N-glycosylation requirements for the AT_{1a} angiotensin II receptor delivery to the plasma membrane

Benoit DESLAURIERS*†, Cecilia PONCE†, Colette LOMBARD†, Renée LARGUIER†, Jean-Claude BONNAFOUS† and Jacky MARIE†[1]

*Laboratoire de Médecine Moléculaire, Centre Hospitalier de l'Université de Montréal, 3840 rue St Urbain, Montréal, Quebec, H2W 158, Canada, and †INSERM U 439, 70 rue de Navacelles, 34090 Montpellier, France

The purpose of this work was to investigate the role of N-glycosylation in the expression and pharmacological properties of the the rat AT_{1a} angiotensin II (AII) receptor. Glycosylation-site suppression was carried out by site-directed mutagenesis (Asn → Gln) of Asn^{176} and Asn^{188} (located on the second extracellular loop) and by the removal of Asn^{4} at the N-terminal end combined with the replacement of the first four amino acids by a 10 amino acid peptide epitope (c-Myc). We generated seven possible N-glycosylation-site-defective mutants, all tagged at their C-terminal ends with the c-Myc epitope. This double-tagging strategy, associated with photoaffinity labelling, allowed evaluation of the molecular masses and immunocytochemical cellular localization of the various receptors transiently expressed in COS-7 cells. We showed that: (i) each of the three N-glycosylation sites are utilized in COS-7 cells; (ii) the mutant with three defective N-glycosylation sites was not (or was very inefficiently) expressed at the plasma membrane and accumulated inside the cell at the perinuclear zone; (iii) the preservation of two sites allowed normal receptor delivery to the plasma membrane, the presence of only Asn^{176} ensuring a behaviour similar to that of the wild-type receptor; and (iv) all expressed receptors displayed unchanged pharmacological properties (K_d for ^{125}I-sarcosine^{1}-AII; sarcosine^{1}-AII-induced inositol phosphate production). These results demonstrate that N-glycosylation is required for the AT_1 receptor expression. They are discussed in the light of current knowledge of membrane-protein maturation and future prospects of receptor overexpression for structural studies.

Key words: COS-7, epitope tagging, mutagenesis, photolabelling.

INTRODUCTION

Angiotensin II receptors are targets for the octapeptide angiotensin II (AII), one of the major components of the renin–angiotensin system [1]. They belong to the G-protein-coupled receptor (GPCR) family, characterized by a seven-trans-membrane-domain topology. Cloned angiotensin receptors have been classified as AT_1 and AT_2 receptor subtypes on the basis of their G-protein coupling and binding of peptidic and non-peptidic drugs [2–5], with the exception of *Xenopus* and turkey receptors, which do not fit with this classification [6]. Extensive studies on structure–function relationships on the AT_1 receptor have already been performed. Molecular modelling and mutagenesis approaches have been widely used to dissect the molecular events underlying recognition of peptide and non-peptide ligands and the process of isomerization between inactive and active conformations involved in receptor activation [7–12].

The amino acid sequence of the rat AT_1 receptor contains three potential sites for N-glycosylation, one at the N-terminus (Asn^{4}) and two on the second extracellular loop (Asn^{176} and Asn^{188}). It has been shown previously that a single mutation of any of these Asn residues preserves angiotensin recognition and hormone-induced inositol phosphate (IP) production [13]. In the present work we report a more complete analysis of the role of glycosylation in rat AT_1 receptor function through the construction of receptors corresponding to all possible combinations of saccharidic chain suppression. Mutagenesis experiments were performed on a receptor tagged with a peptide epitope. The effects of the mutations on the properties of the receptor were analysed by testing the ligand binding and coupling properties of the receptors, evaluating the molecular mass of the mutated proteins and checking their cellular localization after transfection in COS-7 cells. We demonstrate that: (i) the three potential sites of glycosylation of the rat AT_{1a} receptor are utilized in COS-7 cells; (ii) their suppression does not significantly affect the ligand binding and coupling properties of the receptor; (iii) the roles of the various glycosylation sites are not equivalent and specific preservation of one or two of them, according to their localization, is required for the correct delivery of the receptor to the plasma membrane. Furthermore, this work demonstrates that epitope tagging of the AT_1 receptor is suitable not only for cellular visualization and characterization of the receptor but also for its immunoprecipitation, the latter being a prerequisite for purification of the receptor that will be required in future biochemical or biophysical studies.

MATERIALS AND METHODS

Receptor constructs and site-directed mutagenesis

A sequence encoding 10 amino acids (EQKLISEEDL) of the human c-Myc protein [14] was added at the N- or/and C-termini of the rat AT_{1a} receptor using PCR. In order to reduce

Abbreviations used: AII, angiotensin II; GPCR, G-protein-coupled receptor; Sar, sarcosine; IP, inositol phosphate; TBS, Tris-buffered saline; WT, wild-type; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase.

[1] To whom correspondence should be addressed (e-mail marie@u401.montp.inserm.fr).

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the length of the primer encoding the c-Myc-epitope sequence, tagging of the receptor was generated by two steps of amplification with two sets of primers, as described below.

Tagging at the N-terminus

In the first amplification step the primers used were a forward oligonucleotide primer encoding the sequence of the last six amino acids of the c-Myc epitope followed by 21 nucleotides corresponding to amino acids 5–11 of the AT_{1a} receptor, 5′-ATCTCTGAGGAGGACCTGTCTTCTGCTGAAGATGG-TATC-3′ (primer 1) and a reverse primer corresponding to nucleotides 526–546 of the AT_{1a} receptor sequence, 3′-TTAT-AGTGTCACACGCGCAAA-5′ (primer 2). Primer 1 and primer 2 were used to amplify a 0.55 kb fragment using the AT_{1a} receptor cDNA cloned in the PECE vector [15] as a template. PCR conditions were: 30 cycles at 94 °C for 1 min, 67 °C for 1.5 min, 72 °C for 2 min. The amplified fragment was gel purified and used as a template for a second PCR amplification step using a forward oligonucleotide primer encoding a *Bgl*II site followed by an ATG initiation codon, the complete sequence of the c-Myc epitope and the first five nucleotides of the new AT_{1a} receptor sequence, 5′-ACGAAGATCTATGGAGCAGAAGCTGATC-TCTGAGGAGGACCTGTCTTC-3′ (primer 3). The reverse primer was primer 2 and PCR conditions were the same as in the first round of amplification. This second amplified fragment was digested with *Bgl*II and *Dra*III sites. The PECE vector containing the cDNA sequence of the AT_{1a} receptor was also digested with *Bgl*II and *Dra*III, gel-purified and the digested PCR fragment was inserted into the linearized plasmid. This construct contained an ATG initiation codon followed by the c-Myc-epitope sequence and the AT_{1a} receptor sequence starting at the fifth amino acid. The first four amino acids were excluded, including Asn^4, which belongs to the consensus glycosylation site located at the N-terminus. This construct was named myc-N.

