Role of N-Glycosylation in the Expression and Functional Properties of Human AT_1 Receptor†

Pascal M. Lanctôt, Patrice C. Leclerc, Emanuel Escher, Richard Leduc, and Gaétan Guillemette*

Department of Pharmacology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4

Received December 28, 1998; Revised Manuscript Received April 1, 1999

ABSTRACT: The role of N-glycosylation in the pharmacological properties and cell surface expression of AT_1 receptor was evaluated. Using site-directed mutagenesis, we substituted both separately and simultaneously the asparagine residues in all three putative N-linked glycosylation consensus sequences (N-X-S/T) of AT_1 receptor (positions 4, 176, and 188) with aspartic acid. Expression of these mutant receptors in COS-7 cells followed by photolabeling with [^{125}I]-[*p*-benzoyl-Phe^8]AngII and SDS−PAGE revealed ligand−receptor complexes of four different molecular sizes, indicating that the three N-glycosylation sites are actually occupied by oligosaccharides. Binding studies showed that the affinity of each mutant receptor for [Sar^1,Ile^8]Ang II was not significantly different from that of wild-type AT_1 receptor. Moreover, the functional properties of all mutant receptors were unaffected as evaluated by inositol phosphate production. However, the expression levels of the aglycosylated mutant were 5-fold lower than that of the wild-type AT_1 receptor. Use of green fluorescent protein−AT_1 receptor fusion proteins in studying the cellular location of the aglycosylated mutant demonstrated that it was distributed at a much higher density to the ER−Golgi complex than to the plasma membrane in HEK 293 cells. Together, these results suggest an important role of N-glycosylation in the proper trafficking of AT_1 receptor to the plasma membrane.

Angiotensin II (Ang II)[1] is the active component of the renin−angiotensin system. This octapeptide hormone produces a wide variety of physiological effects, including vasoconstriction, aldosterone secretion, decreased glomerular filtration, and cardiac myoproliferation (*1*−*3*). The development of selective analogues of Ang II has led to the identification of two receptor subtypes classified as AT_1 and AT_2 (*4*). Although both receptors bind Ang II with high affinity, AT_1 receptor is preferentially recognized by DuP 753 (losartan), whereas AT_2 receptor is preferentially recognized by PD 123177 (*5*−*7*). The majority of biological effects elicited by Ang II can be antagonized by losartan, suggesting an important physiological role for AT_1 receptor (*8*). This receptor belongs to the large family of G protein-coupled receptors (GPCRs) that possess seven membrane-spanning domains (*9*, *10*). The main effector mechanism activated by the AT_1 receptor is the activation of phospholipase C via the Gq subfamily of G proteins (*11*, *12*), which generates the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (*13*).

A feature found in many GPCRs is their consensus sequences for N-glycosylation (Asn-X-Ser/Thr, where X is not Pro). Three of these motifs are found at asparagines 4, 176, and 188 of the N-terminus and the second extracellular loop of the AT_1 receptor. The glycoprotein nature of AT_1 receptor was initially postulated following photoaffinity labeling studies whereby a "broad band" migration profile was observed on gel electrophoresis (SDS−PAGE) (*14*, *15*). Enzymatic deglycosylation of photolabeled AT_1 receptor, which increased its mobility on SDS−PAGE, led to a more direct demonstration of its glycoprotein nature (*16*).

Glycosylation plays an important role in the structural maturation of many secreted and membrane proteins upon their entry and passage through the endoplasmic reticulum and Golgi network. Although many studies have addressed its functional significance, a specific role for N-glycosylation remains elusive. Reports have suggested its implication in protein conformational stability, folding, intracellular targetting, protection against proteolysis, cell−cell recognition (*17*−*19*), and more recently in protein dimerization (*20*) and association with molecular chaperones (*21*).

In the case of GPCRs, preventing N-linked glycosylation either by treatment of cultured cells with tunicamycin or by site-directed mutagenesis produced unpredictable and non-uniform effects on ligand binding, cell surface expression, and signal transduction. For example, the nonglycosylated form of rhodopsin (*22*), β_2-adrenergic receptor (*23*), AT_2 receptor (*24*), calcium receptor (*25*), VIP receptor (*26*), LH−

† This work was supported by grants from the Medical Research Council of Canada (MRCC). E.E. is recipient of a J. C. Edwards Chair in cardiovascular research. R.L. is a scholar from the Fonds de la Recherche en Santé du Québec.

* To whom correspondence should be addressed: Department of Pharmacology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4. Telephone: (819) 564-5347. Fax: (819) 564-5400. E-mail: gguillem@courrier.usherb.ca.

[1] Abbreviations: Ang II, angiotensin II; AT_1 receptor, angiotensin II receptor subtype 1; AT_2 receptor, angiotensin receptor subtype 2; BSA, bovine serum albumine DMEM, Dulbecco's modified Eagle's medium; LH−RH, luteinizing hormone−releasing hormone; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PNGase, endoglycosidase F; PTH, parathyroid hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; VIP, vasoactive intestinal peptide; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor.

