-19583 and American Heart Association (AHA) Grant 86-1197 with funds from the Massachusettss Affiliate. D. G. S. is supported by AHA Fellowship 13-414856, S. M. L. by NIH National Research Service Award F32 HL07127-01 and NIH First Award NS-24821, and C. D. W. by National Science Foundation Award 86-45896. R. M. G. is an Established Investigator, AHA (82-240).

ABBREVIATIONS: WGA, wheat germ agglutinin; DMN, deoxymannojirimycin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GlcNAc, *N*-acetylglucosamine; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate; LDL, low denisty lipoprotein.

Experimental Procedures

Materials. DDT_1 MF-2 smooth muscle cells were grown in culture to confluency as previously described (12, 13). Carrier-free Na[^{125}I] was purchased from Amersham, Inc. [^{3}H]Prazosin (82 Ci/mmol) was obtained from New England Nuclear, Inc., Boston, MA. [^{125}I]CP65,526 (specific activity 2,175 Ci/mmol) was prepared by radioiodination of the prazosin analog 2-[4-(4-azido-3-iodobenzoyl)piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline (CP63,155) and purified as previously described (11). Cell culture reagents were purchased from Biofluids, Inc., Rockville, MD. Low molecular weight proteins (14,000–94,000 Da) for electrophoresis calibration were obtained from Pharmacia. *N*-Glycanase (peptide-N^4[*N*-acetyl-β-glucosaminyl]asparagine amidase) and (+)-1-DMN were purchased from Genzyme, Inc., Boston, MA. Neuraminidase was obtained from Boerhinger-Mannheim, Inc., Indianapolis, IN. α-Mannosidase (from jackbean), (−)-epinephrine, WGA-Sepharose, swainsonine, and all other chemicals were obtained from Sigma Chemical Company, St. Louis, MO. Digitonin was purchased from Wako Chemical Company, Dallas, TX. Prazosin and CP63,155 were the kind gift of Dr. Hans-Jürgen Hess, Medicinal Chemistry, Pfizer Central Research. Leupeptin, chymostatin, benzamidine, and antipain were obtained from Tranformation Research Inc., Framingham, MA.

Membrane and solubilized receptor preparations. DDT_1 MF-2 smooth muscle cells, grown in 2-liter suspension bottles until a cell density of 5×10^5 cells/ml was achieved, were harvested by centrifugation at 1200 rpm for 10 min and then washed twice in 50-ml volumes of buffer A (150 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, 2.5 mM EGTA; pH 7.4). The final cell pellets (1×10^9 cells) were resuspended in 10–15 ml of buffer A, and cell counts were measured with a hemocytometer. Cell viability was determined by trypan blue exclusion. Plasma membranes from DDT_1 MF-2 cells or rat brain were prepared as described elsewhere (12–14). All buffers used for membrane and solubilized receptor preparations contained the following protease inhibitors: PMSF (1 mM), bacitracin (1 mM), leupeptin (10 μg/ml), chymostatin (10 μg/ml), antipain (20 μg/ml), and benzamidine (10 μg/ml).

The α_1-adrenergic receptor was solubilized from DDT_1 MF-2 cell membranes by incubating in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% digitonin, 1 mM $MgCl_2$, 2.5 mM EGTA, and protease inhibitors for 1 hr at 23°. The resulting suspension was centrifuged for 1 hr at 40,000 rpm in a Beckman Ti-60 rotor (100,000 × *g*) and the solubilized preparation was stored in liquid N_2 until needed.

WGA receptor purification. Solubilized α_1 receptor preparations (10 mg/ml; 7.0 pmol of receptors) were incubated with 2–4 ml of WGA-agarose overnight at 4° in a batch technique (15). The WGA-agarose resin was washed extensively with buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 2.5 mM EGTA, 0.1% digitonin, and protease inhibitors until no protein was detected in the wash buffer. The α_1 receptor was eluted from the WGA-agarose resin by incubation for 1 hr at 4° in the same buffer that included 0.3 M GlcNAc. The GlcNAc elutions were then assayed for protein content and [^{3}H]prazosin binding activity prior to storage at −20° until needed.

[^{3}H]Prazosin binding assays. Equilibrium saturation binding experiments were done by incubating DDT_1 MF-2 cell membranes (100 μg) with increasing concentrations of [^{3}H]prazosin (0.1–6.0 nM) in the absence or presence of 1.0 μM prazosin for 30 min at 23°. Final assay volumes were 150–300 μl, and all assays were done in triplicate. The binding assays were terminated by placing the assay tubes in ice water for 10 min followed by the addition of 4 ml of ice-cold wash buffer (100 mM Tris-HCl, pH 7.4) and vacuum filtration through glass fiber filters. The filters were washed an additional 3 times in 4 ml of buffer and counted for trapped radioactivity in 10 ml of scintillation cocktail. The binding of [^{3}H]prazosin to intact DDT_1 MF-2 cells was done in the same manner except that phentolamine (100 μM) was used as the competing ligand and the glass fiber filters were washed with 100 mM Tris buffer (pH 7.4) at room temperature containing 0.1% bovine serum albumin.

The binding of [^{3}H]prazosin to solubilized receptors was determined by incubating 100–200 μl of solubilized extract with radioligand and buffer or 1.0 μM prazosin for 1 hr at 23°. The unbound radioligand was separated by vacuum filtration through glass fiber filters after precipitation of solubilized receptor protein by incubating at 4° for 10 min with 1 ml of bovine γ-globulin (1 mg/ml) in 50 mM Tris (pH 7.4) and 1 ml of 25% polyethylene glycol 8000 in water. Glass fiber filters were washed in four 4-ml aliquots of ice-cold 8% polyethylene glycol in 50 mM Tris buffer.

