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Comparative analysis of the human angiotensin II type 1a receptor heterologously produced in insect cells and mammalian cells

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Abstract

Angiotensin II type 1a receptor ($AT_{1a}R$) is a member of GPCR superfamily and it plays crucial roles in the regulation of blood pressure, hormone secretion and renal functions. Here, we report functional overexpression and characterization of the human $AT_{1a}R$ in insect cells using the baculovirus system and in mammalian cells using the Semliki Forest virus system. The recombinant receptor was expressed at a level of 29–32 pmol/mg and it binds to angiontensin II with high affinity ($K_d = 0.98$–1.1 nM). Angiotensin II stimulated accumulation of inositol phosphate and phosphorylation of MAP kinase was also observed, which indicated that the recombinant $AT_{1a}R$ could couple to endogenous $G\alpha_q$ protein. Confocal laser scanning microscopy revealed that the recombinant receptor was predominantly localized in the plasma membrane and agonist induced internalization of the recombinant $AT_{1a}R$ was also observed. The recombinant $AT_{1a}R$ was expressed in glycosylated form and *in vivo* inhibition of glycosylation suppressed its surface expression.

Keywords: GPCR; Angiotensin receptor; Overexpression; BHK cells; Insect cells; Glycosylation; Localization

The octapeptide hormone angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) plays regulatory roles in cardiovascular homeostasis, blood pressure, and vascular resistance [1,2]. The target tissues of angiotensin II include adrenals, kidney, brain, vascular smooth muscle cells, and pituitary gland [3]. Although angiotensin II can stimulate two cell surface receptors (AT_1R and AT_2R), it has been found that most of its cellular actions are mediated via the AT_1R [4]. Two distinct subtypes of AT_1R (i.e. AT_{1a} and AT_{1b}) have been identified and they are approximately 95% similar in their amino acid sequence. However, tissue distribution, chromosomal localization, genomic structure, and transcriptional regulation of $AT_{1a}R$ and $AT_{1b}R$ differ significantly [4]. The $AT_{1a}R$ is a member of G protein-coupled receptor superfamily and belongs to class A GPCRs [4]. Like other GPCRs (except rhodopsin), the $AT_{1a}R$ is present in only small amounts (10–100 fmol/mg) in native tissues [5,6]. Transient expression of $AT_{1a}R$ in mammalian cells has allowed biochemical and functional characterization of the receptor [7,8]. However, direct structural studies are required to understand the molecular basis of ligand binding and to facilitate structure based drug design. Structural studies on GPCRs and membrane proteins in general, require milligram amounts of functional protein. Therefore, heterologous expression of recombinant $AT_{1a}R$ is necessary for structural characterization.

In last years, a number of heterologous expression systems have been developed for heterologous expression of GPCRs [9]. The baculovirus mediated expression in insect cells is an efficient way for producing high levels of recombinant membrane proteins [10]. Several GPCRs have been overexpressed and characterized successfully using this system [9–11]. In most cases, the recombinant receptors produced in insect cells exhibit identical ligand binding properties as in native tissues.

For the expression of recombinant human membrane proteins, mammalian cells have the most native like environment. However, generation of stable cells lines and

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transient transfection result in only low expression level of the recombinant proteins. Recent development of Semliki Forest virus based vectors has allowed high-level expression of some GPCRs in mammalian cells [12]. However, detail characterization of the heterologously produced receptor is required to make sure that the recombinant receptor exhibits similar characteristics as its native counterpart.

In this study, we tested the baculovirus mediated expression of $AT_{1a}R$ in insect cells and the Semliki Forest virus mediated expression in mammalian cells. The $AT_{1a}R$ was expressed as a fusion protein with N- and C-terminal tags. Expression level was monitored by immunoblot analysis and ligand binding assay. In addition, the functional coupling of the recombinant receptor to the endogenous G protein was also analysed. The cellular localization and agonist induced internalization of the eGFP tagged $AT_{1a}R$ was analysed using confocal laser scanning microscopy. Glycosylation state of the recombinant receptor as well as the effect of deglycosylation on the surface expression of $AT_{1a}R$ was also investigated.

Materials and methods

Materials. [^{3}H]angiotensin II (50–60 Ci/mmol) and [^{2,3}H]inositol (30 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA, USA). The cell culture medium, glutamine, phosphate-buffered saline (PBS), trypsin–EDTA, and penicillin–streptomycin solution were obtained from Cell Concepts GmbH (Umkirch, Germany) or Sigma (St. Louis, MO, USA). Fetal calf serum was from PAA laboratories (Colbe, Germany). α-Chymotrypsin was from ICN chemicals (CA, USA) and aprotinin from Roche Applied Science (Mannheim, Germany). AG1X8 columns and electroporation cuvettes were obtained from Bio-Rad laboratories (CA, USA). MAP kinase assay kit and 7 mG(ppp)G RNA Cap structure analog was obtained from New England Biolabs (Beverly, MA, USA). SP6 polymerase, transcription buffer, restriction enzymes, PNGaseF, and EndoH were from MBI fermentas (St. Leon-Rot, Germany). Modified baculovirus DNA (BaculoGold) was obtained from Pharmingen (San Diego, CA, USA). Immobilon-P polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA, USA). Anti-his antibody, Anti-Flag M2 antibody, alkaline phosphatase-coupled streptavidin, alkaline phosphatase-coupled secondary antibody, and tunicamycin were from Sigma (St. Louis, MO, USA). Anti-calnexin antibody was from Santa Cruz Biotechnology (Heidelberg, Germany) and Cy3-coupled secondary antibody was from Jackson ImmuoResearch (Hamburg, Germany).