Tagging at the C-terminus

In the first round of amplification, the forward primer corresponded to nucleotides 388–405 of the AT_{1a} receptor cDNA: 5′-ATCGTCCACCCAATGAAG-3′ (primer 4). The reverse primer matched the last 27 nucleotides of the AT_{1a} coding sequence, excluding the TGA stop codon (nucleotides 1056–1077), followed by a sequence encoding the first seven amino acids of the c-Myc epitope: 3′-CGGACGCAGAACAAAACTCCACCTCCTCG-TCTTCGACTAAAGGCTC-5′ (primer 5). Primers 4 and 5 were used to amplify a 0.72 kb fragment using the AT_{1a} receptor cDNA cloned in the PECE vector as a template. PCR conditions was identical to those used for epitope tagging at the N-terminus. The amplified fragment was gel purified and used as a template for amplification, in conditions as already indicated, with primer 4 as the forward primer and a reverse primer matching the last 24 nucleotides of the fragment followed by the sequence encoding the missing part of the c-Myc-epitope sequence, a stop codon and a sequence encoding an *Sma*I site, 3′-CTCCTCGTCT-TCGACTAAAGGCTCCTCCTGGACACTGGGCCCCCT-5′ (primer 6). The amplified fragment was digested with *Dra*III and *Xma*I (isoschizomer of *Sma*I). The PECE plasmid containing the AT_{1a} receptor sequence was digested with *Dra*III and *Xma*I, gel-purified and ligated with the digested amplified fragment. This construct contained the complete AT_{1a} receptor cDNA sequence fused to the c-Myc-epitope sequence at its C-terminus; it was named myc-C.

The cDNA containing the c-Myc epitope at the N- and C-termini was obtained by inserting the digested *Dra*III–*Sma*I fragment of the PECE–myc-C construct into the PECE–myc-N construct, which was digested with *Dra*III and *Xma*I, and gel-purified. It was named myc-N-C.

The chimaeric cDNAs were excised from the PECE plasmid using *Bgl*II and *Xba*I restriction enzymes and subcloned into the *Bam*HI and *Xba*I sites of the M13mp18 vector. Site-directed mutagenesis was performed by using the oligonucleotide-directed *in vitro* 'sculptor' mutagenesis system (Amersham). Oligonucleotides that changed Asn for Gln at positions 176 and 188 were 5′-CGCACACTGTGATTTGGGTGTTCTCGATG-3′ and 5′-GGGAGCGTCGATTGCCGAGACTCATAAT-3′, respectively. The sequences of the tagged cDNA constructs as well as the successful introduction of mutations were verified by sequencing using the dideoxynucleotide chain-termination method (T7 sequencing kit, Pharmacia Biotech). Wild-type (WT) and mutated receptors were subcloned into the *Xma*I site of the pCMV vector [16] for expression in COS-7 cells.

Expression of WT and mutated receptors in COS-7 cells

Receptors were transiently expressed in COS-7 cells by using the electroporation transfection method: briefly, 10^7 cells were resuspended in 300 μl of electroporation buffer (50 mM K_2HPO_4/20 mM KOH/20 mM CH_3COOK) and incubated for 10 min at room temperature in an electroporation cuvette (0.4-cm electrode gap, Bio-Rad) with 20 μg of pCMV carrier and different amounts of pCMV containing cDNA receptor sequences (0.1–2 μg range). Cells were submitted to an electric discharge (950 mF, 280 V, 50 ms) and cultured for 2 days in Dulbecco's modified Eagle's medium/4.5 g/l glucose/10 % fetal calf serum/100 units/ml streptomycin.

Ligand-binding assays

Sarcosine (Sar^1)-AII (Bachem) was radioiodinated as described previously [17]. [^{125}I]Sar^1-AII binding experiments were performed on intact cells cultured in 12-well plates. Transfected COS-7 cells were washed twice with PBS and incubated at 4 °C for 4 h, with various concentrations (0.01–10 nM) of [^{125}I]Sar^1-AII in the presence or absence of a 200-fold excess of Sar^1-AII in PBS (pH 7.4)/5 mM $MgCl_2$/1 mg/ml bacitracin/1 mg/ml BSA. Bound radioactivities were evaluated after washing the cells twice with binding medium and collecting them in 500 μl of 0.1 M NaOH.

IP assays

COS-7 cells expressing the WT and mutant receptors were grown in 6-well plates and labelled for 24 h with *myo*-[2-^{3}H]inositol (1.5 ml/plate, 1 mCi/ml; DuPont NEN) in Medium 199 deprived of serum. Before stimulation, cells were incubated at 37 °C for 1 h in IP buffer [116 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/2.5 mM $CaCl_2$/1.2 mM KH_2PO_4/5 mM $NaHCO_3$/20 mM Hepes/11 mM glucose/0.5 mg/ml bacitracin/0.5 mg/ml BSA (pH 7.4)]. After 15 min of preincubation in IP medium containing 10 mM LiCl, cells were incubated for 15 min at 37 °C, in the same medium in the presence or absence of Sar^1-AII. Pooled IPs were extracted and measured as described previously [18].

Photoaffinity labelling and immunoblotting analysis

Photoaffinity labelling of WT and mutated receptors

COS-7 cells grown on 15-cm plates were washed twice with PBS and incubated in the dark for 4 h at 4 °C in binding buffer containing 3 nM [^{125}I]Sar^1,(4′-N_3)Phe^8-AII [19] in the presence or

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absence of 5×10^{-7} M Sar[1]-AII. Cells were washed twice with PBS/5 mM $MgCL_2$ (pH 7.4). Then the medium was removed and the cell layer was irradiated with UV light (254 nm) for 7 min on ice. Photolysed cells were scraped with a rubber policeman in 1.5 ml of 10 mM Tris/HCl (pH 7.4)/1 mM PMSF/5 mM EDTA/1 % SDS (SDS solubilization buffer), homogenized and incubated for 20 min at room temperature to ensure solubilization of the receptors with a maximal yield (approximately 85 %). Then cell lysates were centrifuged at 14000 ***g*** for 20 min at 15 °C.

