Regulation of Transport of the Angiotensin AT2 Receptor by a Novel Membrane-Associated Golgi Protein

Christoph J. Wruck, Heiko Funke-Kaiser, Thomas Pufe, Heike Kusserow, Mario Menk, Jan H. Schefe, Marie L. Kruse, Monika Stoll, Thomas Unger

Objective—Synthesis and maturation of G protein–coupled receptors are complex events that require an intricate combination of processes including protein folding, posttranslational modifications, and transport through distinct cellular compartments. Little is known concerning the regulation of G protein–coupled receptor transport from the endoplasmic reticulum to the cell surface. Methods and Results—Here we show that the cytoplasmatic carboxy-terminal of the angiotensin AT2 receptor (AT2R) acts independently as an endoplasmic reticulum–export signal. Using a yeast two-hybrid system, we identified a Golgi membrane–associated protein termed ATBP50 (for AT2R binding protein of 50 kDa) that binds to this motif. We also cloned ATBP60 and ATBP135 encoded by the same gene as ATBP50 that mapped to chromosomes 8p21.3. Downregulation of ATBP50 using siRNA leads to retention of AT2R in inner compartments, reduced cell surface expression, and decreased antiproliferative effects of the receptor. Conclusion—Our results indicate that ATBP50 regulates the transport of the AT2R to cell membrane by binding to a specific motif within its cytoplasmic carboxy-terminal and thereby enabling the antiproliferative effects of the receptor.

Key Words: AT2 receptor ■ ATBP50 ■ receptor transport ■ Golgi proteins ■ ERK1/2

The heptahelical G protein–coupled receptors (GPCRs) represent one of the largest structure–function families in eukaryotic cells. A large number of structure–function studies have defined receptor sequences that are essential for ligand binding, G protein coupling, and desensitization. In contrast, little is known concerning the requirements for the transport of these proteins to the plasma membrane. Intracellular accessory proteins can be critical for GPCR biogenesis, including aspects of receptor trafficking. Recent discoveries have identified multiple membrane-associated proteins that dictate the delivery of the receptor to the cell surface.

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The octapeptide hormone angiotensin II (Ang II) exerts a wide variety of physiological actions such as vasoconstriction and aldosterone secretion, but it is also involved in pathological mechanisms of atherosclerosis as well as vascular and cardiac growth. Ang II mainly interacts with two receptor subtypes, designated AT1 and AT2 receptor (AT1R, AT2R), both belonging to the superfamily of GPCRs. The majority of its well-described effects are mediated by the AT1R. Increasing evidence indicates, however, that the AT2R subtype can modulate the effects of AT1R, including those on blood pressure, cardiac and vascular cell growth, and tissue regeneration after injury.

Thus, it has been shown that stimulation or overexpression of AT2R in certain cell lines inhibits cell proliferation induced by growth factors and promotes neuronal differentiation as well as apoptosis. The growth inhibitory effects of the AT2R have been shown to be associated with the activation and/or induction of a series of phosphatases including the protein tyrosine phosphatase SHP-1, mitogen-activated protein kinase phosphatase-1 (MKP-1), and serine/threonine phosphatase 2A (PP2A), which results in the inactivation of AT1R- and/or growth factor–activated extracellular signal regulated kinase (ERK). In PC12W cells, long-term activation of the AT2R increases the synthesis of ceramide, which may lead to the AT2R-mediated apoptosis.

However, besides the growth inhibitory function of AT2R, there is still some controversy, because it has also been reported that AT2R stimulation promotes fibrosis and growth. AT2R activation in AT2R-transfected smooth muscle cells stimulates collagen synthesis. Furthermore, ventricular hypertrophy induced by pressure overload was not observed in AT2R knockout mice.
A peculiarity of the AT2R is its uncommon response to Ang II stimulation, as there is no desensitization or down-regulation in response to ligand binding.\(^3\)\(^0\)\(^3\)\(^1\) In contrast, the cell surface expression of the AT2R undergoes upregulation after stimulation with Ang II, whereas the level of AT2R transcripts does not increase.\(^3\)\(^2\)\(^3\)\(^4\)

Numerous studies have identified different AT2R regions responsible for the interaction with other proteins involved in AT2R activation or regulation. Among these regions, the fifth and sixth transmembrane domains were shown to directly interact with the agonist Ang II.\(^3\)\(^5\)\(^3\)\(^8\)\(^3\)\(^9\) and the third intracellular loop\(^3\)\(^8\)\(^3\)\(^9\)\(^4\)\(^0\) and the cytoplasmic tail were shown to be critical for coupling with G\(_q\) and SHP-1,\(^4\)\(^1\) respectively. Moreover, the cytoplasmic tail was also shown to contain a binding domain for the transcription factor promyelocytic zinc finger protein (PLZF) which affects the AT2R signaling.\(^4\)\(^2\) In an effort to better characterize the signaling mechanisms of the AT2R, we screened for new AT2R interacting proteins.