Generation of the recombinant expression constructs. For heterologous expression of $AT_{1a}R$ in insect cells, two different expression constructs were created (Fig. 1). Both constructs contain a Flag and a His_{10} tags at the N-terminus of $AT_{1a}R$ and either a biotinylation domain of *Propionobacterium shermanii* transcarboxylase (pVLMelFlag$His_{10}AT_{1a}$RBio) or eGFP (pVLMelFlag$His_{10}AT_{1a}$R-eGFP) at the C-terminus. The prepromelittin signal peptide from honeybee was used for proper targeting of the recombinant receptor. In all constructs, the polyhedrin promoter (PHP) drives the expression of recombinant receptor. A PCR was performed with primers AT_{1a}R_Fw (5′-CGGGATCCCCATTCTCA ACTCTTCTACTGAAG-3′) and AT_{1a}R_Rv (5′-CGGAATTCCTCA ACCTCAAAACATGGTGC-3′), using pcDNA3-AT_{1a}R as a template. The resulting DNA fragment was digested with *Bam*HI and *Eco*RI enzymes and ligated into appropriately digested expression vectors.

Two different expression constructs were generated for the expression of $AT_{1a}R$ in mammalian cells (Fig. 1B). These constructs are based on the pSFV2CAP vectors that have been described earlier [13a,b]. A PCR was performed with primers AT_{1a}R_Fw (5′-CGGGATCCCATTCTCA ACTCTTCTACTGAAG-3′) and AT_{1a}R_Rv (5′-CGACTAGTCTCAA CCTCAAAACATGGTGC-3′), using pcDNA3-AT_{1a}R as a template. The PCR product was digested with *Bam*HI and *Spe*I restriction enzymes and ligated into appropriately digested expression vectors. Both vectors contain a subgenomic 26S promoter (PSG) for the expression of $AT_{1a}R$ and a viral capsid sequence that works as translation enhancer. At the C-terminus of $AT_{1a}R$, either a His_{10} tag and the biotinylation domain of *Propionobacterium shermanii* transcarboxylase [14] or eGFP coding region was added. These tags were separated from $AT_{1a}R$ coding region by a TEV (tobacco etch virus) protease cleavage site and they can be removed from the receptor, if required. AT_{1a}R-eGFP fusion construct was created for localization and internalization analysis of the recombinant receptor. The helper plasmid pSFV-helper 2 used for *in vitro* transcription has been described previously [15a,15b]. The constructs were verified by DNA sequencing (SeqLab GmbH, Germany).

Insect cell culture, generation of baculovirus stock and infection. Sf9 (*Spodoptera frugiperda* ovarian cells) were cultured in TNM-FH medium (Grace's basal medium with lactalbumin hydrolysate and yeast olate) supplemented with 5% (v/v) fetal bovine serum and 50 mg/ml gentamycin. High Five (H5) cells (derived from *Trichoplusia ni* egg cell homogenates) were cultured in Express Five medium (Invitrogen). Suspension cultures of Sf9 cells were grown in Erlenmeyer flasks at 27 °C with shaking at 110 rpm.

To generate the recombinant baculovirus stocks, 0.1 μg of linearized BaculoGold DNA (Pharmingen) and 10 μg of recombinant baculovirus

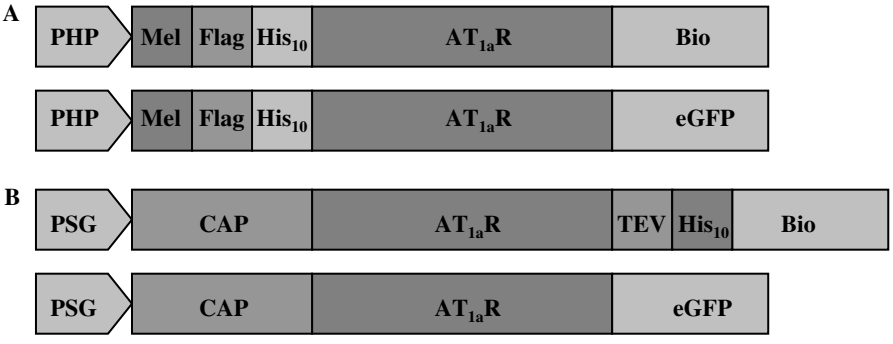

Fig. 1. Schematic representation of the recombinant expression constructs for heterologous production of the human $AT_{1a}R$ in Sf9 cells (A) and BHK cells (B). PHP, polyhedrin promoter; Mel, coding sequence for the prepromelittin signal sequence of honeybee; Flag, coding region for the Flag epitope; $AT_{1a}R$; coding region for the human angiotensin receptor; His_{10}, Histidine 10 tag; Bio, biotinylation domain of *Propionobacterium shermanii* transcarboxylase; eGFP, enhanced green fluorescent protein. PSG, subgenomic 26S promoter; CAP, capsid sequence of Semliki Forest virus; TEV, tobacco etch virus protease cleavage site.

transfer vector were cotransfected in Sf9 cells, using cationic liposomes (Lipofectin) according to the manufacturer's specifications (Life Technologies Inc.). The screening and selection of recombinant baculovirus was performed as described previously [16]. Afterwards, the recombinant baculovirus was amplified and virus titer was determined by end-point dilution [17]. For expression, the ells were grown up to a density of 2.0–2.5×10^6 cells/ml and then infected with recombinant baculovirus at an MOI (multiplicity of infection) of 10.