Photolabelled proteins were separated by SDS/PAGE (10 % gel) under denaturing conditions according to Laemmli's protocol [20]. Gels were fixed in a methanol/glycerol/water mixture (20:1:79, by vol.), dried and exposed to Kodak XAR-5 film at −80 °C.

Immunoprecipitation of photolabelled receptors

COS-7 cells grown on 15-cm plates were washed twice with PBS and then homogenized in 1.5 ml of 10 mM Tris/HCl (pH 8)/1 mM PMSF/5 mM EDTA/137 mM NaCl/10 % glycerol/1 % Triton X-100 (Triton solubilization buffer). Homogenates were incubated at room temperature for 20 min to ensure optimal solubilization of the receptors (receptor solubilization yield approx. 60 %), then centifuged at 14000 ***g*** for 20 min. Solubilized COS-7 cell extracts (500 μl) containing receptors photolabelled with [^{125}I]Sar[1],(4′-N_3)Phe[8]-AII were incubated overnight at 4 °C with a monoclonal anti-c-Myc antibody 9E10 [14] (10 μl of ascites fluid), then for 2 h at 4 °C with 100 μl of a suspension of Protein A–Sepharose beads (2 mg of Protein A/ml of Sepharose beads; Sigma). The sedimented beads were washed three times with 1 ml of cold Triton solubilization buffer, then resuspended in 100 μl of Laemmli buffer and heated at 100 °C for 5 min. Samples were analysed on a 10 % polyacrylamide gel. Immunoprecipitation yields were estimated from the radioactivities in gel slices corresponding to the covalent receptor complexes.

Immunoblotting

Transfected cells grown in 15-cm plates were washed twice with PBS (pH 7.4) and solubilized in 1.5 ml of SDS solubilization buffer, as indicated for the photolabelling experiments. The cell lysates were submitted to SDS/PAGE (10 % gel) and proteins were transferred to a nitrocellulose membrane (0.45 μm; Bio-Rad) in 25 mM Tris/192 mM glycine/20 % methanol. The nitrocellulose membrane was incubated overnight at 4 °C with an anti-c-Myc antibody 9E10 (dilution 1:100 of ascites fluid) in 50 mM Tris/HCl/150 mM NaCl/0.5 mg/ml sodium azide/10 % low-fat milk (pH 7.4; Tris-buffered saline, or TBS buffer). After washing, the membrane was incubated for 1 h at room temperature with anti-mouse IgG antibody (1:4000 dilution) coupled to peroxidase (Boehringer Mannheim) in TBS buffer containing 0.1 % Tween-20. The membrane was washed and proteins were detected by chemiluminescence using the bioluminescence kit from Boehringer Mannheim.

Enzymic deglycosylation

Transfected cells (1.5×10^7) were homogenized in 1 ml of 5 mM Tris/HCl/1 mM $MgCl_2$/protease inhibitors: 10 mg/ml leupeptin/20 mg/ml antipain/100 mg/ml benzamidine/10^5 units of aprotinin (pH 7.4). Homogenates were centrifuged at 100 ***g*** for 5 min at 4 °C and the supernatants were centrifuged at 40000 ***g*** for 10 min at 4 °C. The protein pellets were solubilized in 1 ml of 5 mM Tris/HCl/1 mM $MgCl_2$/1 % Triton X-100/1 mM PMSF (pH 7.4). Samples (50 μl) containing 50 μg of protein were treated with 1.5 units of peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase (PNGase F; Genzyme) for 18 h at 32 °C before SDS/PAGE analysis.

Subcellular localization of tagged receptors in COS-7 cells

Transfected cells were grown on glass coverslips. Cells were fixed with 4 % paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. Fixed cells were washed three times with PBS. Cell permeabilization was carried out by incubating fixed cells for 4 min with 0.2 % Triton X-100 in PBS and washing them three times with PBS. Cells were incubated for 30 min in PBS containing 3 % BSA and incubated overnight with the anti-c-Myc antibody 9E10 (1:100 dilution) in PBS containing 0.5 % BSA. After extensive washings, the cells were incubated for 1 h at room temperature with a goat anti-mouse antibody coupled to Rhodamine (1:500 dilution; GamIg TRITC, Nordic) in PBS containing 0.5 % BSA. After three 30-min washes, the cells were rinsed with PBS. Conventional immunofluorescence microscopy was performed using a Zeiss Axiophot microscope equipped with a Zeiss Neofluar 63 × 1.25 objective.

RESULTS

Expression and pharmacological properties of WT and mutated receptors

The AT_1 receptor amino acid sequence possesses three potential sites for N-glycosylation (Asn-Xaa-Ser/Thr): one at the N-terminal end of the receptor (Asn^4) and two on the second extracellular loop (Asn^{176} and Asn^{188}). Figure 1 shows the different receptors that we used in this study with combined mutations at the three consensus N-glycosylation sites. All modified receptors were generated on the AT_1 receptor bearing a sequence encoding a 10 amino acid epitope (c-Myc) at its C-terminal end (myc-C). The receptor lacking the first potential glycosylation site was generated by deleting the first four amino acids, including Asn^4, and introducing at the N-terminus an additional c-Myc epitope (myc-N-C). The purpose of this modification was to allow receptor detection from the outside of the cell, the absence of proximate bulky saccharidic chains being liable to favour c-Myc-epitope recognition by a specific antibody. As indicated below, elimination of Asn^4 and addition the c-Myc epitope at the N-terminus of the receptor had no effect on function and expression of the receptor and allowed efficient receptor detection.

Epitope tagging of the receptor at the N- or C-termini did not modify the binding properties of the receptor, as shown by unchanged dissociation constants (K_d) for [^{125}I]Sar[1]-AII and maximal binding capacities (B_{max}) of WT and tagged receptors measured on intact transfected COS-7 cells (for details, see Table 1). Expression levels of the various constructs were checked after COS-7 cell transfection with variable amounts of cDNA up to 2 μg (Table 1 and Figure 2). All the receptors possessing two glycosylation sites were expressed at levels similar to that of the WT receptor. The suppression of two glycosylation sites drastically reduced receptor expression with the exception of the receptor possessing Asn^{176} (myc-N-C, Q188) (Table 1 and Figure 2), which emphasizes the essential role of this site. The expression level of the fully defective mutant (myc-N-C, Q176,Q188) was only 3 % of the WT receptor (Table 1 and Figure 2). These results indicate that the glycosylation site at the Asn^{176} residue is sufficient but not necessary for normal expression of the receptor and suggest that N-glycosylation is required for efficient expression of the receptor at the surface of the cell. When binding was detectable, all the defective glycosylation site mutants