**Methods**

For details regarding cell culture, plasmid transfection of cell lines, and mouse tissues, construction of mutant and epitope-tagged ATBP and AT2R expression vectors, design of a fluorescent Golgi-marker (GM) protein, coimmunoprecipitation experiments, immunoblotting, confocal immunofluorescence microscopy, surface expression ELISA, siRNA design and transfection, proliferation assay, Northern blot analysis, RT-PCR analysis, relative quantitation of gene expression by real-time PCR, and in situ hybridization, please see the online Methods, available at http://atvb.ahajournals.org.

**Yeast Two-Hybrid Screening**

The mice AT2R carboxy-terminal tail (residues 313 to 363) GAL4 binding domain fusion protein cloned in the yeast expression vector pGBK7T was cotransformed into the yeast strain AH109 with a GAL4 activation domain fusion library of mouse 11 day embryo cDNA in pACT2 (Clontech, Heidelberg, Germany). Yeast two-hybrid screening was performed according to the Clontech Matchmaker Two-Hybrid System 3 protocol. Clones expressing both the bait and the prey were selected on growth medium lacking Leu and Trp, and the interacting proteins were identified for their adenine auxotroph complementation. The cDNA inserts from positive clones were then sequenced using the dideoxynucleotide method.

**cDNA Cloning of ATBPs**

Total RNA was isolated from mice embryos, and the mRNA was then affinity purified by oligo(dT)-cellulose chromatography. The cDNA synthesis was primed with oligo(dT). A kit for rapid amplification of cDNA ends (RACE) was used to identify the 5' ends of the ATBPs (Life Technologies). The following nested oligonucleotides were used for the polymerase chain reaction (PCR): AP1 primer (Life Technologies), GSP1 (5'-AGGCTCCTGGGATTCTTCTG), and GSP2 (5'-AGGCTCCTGGGATTCTTCTG). Total mice embryo mRNA was used for cDNA synthesis. The PCR products were subcloned into the pC3DNA3.1 vector (Invitrogen) and sequenced.

**Statistical Analysis**

With respect to the epidermal growth factor (EGF) stimulation experiments, a two-tailed Student t test was performed.

**Results**

**Identification of ATBP as Binding Protein of the AT2 Receptor**

To identify AT2-interacting proteins, a yeast two-hybrid screening was set up with the AT2R carboxy-terminal tail (residues 313 to 363) as bait protein and a cDNA library generated from mouse 11 day embryo as prey protein. Of \(2 \times 10^6\) colonies screened, 86 fulfilled all selection criteria (phenotypes: His\(^+\), 3 ATR, galactosidase –, Ura\(^+\), and 5-fluoro-orotic-acid +), and two of these colonies encoded for full-length cDNA termed ATBP50 for AT2 receptor binding protein of 50 kDa. To confirm the result of the two-hybrid screening, coimmunoprecipitation studies were performed with both proteins coexpressed as tagged fusion proteins in COS-7 cells. 3XFlag-tag was fused to the N terminus of AT2R, and V5-tag was fused to the C terminus of ATBP50. Cell lysates were subsequently used for coimmunoprecipitation studies with monoclonal antibodies directed against the respective tags. V5-tagged ATBP50 protein was coimmunoprecipitated with anti-3XFlag antibody when cells were cotransfected with AT2-Flag (Figure 1A, lane 2), but not in the control experiment (Figure 1A, lane 1). The other two isoforms, ATBP60 and ATBP135 (see below), were not able to interact with the AT2R (data not shown).

Furthermore, we were able to demonstrate that a C-terminal deletion mutant of ATBP (ATBP50ΔC), which is discussed below, is still able to interact with the AT2R (Figure 1A, lane 3).
ATBP and AT2 Receptor are Coexpressed in Mouse Tissue

To examine the expression of ATBP50 in embryonic tissue, we performed Northern blots using RNA from mouse embryos with cDNA of ATBP50 as a probe. We found three mRNA isoforms detected by the probe in a comparable intensity (Figure 1B). They had an estimated size of 2.5, 3.6, and 5.9 kb, respectively. To analyze which adult tissues express ATBP, we performed isoform-specific semiquantitative RT-PCRs with RNA from various mouse tissues. A ubiquitous expression of ATBP50 was seen in all tissues tested (Figure 1C). Contrary to ATBP50, ATBP60 and ATBP135 were not detectable in brain and spleen. The strongest expression of ATBP50 was found in the uterus and adrenal gland (Figure 1C, lanes 2 and 3), tissues in which the AT2R is also predominantly expressed (Figure 1C, lanes 2 and 3). In contrast, the AT2R- and ATBP50 expressions in heart, brain, spleen, kidney, gut, and dermis were relatively weak.