10.1021/bi9830516 CCC: $18.00
Published on Web 06/17/1999

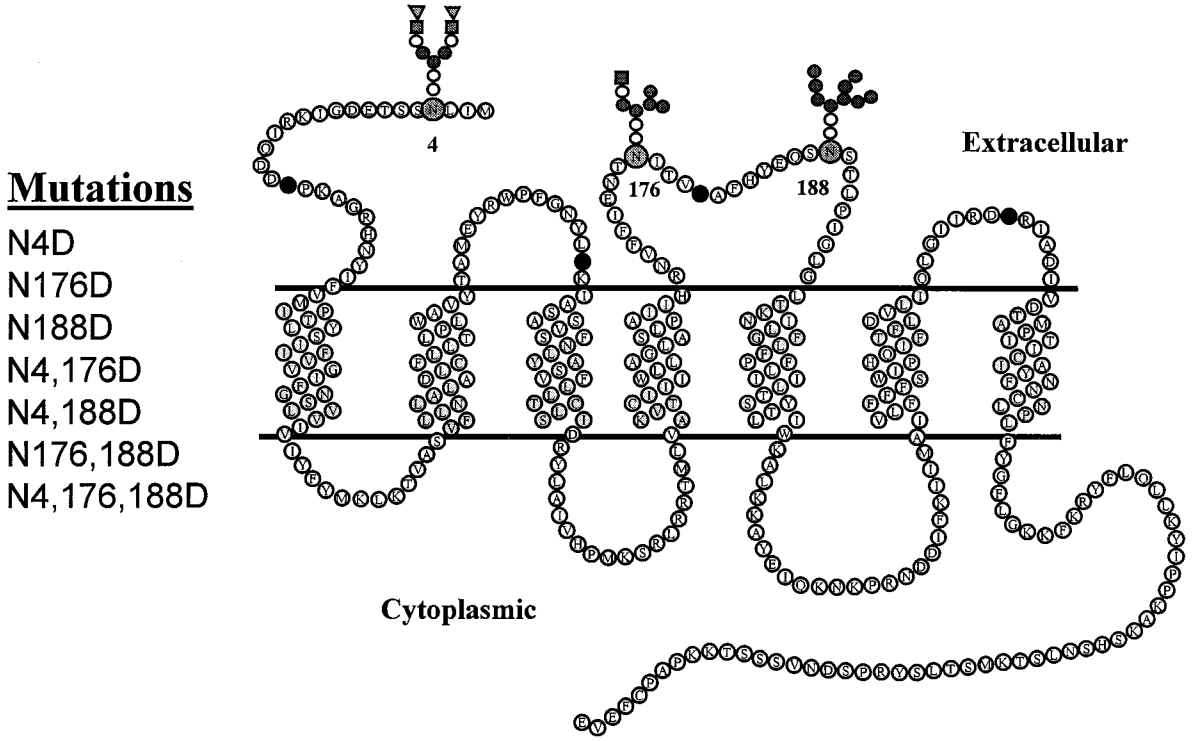

FIGURE 1: Schematic representation of the human AT1 receptor. Amino acid sequence and consensus sites for N-glycosylation (with the three main types of oligosaccharides) are depicted. The AT_1 receptor is part of the GPCR superfamily of proteins which possess seven membrane-spanning domains. Four cysteine residues (●) which potentially form disulfide bridges are shown in the extracellular region of the receptor. Mutations are described (e.g., N4D represents Asn^4 substituted for Asp^4).

RH receptor (*27*), and PAF receptor (*28*) all had lower levels of cell surface expression, while the expressions of m2 muscarinic receptor (*29*), V2 vasopressin receptor (*30*), PTH receptor (*31*), and oxytocin receptor (*32*) were not significantly modified in their aglycosylated form. In most studies addressing the role of N-glycosylation, the researchers have taken advantage of enzymes such as PNGase F to nonspecifically remove oligosaccharides from mature proteins or tunicamycin to prevent the addition of sugars within the ER during protein assembly. Although they have provided valuable information, these approaches have some limitations. For example, PNGase F treatment does not provide insight about the folding or intracellular trafficking of glycoproteins, and tunicamycin treatment which prevents glycosylation of all glycoproteins may indirectly impair the normal function of the protein of interest. In addition, enzyme treatment does not permit a detailed analysis of the contribution of individual glycosylation sites to the properties of the protein. In this study, we used site-directed mutagenesis to circumvent these shortcomings. We created all seven possible glycosylation mutants (Figure 1) to confirm by photaffinity labeling that each of the three putative sites is actually glycosylated. We investigated the ligand binding capacity, functionality, and cell surface expression of the mutant receptors.

MATERIALS AND METHODS

Materials. The cDNA clone encoding the human AT_1 receptor was kindly provided by S. Meloche (University of Montreal, Montreal, PQ) and subcloned in the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). DMEM, lipofectamine, G418, and other cell culture media as well as oligonucleotide primers were obtained from Gibco Life Technologies (Gaithersburg, MD). Ang II, bovine serum albumin (BSA), bacitracin, and soybean trypsin inhibitor (STI) were purchased from Sigma Chemical Co. (St. Louis, MO). The Sculptor in vitro mutagenesis kit and the [^{3}H]-myoinositol (80 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Molecular biology reagents and enzymes, including restriction endonucleases and polymerases, were from Pharmacia (Piscataway, NJ). The AG 1-X8 resin was purchased from Bio-Rad (Richmond, CA). [Sar^1,Ile^8]Ang II was obtained from Bachem (Torrance, CA). [^{125}I]Ang II and [^{125}I]-[Sar^1,Ile^8]Ang II (1000 Ci/mmol) were prepared with iodogen as previously described (*33*) and purified by high-performance liquid chromatography (HPLC) on a C-18 column. The specific activity was determined by self-displacement in our binding system. Briefly, a saturation curve with increasing concentrations of the radioactive ligand and a dose-displacement curve with a fixed concentration of the radioactive ligand inhibited by increasing concentrations of unlabeled homologous peptide were performed simultaneously using a bovine adrenal cortex membrane preparation. We estimated the amount of radioactive ligand necessary to obtain an occupation ratio in the saturation curve corresponding to the occupation ratio obtained with a known amount of unlabeled peptide in the dose-displacement curve.