Mammalian cell culture, generation of virus stock and infection. Baby hamster kidney cells (BHK-21) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 0.1% streptomycin–penicillin. BHK cells in suspension were cultured using GMEM (Glasgow minimal essential medium) supplemented with 5% FCS, 2 mM glutamine, 10 mM tryptose phosphate broth, and 0.1% streptomycin–penicillin. Cells were maintained in a humified atmosphere at 37 °C with 5% CO_2.

The protocol for generation of recombinant virus particles has been published previously [13a,b]. Briefly, the expression vector and the helper vectors were linearized with *Nru*I and *Spe*I, respectively. *In vitro* transcription of RNA was performed using SP6 polymerase and it was electroporated into BHK cells (1500 V, 25 μF, ∞ Ω). Virus particles were collected after 24 h, filtered though a 0.22 μm filter, and stored at −70 °C until used. Before infection, the virus particles were activated with α-chymotrypsin (final concentration of 0.5 mg/ml) for 20–25 min at room temperature and then, α-chymotrypsin was inactivated by adding aprotinin (final concentration of 0.25 mg/ml). For expression, virus was added either to monolayer cells grown up to 80% confluency or suspension culture of BHK cells (2×10^6 cells/ml).

Membrane preparation. The cells were harvested (Sf9 cells-72 h post-infection and BHK cells-24 h post-infection), washed in cold breaking buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, and Complete protease inhibitor cocktail), and lysed by nitrogen cavitation (Parr Instruments, Moline, IL, USA). The crude lysate was centrifuged (3000*g*, 10 min, 4 °C) to remove the nuclei and cell debris. Subsequently, the membranes were pelleted by ultracentrifugation (100,000*g*, 1 h, 4 °C). The membrane pellet was resuspended in storage buffer (breaking buffer supplemented with 5% glycerol) at a protein concentration of 5–10 mg/ml, flash-frozen in liquid nitrogen, and stored at −70 °C. The protein concentration was determined using the bicinchoninic acid reagent (Pierce, Rockford, USA) and BSA as standard.

[^{3}H]angiotensin II binding assay. Single point measurements were performed by incubating membranes (7–3 μg of total protein per assay point) with 25 nM [^{3}H]angiotensin II in the binding buffer (30 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA and Complete protease inhibitor cocktail) for 45–60 min at 25 °C. For saturation binding analysis, different concentration of [^{3}H]angiotensin II (0.1–50 nM) were used. Non-specific interactions were determined in the presence of 1 μM angiotensin II. After incubation, the reaction was terminated by rapid filtration through GF/F glass-fiber filters which were presoaked in 0.3% (v/v) polyethyleneimine. Filters were quickly washed four times with ice cold binding buffer and subsequently transferred to counting vials. Radioactivity was measured by liquid scintillation counting. Dissociation constant (K_d) and maximum expression level (B_{max}) was calculated with the "KaleidaGraph" software by non-linear regression using a single site model.

Western blot analysis and deglycosylation. Proteins were separated using 10% SDS–PAGE and subsequently transferred to a PVDF membrane as described previously [13]. The membranes were incubated in 5% (w/v) nonfat dried milk powder in TBST buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% Tween 20) for 1 h at room temperature to block the non-specific interactions. Anti-his antibody, anti-Flag M2 antibody, alkaline phosphatase-coupled streptavidin, and alkaline phosphate-conjugated secondary antibody were used according to the manufacturer's protocol (Sigma). Blots were developed in 10 ml alkaline phosphatase buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 66 μl of BCIP (5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidinium salt, stock solution 50 mg/ml in dimethylformamide) and 33 μl of NBT (nitro blue tetrazolium chloride, stock solution 50 mg/ml in 70% dimethylformamide).

For *in vivo* inhibition of glycosylation, tunicamycin (final concentration 10 μg/ml) was added to the culture medium during expression. Enzymatic deglycosylation was performed by incubating 50 μg of membranes (5 mg/ml) with 1–2 U of PNGaseF or EndoH at 37 °C for 2 h followed by Western blot analysis.

Total inositol phosphate release and MAP kinase activation. Angiotensin II induced accumulation of total inositol phosphates was determined as described previously [13a]. Briefly, the BHK cells expressing $AT_{1a}R$ were incubated with 2 μCi of myo[^{2,3}H]inositol/ml for 18 h and then incubated with serum-free medium for an additional 2 h. Subsequently, the cells were stimulated with 1 μM angiotensin II for 10 min. Afterwards, the medium was replaced with 1:2 mixtures of chloroform/methanol and then, 0.5 ml H_2O and 1 ml of chloroform was added to terminate the reaction. The aqueous fraction containing total inositol phosphates was incubated at 50 °C for 10 min to remove residual chloroform and subsequently it was loaded on pre-washed (with 5 mM myo-inositol in water) AG1X8 columns (mesh size 100–200). The columns were washed further with 5 mM myo-inositol and then total inositol phosphates were eluted with 1.0 M ammonium formate/0.1 M formic acid. Radioactivity was measured as described earlier.