Protein concentrations were determined by the method of Bradford (16).

Photoaffinity labeling of α_1-adrenergic receptor. DDT_1 MF-2 and rat brain membranes (100–150 μg) in 400 μl of buffer (10 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 2.5 mM EGTA, protease inhibitors, pH 7.4) were incubated with 50 μl of [^{125}I]CP65,526 (0.3 nM) and 50 μl of buffer or competing ligand for 45 min at 23° in the dark. The samples were then photolyzed for 15 min with a hand-held, long wave ultraviolet lamp. Following photolysis, 500 μl of ice-cold buffer containing 1 mM glutathione were added to each sample, the samples were centrifuged for 5 min in an Eppendorf Microfuge, and the supernatants were discarded. The membrane pellets were resuspended and washed in the appropriate buffer for the glycohydrolase experiments described below. The final membrane pellets were resuspended in 100 μl of high sucrose quench buffer [30% (w/v) sucrose, 0.45 M dithiothreitol, 6% (w/v) SDS, 60 mM EDTA, and bromophenol blue] and allowed to solubilize overnight in the dark before SDS-PAGE analyses on 10% (w/v) polyacrylamide gels (17). SDS-PAGE gels were dried on a Bio-Rad gel drier, and autoradiography was performed by exposing Kodak XAR-5 film on the dried gels at −70° for up to 1 week. Photoaffinity labeling of the α_1-adrenergic receptor in intact DDT_1 MF-2 cells was done as previously described (13). Solubilized receptor preparations were photolabeled in the same manner and the samples were exchanged into the appropriate buffer by gel filtration over 10-ml Sephadex G-50 columns.

Exoglycohydrolase experiments. [^{125}I]CP65,526-labeled DDT_1 MF-2 and rat brain membranes (100–150 μg) were resuspended in 400 μl of 100 mM sodium acetate buffer containing 10 mM EDTA and protease inhibitors at pH 4.5 for neuraminidase and pH 5.0 for α-mannosidase. Neuraminidase (0.12 unit/ml final concentration) or α-mannosidase (25 units/ml final concentration) was added to the photolabeled membranes and incubated overnight at 37° in a final assay volume of 500 μl. The membranes were resuspended to 1 ml with phosphate-buffered saline, pelleted in a Microfuge, and resuspended in 100 μl of high sucrose quench buffer for SDS-PAGE analysis.

***N*-Glycosidase experiments.** Photolabeled DDT_1 MF-2 cell membranes were resuspended in 120 mM sodium phosphate buffer (pH 8.6) in the presence of protease inhibitors and incubated overnight with *N*-Glycanase (40 units/ml) at 37°. The membranes were washed in 1 ml of phosphate-buffered saline and solubilized as above for SDS-PAGE analysis.

WGA-purified α_1 receptors (950 fmol/mg; 50 μg) were photolabeled as described above and exchanged into a buffer containing 10 mM Tris, 0.02% digitonin, and protease inhibitors (pH 8.6) by gel filtration. *N*-Glycanase (250 milliunits/50 μg of protein final concentration) was added to 1.5-ml aliquots of solubilized photolabeled receptor preparations, and the samples were incubated overnight at 37°. The samples were lyophilized and the residue resuspended in 100 μl of high sucrose quench buffer for SDS-PAGE analysis.

Inhibition of α_1-adrenergic receptor glycosylation. Fifty ml of confluent DDT_1 MF-2 cells (5×10^5 cells/ml) were incubated with phenoxybenzamine (10 μM) for 1 hr at 37° to irreversibly inactivate native α_1 receptors (in preliminary studies this treatment was found to inactivate >95% of the α_1-adrenergic receptor sites). The cells were washed once with 50 ml of Dulbecco's modified Eagle's medium and then resuspended in 20 ml of media in the absence or presence of either (+)-1-DMN (0.5 mM) or swainsonine (1.0 μM). After 48 hr in culture, the cells were harvested and washed in 50 ml of buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 2.5 mM EGTA, 0.02% NaN_3

and 0.1 mM PMSF) and then resuspended in buffer to a final density of 2–4 × 10^6 cells/ml. Intact DDT_1 MF-2 cells were then assayed for [^{3}H]prazosin binding activity, and the α_1-adrenergic receptors from cells treated with each agent were photolabeled with [^{125}I]CP65,526 as described above.

Data analysis. Radioligand binding data were evaluated with the mass action-based, weighted, nonlinear curve-fitting procedure LIGAND (18), on a VAX Digital computer system. Molecular weights of the photolabeled peptides were determined by linear regression analysis based on the mobility of protein standards electrophoresed simultaneously.

Results and Discussion

WGA binds to GlcNAc and sialic acid residues in the carbohydrate chains affixed to glycoproteins (19). The solubilized α_1-adrenergic receptor from DDT_1 MF-2 cell membranes binds to WGA-agarose and can be eluted by GlcNAc (Fig. 1A). Typically, a 15- to 30-fold purification of the receptor can be demonstrated with solubilized DDT_1 MF-2 and rat hepatic α_1 receptor preparations (Table 1). The affinity of the WGA-purified receptor from DDT_1 MF-2 membranes for [^{3}H]prazosin (89 ± 25 pM; n = 3) is similar to that in DDT_1 MF-2 membrane (120 ± 10 pM; n = 3) or whole cell preparations (140 ± 20 pM; n = 3). Based on photoaffinity labeling studies with [^{125}I]CP65,526, the WGA-purified α_1 receptor maintains its specificity for adrenergic ligands. As can be seen in Fig. 1B, prazosin and (−)-epinephrine inhibit the labeling of the partially purified receptor, whereas (+)-epinephrine and the β-adrenergic receptor antagonist, (−)-alprenolol, do not. These results indicate that the α_1-adrenergic receptor is a glycoprotein.