Construction of Glycosylation Mutants and GFP Fusion Proteins. The pcDNA3 vector containing the human AT_1 receptor cDNA was digested with *Hin*dIII and *Xba*I, and the resulting 1250 bp fragment was subcloned into the *Hin*dIII−*Xba*I-digested RF form of M13 mp19. Three oligonucleotides were synthesized to substitute asparagines 4, 176, and 188 for aspartic acids. The mutagenesis oligonucleotides that were used are listed below (altered nucleotides are underlined): N4D (5′-AGTAGAAGAGT**C**CGAGAATCATTT-3′), N176D

(5′-ACAAACTGTAATAT**C**GGTGTTCTCA-3′), and N188D (5′-AGGGTTGAAT**C**TTGGGACTCA-3′). The N4,176D, N4,188D, N176,188D, and N4,176,188D mutants were constructed by performing mutagenesis on the single mutants with various combinations of the three oligonucleotides. The AT_1−GFP fusion proteins (wild-type AT_1−GFP and aglycosylated AT_1−GFP) were constructed by the PCR method using the T7 (5′-TAATACGACTCACTATAGGG-3′) and PCRBamAT_1 (5′-TACAACGGATCCTCAACCTCAAAACATGGTG-3′) oligonucleotides on pcDNA3-hAT_1. To allow proper fusion of the two genes, PCRBamAT_1 was designed to remove the stop codon of the receptor so a *Bam*HI restriction site could be inserted. The resulting DNA was digested with *Hin*dIII and *Bam*HI restriction enzymes, and the 1250 bp amplified fragment was cloned into the *Hin*dIII−*Bam*HI-digested pEGFP-N1 (Clonetech, PaloAlto, CA).

Cell Culture and Transfections. COS-7 cells and HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. When the cells reached approximately 50% confluence, they were washed and transfected in serum-free DMEM containing 4 μg of DNA and 25 μL of lipofectamine (in 100 mm dishes) or 0.5 μg of DNA and 4 μL of lipofectamine (in six-well plates). Cells were incubated for 5 h at 37 °C, after which the medium was replaced with serum-rich DMEM. Forty-eight hours after initial transfection, cells were washed once in PBS and used immediately for functional assays or stored at −80 °C for binding experiments.

Binding Experiments. COS-7 cells were grown for 48 h posttransfection in 100 mm dishes, washed once with PBS, and subjected to one freeze−thaw cycle. Broken cells were then gently scraped in PBS, centrifuged at 2500*g* for 15 min at 4 °C, and resuspended in binding buffer [25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 100 mM NaCl, 0.1% BSA, and 0.01% bacitracin]. Broken cells (25−50 μg of protein) were incubated for 1 h at room temperature in binding buffer containing increasing concentrations of [^{125}I]-[Sar^1-Ile^8]Ang II in a final volume of 0.5 mL. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked for 2 h in binding buffer. The level of nonspecific binding was measured in the presence of 1 μM unlabeled Ang II. Receptor-bound radioactivity was evaluated by γ counting.

Photoaffinity Labeling. Transfected COS-7 cells were photolabeled as previously described (*34*). Briefly, cells were incubated with 10 nM [^{125}I]-[L-Bpa^8]Ang II in 1 mL of medium containing 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, and 0.1% BSA. After 45 min at room temperature, cells were washed twice with PBS and irradiated for 30 min at 0 °C under filtered UV light (365 nm). Labeled cells were solubilized in 150 μL of medium containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride. After centrifugation at 15000*g* for 10 min to remove insoluble material, the supernatant was mixed with an equal volume of 2× Laemmli solution and incubated for 60 min at 37 °C followed by electrophoresis on a 10% polyacrylamide gel at 200 V. Gels were dried under vacuum before autoradiography for different periods of time on Kodak Biomax MS.

Phospholipase C Assay. COS-7 cells were seeded in six-well plates, transfected, and labeled for 16 h in inositol-free DMEM containing 10 μCi/mL of [^{3}H]myoinositol. After the cells had been washed twice with PBS 0.1% dextrose, inositol trisphosphate production was induced with 100 nM Ang II for 5 min in stimulation buffer (M199, 25 mM HEPES and 0.1% BSA) at 37 °C. Incubations were terminated by the addition of ice-cold perchloric acid (5% v/v). Cells were scraped and centrifuged at 15000*g* for 5 min. Water-soluble inositol phosphates were then extracted with an equal volume of the 1:1 mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine. The samples were vigorously mixed and centrifuged at 15000*g* for 1 min. The upper phase containing inositol phosphates was applied to an AG 1-X8 resin column. Inositol phosphates were sequentially eluted by the addition of an ammonium formate/formic acid solution of increasing ionic strength (*35*).

Fluorescence and Confocal Microscopy. Transiently transfected HEK 293 cells were used for microscopy experiments. They were grown on 25 mm diameter glass coverslips in multiwell dishes. Cell fluorescence was evaluated with a Leica Leitz DMR phase contrast and fluorescence microscope equipped with a Wild MPS 48/52 photomicrographic system. Confocal microscopy was performed with a Molecular Dynamics Multi Probe 2001 confocal argon laser scanning (CSLM) system equipped with a Nikon Diaphot epifluorescence inverted microscope and a 60× (1.4 NA) Nikon Oil Plan achromat objective. The 488 nm argon laser line (9.0 mV) was directed to the samples via a 510 nm primary dichroic filter and attenuated with a 1−3% neutral density filter to reduce the extent of photobleaching. The pinhole size was set at 50 μm, and the image size was set at 512 pixels × 512 pixels with a pixel size of 0.08 μm. In all experiments, laser line intensity, photometric gain, PMT settings, and filter attenuation were kept constant. Fluorescence intensity was reported on a pseudocolor scale in which computer analysis of each pixel (0.08 μm) tranforms fluorescence intensity to a color scale from black (0 being the lowest fluorescence intensity) to white (255 being the maximum fluorescence intensity).