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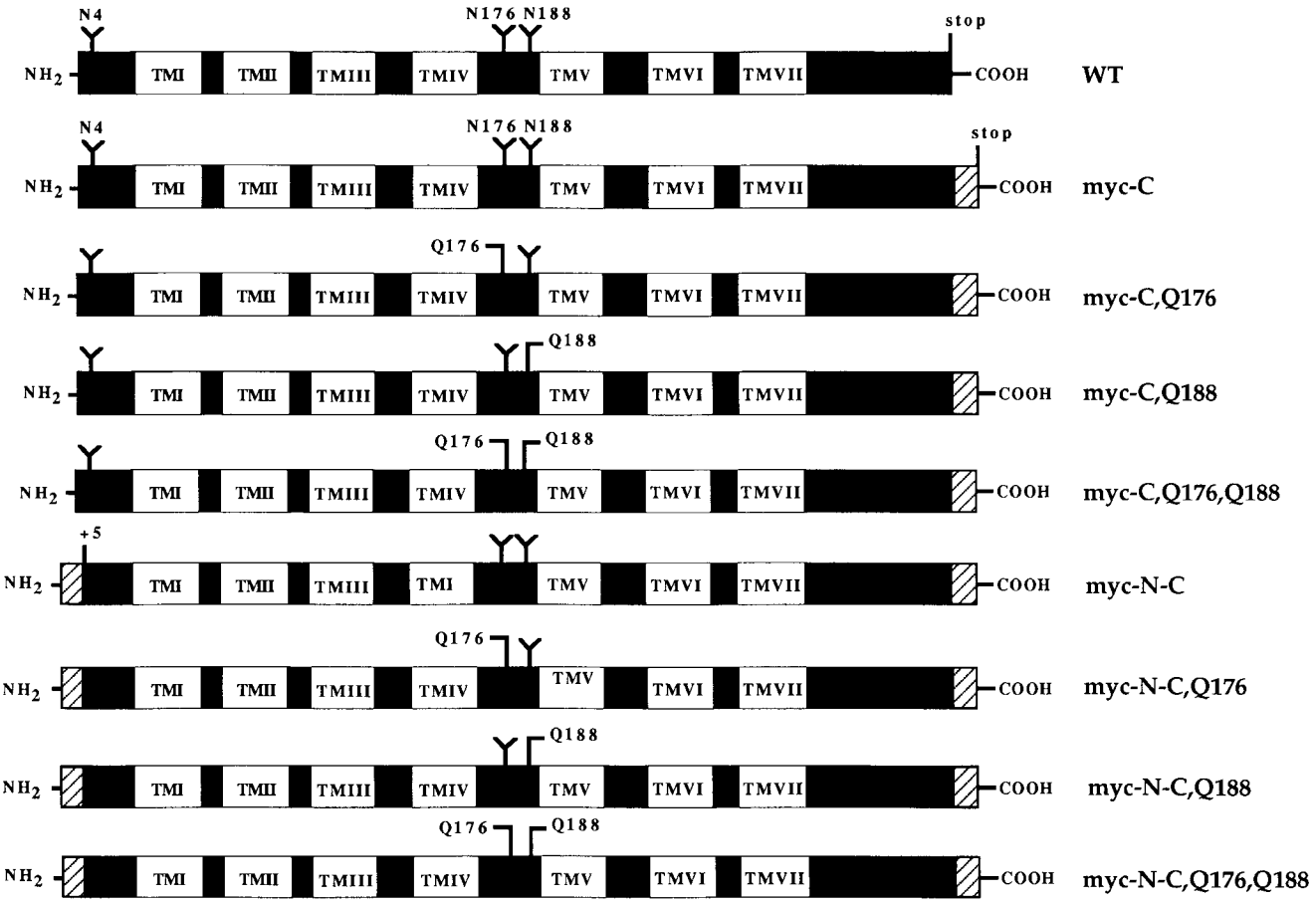

Figure 1 Schematic representation of N-glycosylation-site-defective receptors

This Figure represents the WT and N-glycosylation-site-defective mutant receptors with their seven transmembrane domains (TM). All the receptors were eptitope-tagged with the peptide c-Myc (hatched boxes on right) at their C-termini (myc-C). Asn residues of the consensus sequence Asn-Xaa-Ser/Thr for potential N-glycosylation are Asn^4 (N4), Asn^{176} (N176) and Asn^{188} (N188). Asn^4-site-defective mutant was obtained by deletion of the first four amino acids [the AT_1 receptor sequence starting at its fifth amino acid (+5)] and addition of a second c-Myc epitope (myc-N-C; hatched boxes on left). Q176, Q188 and Q176,Q188 represent Asn → Gln mutations at positions 176, 188 or both, respectively.

displayed unchanged binding affinities for [^{125}I]Sar^1-AII as compared with the WT receptor (see K_d values shown in Table 1).

Angiotensin-stimulated IP production of cells transfected with WT and mutant receptors

COS-7 cells were transfected with 100 ng of cDNA of WT, myc-C, myc-N-C, myc-N-C, Q176 and myc N-C, Q188 and 2 μg of cDNA of myc-N-C, Q176,Q188 in order to obtain similar expression levels of the WT and mutant receptors [$(2–3) \times 10^5$ sites/cell]. As shown in Table 1, the extent of maximal IP production was very similar for WT and mutant receptors when stimulated with Sar^1-AII (10^{-7} M), suggesting that N-glycosylation does not play a significant role in AII-stimulated IP production via receptor coupling to phospholipase C.

Evaluation of carbohydrate addition at the potential N-glycosylation sites by molecular-mass determination of WT and mutant receptors

Analysis of photolabelled receptors by SDS/PAGE and autoradiography

Photoaffinity labelling of WT and mutant receptors were carried out using the iodinated photoreactive probe [^{125}I]Sar^1,(4′-N_3)Phe^8-AII. This agonist probe of the AT_1 receptor has been shown be suitable for specific covalent labelling of the AT_1 receptor [19] and has been used for molecular-mass determination of AT_1 receptors from different tissues and species [19,21,22].