To measure the MAP kinase activation, the BHK cells expressing the recombinant $AT_{1a}R$ were stimulated with angiotensin II (1 μM) for 10 min. Subsequently, phosphorylated and total MAP kinase were detected using the MAP kinase assay kit as per manufacturer's protocol (NEB).

Confocal microscopy. To check the cellular localization of the recombinant receptor, the cells were grown on poly-L-lysine coated cover slips in 6-well plates and infected with $AT_{1a}R$-eGFP virus. Sixteen hours after infection (BHK cells) or 66 h after infection (Sf9 and H5 cells), the cells were washed twice in PBS and then fixed with 4% (v/v) paraformaldehyde for 15 min at room temperature. Subsequently, the cells were washed three times with PBS, three times with water and then mounted onto glass slides using gel mount medium (Sigma). B_2R-eGFP fusion protein was visualized using a Leica confocal laser-scanning microscope (488 nm). For calnexin staining, the cells were permeabilized using digitonin (50 μg/ml) and anti-calnexin antibody followed by Cy3-coupled secondary antibody was used.

Receptor internalization analysis. To investigate the agonist induced internalization of the receptor, BHK cells expressing the recombinant $AT_{1a}R$-eGFP fusion protein were stimulated with 1μM angiotensin II at 37 °C for different time intervals. Subsequently, the cells were fixed and visualized using a Leica confocal laser scanning microscope (488 nm).

Results and discussion

Constructs for expression of the recombinant $AT_{1a}R$

For expression of the human $AT_{1a}R$ in insect cells, we created two recombinant $AT_{1a}R$ constructs which are shown in Fig. 1A. The expression of recombinant receptor is driven by a strong polyhedrin promoter. These constructs contain an N-terminal in-frame fusion of the prepromelittin signal sequence. This signal sequence is expected to lead proper targeting of the recombinant receptors. A positive effect of the prepromelittin signal sequence on the functional expression of the oxytocin receptor [18], the $5HT_{5A}$ serotonin receptor [19] and the D_{2S} dopamine receptor [20] has been reported in baculovirus infected Sf9 cells. Additionally, the recombinant constructs contain Flag and His_{10} tag at the N-terminus of $AT_{1a}R$. At the C-terminus of $AT_{1a}R$, either the biotinylation domain of

Propionibacterium shermanii transcarboxylase [22], or enhanced green fluorescent protein (eGFP) was added.

Two recombinant SFV expression constructs based on previously described SFV vectors [13,21] were generated for the production of the human $AT_{1a}R$ in mammalian cells (Fig. 1B). Here, the expression of the recombinant receptor is driven by a subgenomic 26S promoter (PSG). A self-cleavable capsid sequence (a translation enhancer element from 5′ end of the capsid gene from Semliki Forest virus) was fused at the N-terminus of the receptor-coding region. It has been reported previously that fusion of capsid sequence leads to an increase in expression of the transferrin receptor [15a], the neurokinin receptor [15b] and the bradykinin receptor [13a]. At the C-terminus of $AT_{1a}R$, a His_{10} tag and the biotinylation domain of *Propionibacterium shermanii* transcarboxylase [14] or eGFP coding region was fused.

Flag tag, His tag, and Bio tag can be used for immunodetection and purification of the recombinant receptor. Another advantage of the bio tag at the C-terminus is its positive effect on the functional expression of recombinant GPCRs [13,22]. The $AT_{1a}R$-eGFP fusion construct can be used for localization and internalization analysis of the recombinant receptor.

Western blot analysis of the recombinant $AT_{1a}R$

To check the expression of recombinant $AT_{1a}R$, membranes from Sf9 cells and BHK cells expressing the recombinant receptor were analysed by Western blot using anti-Flag M2 antibody, anti-his antibody or alkaline phosphatase-conjugated streptavidin. The anti-Flag M2 antibody and AP-conjugated streptavidin recognized a main band of ~55 kDa in the membranes of Sf9 cells expressing the $AT_{1a}R$ (Fig. 2A and B). The size of this band corresponds well with the calculated size of recombinant $AT_{1a}R$ fusion protein (51.2 kDa). Staining of the $AT_{1a}R$ fusion protein by AP-conjugated streptavidin suggests that *in vivo* biotinylation of the *Propionobacterium shermanii* transcarboxylase domain fused to the receptor took place. However, in addition to the main receptor band, some additional bands of smaller sizes were also observed on the immunoblots. These additional bands represent the proteolytic degradation products of recombinant $AT_{1a}R$. Similar proteolysis has been reported previously for the human beta 2-adrenergic receptor [23], the human histamine H_1 receptor [24] and human $CCXR_1$ chemokine receptor [25] in Sf9 cells. The long expression time might be responsible for the proteolytic cleavage of recombinant GPCRs expressed in insect cells. In case of the human histamine H_1 receptor, rapid proteolysis of the receptor was efficiently suppressed by including the protease inhibitor leupeptin in the culture medium [35]. A similar possibility remains to be explored for the recombinant $AT_{1a}R$ also, although we have observed that addition of leupeptin in the culture medium does not prevent proteolytic degradation of recombinant bradykinin receptor in Sf9 cells [13c].