RESULTS

All Three N-Glycosylation Sites on the AT_1 Receptor Are Glycosylated. To verify whether the three putative N-glycosylation sites of AT_1 receptor are occupied by oligosaccharides, the recombinant receptors were photoaffinity labeled with [^{125}I]-[Bpa^8]Ang II and analyzed by SDS−PAGE and autoradiography. The wild-type AT_1 receptor expressed in COS-7 cells migrated as a broad band with an average apparent molecular mass (M_r) of 116 kDa (Figure 2) in accordance with the previously reported M_r of this receptor (*36*). The broadness of the band was consistent with the glycoprotein nature of the AT_1 receptor. The three mutant receptors lacking a single glycosylation site (N4D, N176D, and N188D) exhibited a slight increase in electrophoretic mobility with average M_r values varying between 102 and 106 kDa (Figure 2A). These results indicate that all three consensus sites on the AT_1 receptor are actually glycosylated in COS-7 cells. Mutant receptors lacking two glycosylation sites (N4,176D, N4,188D, and N176,188D) exhibited an important increase in electrophoretic mobility with average M_r values of 56 kDa (Figure 2B). As expected, the triple-

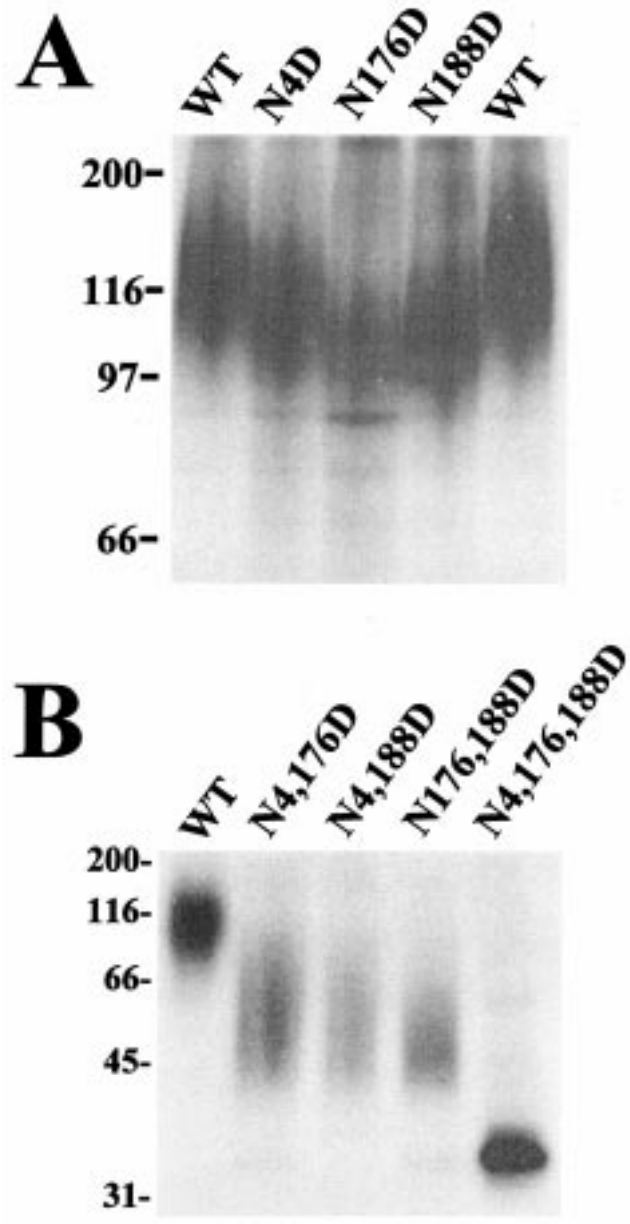

FIGURE 2: Photoaffinity labeling of aglycosylated AT_1 receptors. COS-7 cells expressing the wild-type AT_1 receptor (WT) and various mutant receptors were incubated in the presence of 5 nM [^{125}I]-[L-Bpa^8]Ang II for 1 h at room temperature. Cells were then irradiated under 365 nm filtered UV light for 45 min at 0 °C. After solubilization, samples were resolved by 10% SDS−polyacrylamide gel electrophoresis followed by autoradiography as described in Materials and Methods. A 3-fold larger volume of the samples containing mutant receptors missing the N176 oligosaccharide (N176D, N4,176D, N176,188D, and N4,176,188D) was loaded onto the gel. Protein standards with the indicated molecular masses were run in parallel (representative of at least three independent experiments).

mutant receptor lacking all three glycosylation sites migrated with an M_r of 34 kDa, similar to the M_r previously observed after complete enzymatic deglycosylation of the AT_1 receptor (*16*). These results indicate that all seven glycosylation mutants of the AT_1 receptor are expressed (although at different levels) at the surface of COS-7 cells.