As shown in Figure 3, photoaffinity labelling of c-Myc-epitope-tagged receptor expressed in COS-7 cells followed by SDS/PAGE and autoradiography analysis led to the detection of a protein with a molecular mass of 153 ± 12 kDa, similar to the WT receptor (Figure 3A, lanes a and b). This labelling was specific, and was suppressed when performed in the presence of excess Sar^1-AII. The molecular mass of the detected protein, which was much higher than the one calculated from its amino acid sequence [41 kDa (WT receptor) + 1.2 kDa (c-Myc epitope)], as well as the breadth of the band, indicated that the AT_1 receptor was highly glycosylated when expressed in COS-7 cells. The molecular mass of the photolabelled mutant lacking Asn^4 (myc-N-C) was reproducibly lower than that of myc-C, but the shift could not be accurately estimated due to the low resolution of polyacrylamide gels in the corresponding range of molecular mass (Figure 3A). The contribution of Asn^4 to the shift in molecular mass could more easily be seen for mutants lacking an additional glycosylation site [compare myc-N-C, Q176 and myc-N-C, Q188 with myc-C, Q176 and myc-C, Q188 (Figure 3B)]; it was estimated to

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Table 1 Ligand binding and coupling properties of WT and mutant receptors expressed in COS-7 cells

1[^{125}I]Sar^1-AII binding was performed on intact COS-7 cells transfected with cDNA amounts that ensured maximal expression levels for each receptor (2 μg of cDNA for WT, myc-C, myc-N-C, myc-N-C, Q188 and myc-N-C, Q176,Q188 and 1 μg for myc-N-C, Q176) as indicated in the Materials and methods section. Dissociation constants (K_d) and maximal binding capacities (B_{max}) were determined by Scatchard analysis. Values are means $\pm$ S.D. of four independent experiments performed in triplicate. Total (T) and non-specific (NS) binding determined for 1×10^5 cells incubated with a saturating concentation of [^{125}I]Sar^1-AII (10 nM, 180 Ci/mmol) were: T, 195000 $\pm$ 1000 c.p.m. and NS, 4360 $\pm$ 350 c.p.m. for the WT receptor; T, 37500 $\pm$ 500 c.p.m. and NS, 3500 $\pm$ 550 c.p.m. for the myc-N-C, Q176 mutant; and T, 9200 $\pm$ 650 c.p.m. and NS, 3400 $\pm$ 400 c.p.m. for the myc-N-C, Q176,Q188 mutant (values are means $\pm$ S.D. of triplicates obtained in a representative experiment). Sar^1-AII-induced IP production was measured in COS-7 cells expressing the WT or N-glycosylation-site-defective-mutant receptors [(200–300) $\times 10^3$ sites/cell]. Cells were stimulated for 15 min by 10^{-7} M Sar^1-AII and pooled IPs were extracted and measured as described in the Materials and methods section. Values are means $\pm$ S.D. of three independent experiments performed in triplicate. Basal and Sar^1-AII-stimulated IP production values were: 478 $\pm$ 28 (basal) and 2052 $\pm$ 200 (stimulated) d.p.m. and 608 $\pm$ 100 (basal) and 1880 $\pm$ 150 (stimulated) d.p.m. for cells expressing WT receptor (200×10^3 sites/cell) and myc-N-C, Q176 mutant receptor (195×10^3 sites/cell), respectively (means $\pm$ S.D. from one typical experiment performed in triplcate). ND, non-detectable binding; –, not determined.

Receptor	[^{125}I]Sar^1-AII binding		IP production (% of WT maximal)
	K_d (nM)	B_{max} (% of WT)	
WT	0.79 $\pm$ 0.25	100	100
myc-N	0.82 $\pm$ 0.23	98 $\pm$ 15	—
myc-C	0.46 $\pm$ 0.09	100 $\pm$ 5	95 $\pm$ 4
myc-C, Q176	0.50 $\pm$ 0.15	97 $\pm$ 19	—
myc-C, Q188	0.63 $\pm$ 0.12	95 $\pm$ 17	—
myc-C, Q176,Q188	ND	ND	—
myc-N-C	0.79 $\pm$ 0.12	83 $\pm$ 5	97 $\pm$ 7
myc-N-C, Q176	0.74 $\pm$ 0.18	16 $\pm$ 5	90 $\pm$ 1
myc-N-C, Q188	0.63 $\pm$ 0.12	86 $\pm$ 13	150 $\pm$ 49
myc-N-C, Q176,Q188	0.84 $\pm$ 0.10	3 $\pm$ 0.5	92 $\pm$ 28

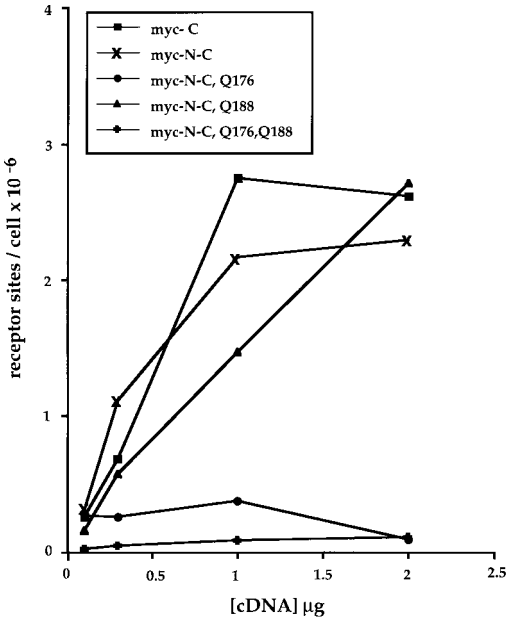

Figure 2 Expression of mutant receptors in COS-7 cells

COS-7 cells were transfected with the indicated amounts of cDNAs encoding the various N-glycosylation-site-defective mutants and the expression levels were determined in intact cells incubated with a saturating concentration of [^{125}I]Sar^1-AII (10^{-8} M). Non-specific binding was estimated in the presence of a 100-fold excess of unlabelled Sar^1-AII as indicated in the Materials and methods section. Similar results were obtained in three separate experiments.