Analysis of the carbohydrate moieties of glycoproteins usually requires a minimum of μg quantities of purified material (20). To circumvent this problem, we have taken advantage of the ability to specifically label the hormone-binding site with the photoaffinity probe [^{125}I]CP65,526. By treating the photolabeled receptor with endo- and exoglycohydrolases and evaluating the resulting changes in the electrophoretic mobility of the receptor on SDS-polyacrylamide gels, the carbohydrate composition of sugars N-linked to the α_1 receptor via asparagine residues can be evaluated.

In order to characterize more completely the type of carbohydrate chains associated with the receptor, photolabeled α_1 receptors from DDT_1 MF-2 and rat brain membranes were treated with neuraminidase and α-mannosidase. Neuraminidase, which cleaves terminal sialic acid residues from complex type carbohydrate chains (10, 21), caused an increase in receptor mobility on 10% SDS-polyacraylamide gels (Fig. 2, *lanes 5* and *11*). This increase in mobility corresponds to a 6,000-Da decrease in the molecular weight of the receptor. Similar results were observed for the rat hepatic membrane α_1-adrenergic receptor (data not shown). In contrast, α-mannosidase, which removes terminal mannose residues from hybrid and high mannose type carbohydrate chains, had no effect on the mobility of the photolabeled receptor (Fig. 2, *lanes 8*, *13*, and *14*). It is of interest to note that a nonspecifically labeled peptide at 35,000–40,000 Da in DDT_1 MF-2 membranes (Fig. 2A; *lanes 7* and *8*) is a substrate for α-mannosidase. The decrease in molecular weight of this low molecular weight peptide by α-mannosidase suggests that the enzyme is active under the experimental conditions used in these studies. In addition, when a 4-fold greater concentration of enzyme was used in rat brain membranes (Fig. 2B, *lane 14*), still no effect was observed. These results suggest that the carbohydrate chains on the α_1-adrenergic receptor are of the complex type.

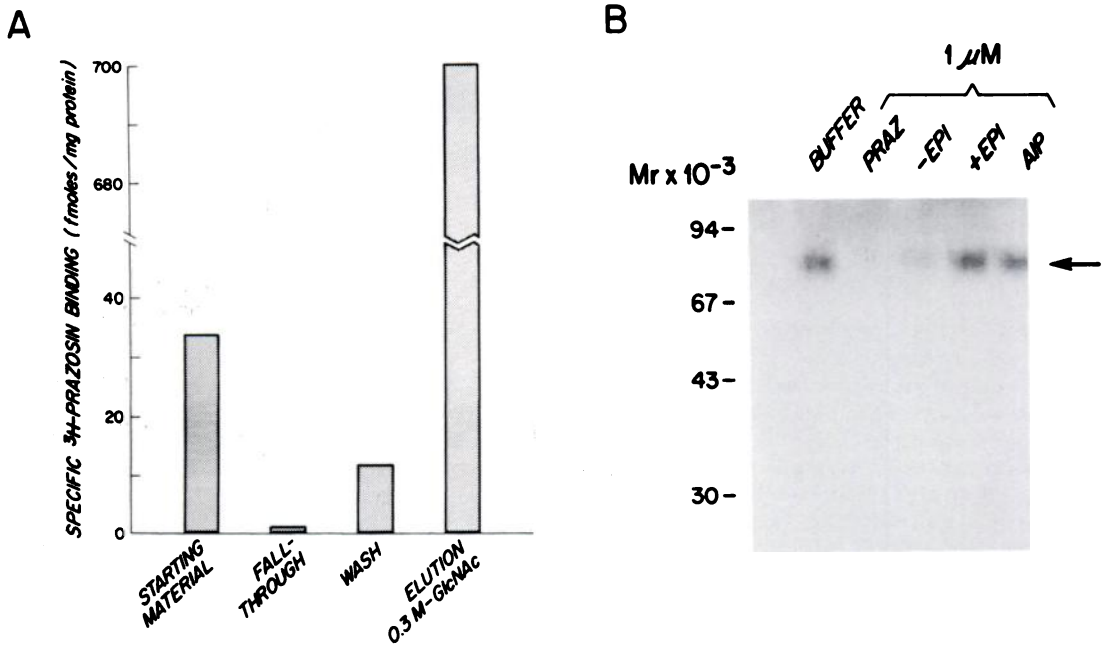

Fig. 1. WGA-agarose purifications of solubilized DDT_1 MF-2 α_1-adrenergic receptors. A. Solubilized α_1-adrenergic receptor was adsorbed to a WGA-agarose resin and eluted with 0.3 M GlcNAc as described under Experimental Procedures. Specific [^{3}H]prazosin binding (fmol/mg of protein) to each fraction was then determined. B. Demonstration of specificity in the photoaffinity labeling of the DDT_1 MF-2 α_1 receptor with [^{125}I]CP65,526 following WGA chromatography. ←, the photolabeled receptor band between 75,000 and 80,000 M_r. Molecular weights of known protein standards, electrophoresed simultaneously, are shown. *PRAZ*, prazosin; *EPI*, epinephrine; *AIP*, (−)-alprenodol. The data presented in A and B are from two different receptor preparations.

