The Trafficking Protein GABARAP Binds to and Enhances Plasma Membrane Expression and Function of the Angiotensin II Type 1 Receptor

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Abstract—Proteins that bind to the intracellular expanses, particularly cytoplasmic tail regions, of heptahelical integral membrane receptors are of particular interest in that they can mediate or modulate trafficking or intracellular signaling. In an effort to distinguish new proteins that might promote angiotensin II type 1 (AT₁) receptor intracellular events, we screened a yeast 2-hybrid mouse brain library with the rat AT₁A receptor (AT₁R) carboxyl terminus and identified GABARAP, a protein involved in intracellular trafficking of the GABA₃ receptor, as a binding partner for the AT₁R. Interaction of GABARAP with the AT₁R carboxyl terminus was further substantiated using GST pull-down assays, and binding of the full-length tagged AT₁R to GABARAP was verified using coimmunoprecipitation. Bioluminescence resonance energy transfer assays further confirmed specific interaction of GABARAP with AT₁R. Moreover, GABARAP clearly increased the steady-state level of plasma membrane-associated AT₁R in PC-12 cells. Cotransfection of GABARAP with an AT₁R fluorescent fusion protein increased PC-12 cell surface expression of the AT₁R more than 6-fold when standardized to the level of intracellular expression. Furthermore, GABARAP overexpression in CHO-K1 cells engineered to express AT₁R increased angiotensin II binding sites 3.7-fold and angiotensin II–induced phospho–extracellular signal-regulated kinase 1/2 and cellular proliferation significantly over levels obtained with AT₁R overexpression alone. In addition, small interfering RNA–mediated knockdown of GABARAP reduced the steady-state levels of the AT₁R fluorescent fusion protein by 43% and its cell surface expression by 84%. Immunoblot analyses confirmed the quantitative image data. We conclude that GABARAP binds to and promotes trafficking of the AT₁R to the plasma membrane. (Circ Res. 2008;102:1539-1547.)

Key Words: angiotensin receptor ■ AT₁A ■ GABARAP ■ yeast 2-hybrid ■ protein binding

The angiotensin (Ang) II type 1 (AT₁) and 2 (AT₂) receptors are 7-transmembrane G protein–coupled receptors (GPCRs) of the largest GPCR subfamily, family 1, or the rhodopsin-like family. The GPCR superfamilly has more than 860 members¹ and more than 50 “GPCR-associated” proteins have now been discovered, the majority of which interact with GPCR cytoplasmic carboxyl termini.² Most of these are involved in trafficking, subcellular targeting, and intracellular signaling. Our preliminary studies were designed to identify proteins that bind to the cytoplasmic carboxyl terminus of the AT₁ receptor (AT₁R), the most prevalent and best characterized of the Ang receptors. Such proteins are expected to be involved in trafficking of the AT₁R through the secretory pathway and to the plasma membrane, as well as in ligand-mediated internalization and recycling. Moreover, our recent published studies suggest that the AT₁R is cleaved in a ligand-dependent manner to liberate the cytoplasmic domain, a significant quantity of which traffics to the nucleus.³ Presumably, this nuclear trafficking event also involves sequence-specific binding proteins. Using a yeast 2-hybrid (Y2H) approach to screen a mouse brain library, we have identified several proteins which bind to the AT₁R, the most prevalent of which are GABARAP (γ-aminobutyric acid [GABA] receptor–associated protein) and the related protein GABARAPL1 (L1 indicates like-1). Of 40 clones isolated, approximately one-half were identified by sequence analysis, as GABARAP or GABARAPL1, both members of the microtubule-associated protein (MAP) family. GABARAP was originally identified through its binding to one subunit of the pentameric ionotropic GABA₃ receptor. It is involved in trafficking of the GABA₃ receptor to the plasma membrane via microtubule tracks and affects both clustering and kinetic properties of the receptor. GABA is the major inhibitory neurotransmitter in the brain and acts through the ionotropic GABA₃ and GABAₓ receptors and the metabotropic GABA_{μ} receptor.⁴ Of these, GABARAP is known to bind only to the GABA₃ receptor. Postsynaptic binding of GABA to the GABA₃ receptor opens chloride ion channels and leads to hyperpolarization, thereby slowing neuroelectrical impulses. Coexpression of GABARAP has been shown to increase the

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level of GABA<sub>A</sub> receptors detected at the plasma membrane and to cluster recombinant GABA<sub>A</sub> receptors,<sup>5–8</sup> the net effect of which is to modulate neuroelectrical inhibition.