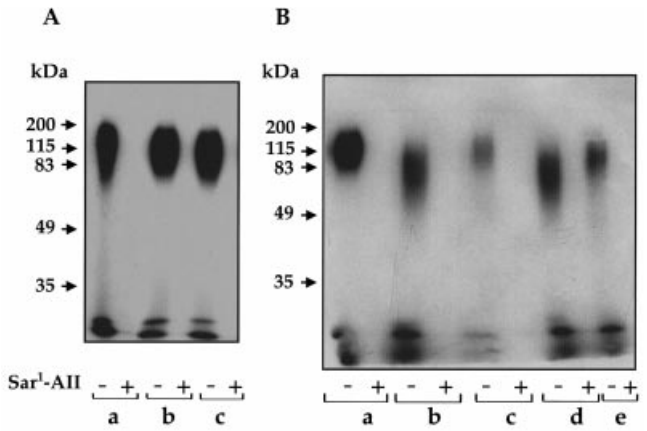

Figure 3 Electrophoresis pattern of photolabelled mutant receptors

COS-7 cells expressing the receptors at maximal levels were photolabelled with 5 nM [^{125}I]Sar^1,(4′-N_3)Phe^8-AII in the presence (+) or absence (—) of a 100-fold excess of Sar^1-AII. Cell extract containing 10–15 fmol of labelled receptors submitted to photolysis [(40–60) $\times 10^3$ d.p.m.] were prepared and analysed by SDS/PAGE (10% gel) followed by autoradiography as described in the Materials and methods section. Prestained molecular-mass markers (Bio-Rad) are indicated by arrows. (**A**) Electrophoretic pattern of WT and c-Myc-epitope-tagged receptors. Lane a, WT receptor; lane b, receptor tagged at its C-terminus (myc-C); and lane c, receptor tagged at both N- and C-termini (myc-N-C). (**B**) Electrophoretic pattern of N-glycosylation-site-defective mutants. Lane a, myc-N-C; lane b, myc-N-C, Q176; lane c, myc-C, Q176; lane d, myc-N-C, Q188; and lane e, myc-C, Q188.

25–30 kDa. N176Q and N188Q mutations induced decreases in the molecular masses of the photolabelled receptors (85 $\pm$ 6 and 80 $\pm$ 1 kDa for myc-N-C, Q176 and myc-N-C, Q188 mutants respectively; Figure 3B), indicating that Asn^{176} and Asn^{188} are sites for carbohydrate addition. Taken together these data demonstrate that each of the three potential N-glycosylation sites is utilized in COS-7 cells.

As the fully glycosylation-defective mutant (myc-N-C, Q176,Q188) was poorly expressed at the plasma membrane, its molecular mass could not be evaluated through photolabelling and autoradiography.

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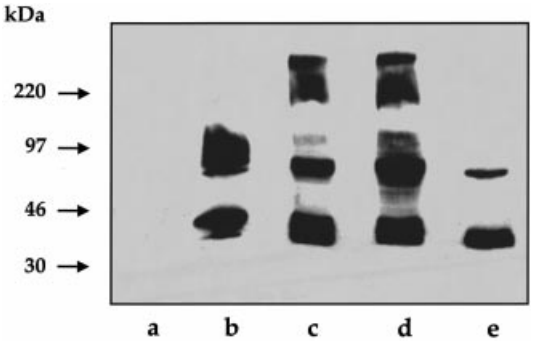

Figure 4 Western-blot analysis of WT and mutant receptors

COS-7 cells expressing the WT or epitope-tagged mutant receptors were homogenized in SDS solubilization buffer. Extracts (40–50 μg of protein) were submitted to SDS/PAGE (10% gel) and proteins were electroblotted on to a 0.45 μm nitrocellulose membrane. Epitope-tagged proteins were revealed by immunoblotting using anti-c-Myc antibody with chemiluminescence detection as described in the Materials and methods section. Arrows indicate the position of prestained markers (rainbow markers from Amersham). Lane a, WT; lane b, myc-N-C; lane c, myc-N-C, Q176; lane d, myc-N-C, Q188; and lane e, myc-N-C, Q176,Q188. This experiment is representative of four independent experiments which gave similar results.

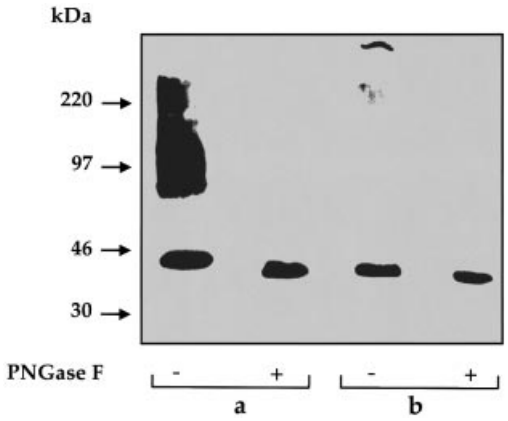

Figure 5 Enzymic deglycosylation of the receptors

Cell lysates were prepared from COS-7 cells as described in the Materials and methods section. Samples (50 μg of protein) were incubated for 18 h at 32 °C in the presence (+) or absence (−) of 1.5 units of PNGase F. Samples were submitted to SDS/PAGE (10% gel) and immunoblotted with an anti-c-Myc antibody with chemiluminescence detection as described in the Materials and methods section. Lane a, myc-N-C; and lane b, myc-N-C, Q176,Q188. Prestained markers (rainbow markers, Amersham) are indicated by arrows.

Immunoblotting detection of WT and mutant receptors

Single tagging (N- or C-terminal) of the AT_1 receptor did not allow its efficient detection through SDS/PAGE analysis of COS-7 cell extracts and immunoblotting. However, the epitope-tagged receptors solubilized in 0.1% Triton X-100 detergent could be immunoprecipitated with anti-c-Myc antibodies adsorbed on Protein A–Sepharose beads, with a yield of 40% (results not shown). These results indicate that the epitope was not easily accessible when the protein was denatured in SDS and transferred to nitrocellulose membrane. Double tagging of the receptor (myc-N-C) not only increased the yield of receptor immunoprecipitation up to 60% (results not shown), but also increased the sensitivity of immunoblotting detection. Thus only receptors bearing the epitope at the two N- and C-terminal ends (lacking Asn^4) were analysed by Western blotting. As shown in Figure 4, two major bands were detected by the c-Myc antibody for myc-N-C, myc-N-C, Q176 and myc-N-C, Q188: low-molecular-mass forms (35–43 kDa), present in the three mutants, and higher-molecular-mass forms with sizes varying with the analysed receptor. As shown in Figure 4 (lane a), the staining was specific for the c-Myc epitope; no band was detected in cell extract containing the WT receptor. The high-molecular-mass bands detected by the c-Myc antibody corresponded to those detected by receptor photolabelling; they probably represented molecular forms of receptors that had reached the plasma membrane and were accessible to labelling on intact cells. As already found in photolabelling experiments (Figure 3), a shift of these immunodetected bands was observed for the N176Q and N188Q mutants (Figure 4, lanes c and d), confirming that Asn^{176} and Asn^{188} bear carbohydrate chains. The low-molecular-mass band was heterogeneous except for the case of the fully defective glycosylation-site mutant (myc-N-C, Q176,Q188; Figure 4, lane e), this latter was characterized by a 35 kDa entity corresponding to the lower-molecular-mass component detected for the other mutants (Figure 4, lanes b, c and d). This band is characteristic of the fully deglycosylated receptor, as it was not significantly shifted upon PNGase F treatment (Figure 5, lane b); it was obtained through enzymic treatment of the glycosylated receptor (Figure 5, lane a). One must notice that this value is significantly lower than expected from the amino acid sequence of the tagged receptor (41 kDa and 1.8 kDa for the AT_1 receptor and c-Myc epitope, respectively). Such a difference between theoretical and experimental molecular masses has already been observed for angiotensin receptors [23–25] and histamine H_2 receptors [26]. Besides a possible inaccurate estimation of molecular masses by SDS/PAGE, one cannot exclude proteolysis of the receptor, which might be facilitated in the absence of N-linked sugars and would agree with the well-known protecting role of saccharidic chains [27,28]. Such a hypothesis would imply the loss of one of the two c-Myc epitopes; immunoblotting detection in this situation would be facilitated by increased accessibility of the remaining epitope, as compared with the detection of the glycosylated receptor, which required both tags.