Binding and Functional Properties of the Mutant AT_1 Receptors. To assess their pharmacological properties, pcDNA3 vectors encoding the various AT_1 receptors were transiently transfected into COS-7 cells. Binding properties

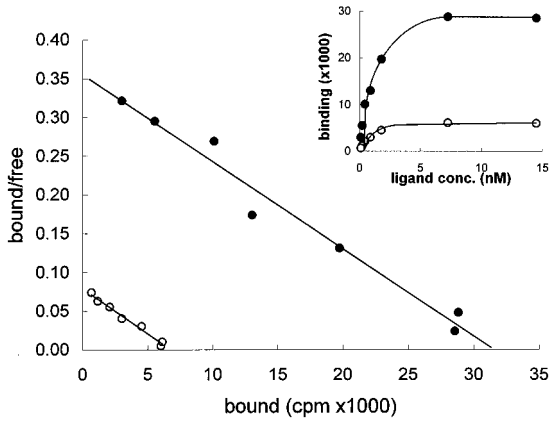

FIGURE 3: Binding properties of algycosylated AT_1 receptors. Broken COS-7 cells (50 μg of protein) expressing wild-type (●) and aglycosylated (○) AT_1 receptors were incubated for 1 h at room temperature with increasing concentrations of [^{125}I]-[Sar^1,Ile^8]Ang II. Nonspecific binding was assessed in the presence of 1 μM unlabeled [Sar^1,Ile^8]Ang II. Each point represents the mean of triplicate values. K_d and B_{max} values were evaluated by Scatchard analysis of the saturation curve data shown in the inset (representative of three independent experiments).

were evaluated with increasing concentrations of [^{125}I]-[Sar^1,Ile^8]Ang II in the presence or absence of 10 μM unlabeled [Sar^1,Ile^8]Ang II. Figure 3 (inset) shows representative binding isotherms for the wild-type receptor (●) and the triple-mutant receptor (○). Scatchard plot analysis of these data (Figure 3) yielded K_d values of 1.22 ± 0.26 and 0.75 ± 0.27 nM and B_{max} values of 4.44 ± 0.28 and 0.86 ± 0.14 pmol/mg of protein (three experiments) for the wild-type receptor and the triple-mutant receptor, respectively. These results indicate that the level of glycosylation of the AT_1 receptor does not affect its ligand binding properties but greatly influences (by about 5-fold) its level of cell surface expression. Figure 4A shows the relative level of cell surface expression (compared with that of the wild-type receptor) of all seven mutant receptors. Except for the N4D mutant, removal of a single glycosylation site caused a significant decrease in the level of cell surface expression of the AT_1 receptor. Interestingly, all mutants lacking the glycosylation site at position 176 showed an importantly decreased level of expression. The binding affinity of all mutant receptors was not significantly different from that of the wild-type AT_1 receptor (data not shown).

The functional properties of wild-type and mutant AT_1 receptors were evaluated by assessment of the Ang II-induced inositol phosphate accumulation within transiently transfected COS-7 cells. Figure 4B shows the relative amount of inositol trisphosphate accumulation after maximal activation (100 nM Ang II for 5 min) of mutant receptors. Although all receptors significantly increased the level of inositol trisphophates above the basal level, mutants lacking the position 176 oligosaccharide showed a decreased phospholipase C activating capacity. It is noteworthy that the relative functional properties of all the mutant receptors almost mirror their relative level of expression.

Visualization of the AT_1−GFP Fusion Proteins. To determine the cellular location of the receptors, we constructed AT_1 receptors which possess the added feature of autofluorescence. GFP was fused to the intracellular C-terminus of the AT_1 receptor. The presence of the GFP

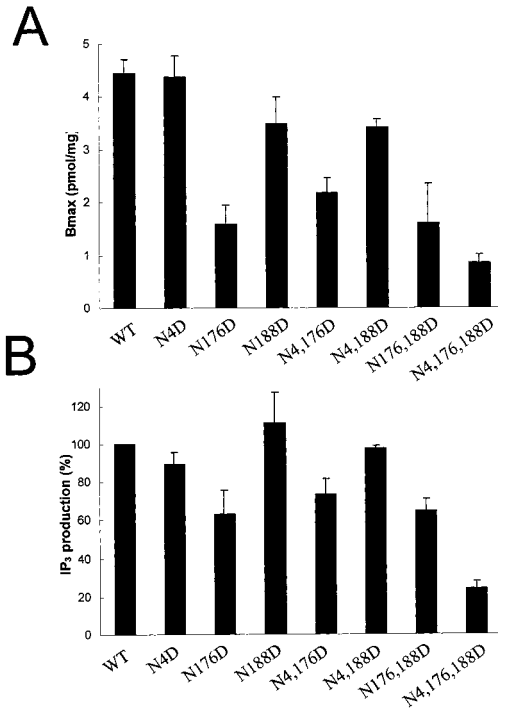

FIGURE 4: Cell surface expression and functional properties of mutant AT_1 receptors. (A) Broken COS-7 cells transiently expressing the different mutant receptors were incubated with increasing concentrations of [^{125}I]-[Sar^1,Ile^8]Ang II for 1 h at room temperature. Nonspecific binding was assessed in the presence of 1 μM unlabeled [Sar^1,Ile^8]Ang II. B_{max} and K_d values were evaluated by Scatchard analysis of the binding data. (B) COS-7 cells transiently expressing the seven glycosylation mutants were labeled for 18 h with 10 μCi of [^{3}H]myoinositol. They were then stimulated with 100 nM Ang II for 5 min. Inositol phosphates were extracted and sequentially eluted by ion exchange chromatography with increasing concentrations of ammonium formate/formic acid solutions. Results are expressed as a percentage of the wild-type AT_1 receptor IP production (100% = 5649 ± 2080 cpm) (mean of three independent experiments).