TABLE 1

Wheat germ lectin purification of α_1-adrenergic receptors from solubilized liver and DDT$_1$ MF-2 cell membranes

α_1-Adrenergic receptors were solubilized and then purified by WGA-agarose chromatography as described under Experimental Procedures. These data are the means and standard deviations of three preparations for each tissue.

	Specific activity	Fold purification
	fmol/mg	
DDT$_1$ MF-2 membranes		
Digitonin-solubilization	35.0 (2.0)	
WGA GlcNAc elution	598.0 (62.9)	17.3 (1.8)
Liver membranes		
Digitonin-solubilization	18.9 (1.1)	
WGA GlcNAc elution	591.2 (121.7)	31.4 (6.7)

The next series of experiments was conducted to gain an approximation of the molecular weight of the receptor's peptide backbone. When the photolabeled α_1 receptor in DDT$_1$ MF-2 membranes was treated with peptide-N^4[*N*-acetyl-β-glucosaminyl]asparagine amidase (*N*-Glycanase), an enzyme which selectively removes all classes of *N*-linked carbohydrate chains from glycoproteins (22), a marked decrease in apparent molecular weight of the receptor was observed (Fig. 3A, *lane 4*). The major receptor subunit at 75,000–80,000 Da migrated to a position between 50,000 and 55,000 Da following enzyme treatment.

A similar pattern was observed when WGA partially purified α_1 receptor was photolabeled with [^{125}I]CP65,526 and then treated with *N*-Glycanase. Photolabeled receptor treated for 4 or 18 hr at 37° in the absence of enzyme (Fig. 3, *lanes 5* and *9*), migrated as a broad band with an apparent molecular weight of 80,000. After 4 hr of *N*-Glycanase treatment, a second band at 65,000–70,000 Da is apparent (Fig. 3B, *lane 7*). After 18 hr of enzyme treatment, a single, narrow band at 50,000–55,000 Da is observed (Fig. 3B, *lane 11*). No photolabeled receptor was observed under any condition when 10 μM prazosin was included during the photolabeling step. The decrease in receptor molecular weight observed with *N*-Glycanase is not likely to be due to proteolysis since 1) a battery of protease inhibitors (see Experimental Procedures) was included in all buffers to ensure against proteolytic activity and 2) incubation of bovine serum albumin with *N*-Glycanase under the same experimental conditions did not result in a decrease in the molecular weight of the albumin following SDS-PAGE.

The significant decrease in molecular weight caused by *N*-Glycanase (≥25% of the native receptor) suggests that a large component of the carbohydrate chains coupled to the α_1 receptor is *N*-linked at asparagine residues. In agreement with our studies, a preliminary communication by Terman and Insel (23) suggests that the molecular weight of the deglycosylated α_1 receptor is 51,000, based on endoglycosidase F cleavage of *N*-linked carbohydrates from solubilized photoaffinity-labeled receptors of BC3H$_1$ smooth muscle cells. The large increase in mobility of the *N*-deglycosylated receptor may not necessarily reflect an equivalent decrease in molecular weight of the receptor. For example, it has been shown that the LDL receptor appears as a larger molecular weight species following the processing of *O*-linked sugars due to anomalous behavior during SDS-PAGE (24). It is possible, therefore, that some carbohydrate chains *O*-linked to serine or threonine residues may remain following *N*-glycanase treatment. Characterization of *O*-linked sugars by evaluating molecular weight shifts in the photolabeled receptor is problematic since chemical cleavage techniques for evaluating *O*-linked sugars, such as the standard technique of Iyer and Carlson (25) (which employs 50 mM NaOH and 2 M $NaBH_4$), can hydrolyze peptides and/or cause a chemical cleavage of the photolabel from the peptides (data not shown). However, the appearance of a narrow photolabeled band after *N*-Glycanase treatment (Fig. 3B, *lane 11*) suggests that either there is little microheterogeneity of *O*-linked sugars or that the receptor is completely deglycosylated.

The exact function of the carbohydrate component of the α_1 receptor-glycoprotein is not clear. Glycosylation is an important step in the structural maturation of cell-surface proteins (9). Inhibition of *N*-glycosylation impairs the normal function of many cell-surface receptors including the receptor for epidermal growth factor (26), acetylcholine (27), and insulin (28). Further evidence in support of this hypothesis was recently demonstrated in LDL-D14 cells, a mutant Chinese hamster