Surprisingly, in some experiments a 64 ± 5 kDa molecular-mass band was detected in cellular extracts prepared from cells expressing the receptor lacking the three consensus N-glycosylation sites (myc-N-C, Q176,Q188; Figure 4, lane e). One can speculate that this band corresponds to aggregated forms of the receptor (its size is consistent with that of a dimer); this hypothesis is supported by data reporting the existence of multimeric forms of GPCR resistant to denaturing conditions [29–31]. The very-high-molecular-mass bands detected for other mutants might reflect the presence of aggregated forms of fully or partially glycosylated receptors.

Subcellular localization of WT and N-glycosylation-site-defective receptors

In order to verify the fate of mutant receptors that could not be detected through radioligand binding, their expression was checked in intact COS-7 cells by indirect immunostaining using a monoclonal anti-c-Myc antibody and a Rhodamine-labelled anti-mouse antibody. Staining of COS-7 cells transfected with the receptor bearing the c-Myc epitope at its C-terminus was only visible when they were permeabilized (Figure 6, row 2). The intracellular staining, localized at the inner face of the plasma membrane and inside the cell, was specific, as shown by its absence on cells transfected with the WT receptor (Figure 6, row

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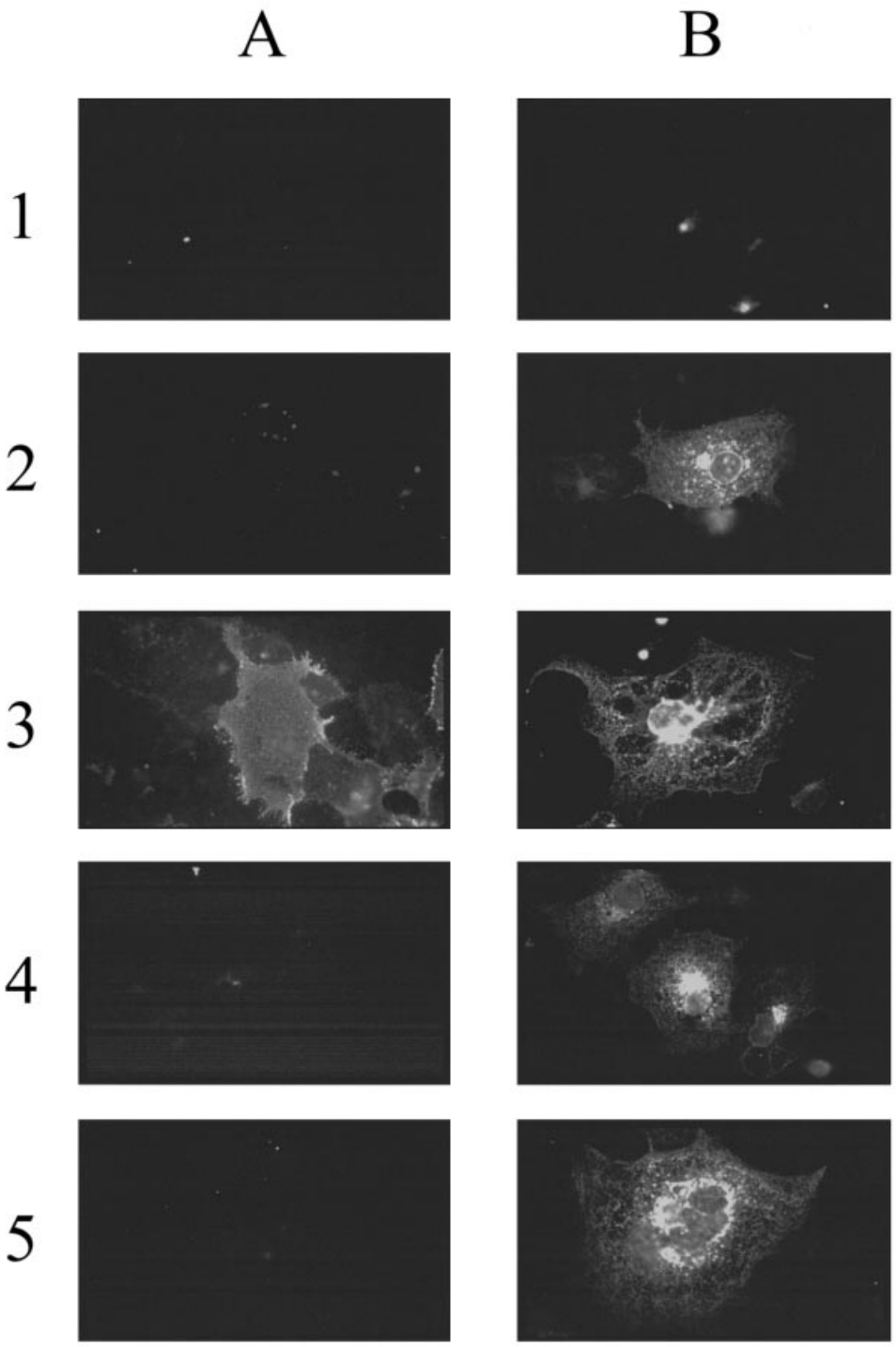

Figure 6 Cellular localization of the WT and mutant receptors

COS-7 cells expressing the WT or epitope-tagged mutant receptors were incubated with the anti-c-Myc antibody 9E10. Immunostaining was performed with an anti-mouse antibody coupled to Rhodamine, as described in the Materials and methods section. Row 1, WT; row 2, myc-C; row 3, myc-N-C; row 4, myc-C, Q176,Q188; and row 5, myc-N-C, Q176,Q188. (**A**) Non-permeabilized cells; (**B**) permeabilized cells. Photographs were all taken at the same magnification with the same objective and are samples from the same experiment. They are representative of a large number of specimens obtained from three independent experiments. Magnification $\times 630$.