More detailed investigations regarding coexpression of ATBP and AT2R were performed by in situ hybridization to evaluate the distribution patterns within different tissues using a probe common for all three isoforms. For example, in the adrenals (Figure IIA, available online at http://atvb.ahajournals.org) the expression patterns of ATBP and AT2 were strikingly similar, compatible with coexpression of both genes. This was also true for kidney, lung, heart, and brain (data not shown). We further examined tissues of AT2R-deficient mice by in situ hybridization. Although the adrenal glands of wild-type mice coexpressed the AT2 receptor and ATBP, the ATBP expression in this tissue was drastically reduced in AT2 receptor–deficient mice (Figure 1C). To substantiate this finding, we performed real-time PCR analysis on uterine tissue of wild-type– and AT2R-deficient mice, respectively. Uterus was chosen because of the strong coexpression of both genes. This was also true for kidney, lung, heart, and brain (data not shown). We further examined tissues of AT2R-deficient mice by in situ hybridization. Although the adrenal glands of wild-type mice coexpressed the AT2 receptor and ATBP, the ATBP expression in this tissue was drastically reduced in AT2 receptor–deficient mice (Figure 1C). To substantiate this finding, we performed real-time PCR analysis on uterine tissue of wild-type– and AT2R-deficient mice, respectively. Uterus was chosen because of the strong coexpression of both genes in previous experiments. The ATBP mRNA expression in AT2 receptor knockout mice was reduced to <10% compared with wild-type mice (Figure IIB).

Genomic Organization of the ATBP Gene

The presence of three differently sized transcripts suggested that the ATBP gene encodes for two more transcripts than expected. A genomic BLAST search with the cDNA sequence of ATBP50 (GenBank accession number AY626781) in the mouse genome database at NCBI showed that the gene mapped to the minus strand of the mice chromosome 8p21.3 with 10 exons spanning >47.7 kb. A search with the WGS supercontig Mm8_WIFeb01_187 sequence upstream of the ATBP50 stop codon in GENSCAN Web Server at MIT showed the whole gene of ATBP, spanning a region of 90.5 kb genomic DNA with 15 exons and 14 introns, codes for two additional transcripts, ATBP60 (GenBank accession number AY626782) and ATBP135 (GenBank accession number AY626781), respectively. The 5’ terminus and the 3’ terminus of every intron exhibit the GT-consensus and AG-AY626781, respectively. The 5’ end of the mRNAs. ATBP50 mRNA additionally contains exon 6 (358 bp) with the translation initiation start. Exons 4 (133 bp) and 5 (41 bp) are expressed in both ATBP60 and ATBP135 mRNA, whereas exon 3 (238 bp) is only expressed in ATBP60 mRNA and bears its translation initiation codon. Exons 1 (2311 bp) and 2 (119 bp) are exclusively expressed in ATBP135 mRNA with the translation initiation start in exon 1 (Figure 2A and 2B). Analysis of the sequence surrounding the potential translation initiation start revealed a favorable Kozak sequence for ATBP50 (5’-GAAGAGATGC) and ATBP135 (5’-TTTATGTTT) but not for ATBP60 (5’-TTTTATGTTT). A poly(A) tail and a polyadenylation signal (AATAAA) 21 nucleotides upstream from the poly(A) tail were detected in all three cDNA sequences.

Sequence and Structural Analysis of the ATBP Proteins

The predicted protein sequences encoded by the ATBP gene are shown in Figure 2B. Mice ATBP50, ATBP60, and ATBP135 are proteins with 440, 520 and 1209 residues and a calculated mass of 450, 60, and 135 kDa, respectively. A PHI- and PSI-BLAST search with the three protein sequences in the NCBI database showed no significant homologies to other proteins except proteins with a long coiled coil region like Golgi matrix proteins such as GM130/golgin-95, Golgi170, golgin-160, and p15. An identity...
was found with the human mitochondrial tumor suppressor gene 1 (MTSG1) protein and the human ATIP. In the protein family database Pfam, we found a homology of the ATBP proteins to the CDD:pfam05483 named HOOK protein. It has been demonstrated that endogenous HOOK3 binds to Golgi.

The ATBP proteins could be divided into two parts: an N-terminal “head” region of isoform-specific length, assumed to have a random coiled structure, and a C-terminal part of 400 amino acids identical in all three proteins. Using the program coils (version 2.1) at EMBl^, the C-terminal part of the ATBP proteins were predicted to have an a-helical coiled-coil motif, starting at position 214 (relative to the common part of all isoforms) and interrupted at position 260.

The C-terminus ends in a noncoiled-coil serine/proline-rich tail of 38 amino acids (Figure 2B). Using the program 2ZIP at DKFZ, Heidelberg, the coiled-coil region was predicted to contain two potential leucine zippers, a specialized form of coiled-coil dimerization motifs. A hydropathy plot illustrated that the ATBP proteins were highly hydrophilic without any potential transmembrane domain.