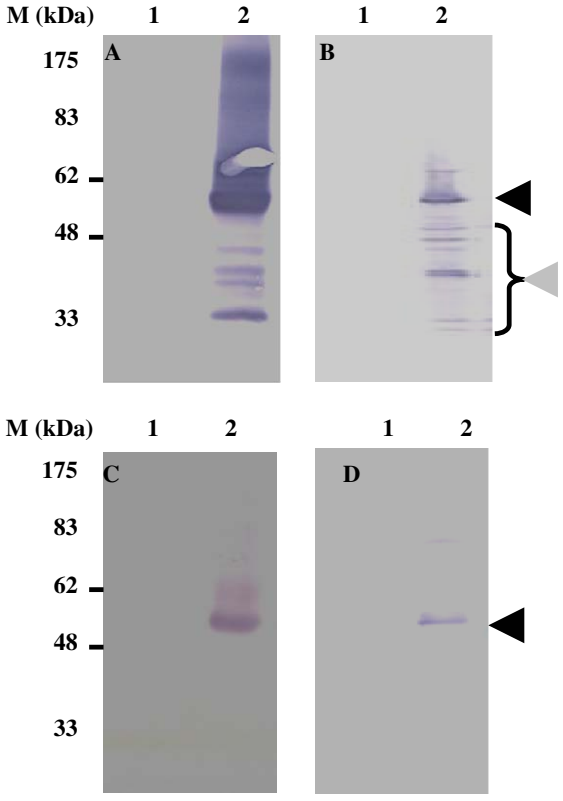

Fig. 2. Western blot analysis of the recombinant $AT_{1a}R$. Membranes from Sf9 cells expressing the recombinant $AT_{1a}R$ were probed with anti-Flag M2 antibody (A) or alkaline phosphatase-coupled streptavidin (B) and membranes from BHK cells expressing $AT_{1a}R$ were probed with anti-his antibody (C) or alkaline phosphatase-coupled streptavidin (D). Lane 1, membranes from non-infected Sf9/BHK cells and lane 2, 10 μg membranes from Sf9/BHK cells expressing the recombinant $AT_{1a}R$. The black arrowhead shows $AT_{1a}R$ and the grey arrowhead indicates degraded receptor.

In the membranes of BHK cells expressing $AT_{1a}R$, both anti-his antibody and alkaline phosphatase-conjugated streptavidin recognized a band of ~55 kDa, which corresponds well to the calculated size of $AT_{1a}R$ fusion protein (50.9 kDa) (Fig. 2C–D). Again, staining of the $AT_{1a}R$ band with AP-conjugated streptavidin suggested that *in vivo* biotinylation of the bio domain took place. Additionally, size of the recombinant receptor band suggested that efficient autocatalytic cleavage of the capsid sequence occurred in BHK cells. More importantly, there was no proteolytic degradation of the recombinant receptor in BHK cells.

Saturation binding analysis

Human $AT_{1a}R$ is expressed at low levels in native tissues such as human skin fibroblasts (18–20 fmol/mg), smooth muscle cells (350 fmol/mg) and renal cortex (16 fmol/mg) [5,6]. In order to calculate the functional expression level of $AT_{1a}R$ in Sf9 cells and BHK cells, a saturation binding analysis was performed using [^{3}H]angiotenisn II. As shown in Fig. 3A and B, the ligand binding was saturable and a