1). As expected, the same pattern was obtained with cells transfected with receptor tagged at both ends (myc-N-C; Figure 6B, row 3). However, this latter could be detected from the outside without cell permeabilization (Figure 6A, row 3). These results indicate that the receptor lacking the Asn^4 residue was normally expressed at the plasma membrane, thus confirming the [^{125}I]Sar^1-AII-binding experiments. As shown in Figure 6, receptors mutated at both Asn^{176} and Asn^{188} (myc-C, Q176,Q188)

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or lacking the three consensus glycosylation sites (myc-N-C, Q176,Q188) were not detected on the surface of non-permeabilized cells, but could be stained inside permeabilized cells. These data are in agreement with the very low or undetectable [^{125}I]Sar^1-AII binding (see binding experiments, Table 1), demonstrating that these mutants displayed impaired trafficking to the plasma membrane. Although the identification of the stained intracellular compartments requires further studies, the perinuclear localization of these mutants suggests that they might be sequestered to the endoplasmic reticulum.

DISCUSSION

The role of N-glycosylation in the pharmacological properties and expression of GPCR has been investigated for some members of this family [26,32–39]. As do most heptahelical receptors, the rat AT_{1a} AII receptor possesses several potential consensus sites for N-glycosylation [6,9], one located at the N-terminus (Asn^4) and two located in the second extracellular loop (Asn^{176} and Asn^{188}). A preliminary study of Yamano et al. [13] reported that individual mutations of these sites had no effect on receptor function. The purpose of the more detailed mutagenesis study reported in the present work was to investigate the role of N-linked saccaridic chains on receptor functions: expression at the plasma membrane, AII recognition and phospholipase C-coupling properties. The work involved photaffinity labelling, and epitope tagging of the WT and mutant receptors for their detection through immunoblotting and immunocytochemical cellular localization. All the mutants were generated on a receptor bearing a 10 amino acid c-Myc epitope at the C-terminal end of the receptor. Asn^{176} and Asn^{188} were mutated into Gln while Asn^4 was removed by deleting the first four amino acids and replacing them with an additional c-Myc sequence. This double tagging was shown to amplify the sensitivity of receptor immunodetection by Western blotting. The introduction of these tags, including N-terminal tagging associated with Asn^4 suppression, did not modify [^{125}I]Sar^1-AII binding, phospholipase C coupling or processing properties of the receptor. SDS/PAGE analysis of photolabelled receptors and immunoblotting detection together with enzymic deglycosylation demonstrated that the three potential N-glycosylation sites actually bear carbohydrate chains in COS-7 cells, as assessed by the striking changes in the molecular masses of mutant receptors. An extensive analysis of the properties of mutant receptors lacking one or several glycosylation sites allowed us to draw conclusions about the requirements for normal expression. N-glycosylation is required for receptor expression at the plasma membrane as the receptor lacking the three sites could be detected neither by ligand binding nor by immmunocytochemical detection in non-permeabilized intact cells. We also demonstrated that the presence of two sites, irrespective of their localization, allowed an expression level similar to that of the WT receptor. Interestingly, only the preservation of Asn^{176} insured optimal expression. The same was not found for Asn^4 and Asn^{188}, demonstrating that the roles of the saccharidic chains at the various sites are not equivalent. All the mutant receptors that were expressed at the plasma membrane displayed unchanged pharmacological properties (K_d for ^{125}I-Sar^1-AII binding; Sar^1-AII-induced IP production) as compared with the WT receptor.

Epitope tagging has allowed us to monitor the cellular localization of the various receptors. The validity of the strategy and experimental conditions can be verified; N-terminal tagging with the c-Myc epitope proved to be appropriate for the specific detection of expressed receptors from the outside of the cell, whereas intracellular receptors were detected in permeabilized cells. We confirmed that mutant receptors that were undetectable by ligand binding to intact cells were not expressed at the plasma membrane; moreover they were normally detected intracellularly, indicating that their biosynthesis was not affected by glycosylation-site suppression. The immunochemical staining was more concentrated near the nucleus, suggesting that the protein accumulates in the endoplasmic reticulum of the cell. As a consequence, the lack of delivery of some glycosylation-deficient mutants can be interpreted as resulting from their impaired folding. Indeed, the role of N-glycosylation on protein maturation has been documented widely and a folding process based on the binding of the lectins calnexin and calreticulin to the newly synthesized glycoprotein has been described [40,41]. Although calnexin-dependent and -independent mechanisms have been postulated to participate in the conformational maturation of the vasoactive intestinal peptide 1 receptor [35], studies on the relationships between N-glycosylation and molecular chaperones, such as lectins present in the ER, remain to be extended to other GPCRs. Up to now, a general role for N-glycosylation within this family has not been clarified. Whereas N-glycosylation requirements for the trafficking of the β_2 adrenergic receptor [32–34], vasoactive intestinal peptide 1 receptor [35], rhodopsin [36] and gonadotropin-releasing hormone receptor [37] have been reported, N-glycosylation is not essential for the processing of m_2 muscarinic receptor [38], histamine H_2 receptor [26] or parathyroid receptor [39]. The role of N-glycosylation in the AT_1-receptor trafficking might explain the difficulties we encountered for its expression in SF9 insect cells (D. Solier, J. C. Bonnafous and J. Marie, unpublished work) and *Escherichia coli* (B. Bertin, J. Marie and D. Strosberg, personal communication), which are cell systems in which oligosaccharide processing is lacking (*E. coli*) or differs from that of mammalian cells (SF9 cells) ([42] and references cited therein). The difficulty of expressing functional receptors in *E. coli* could be overcome by forcing their accumulation in inclusion bodies and reconstituting them in lipid vesicles, as shown by an elegant study of an olfactory receptor [43]. Nevertheless, the development of eukaryotic systems for GPCR overexpression appears the most straightforward, as shown for rhodopsin [44]. The development of overexpression and purification systems [42] remains, to date, a challenge for most of the laboratories working on structure–function relationships of GPCRs, including their physicochemical or biophysical characterization. In this respect, the present work constitutes a prerequisite for future structural studies, inasmuch as it has delineated a set of receptor constructs that allow preservation of expression properties and are adapted to their purification using c-Myc antibodies.

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