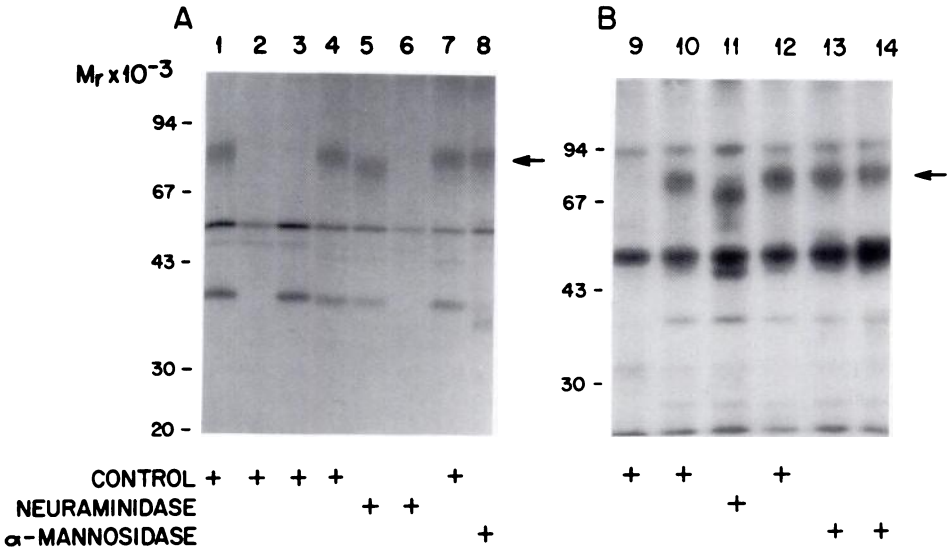

Fig. 2. The effects of neuraminidase and α-mannosidase on photolabeled α_1-adrenergic receptors. α_1-Adrenergic receptors in DDT$_1$ MF-2 (A) and rat brain (B) membranes (100–150 μg) were photolabeled with [^{125}I] CP65,526 (0.3 nM) in the absence (*lanes 1, 4, 5, 7, 8,* and *10–14*) and presence of 10 μM prazosin (*lanes 2* and *9*) or 100 μM (−)-epinephrine (*lane 3*). The photolabeled membranes were treated with neuraminidase and α-mannosidase as indicated and as described under Experimental Procedures. The final neuraminidase concentration was 0.12 unit/ml and that of α-mannosidase was 25 units/ml in *lanes 8* and *13*; and 100 units/ml in *lane 14*. ←, the α_1 receptor hormone-binding subunit. The data are representative of two to five experiments for each tissue. Molecular weights of known standards, electrophoresed simultaneously, are shown.

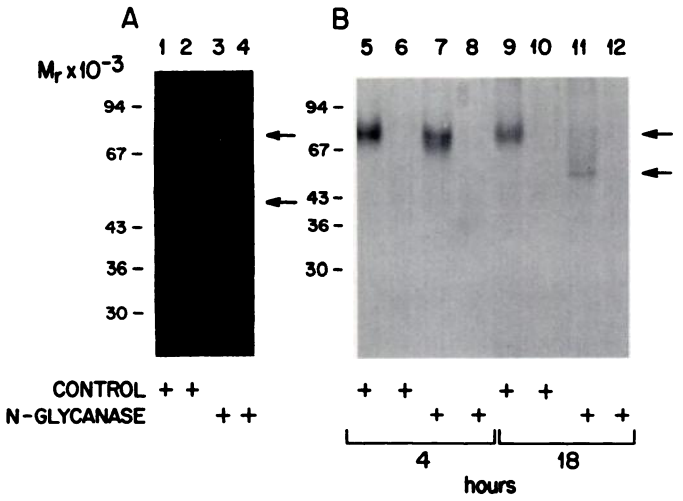

Fig. 3. The effect of *N*-Glycanase (peptide-N⁴[*N*-acetyl-β-glucosaminyl] asparagine amidase) on photolabeled α_1 receptors in DDT_1 MF-2 membranes (A) and WGA-purified (B) preparations. DDT_1 MF-2 membranes and solubilized α_1 receptor were photolabeled with [^{125}I]CP65,526 (0.3 nM) in the absence (*lanes 1, 3, 5, 7, 9,* and *11*) or presence (*lanes 2, 4, 6, 8, 10,* and *12*) of 10 μM prazosin. The receptor preparations were then treated with *N*-Glycanase as described under Experimental Procedures for the indicated period of time at 37°. Photolabeled membranes were treated with enzyme for 18 hr. *Arrows* indicate the position of the labeled receptor before and after *N*-Glycanase treatment and following SDS-PAGE on 10% acrylamide gels. Molecular weights of known standards, electrophoresed simultaneously, are shown. The data presented are representative experiments of three membrane and five solubilized α_1 receptor preparations.

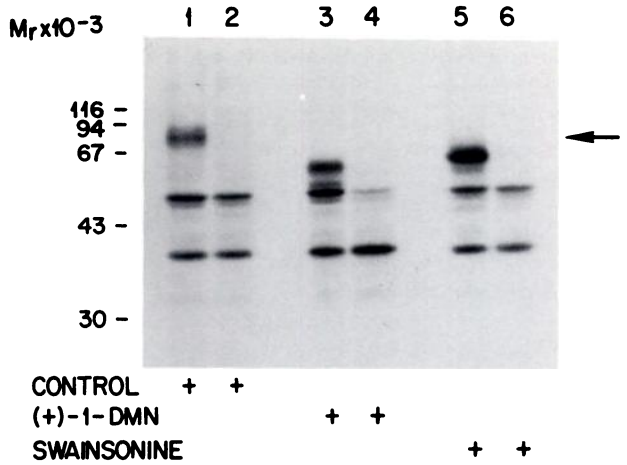

Fig. 4. The effect of (+)-1-DMN and swainsonine on the size of the α_1-adrenergic receptor. DDT_1 MF-2 cells were treated with (+)-1-DMN and swainsonine as described under Experimental Procedures. The α_1 receptor from control and treated intact DDT_1 MF-2 cells was photolabeled with [^{125}I]CP65,526 (0.3 nM) in the absence (*lanes 1, 3,* and *5*) or presence (*lanes 2, 4,* and *6*) of 100 μM phentolamine. ←, the positions of the photolabeled receptor before (+)-1-DMN and swainsonine treatment, following SDS-PAGE on 10% acrylamide gels. Molecular weights of known standards, electrophoresed simultaneously, are shown. The data presented are typical of three similar experiments.

ovary cell line that lacks LDL receptor activity because of a deficiency in the enzymes UDP-galactose-4-epimerase and UDP-galactosamine-4-epimerase (29). Incorporation of the enzyme into this mutant cell line results in the restoration of glycosylation and the expression of LDL receptor activity (30).