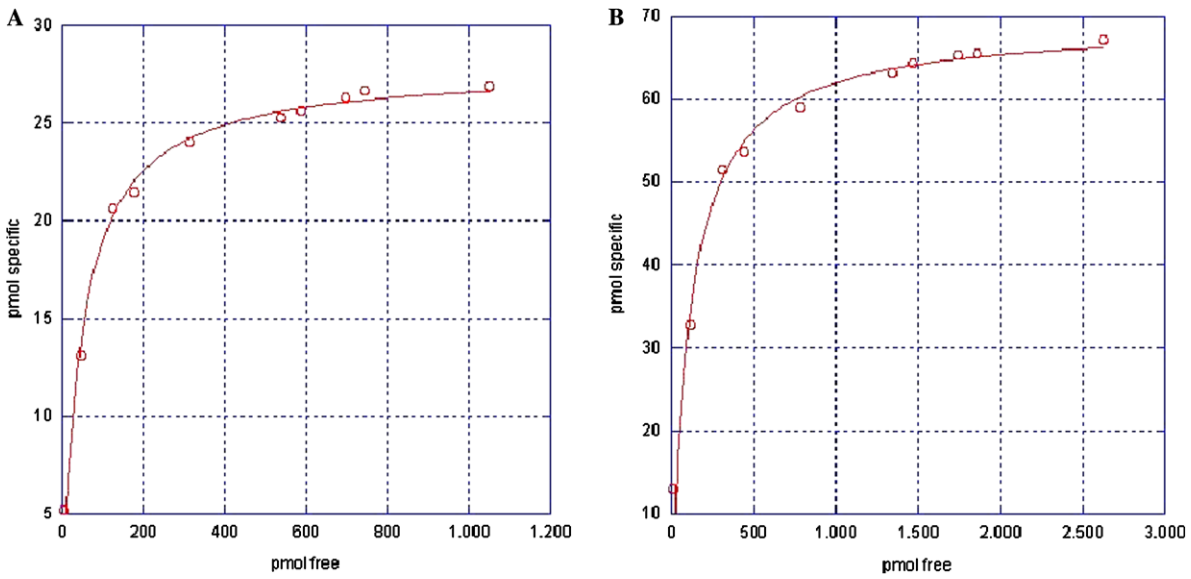

Fig. 3. Saturation binding analysis of the recombinant receptor. [^{3}H]angiotensin II binding assay was performed on membranes prepared from the Sf9 cells (A) and BHK cells (B), expressing $AT_{1a}R$-Bio fusion protein. The curves were generated by KaleidaGraph software using non-linear regression. The values are an average of two independent experiments.

single binding site was observed in both Sf9 cells and BHK cells. An expression level of 29 pmol recombinant receptor per milligram of total membrane protein was measured in the Sf9 cells while BHK cells showed an expression level 32 pmol/mg. These expression levels are significantly higher than $AT_{1a}R$ expression in native tissues (as mentioned above) and the previously reported values of $AT_{1a}R$ expression in mammalian cells (0.5–0.7 pmol/mg in CHO cells) (1–2 pmol/mg in HEK cells) [7,8]. The recombinant $AT_{1a}R$ binds to its cognate ligand with high affinity and the K_d values observed here (0.98 nM for Sf9 cells and 1.1 nM for BHK cells) are in good agreement with that of $AT_{1a}R$ in native tissues (0.1–2 nM) [5,6]. This result indicated that the tags and fusions introduced at the N- and C-terminus of $AT_{1a}R$ had no detrimental effect on its ligand binding properties.

The expression levels of $AT_{1a}R$-eGFP constructs was four to fivefold lower compared to $AT_{1a}R$-Bio constructs. Similar effect of eGFP fusion on the expression level of human bradykinin receptor has been observed in Sf9 cells and BHK cells ([13] and a & c). Therefore, for further biochemical characterization, $AT_{1a}R$-Bio fusion construct was used and $AT_{1a}R$-eGFP construct was used for localization and internalization analysis of the receptor.

Receptor localization

In order to check the cellular localization of the recombinant $AT_{1a}R$, Sf9 cells and BHK cells were infected with recombinant virus encoding $AT_{1a}R$-eGFP fusion protein. Subsequently, the cells were fixed and analysed by confocal microscopy. As shown in Fig. 4A–C, the recombinant $AT_{1a}R$ was expressed in plasma membrane in both BHK cells and insect cells. This is particularly interesting because in many cases, such as the human CXCR1 chemokine receptor [35], the murine CB_2 cannabinoid receptor [26], the human D_2S dopamine receptor [20], the rat M_3 muscarinic acetylcholine receptor [27] and the human bradykinin B_2 receptor [13,28], heterologously expressed receptors exhibit intracellular retention in host cells. It is generally assumed that high-level overexpression of recombinant GPCRs leads to intracellular accumulation of the receptors probably due to the saturation of trafficking machinery of the host cell. However, some receptors, e.g., the human melanocortin 4 receptor [29], the human μ opioid receptor [30] and human $AT_{1a}R$ (this study), are present in the plasma membrane even after heterologous expression. Taken together, these results suggest that the localization of recombinant GPCRs depend on the individual receptor.

Glycosylation analysis

Based on the conserved *N*-glycosylation motif, three putative N-linked glycosylation sites (i.e. N^4, N^{176} and N^{188}) have been identified in the human $AT_{1a}R$. It has been previously reported that N-linked glycosylation plays an important role in the proper trafficking of $AT_{1a}R$ but it does not affect the ligand binding properties of the receptor [7]. In order to determine the glycosylation state of the recombinant receptor, enzymatic deglycosylation using PNGaseF and EndoH was performed on the membranes of Sf9 cells and BHK cells expressing $AT_{1a}R$. PNGaseF is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins while EndoH is a recombinant glycosidase which cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. As shown in Fig. 4D and E, the 55 kDa band was shifted downwards