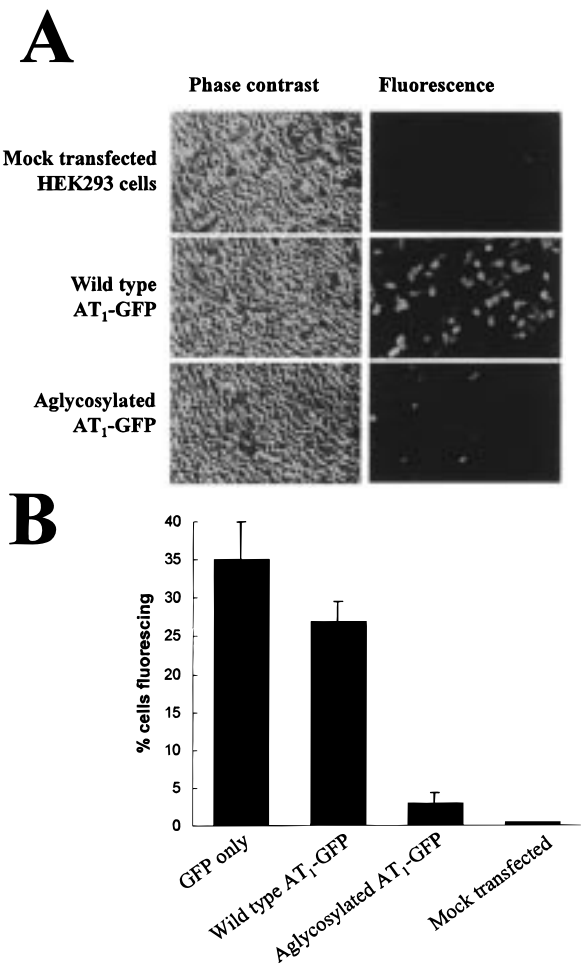

FIGURE 5: Expression of AT_1−GFP fusion proteins. (A) HEK 293 cells were grown and transfected on circular (25 mm diameter) coverslips. Forty-eight hours post-transfection, coverslips were placed on slides for direct microscopic visualization. All photomicrographs were taken under identical conditions at a magnification of 200×. (B) HEK 293 cells were transiently transfected with GFP only, with wild-type AT_1−GFP, with aglycosylated AT_1−GFP, and with an empty vector. All cells emitting a visually detectable fluorescence were counted. % represents the number of fluorescent cells in each caption over the total number of cells evaluated under phase contrast in the same caption (mean of three independent experiments).

protein did not significantly alter the pharmacological or functionnal properties of the AT_1 receptor (data not shown). Conventional fluorescence microscopy revealed that a large proportion of HEK 293 cells expressing the wild-type AT_1 receptor−GFP fusion protein emitted at variable levels of intensity, reflecting the variable level of protein expression in a transiently transfected cell population (Figure 5A). Under identical conditions, a much lower proportion of HEK 293 cells transfected with the aglycosylated AT_1 receptor−GFP fusion protein emitted at a detectable level of fluorescence intensity. Quantitative estimates revealed that under our experimental conditions about 35% of the HEK 293 cells transfected with GFP alone, 27% of the cells transfected with wild-type AT_1−GFP, and only 3% of the cells transfected with the aglycoslated AT_1−GFP emitted at a detectable level of fluorescence intensity (Figure 5B). Since all transfections were carried out under identical conditions, these results suggest that the transfected proteins were expressed at different levels.

Confocal fluorescence microscopy revealed that, in HEK 293 cells, the wild-type AT_1−GFP receptor was mostly distributed to the plasma membrane but also (at about the same level of fluorescence intensity) within an intracellular compartment corresponding to the endoplasmic reticulum (Figure 6). Interestingly, in HEK 293 cells expressing the aglycosylated AT_1−GFP receptor, fluorescence also distributed to the plasma membrane but was much more intense within the intracellular compartment (Figure 6). Regions of intense fluorescence observed in the intracellular compartment of cells expressing the aglycosylated mutant colocalized with a specific marker of the endoplasmic reticulum ($DiOC_6$) (data not shown). These results suggest that the aglycosylated receptor is not trafficking properly within HEK 293 cells. Similar results were also obtained in COS-7 cells (data not shown).

DISCUSSION

Several studies have verified the effects of enzymatically deglycosylating a receptor on its pharmacological profile (*24*, *31*). Another report showed that monosubstitutions of the asparagine residue within the three putative glycosylation sites of the AT_1 receptor did not affect its affinity for [^{125}I]-[Sar^1-Ile^8]Ang II (*37*). In this study, we have used a site-directed mutagenesis approach to confirm the presence and

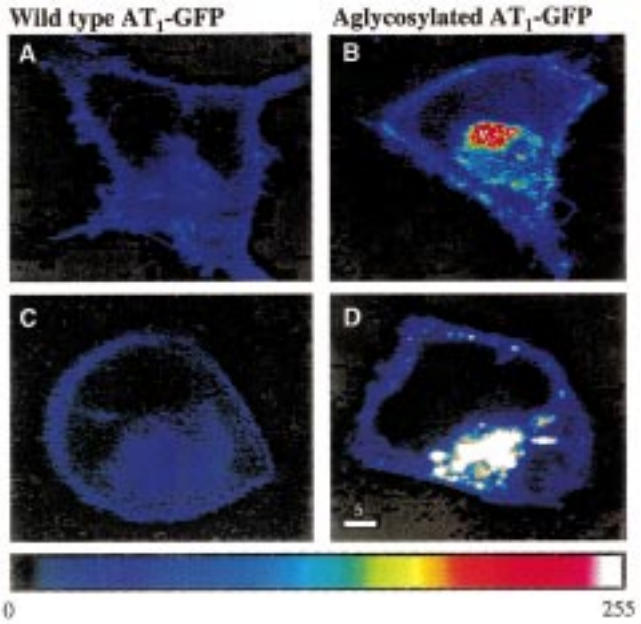

FIGURE 6: Cellular localization of the aglycosylated AT_1−GFP receptor. Transiently transfected (A and B) and stable HEK 293 cell lines (C and D) expressing the wild-type (A and C) and aglycosylated AT_1−GFP receptor (B and D) were grown and transfected on circular (25 mm diameter) coverslips and observed by confocal microscopy. The fluorescence intensity is reported on a pseudocolor scale from black (0 being the lowest fluorescence intensity) to white (255 being the maximal fluorescence intensity). Note the high fluorescence intensity at the ER−Golgi complex in cells expressing the aglycosylated receptor (representative of at least three independent experiments).