The next series of experiments was conducted to determine if altering the carbohydrate chains on the α_1 receptor resulted in a change in receptor function as assessed by ligand binding. DDT_1 MF-2 cells were grown in the presence of (+)-1-DMN or swainsonine after inactivation of native receptors with phenoxybenzamine. DMN interferes with the cellular processing of *N*-linked complex-type carbohydrate chains by inhibiting the action of mannosidases IA and IB (31). Swainsonine blocks completion of *N*-linked glycosylation of carbohydrate chains by inhibiting the enzyme Golgi mannosidase II (32, 33). Based on their mechanisms of action, one would expect the α_1 receptor to appear as a lower molecular weight species following incubation with either DMN or swainsonine. Fig. 4 demonstrates that the photolabeled α_1 receptor migrates with an apparent molecular weight of 60,000 following DMN treatment and 62,000 after exposure to swainsonine. These values are signifanctly lower than that of the native receptor in control DDT_1 MF-2 cells which migrates as an 80,000-Da species. In addition, following DMN or swainsonine treatment, the DDT_1 MF-2 α_1 receptor is sensitive to α-mannosidase (data not shown), whereas the native receptor is not (Fig. 2). This would be predicted since DMN-treated cells synthesize increased amounts of high mannose chains on glycoproteins (31) and swainsonine-treated cells synthesize "hybrid" chains (34), both of which contain terminal mannose sugars.

There was no change, however, in the number of α_1-adrenergic receptors or their affinity for [^{3}H]prazosin based on Scatchard analysis of saturation binding data. The affinities of the α_1 receptor for [^{3}H]prazosin in control and in DMN- and swainsonine-treated cells were 71 pM, 45 pM, and 109 pM, respectively, and the number of binding sites ranged from 32,000 to 37,000/cell. These results indicate that altering the carbohydrate composition and, therefore, the subunit molecular weight of the α_1-adrenergic receptor in DDT_1 MF-2 cells, does not acutely alter the steady state number of α_1-adrenergic receptors in the cell membrane or their ability to bind the antagonist radioligand, [^{3}H]prazosin. It is still possible, however, that altering the carbohydrate composition of the receptor with DMN or swainsonine may influence agonist interactions with the receptor, as suggested by Terman and Insel (23), or may alter the kinetics of receptor cycling.

The data presented in this paper indicate that the mammalian α_1-adrenergic receptor is a glycoprotein containing complex-type carbohydrate chains. The molecular weight of the *N*-deglycosylated peptide backbone is 50,000–55,000. This molecular weight may closely approximate the actual molecular

TABLE 2

Comparison of molecular weights for native and deglycosylated mammalian neurotransmitter receptors

Neurotransmitter receptor	Native subunit ($M_r \times 10^{-3}$)[a]	Deglycosylated receptor ($M_r \times 10^{-3}$)[a]	Calculated molecular mass($\times 10^{-3}$)[b]
α_1-Adrenergic	75–80 (1)[c]	50–55 (23)	ND[d]
α_2-Adrenergic	62–64 (14,38)	44–48[e]	ND
β_1-Adrenergic	67 (35)	57 and 44 (7)	ND
β_2-Adrenergic	64–67 (36,37)	49 (6)	46 (39,40)
M_1-Muscarinic acetylcholine	70 (41)	ND	51 (42)
M_2-Muscarinic acetylcholine	78 (43)	50–52 (44)	51 (45)
A_1-Adenosine	38 (4)	32 (4)	ND

[a] Based on SDS-PAGE analysis of purified or photoaffinity-labeled receptor preparations.
[b] Based on amino acid sequence predictions from isolated cDNA clones.
[c] Present study. Values for each receptor molecular weight are taken from the literature as indicated in parentheses.
[d] ND, not determined.
[e] Lanier *et al.*, unpublished observation.

weight of the receptor's peptide backbone, since the M_r determined following deglycosylation of several other neurotransmitter and hormone receptors is similar to the molecular mass calculated from nucleotide sequence data (Table 2). Both of the muscarinic receptor subtypes (M_1 and M_2), for example, have native subunit M_r of 70,000–80,000 and, based on cDNA sequence predictions, have peptide backbones of 50,000–52,000 Da. In a similar manner, the mammalian β-adrenergic receptors have subunit M_r of 64,000–67,000 and predicted peptide backbones of 45,000–50,000 Da (35–37). Studies with the human platelet α_2-adrenergic receptor and rat brain adenosine-A_1 receptor (4) also indicate significant degrees of *N*-glycosylation and somewhat smaller peptide backbones (M_r ≤38,000 and ≤32,000, respectively).

Further work necessary to ascertain the functional role of the carbohydrate chains, if any, in either transmembrane signaling or mobilization of second messengers such as inositol triphosphate and Ca^{2+}, which are associated with α_1 receptor activation, is currently in progress.

Acknowledgments

The authors wish to thank Dr. Charles Homcy for valuable discussions and for reviewing the manuscript, Dr. L. E. Cornett for the generous supply of DDT$_1$ MF-2 cells, A. Wechsler for technical assistance, and D. Rollins for typing the manuscript.