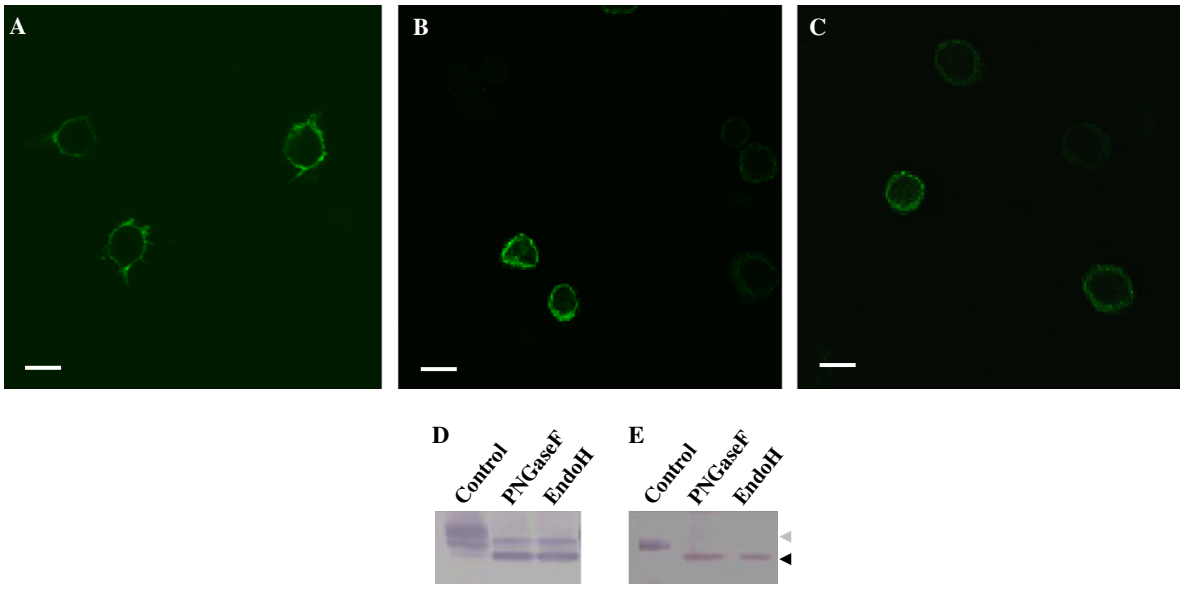

Fig. 4. (A–C) Localization analysis of the recombinant $AT_{1a}R$. BHK cells (A), and Sf9 cells (B) H5 cells (C) expressing the $AT_{1a}R$-eGFP fusion protein were visualized by confocal laser scanning microscopy (488 nm). (D,E) Glycosylation analysis of the recombinant $AT_{1a}R$. Enzymatic deglycosylation was performed on membranes (20–40 μg) from Sf9 cells (D) or BHK cells (E) using PNGaseF (lane 2) and EndoH (lane 3). Immunodetection was performed with anti-Flag M2 antibody or anti-his antibody. The black arrow head indicates deglycosylated receptor and grey arrow head indicates glycosylated receptor.

in response to the enzymatic deglycosylation (lanes 2 and 3). Thus, the 55 kDa band represented glycosylated form of the recombinant receptor. Glycosylation in insect cells remains mostly very simple of the high mannose type and EndoH digestion indicates that $AT_{1a}R$ also had high mannose type glycosylation when expressed in insect cells.

As mentioned above, glycosylation of $AT_{1a}R$ plays a crucial role in surface trafficking. Effect of glycosylation on surface trafficking of other GPCRs such as the human bradykinin receptor [31], the FSH receptor (46) and the β_2-AR [32] have also been reported. On the other hand, in some cases such as the α_1-AR [33], the H_2 histamine receptor [34] and the M_2-muscarinic receptor [35] glycosylation does not significantly influence their ability to be expressed at the cell surface. In order to check if the surface expression of $AT_{1a}R$ is dependent on glycosylation in Sf9 cells and BHK cells also, we performed *in vivo* deglycosylation (by adding tunicamycin in the culture medium) followed by localization analysis. As shown in Fig. 5A–C, upon tunicamycin treatment, the recombinant receptor was localized mainly in the endoplasmic reticulum (colocalized with calnexin) in Sf9 cells and it could not reach the plasma membrane. Similarly, in tunicamycin treated BHK cells also, the recombinant receptor was present in perinuclear membranes and not at the cell surface (Fig. 5D–F). These results confirm that glycosylation of $AT_{1a}R$ is required for its optimal cell surface expression.

G protein coupling and MAP kinase activation

Like many other GPCRs, the $AT_{1a}R$ primarily couples to $G_{q/11}$ protein and activates phosphoinositide hydrolysis, inositol phosphate accumulation, calcium release, and activation of protein kinase C [36]. By measuring the total phosphoinositol accumulation, we analysed if the recombinant $AT_{1a}R$ can couple to the endogenous $G\alpha_q$ protein of BHK cells. As shown in Fig. 6A, a significant increase in inositol phosphate accumulation was observed in response to angiotensin II stimulation in BHK cells expressing the $AT_{1a}R$. The level of inositol phosphate accumulation was much lower in control cells (in non-stimulated cells and non-infected cells).

Angiotensin II induced phosphorylation of MAP kinase in vascular smooth cells [37] and transfected cells [8] have been reported earlier. During this study, we also observed angiotensin II stimulated MAP kinase phosphorylation (ERK-1/2) in $AT_{1a}R$ expressing BHK cells (Fig. 6B). No phosphorylation of MAP kinase was observed in non-stimulated and non-infected cells. Taken together these results suggest that the recombinant $AT_{1a}R$ in BHK cells is functional in terms of its intracellular signaling.

Agonist induced internalization of $AT_{1a}R$

Stimulation with angiotensin II (agonist) leads to rapid internalization of $AT_{1a}R$ in native tissues and transfected cells [6,8]. Here, we also observed that angiotensin II stimulation leads to robust internalization of $AT_{1a}R$ in BHK cells within 5 min (Fig. 6 C). This result further confirms the native like behaviour of the recombinant $AT_{1a}R$.