evaluate the role of individual consensus sequences for N-glycosylation on the AT_1 receptor. We have shown that all three asparagines at positions 4, 176, and 188 are glycosylated in COS-7 cells. The aglycosylated triple mutant (N4,176,188D) migrated with an M_r of 34 kDa which compares favorably with the value from a previous study in which enzymatic deglycosylation of the AT_1 receptor revealed an M_r of 32 kDa (*16*). The photoaffinity labeling approach confirmed that each of the seven mutants was expressed in COS-7 cells and that the three consensus sequences for N-linked glycosylation were actually occupied by oligosaccharides.

Binding experiments showed that removal of the N176 sugar moiety on the second extracellular loop caused an important decrease whereas removal of all three sugar moieties caused a 5-fold reduction in the level of cell surface expression of the receptor. Most GPCRs studied to date showed decreased levels of cell surface expression upon mutagenesis-induced deglycosylation (*22*, *23*, *25*−*28*). Nonetheless, the expression level and biological activity of some GPCRs were not affected by removal of oligosaccharides (*29*, *30*, *32*). Not surprisingly, when the functional properties of each mutant AT_1 receptor were assayed, production of the second messenger $InsP_3$ correlated favorably with the level of cell surface expression. These results suggest that the mutant receptors couple efficiently with the intracellular machinery and that the decreased level of $InsP_3$ production observed is simply due to a decreased level of cell surface receptors.

Our results are in agreement with those of Yamano et al. (*37*), who showed that individual substitution of the three consensus sequences on AT_1 receptor did not alter its binding affinity. Yamano et al. did not verify the effects of double or triple mutations on the pharmacological properties and the expression level of the receptor. On one hand, our more extended study showed that glycosylation plays a role in the proper expression of the receptor. Indeed, removal of the consensus sequence at position 176 caused a significant decrease, whereas removal of all three glycosylation sites caused an 80% decrease in the level of cell surface expression of the receptor. On the other hand, our results also suggest that glycosylation is not absolutely essential for the proper expression and function of the receptor since some aglycosylated receptors were functionally expressed at the cell surface.

Using GFP fusion technology, we showed by conventional fluorescence microscopy that the fluorescence intensity of cells expressing the wild-type receptor was much higher than that of cells expressing the aglycosylated mutant. Confocal microscopy allowed a more detailed evaluation of the fluorescence distribution. We showed that the wild-type receptor is distributed to the plasma membrane and to the endoplasmic reticulum at a similar level of fluorescence intensity. The aglycosylated receptor distributed more intensely to the endoplasmic reticulum than to the plasma membrane. It is important to mention that, to analyze the fluorescence of cells expressing the wild-type receptor, we had to select cells with low expression levels. Actually, most HEK 293 cells transfected with wild-type AT_1−GFP emitted at high fluorescence levels, which is inappropriate for the accurate resolution of the fluorescence signal. To resolve this problem, only cells that had discernible fluorescence at the plasma membrane were selected. In the case of cells expressing the aglycosylated AT_1 receptor, we selected among cells that fluoresced at the highest level, again highlighting the low expression level of the aglycosylated AT_1 receptor. A previous study has shown that impairment of glycosylation can produce a protein which is retained in the perinuclear organelles and prevented from reaching the plasma membrane (*38*). Our results suggest that the decrease in the level of cell surface expression of the aglycosylated AT_1 receptor can be explained by improper trafficking to the plasma membrane. Indeed, the aglycosylated receptor appears to accumulate in the endoplasmic reticulum. Our results cannot exclude the possibility that the retention of the aglycosylated receptor in the endoplasmic reticulum is due to a local misfolding caused by the introduction of substituted residues rather than to its lack of N-linked carbohydrate. To address this question, we abolished the N-glycosylation motif (N-X-T) at position 176 by substituting Thr 178 for Ala. The maximal binding capacity of COS-7 cells expressing the mutant T178A was 4-fold lower than the maximal binding capacity of COS-7 cells expressing the wild-type AT_1 receptor. Again, although these results cannot definitively exclude the possibility of a misfolded receptor due to the substitution at position 178, they strengthen the suggestion of a role of N-glycosylation in the targeting of the AT_1 receptor to the cell surface.

In conclusion, we have shown that N-glycosylation does not play a significant role in the ligand recognition and in the functional properties of the AT_1 receptor. Rather, N-glycosylation appears to be involved in the trafficking of the AT_1 receptor from the endoplasmic reticulum to the

plasma membrane. The low level of expression of the aglycosylated AT_1 receptor further suggests that it hardly escapes a cellular quality control process routing it to a degradation pathway.

