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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

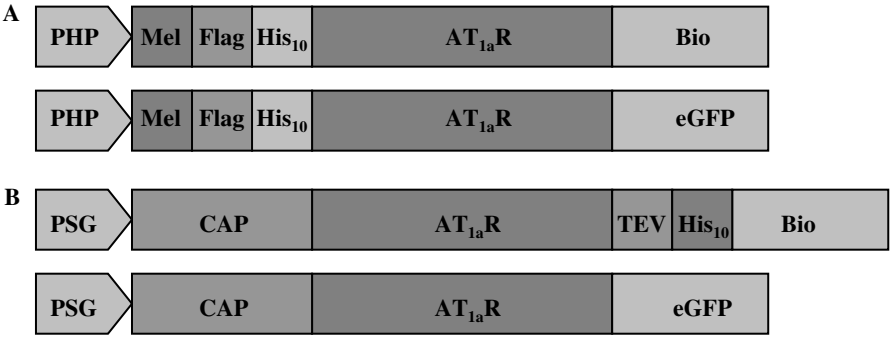

Fig. 1. Schematic representation of the recombinant expression constructs for heterologous production of the human $AT_{1a}R$ in Sf9 cells (A) and BHK cells (B). PHP, polyhedrin promoter; Mel, coding sequence for the prepromelittin signal sequence of honeybee; Flag, coding region for the Flag epitope; $AT_{1a}R$; 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.