ATBP Proteins Are Localized at the Golgi Matrix Through Their C-Terminal Domains

To examine the subcellular distribution of the ATBP proteins, we compared the localization of ATBP proteins with the localization of a Golgi marker (GM). For this purpose, we transiently cotransfected COS-7 cells with a DsRed2-coupled GM and one of the Flag-tagged ATBP proteins (wtATBP50 or one of the three ATBP50 deletion mutants described below, respectively). The Flag-tagged ATBP proteins were detected with fluorescein isothiocyanate (FITC)-coupled anti-Flag antibody and imaged by confocal microscopy. WtATBP50 (Figure 3A) as well as the GM (Figure 3B) were primarily located in the perinuclear region with a small amount of diffuse background staining throughout the cell. This membrane distribution is similar to what has been reported for other Golgi-Network resident proteins. To examine the role of ATBP domains in Golgi targeting, three EGFP-coupled deletion mutants of the ATBP50 protein were created. DsRed2-coupled GM and the mutants were cotransfected in COS-7 cells, and localization was examined by confocal microscopy. WtATBP50 (Figure 3A) as well as the GM (Figure 3B) were primarily located in the perinuclear region with a small amount of diffuse background staining throughout the cell. This membrane distribution is similar to what has been reported for other Golgi-Network resident proteins.

ATBP Proteins Appear as Homo- and Heterodimerize in vivo.

To analyze putative dimerizations we performed coimmunoprecipitation studies with coexpressed ATBP proteins. ATBP50 was Flag-tagged and coexpressed in COS-7 cells with V5-tagged ATBP50, ATBP60, and ATBP135. Total cell extracts were immunoprecipitated (IP) with an anti-Flag antibody and electrophoresed, and the resulting blots were probed with anti-V5 antibodies to detect the various immunoprecipitated epitope-tagged ATBP proteins.

ATBP Proteins Are Required for AT2 Receptor Cell Surface Expression

To determine the physiological significance of the ATBP/AT2R protein interaction, we used siRNA technology to...
We then examined whether the results of reduced ERK1/2 inhibition of AT2R in the absence of ATBP50 would be reproducible in a proliferation assay with N1E-115 cells in the same experimental setup. After two days of EGF (50 ng/mL) treatment the cell proliferation rate nearly doubled compared with untreated cells (Figure IB, columns 1 and 2). This proliferation induction could be blocked with Ang II treatment (50 nM), whereas preincubation with PD123319 (10 μM) was able to prevent this inhibitory effect (Figure IB, columns 3 and 4). Pretreatment of N1E-115 cells with ATBP50 specific siRNA 2 days before Ang II stimulation caused a significant reduction of the inhibitory effect of AT2R. Taken together, these data indicate that the inhibitory effect of the AT2R on EGF-induced cell proliferation requires ATBP50.

Stimulation of PC12W Cells With Ang II Leads to Strong Induction of ATBP50 mRNA

PC12W rat pheochromocytoma cells, which endogenously express both AT2R and ATBP50, were stimulated with 100 nM Ang II. A continuous increase in ATBP50 mRNA levels was observed, starting 30 minutes after stimulation (Figure IIC).

To assess whether Ang II also affects the cell surface expression of the AT2R we performed a further surface expression ELISA. In the experimental setting used, Ang II did not alter the amount of transient transfected Flag-tagged AT2R at the cell membrane (Table II, available online at http://atvb.ahajournals.org). This experiment is consistent with an additional coimmunoprecipitation experiment, where Ang II incubation did not alter the amount of ATBP50 coprecipitated with Flag-tagged AT2R (Figure 1A, lane 4).

In addition, the data shown in Table II indicate, that—besides Ang II—cotransfection of ATBP50 or ATBP50ΔC also did not alter the amount of transient transfected Flag-tagged AT2R at the cell membrane in this experimental system.

Discussion

In this study, we have identified the previously unknown ATBP50 as a new AT2R interacting protein by yeast two-hybrid screening. ATBP50 was shown to directly interact with the carboxyl-terminal tail of this receptor, and the specificity of this association was demonstrated by coimmunoprecipitation experiments. Three different ATBP transcripts (2.5 kb, 3.6 kb, and 5.9 kb) were detected by Northern blot analysis in mouse embryo tissues. These ATBP isoforms also show tissue-specific expression levels using RT-PCR analysis of multiple organs. In the adult mouse, the ATBP50 transcripts were present in all tissues tested, with abundant expression in uterus and adrenal correlating with the expression pattern of the AT2R in the adult mouse. The weak expression of ATBP50 in the liver, where the AT2R is not detectable, and the strong ATBP50 expression in the lung compared with AT2R expression suggest that ATBP has further functions that are independent of the AT2R. The putative polypeptides encoded by these gene have predicted molecular masses of 50 kDa (ATBP50), 60 kDa (ATBP60), and 135 kDa (ATBP135), respectively. Even though the N termini of all ATBPs are of different size and show no
homologies, the main structural feature of the ATBP proteins is their predicted ability to form coiled-coils in the region of 400 amino acids which is identical in all isoforms. Coimmunoprecipitation experiments suggested that all three ATBP proteins are able to form hetero- and homodimers with each other, thus indicating possible complex functions besides the receptor trafficking described below.