References

1. Graham, R. M., and S. M. Lanier. Identification and characterization of alpha-adrenergic receptors, in *The Heart and Cardiovascular System* (H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz, and H. E. Morgan, eds.). Raven Press, New York, 1059–1095 (1986).
2. Meier, K. E., D. R. Sternfeld, and P. A. Insel. Alpha$_1$- and beta$_2$-adrenergic receptors are co-expressed on cloned MDCK cells and are distinct glycoproteins. *Biochem. Biophys. Res. Commun.* **118**:73–81 (1984).
3. Lomasney, J. W., L. M. F. Leeb-Lundberg, S. Cotecchia, J. W. Regan, J. F. DeBernardis, M. G. Caron, and R. J. Lefkowitz. Mammalian α_1-adrenergic receptor: purification and characterization of the native receptor ligand binding subunit. *J. Biol. Chem.* **261**:7710–7716 (1986).
4. Stiles, G. Photoaffinity cross-linked A_1 adenosine receptor-binding subunits. Homologous glycoprotein expression by different tissues. *J. Biol. Chem.* **261**:10839–10843 (1986).
5. Shirakawa, O., T. Kuno, and C. Tanaka. The glycoprotein nature of solubilized muscarinic acetylcholine receptors from bovine cerebral cortex. *Biochem. Biophys. Res. Commun.*115:814–819 (1983).
6. Stiles, G. L., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Mammalian β-adrenergic receptors: distinct glycoprotein populations containing high mannose or complex type carbohydrate chains. *J. Biol. Chem.* **259**:8655–8663 (1984).
7. George, S. T., A. E. Ruoho, and C. C. Malbon. *N*-Glycosylation in expression and function of β-adrenergic receptors. *J. Biol. Chem.* **261**:16559–16564 (1986).
8. Lanier, S. M., C. J. Homcy, J. M. Nunnari, L. E. Limbird, and R. M. Graham. Carbohydrate moieties of porcine brain α_2-adrenergic receptor: glycosylation via asparagine-linked oligosaccharides. *Fed. Proc.* **46**:1309 (1987).
9. Kornfeld, R., and S. Kornfeld. Assembly of asparagine linked oligosaccharides. *Annu. Rev. Biochem.* **54**:631–664 (1985).
10. Kobata, A. The carbohydrates of glycoproteins, in *The Biology of Carbohydrates* V. Ginsberg and P. W. Robbins, eds.), Vol. 2. John Wiley and Sons, Inc., New York, 87–161 (1984).
11. Seidman, C. E., H.-J. Hess, C. J. Homcy, and R. M. Graham. Photoaffinity labeling of the alpha-adrenergic receptor using an ^{125}I-labeled aryl azide analog of prazosin. *Biochemistry* **23**:3765–3770 (1984).
12. Cornett, L. E., and J. S. Norris. Characterization of the α_1-adrenergic receptor subtype in a smooth muscle cell line. *J. Biol. Chem.* **257**:694–697 (1982).
13. Sawutz, D. G., L. Sena, L. E. Cornett, and R. M. Graham. Alpha$_1$-adrenergic receptor photoaffinity labeling of intact cells. *Biochem. Pharmacol.*, in press.
14. Lanier, S. M., R. M. Graham, H.-J. Hess, A. Grodski, M. G. Repaske, J. M. Nunnari, L. E. Limbird, and C. J. Homcy. Photoaffinity labeling of the porcine brain α_2-adrenergic receptor using a radioiodinated arylazide derivative of rauwolscine: identification of the hormone-binding subunit. *Proc. Natl. Acad. Sci. USA* 83:9358–9362 (1986).
15. Hedo, J. A. Lectins as tools for the purification of membrane receptors, in *Receptor Biochemistry and Methodology* (J. C. Venter and L. C. Harrison, eds.), Vol. 2. Alan R. Liss, Inc., New York, 45–61 (1984).
16. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **73**:248–254 (1976).
17. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
18. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–229 (1980).
19. Yamamoto, K., T. Tsuji, I. Matsumoto, and T. Osawa. Structural requirements for the binding of oligosaccharides and glycopeptides to immobilized wheat germ agglutinin. *Biochemistry* **20**:5894–5899 (1981).
20. Bauman, H., and D. Doyle. Determination of carbohydrate sturctures in glycoproteins and glycolipids, in *Molecular and Chemical Characterization of Membrane Receptors* (J. C. Venter and L. C. Harrison, eds.). Alan R. Liss, Inc., New York, 125–160 (1984).
21. Bhavanandan, V. P., and A. W. Katlic. The interaction of wheat germ agglutinin with sialoglycoproteins: the role of sialic acid. *J. Biol. Chem.* **254**:4000–4008 (1979).
22. Plummer, T. H., J. H. Elder, S. Alexander, A. W. Phelan, and A. L. Tarentino. Demonstration of peptide: *N*-glycosidase F activity in endo-β-*N*-acetylglucosaminidase F preparations. *J. Biol. Chem.* **259**:10700–10704 (1984).
23. Terman, B. I., and P. A. Insel. Oligosaccharide moieties contribute to structure and localization of alpha$_1$-receptors in muscle cells. *Clin. Res.* **35**:576A (1987).
24. Cummings, R. D., S. Kornfeld, W. J. Schneider, K. K. Hobgood, H. Tolleshaug, M. S. Brown, and J. L. Goldstein. Biosynthesis of *N*- and *O*-linked ologosaccharides of the low density lipoprotein receptor. *J. Biol. Chem.* **258**:15261–15273 (1983).