In conclusion, we have established high-level expression of an affinity tagged human $AT_{1a}R$ in Sf9 cells and BHK cells. The recombinant receptor was characterized in terms of ligand binding, localization in host cells, glycosylation

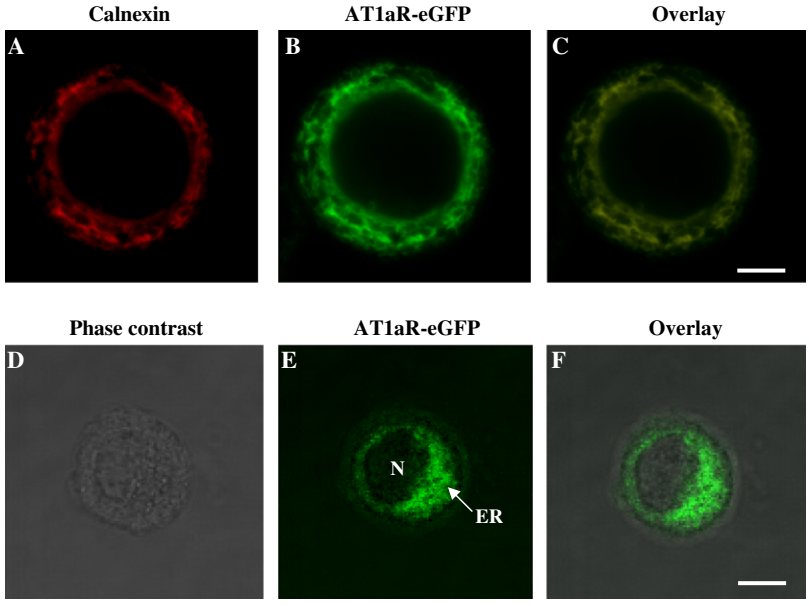

Fig. 5. Effect of *in vivo* deglycosylation on the cell surface expression of recombinant $AT_{1a}R$. Tunicamycin (10 μg/ml) was added to the Sf9 cells (A–C) and BHK cells (D–F) expressing the $AT_{1a}R$-eGFP fusion protein. Subsequently, the cells were fixed, permeabilized, and visualized by confocal laser scanning microscopy (488 nm for eGFP and 550 nm for Cy3).

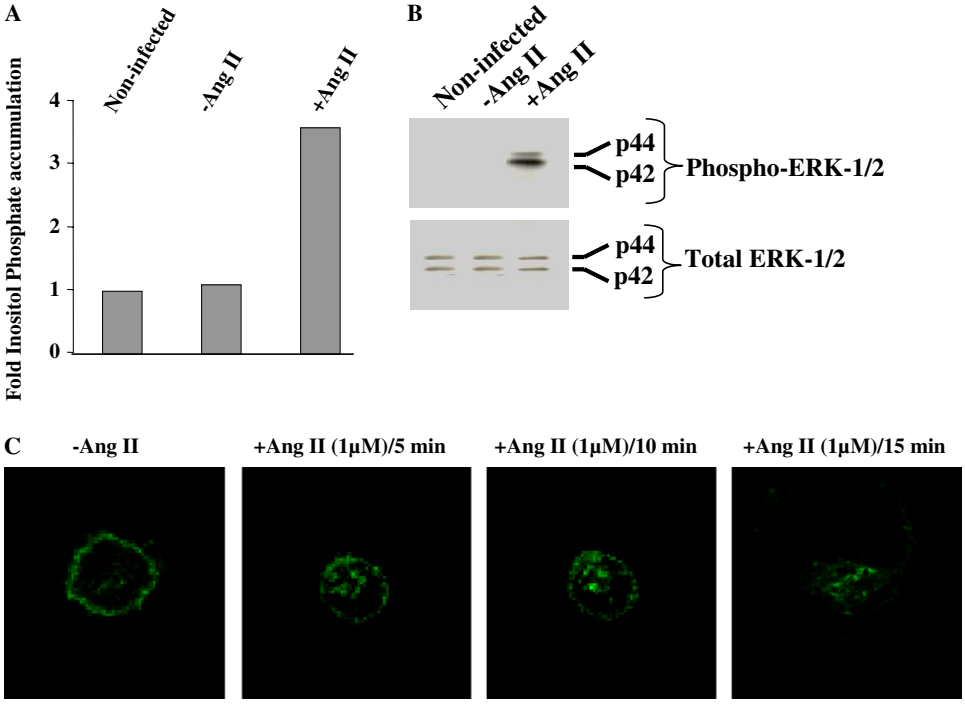

Fig. 6. (A,B) Angiotensin II induced accumulation of inositol phosphate (A) and activation of MAP Kinase (B) in BHK cells expressing the recombinant $AT_{1a}R$. The cells were stimulated with 1 μM of angiotensin II for 10 min and subsequently IP accumulation and MAP kinase activation was analysed. (C) Agonist induced internalization of $AT_{1a}R$. The BHK cells expressing $AT_{1a}R$-eGFP fusion protein were stimulated with 1 μM angiotensin II for 5–15 min. Subsequently, the cells were fixed and analysed by confocal laser scanning microscope (488 nm).

state and coupling to endogenous G protein. To our knowledge, this is the first report of heterologous overexpression of an affinity tagged recombinant $AT_{1a}R$ and it should pave the way for direct structural characterization of this receptor.

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