Our study provides evidence for a functional relationship between ATBP and AT2R concerning two different aspects: First, we have shown by confocal fluorescence microscopy and ATBP50 specific siRNA that ATBP50 promotes cell surface expression of AT2R. These results were confirmed by our cell surface ELISA experiments. Together these data suggest that ATBP50 functions as a regulatory protein to modulate the plasma membrane routing and thereby the functionality of the AT2R. With respect to functionality, we observed that downregulation of ATBP50 reduced the maximal inhibitory effect of AT2R on EGF-stimulated ERK1/2 activation in N1E-115 cells. This could be a direct result of a reduced presence of the receptor in the plasma membrane, a finding in accordance with Miura and Karnick who showed that the level of receptor protein expression is critical for induction of antiproliferation and apoptosis. The presence and expression level of ATBP50 has the potential to alter the AT2R signaling. We offer a putative functional explanation for the effects of ATBP on AT2R mediated ERK1/2 inhibition by demonstrating that ATBP is necessary for AT2R cytoplasmic membrane transport.

Our results indicate that ATBPs can be added to a growing list of proteins that have been identified as GPCR-binding proteins managing receptor trafficking to the cell surface. For instance, the actin binding protein filamin A (ABP-280) was found to modulate the cell surface expression of D2 and D3 receptor subtypes. Furthermore, association of the somatostatin receptor subtype 1 with Shk1 kinase–binding protein are required for targeting the receptor to the cell surface. In addition, Leclerc et al predicted regulatory proteins modulating the routing of the AT1R to the cell surface.

Consistent with our bioinformatic results (eg, homology to HOOK protein), using confocal microscopy we were able to show that ATBP is located at the Golgi apparatus. Although ATBP proteins are all hydrophilic, lacking obvious hydrophobic regions capable of serving as membrane anchors, the ATBP appends to the Golgi through its C-terminal tail as evidenced by our mutation experiments.

Secondly, we demonstrate a functional interaction between ATBP and AT2R in vivo, because ATBP mRNA was markedly reduced in AT2R knockout mice, as shown by in situ hybridization and real-time PCR analysis. These results are consistent with our complementary observation that Ang II can induce ATBP50 mRNA in PC12W cells, which exclusively express AT2R but not AT1R. Therefore, ATR2 exerts positive feedback on ATBP mRNA expression. In this context it is important to note that ATBP isoforms are encoded by a single gene and characterized by N-terminal isoform-specific exons, implying that these transcripts are controlled by three different promoters. The complex mechanisms of this feedback of AT2 receptor signaling on the isoform-specific ATBP promoters and/or isoform-specific mRNA half-lives will be examined in a further project.

Therefore, our data point to the existence of a two-directional interaction between ATBP and AT2R: On the one hand, ATBP is necessary for the trafficking and function of AT2R as described above; on the other hand, AT2R positively regulates ATBP mRNA expression.

In this context it is of interest to note that besides ATBP, the C-terminus of the AT2R is able to bind the recently described promyelocytic zinc finger protein (PLZF). After Ang II stimulation PLZF colocalizes with AT2R at the plasma membrane, followed by internalisation of AT2R and PLZF and nuclear translocation of PLZF. Therefore, it is conceivable that the transcriptional upregulation of ATBP by Ang II described here might be mediated by PLZF.

The interaction between ATBP50 and the AT2R may have direct implementations for human disease. For instance, Vervoort and coworkers have shown that mutations of the AT2R are linked to mental retardation; in particular, mutations in the receptor cytoplasmic C terminus are associated with this innateness. It is conceivable that dysfunctional interaction between AT2R and ATBP50 may be responsible for this disease. Another potential role of ATBP gene in human disease is suggested by its chromosomal localization at 8p21.3 as abnormalities in this region have been reported in various cancer types, thus implicating ATBP as a positional candidate gene in cancer.

This hypothesis is further supported by the well documented antiproliferative properties of the AT2R that are, according to our results, closely linked to the AT2R-ATBP50 interaction.

Furthermore, Seibold et al reported that the mitochondrial tumor suppressor gene 1 (MTSG1) protein, which is identical with ATBP50, is a mitochondri/resident protein. However, we were not able to demonstrate that any of the ATBP proteins are localized in mitochondrion. Possible explanations for this discrepancy might be the different cellular systems used (pancreatic tumor cells versus COS-7 cells) and the observation that the type of heterodimerization can influence the subcellular distribution.

In conclusion, we have identified a novel, Golgi-localized protein, ATBP50, that interacts specifically with the carboxyl-terminal cytoplasmic domain of the AT2R and is necessary for the cell surface expression of the AT2R. ATBP50 influences the receptor-mediated signaling by regulating receptor cell surface expression. Further functions of the ATBP isoforms and the nature of their associated proteins are now under investigation. Given that the AT2R is an important mediator of the renin-angiotensin system, ATBP50 may play a significant role in cardiovascular physiology and pathophysiology as well as other conditions that are genetically linked to the AT2R, such as mental retardation.

While this manuscript was in preparation, Nouet et al have described a protein named ATIP, which is identical to ATBP50, as an AT2 receptor interacting protein.

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References


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Figure I.

ATBP50 Knockdown with siRNA prevents Ang II stimulated AT2R mediated Antiproliferation.