REFERENCES

1. Peach, M. J. (1981) *Biochem. Pharmacol. 80*, 2745−2751.
2. Vallotton, M. B. (1987) *Trends Pharmacol. Sci. 8*, 69−74.
3. Powell, J. S., Müller, R. K. M., Rouge, M., Kuhn, H., Hefti, F., and Baumgartner, H. R. (1990) *J. Cardiovasc. Pharmacol. 16*, S42−S49.
4. Bumpus, F. M., Catt, K. J., Chiu, A. T., de Gasparo, M., Goodfriend, T., Husain, A., Peach, M. J., Taylor, D. G., and Timmermans, P. B. M. W. M. (1991) *Hypertension (Dallas) 17*, 720−723.
5. Chang, R. S. L., and Lotti, V. J. (1990) *Mol. Pharmacol. 3*, 347−351.
6. Whitebread, S., Mele, M., Kamber, B., and de Gasparo, M. (1989) *Biochem. Biophys. Res. Commun. 163*, 284−291.
7. Chiu, A. T., Herblin, W. F., McCall, D. E., Ardecky, R. J., Carini, D. J., Duncia, J. V., Pease, L. J., Wong, P. C., Wexler, R. R., Johnson, A. L., and Timmermans, P. B. M. W. M. (1989) *Biochem. Biophys. Res. Commun. 165*, 196−203.
8. Timmermans, P. B. M. W. M., Wong, P. C., Chiu, A. T., Herblin, W. F., Renfield, P., Carini, D. J., Lee, R. J., Wexler, R. R., Saye, J. M., and Smith, R. D. (1993) *Pharmacol. Rev. 45*, 205−251.
9. Murphy, T. J., Alexander, R. W., Griendling, K. K., Runge, R. S., and Bernstein, K. E. (1991) *Nature 351*, 233.
10. Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J. J., Hasegawa, M., Matsuda, Y., and Inagami, T. (1991) *Nature 351*, 230−233.
11. Catt, K. J., Sandberg, K., and Balla, T. (1993) in *Cellular and Molecular Biology of the Renin-Angiotensin System* (Raizada, M. K., Phillips, M. I., and Sumners, C., Eds.) pp 307−356, CRC Press, Boca Raton, FL.
12. Spat, A., Enyedi, P., Hajnoczky, G., and Hunyady, L. (1993) *Exp. Physiol. 76*, 859−885.
13. Balla, T., Hausdorff, W. P., Baukal, A. J., and Catt, K. J. (1989) *Arch. Biochem. Biophys. 270*, 398−403.
14. Capponi, A. M., and Catt, K. J. (1980) *J. Biol. Chem. 255*, 12081−12086.
15. Guillemette, G., and Escher, E. (1983) *Biochemistry 22*, 5591−5596.
16. Carson, M. C., Leach Harper, C. M., Baukal, A. J., Aguilera, G., and Catt, K. J. (1987) *Mol. Endocrinol. 1*, 147−153.
17. Olden, K., Parent, J. B., and White, S. L. (1982) *Biochim. Biophys. Acta 57*, 209−232.
18. Breitfeld, P. P., Rup, D., and Schwartz, A. L. (1984) *J. Biol. Chem. 259*, 10414−10421.
19. Rademacher, T. W., Parekh, R. B., and Dwek, R. A. (1988) *Annu. Rev. Biochem. 57*, 785−838.
20. Opdenakker, G., Rudd, P. M., Wormald, M., Dwek, R. A., and Van Damme, J. (1995) *FASEB J. 9*, 453−457.
21. Hebert, D. N., Foellmer, B., and Helenius, A. (1995) *Cell 81*, 425−433.
22. Kaushal, S., Ridge, K. D., and Khorana, H. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4024−4028.
23. Rands, E., Candelore, M. R., Cheung, A. H., Hill, W. S., Strader, C. D., and Dixon, R. A. F. (1990) *J. Biol. Chem. 265*, 10759−10764.
24. Servant, G., Dudley, D. T., Escher, E., and Guillemette, G. (1996) *Biochem. J. 313*, 297−304.
25. Gaofeng, F., Goldsmith, P. K., Collins, R., Dunn, C. K., Krapcho, K. J., Rogers, K. V., and Spiegel, A. M. (1997) *Endocrinology 138*, 1916−1922.
26. El Battari, A., Forget, P., Fouchier, F., and Pic, P. (1991) *Biochem. J. 278*, 527−533.
27. Liu, X., Davis, D., and Segaloff, D. L. (1993) *J. Biol. Chem. 268*, 1513−1516.
28. Rodriguez, C. G., Cundell, D. R., Tuomanen, E. I., Kolakowski, L. F., Jr., Gerard, C., and Gerard, N. P. (1995) *J. Biol. Chem. 270*, 25178−25184.
29. van Koppen, C. J., and Nathanson, N. M. (1990) *J. Biol. Chem. 265*, 20887−20892.
30. Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1996) *Mol. Pharmacol. 50*, 467−473.
31. Bisello, A., Greenberg, Z., Behar, V., Rosenblatt, M., Suva, L. J., and Chorev, M. (1996) *Biochemistry 35*, 15890−15895.
32. Kimura, T., Makino, Y., Bathgate, R., Ivell, R., Nobunaga, T., Kubota, Y., Kumazawa, I., Saji, F., Murata, Y., Nishihara, T., Hashimoto, M., and Kinoshita, M. (1997) *Mol. Hum. Reprod. 3*, 957−963.
33. Boulay, G., Chretien, L., Richard, D. E., and Guillemette, G. (1994) *Endocrinology 135*, 2130−2136.
34. Servant, G., Boulay, G., Bossé, R., Escher, E., and Guillemette, G. (1993) *Mol. Pharmacol. 43*, 677−683.
35. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P., and Irvine, R. F. (1983) *Biochem. J. 212*, 473−482.
36. Laporte, S. A., Servant, G., Richard, D. E., Escher, E., Guillemette, G., and Leduc, R. (1996) *Mol. Pharmacol. 49*, 89−95.
37. Yamano, Y., Ohyama, K., Chaki, S., Guo, D.-F., and Inagami, T. (1992) *Biochem. Biophys. Res. Commun. 187*, 1426−1431.
38. Buteau, H., Pezet, A., Ferrag, F., Perrot-Applanat, M., Kelly, P. A., and Edery, M. (1998) *Mol. Endocrinol. 12*, 544−555.

BI9830516