25. Iyer, R. M. and D. M. Carlson. Alkaline borohydride degredation of blood group H substance. *Arch. Biochem. Biophys.* **142**:101–105 (1971).
26. Soderquist, A. M. and G. Carpenter. Glycosylation of the epidermal growth factor receptor in A-431 cells: The contribution of carbohydrate to receptor function. *J. Biol. Chem.* **259**:12586-12594 (1984).
27. Pruies, J. and D. Bar-Sagi. Effect of tunicamycin, an inhibitor of glycosylation, on the biological properties of acetylcholine receptor in cultured muscle cells. *J. Biol. Chem.* **258**:1775–1780 (1983).
28. Sleiker, L. J. and M. D. Lane. Post-translational processing of the epidermal growth factor receptor: Glycosylation dependent aquisition of ligand binding capacity. *J. Biol. Chem.* **260**:687–690 (1985).
29. Kingsley, D. M., K. F. Kozarsky, L. Hobbie, and M. Krieger. Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-gal/UDP-galNAc 4-epimerase deficient mutant. *Cell* **44**:749–759 (1986).
30. Hobbie, L., D. M. Kingsley, K. F. Kozarsky, R. W. Jackman, and M. Krieger. Restoration of LDL receptor activity in mutant cells by intercellular junctional communication. *Science (Wash. D. C.)* **235**:69–73 (1987).
31. Fuhrmann, U., E. Bause, G. Leglar, and H. Ploegh. Novel mannosidase inhibitor blocking conversion of high mannose to complex oligosaccharides. *Nature (Lond.)* **307**:755–758 (1984).
32. Elbein, A. D., P. R. Dorling, K. Vosbeck, and M. Harisberger. Swainsonine prevents the processing of the oligosaccharide chains of influenza virus hemagglutinin. *J. Biol. Chem.* **257**:1573–1576 (1982).
33. Tulsiani, D. R. P., T. M. Harris, and O. Toustar. Swainsonine inhibits the biosynthesis of complex glycoproteins by inhibition of Golgi mannosidase II. *J. Biol. Chem.* **257**:7936–7939 (1982).
34. Tulsiana, D. R. P., and O. Touster. Swainsonine causes the production of hybrid glycoproteins by human skin fibroblasts and rat liver Golgi preparations. *J. Biol. Chem.* **258**:7578–7585 (1983).
35. Cubero, A., and C. C. Malbon. The fat cell beta-adrenergic receptor: purification and characterization of a mammalian beta$_1$-adrenergic receptor. *J. Biol. Chem.* **259**:1344–1350 (1984).
36. Insoft, R., D. G. Sawutz, and C. J. Homcy. Purification and characterization of the beta$_2$-adrenergic receptor from calf lung. *Biochim. Biophys. Acta* **861**:345–352 (1986).
37. Benovic, J. L., R. G. L. Shorr, M. G. Caron, and R. J. Lefkowitz. The mammalian beta$_2$-adrenergic receptor: purification and characterization. *Biochemistry* **23**:4510–4518 (1984).
38. Regan, J. W., H. Nakata, R. M. DeMarinis, M. G. Caron, and R. J. Lefkowitz. Purification and characterization of the human platelet alpha$_2$-adrenergic receptor. *J. Biol. Chem.* **261**:3894–3900 (1986).
39. Dixon, R. A. F., B. K. Kobilka, D. J. Strader, J. L. Benovic, H. G. Dohlman, T. Frielle, M. A. Bolanowski, C. D. Bennett, E. Randa, R. E. Diehl, R. A. Mumford, E. E. Slater, I. S. Sigal, M. G. Caron, R. J. Lefkowitz, and C. D. Strader. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature (Lond.)* **321**:75–79 (1986).
40. Kobilka, B. K., R. A. F. Dixon, T. Frielle, H. G. Dohlman, M. A. Bolanowski, I. S. Sigal, T. L. Yang-Feng, U. Francke, M. G. Caron, and R. J. Lefkowitz. cDNA for the human beta$_2$-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc. Nat. Acad. Sci. USA* **84**:64–50 (1987).
41. Haga, K., and T. Haga. Purification of the muscarinic acetylcholine receptor from porcine brain. *J. Biol. Chem.* **260**:7927–7935 (1985).
42. Kubo, T., K. Fukuda, A. Mikami, A. Maeda, H. Takahashi, M. Mishina, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. Cloning, sequencing and expression of complementary DNA

encoding the muscarinic acetylcholine receptor. *Nature (Lond.)* **232**:411–416 (1986).
43. Peterson, G. L., G. S. Harron, M. Yamaki, D. S. Fullerton, and M. I. Schimerlik. Purification of the muscarinic acetylcholine receptor from porcine atria. *Proc. Natl. Acad. Sci. USA* **81**:4993–4997 (1984).
44. Peterson, G. L., L. C. Rosenbaum, D. J. Broderick, and M. I. Schimerlik. Physical properties of the purified cardiac muscarinic acetylcholine receptor. *Biochemistry* **25**:3189–3202 (1986).
45. Peralta, E. G., J. W. Winslow, G. L. Peterson, D. H. Smith, A. Ashkenazi, J. Ramachandran, M. I. Schimerlik, and D. J. Capon. Primary structure and biochemical properties of an M_2 muscarinic receptor. *Science (Wash. D. C.)* **236**:600–605 (1987).

Send reprint requests to: Dr. Robert M. Graham, Cellular and Molecular Research Laboratory, Cardiac Unit, Jackson 12, Massachusetts General Hospital, 32 Fruit Street, Boston, MA 02114.