A. N1E-115 cells were treated as described, cell lysates were immunoblotted and the activation of ERK1/2 were analysed using phospho-ERK1/2 specific monoclonal antibody.

B. N1E-115 cells were treated for 48 h at 37 °C with (+) or without (-) EGF (50 ng/ml) and Ang II (50 nM), and proliferation was measured using Cyquant as indicated in methods. The y-axis shows the relative fluorescence to control (100%). Means and standard deviations (n= 8) as well as P-values (student's t test compared to control) are shown.
Figure II.

A. Tissue expression of AT2R and ATBP50 mRNA. In situ hybridisation was performed on adrenal tissue of wildtype and AT2R deficient mice using antisense probes specific for AT2R and ATBP50. Sense probes served as control.

B. Real-time PCR Analysis for ATBP50 on Mouse Uterus Tissue in Wildtype and AT2R Deficient Animals. The expression level of wildtype mice was set to 100%. Data represents 4 animals in each group.

C. ATBP50 mRNA Expression after AT2R Stimulation of PC12W Cells. PC12W cells were incubated with 100 nM Ang II. RNA was extracted at indicated time points after Ang II stimulation. RT-PCR was performed for ATBP50 and standardized with HPRT.
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Table I.

N1E-115 cells were transfected and the number of receptors expressed at the cell surface was measured using a surface expression ELISA as described in methods. Optical density (O.D.) was measured at 490 and 650 nm. WT receptor without the Flag epitope at the N-terminus and control siRNA were used as controls.
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</tr>
<tr>
<td>AT2R 0.5</td>
<td>+</td>
<td></td>
<td></td>
<td>0.913</td>
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</tr>
<tr>
<td>AT2R 1</td>
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<td></td>
<td></td>
<td>0.915</td>
<td>+</td>
</tr>
<tr>
<td>AT2R 3</td>
<td>+</td>
<td></td>
<td></td>
<td>0.924</td>
<td>+</td>
</tr>
<tr>
<td>AT2R 6</td>
<td>+</td>
<td></td>
<td></td>
<td>0.912</td>
<td>+</td>
</tr>
</tbody>
</table>

Table II.

COS-7 cells were cotransfected with the indicated constructs (2 µg of each plasmid/well). 48 h later Ang II stimulation (100 nM) was performed with the indicated durations followed by cell surface ELISA as in table 1.
Methods

Cell Culture, Plasmid Transfection of Cell Lines and Mouse Tissues

PC12W cells (rat pheochromocytoma) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose (GibcoBRL, Eggenstein, Germany) supplemented with 5% fetal calf serum (GibcoBRL) and 10% horse serum (GibcoBRL). COS-7 cells were grown in DMEM containing 4.5 g/l glucose (GibcoBRL) supplemented with 10% fetal calf serum (GibcoBRL) and 5 mM glutamine (GibcoBRL), and 100 U of penicillin and 100 µg of streptomycin per ml. N1E-115 cells (mouse neuroblastoma) were grown accordingly but without sodium pyrovate. N1E-115 cells were transiently transfected with LipofectAMINE 2000 transfection reagent (Invitrogen, Karlsruhe, Germany) and COS-7 cells with PolyFect (Qiagen, Hilden, Germany).

AT2 receptor knockout mice and controls were gratefully obtained from Michael Bader, MDC, Berlin, Germany.

Construction of Mutant and Epitope-tagged ATBP and AT2R Expression Vectors

ATBP50, ATBP60 and ATBP135 proteins were tagged with three repeats of a Flag sequence (DYKDDDDK) using p3XFlag-CMV expression vector (Sigma, Taukirchen, Germany) at the N-terminus or with a V5 epitope (GKPIPNPLLGLDST) using pcDNA3.1-V5-polyHis expression vector at the C-terminus, respectively. To truncate the first 40 aa in order to build a ATBPΔN mutants, the translation start was shifted behind the original start codon to the first nucleotide identical in all ATBPs. To truncate the last 40 aa in order to build a ATBPΔC mutant, a translation stop signal was introduced 120 nucleotides upstream of the endogenous stop codon. The ATBPΔNΔC mutant was created using the ATBP50ΔN mutant with an additional translation stop signal 1203 nucleotides downstream the
translation start codon. AT2R was Flag- or EGFP-tagged at the N-terminus (pEGFP-C3 vector, Clontech), with an additional N-terminal interleukin 1 receptor signal peptide (IL1-SP) upstream the tagged construct to ensure a correct membrane targeting. To truncate the last 60 aa in order to build a AT2ΔC mutant, a translation stop signal was introduced 180 nucleotides upstream. Sequences of the primers used are:

ATBP50 sense: 5´-CTCAAGCTTCACCATGCTGGTGTCTCCCAAATTCTCCTTA;
ATBP60 sense: 5´-CACCAGATCTCATGACTATACCAGGAGGATTTCGCAGTTG;
ATBP135 sense: 5´-CACCTCTAGAATGATGATAATTCAGATAGGACAG;
ATBP antisense: 5´-CTCTCTAGATCTGGGAGATGCTGCTGGGAGAGAG;
ATBP50ΔN sense: 5´-
CACCAGATCTATGGTTGAAAAAGGCGAGGCGAGAAGATCCC;
ATBP50ΔC antisense: 5´-CTCGGATCCCCACAGAAGGCTATCCTGCTCCATGGAC;
AT2R sense: 5´-CACCTCTAGAATGAAAGGACAACTTCAGTTTTGCTGCCACC;
AT2R antisense: 5´-CTCTCTAGATTAAGACACAAAGGTTCATTTCTCTAAG;
IL1-SP sense: 5´-
GCTAGCGCAGCGGCACCATGGGAGATATGAAAGTGCTGCTGCTGGGCTACTTTGTCTCATGGTGCCTCTGCTGTCGTTACCGGT;
IL1-SP Antisense: 5´-
GGCCATTGCTGCTGGTCTCCGTTACTCTGTTTACTCGGGGTCATCGTGAAAGTATAAGAGGTACCACGCCGCGC.
All sequences were confirmed by nucleotide sequencing.

Design of a fluorescent Golgi-marker (GM) protein

The N-terminal 119 aa, representing the cytoplasmic and membrane spanning domains and also including the membrane-anchoring signal peptide, of the type II membrane-anchored
mice protein galactosyltransferase (UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase; GenPept NP_071641) were fused N-terminal to the fluorescent DsRed2 peptide encoded by the vector pDsRed2-N1 (Clontech). This construct has been used as a marker of the Golgi apparatus (Nilsson T et al 1991; Llopis J. et al. 1998). Sequences of the primers used are: Forward 5´-CTCGCTAGCCATGAGGTTTCGTGAGCAGTTCCTGGGCGG; Reverse 5´-GAGGGTACCGCACCCCGCGGCCGCTGCTCTCCTGCGGGG.

Co-immunoprecipitation Experiments
COS-7 cells were transiently co-transfected with Flag-AT2R and V5-ATBP and then incubated an additional 30 min in the presence of a thio-cleavable chemical cross-linker bis(N-succimidyl)3,3′-dithiodipropionate (2.5 mM final in 10% DMSO; Sigma). The cells were then washed twice with ice-cold phosphate-buffered saline and were lysed in cold lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton X-100 and Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). The lysate was treated with Flag M2-agarose affinity beads (Sigma). V5-tagged ATBP was detected via Western blotting with an anti-V5 rabbit polyclonal antibody. For the co-immunoprecipitation assay of homo- and heterodimerisation of ATBP proteins, COS-7 cells were cotransfected with Flag- and V5-tagged ATBP-cDNAs, respectively.

Immunoblotting
After washing, lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40) was added, the cells were scraped from the plates, transferred to a microcentrifuge tube, and boiled for 5 min. Total protein content in the supernatant was determined by the bicinchoninic acid method (Pierce). An equal amount of protein (10–30 µg) per well was
loaded onto 10% SDS–PAGE gels. Western blot analysis was performed with primary antibodies as specified in each case. Horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescent substrate system (Amersham) were used as a detection system.

**Confocal Immunofluorescence Microscopy**

Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 63× 1.3 NA oil immersion lens. COS-7 cells were seeded on glass coverslips and transfected using the method described above. The cells were then fixed and permeabilized with ice-cold methanol for 10 min at –20 °C. ATBP receptor was detected with the cyanine dye Cy3-conjugated anti-Flag M2 monoclonal mouse antibody (Sigma).

**Surface Expression ELISA**

COS-7 cells were transiently transfected with the wild type AT2R or the receptor containing the 3xFlag epitope at the N-terminus. On day one the cells were plated at a density of 1 x 10⁵ cells per well in a 24-well plate and transfected with ATBP50 specific siRNA 12 h later. On day two the cells were transfered in a polylysine-coated 96-well plate at a density of 5 x 10⁴ cells per well followed by transfection with an AT2R construct 12 h later. On day three, after washing and fixation (4% formaldehyde in PBS), 1 μg mouse alkaline phosphatase-conjugated M2-Flag antibody in a total of 100 μl DMEM containing 10% FBS was added for 1 h at 37°C. The plates were then incubated with the substrate p-nitrophenyl phosphate disodium (pNPP) that was subsequently hydrolyzed by alkaline phosphatase-conjugated antibody to produce a yellow-colored solution that can be read spectrophotometrically at 405 nm. The ELISA shown in table II was performed in 6-well plates with 1 ml medium/well.
siRNA Design and Transfection

Selection of mouse siRNAs was based on the characterization of siRNA by Elbashir et al. (Genes Dev. 2001;15:188-200, and Nature. 2001;411:494-498). RNA interference of ATBP was performed using a 21 base pair siRNA duplex including a d(T)d(T) overhang purchased from Qiagen-Xeragon (Germantown, USA). The coding strand for ATBP siRNA was 5’-AAAGGACTGCTTCGAAACCTCdTdT. For transfection, N1E-115 cells were transfected at 50% confluency with siRNA duplexes using Oligofectamine according to the manufacturers protocol (Invitrogen, Carlsbad, CA) 48 h before application according to the manufacturers recommendations. Cells treated with Oligofectamine (mock) or transfected with a control Fluorescein non-silencing siRNA duplex (Qiagen-Xeragon, Germantown, USA; 5´-AATTCTCCGAACGTGTCACGT) were used as controls. After 48 h of transfection, cells were treated and assayed as indicated in results.

Proliferation Assay

N1E-115 cells were seeded at a density of 2.0x10^5 in 12-well plates in 2 ml DMEM with 10% fetal calf serum and incubated for 48 h. The cells were then washed with serum-free DMEM and kept in serum-free DMEM for an additional 72 h to obtain a quiescent state. EGF (50 ng/ml) and Ang II (50 nM) were added in 2 mL fresh, serum-free medium. PD123319 (10µM) was added 30 min prior stimulation. The cells were incubated for 72 h, rinsed twice with PBS, and harvested with trypsin-EDTA (0.05% trypsin and 0.02% EDTA) solution. Cell number was determined directly by using CyQuant Proliferation Assay (Molecular Probes, Leiden, Netherlands).
Northern Blot Analysis

The ATBP50 cDNA probe was agarose gel purified (Qiaex purification resin; Qiagen) and $^{32}$P-labeled using Ready-To-Go DNA Labelling Beads (Pharmacia). Total RNA was isolated using the RNeasy Protect Kit (Qiagen). Denatured RNA samples (2 µg) were electrophoresed in 1.0% agarose-formaldehyde gels, transferred to ZetaProbe GT membrane (Bio-Rad), fixed by UV irradiation, and hybridized. After washing, membranes were exposed to x-ray films at -70°C with an intensifying screen.

RT-PCR Analysis

RNA was isolated using the absolutelyRNA kit (including the RNase-Free DNase set; Stratagene, Germany). Total RNA (1 µg) was reverse transcribed in 25 µl final volume for 1 h at 37°C using 100 U MLV Reverse Transcriptase and dT18V primer according to the manufacturers manual. cDNAs were subjected to PCR reactions using Invitek Taq Polymerase (Berlin, Germany) and following primer pairs:

ATBP50 forward: 5´-CACCATCCACGTCCGCTAA;
ATBP50 reverse: 5´-CTGATGCTGCTGGTTTAGTTTC;
ATBP60 forward: 5´-GTGAGACACATTCACACGGC;
ATBP60 reverse: 5´-GTTGTAACTTGCGCAGCCTG;
ATBP135 forward: 5´GGAGCATCTCAGACTACAGTTG;
ATBP135 reverse: 5´-GAGCATCTCAGACTACAGTTG;
AT2R forward: 5´-CTGGCACCAATGAGTCCGCC;
AT2R reverse: 5´-GCAGCTGCCATCTTCAGGAC;
HPRT forward: 5´ GCTGGTGAAAAGGACCTCT;
HPRT reverse: 5´ CACAGGACTAGAACACCTG.
Relative Quantitation of Gene Expression by Real-time PCR

RNA was isolated and reverse transcribed as described above. Relative quantitation of gene expression was performed with the SYBR Green PCR Mastermix (Applied Biosystems, Weiterstadt, Germany) in a total volume of 25 µl. Murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the endogenous control.

ATBP primer: 5’-TGAATCCAAGTCTCTATTTGGTGGG (forward); 5’-CTACTCTCGCTCTTCCTCAGG (reverse).

GAPDH primer: 5’-TCAAGAAGGTGGTGAAGCAG (forward), 5’-CATCGAAGGTGGAAGAGTGG (reverse). Runs were performed on a ABI 7000 sequence detection system (Applied Biosystems) using the standard curve method.

In situ Hybridisation

Prior to in situ hybridization, mouse tissues, embedded in paraffin, were cut (4 µm sections), dried overnight at 60°C and deparaffinized. Fixation was performed using 4 % paraformaldehyde. Slides were incubated with proteinase K for 10 minutes and rinsed with PBS twice. For riboprobe synthesis the fragment was transcribed in vitro using DIG-UTP labelling kit (Roche, Mannheim, Germany).

For AT2R detection a probe corresponding to nucleotides 1 to 1367 (GenBank accession number BC003811) and for ATBP detection a probe corresponding to the first 559 nucleotides coding for ATBP50 was used. The sections were then prehybridized at RT for at least 4 h in hybridization buffer (50 % formamide, 5 x SSC, 5 x Denhardt’s, 200 µg/ml yeast tRNA, 100 µg/ml salmon sperm DNA). Hybridization was performed overnight at 72°C after adding 750 ng/ml denatured probe. The slides were then washed with 5 x SSC, RNase treated and washed again at higher stringency at 72°C in 0.2 x SSC.

Immunohistochemical detection was performed using the DIG-UTP labelling kit (Roche).
Sections were then mounted with Hydromount (National Diagnostics, Oldendorf, Germany).