GABARAPL1 (GEC1), originally identified as an estrogen-induced protein homologous to GABARAP,<sup>9</sup> has since been found to bind to the GABA<sub>A</sub> receptor<sup>10</sup> and to the carboxyl terminus (C terminus) of the metabotropic k-opioid receptor (KOR) and to facilitate receptor trafficking of the KOR from the endoplasmic reticulum/Golgi to the plasma membrane.<sup>11</sup> When expressed in CHO cells, GABARAP/1 coimmunoprecipitates with KOR and greatly increases total and cell surface KOR opioid receptors but not µ- or δ-opioid receptors. Both of the MAPs, GABARAP and GABARAPL1, therefore, are involved in plasma membrane-directed protein trafficking.

The vital importance of accessory proteins, such as GABARAP, that are involved in intracellular trafficking is exemplified by the development of kidney hypertrophy and hypertension in transgenic mice overexpressing Ang II receptor–associated protein 1 (ARAP1), a protein that is involved in AT<sub>R</sub> “recycling” in the kidney.<sup>12</sup> The studies described herein were designed to confirm the observed AT<sub>R</sub>:GABARAP interaction in yeast and to investigate the nature and function of the intermolecular association.

**Materials and Methods**

**Y2H Analysis**

An adult mouse brain cDNA library cloned into a GAL4 activation domain vector (pGADT7-Rec) and transformed into yeast strain Y187 was obtained from Clontech (catalog no. 638863) and used for Y2H screening as recommended by the manufacturer.

The AT<sub>R</sub> C terminus was ligated in-frame into pGBK7 to produce a Gal4-DBD:AT<sub>R</sub> C terminus fusion protein. The Y2H target library consisted of mouse brain sequences ligated into pGADT7-Rec to produce Gal4-AD fusion sequences (library from Clontech) (DBD indicates DNA-binding domain; AD, activation domain).

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Results**

Note that the term GABARAP(X) refers to the combined terms GABARAP and GABARAPL1.

**Y2H Screen**

A Y2H screen of a mouse brain cDNA library was conducted to identify proteins that interact with the cytoplasmic C terminus of the AT<sub>R</sub> (amino acids 306 to 359). Of 1.3×10<sup>6</sup> clones screened, 40 clones interacted with AT<sub>R</sub> as judged by growth on selective media. Sequence analyses revealed that 21 of the 40 recovered clones encoded GABARAP or the related protein GABARAPL1. Both GABARAP and GABARAPL1 (data not shown for the latter) interacted specifically with AT<sub>R</sub>:R<sub>ct</sub>; interactions with heme oxygenase-1 and biliverdin reductase (BvR) do not interact with GABARAP and, hence, do not permit yeast growth on restrictive media.

GABARAP and GABARAPL1 encode very similar proteins belonging to a new MAP family and possess aminoterminal tubulin-binding domains (residues 1 to 27).<sup>13</sup> Not surprisingly, all GABARAP(X) clones recovered from the Y2H screen encode partial proteins (see Figure 1 in the online data supplement for more information).

**GABARAP Interacts With the AT<sub>R</sub> C-Terminal Cytoplasmic Sequence in Mammalian Cells**

The AT<sub>R</sub>:GABARAP interaction observed in yeast was initially verified in mammalian cells using GST pull-down assays (Figure 2A). CHO-K1 cells were transiently transfected with pCMV/HA/GABARAP, pGST/AT<sub>R</sub>:R<sub>ct</sub>, or both plasmids, and cell extracts were prepared at 24 hours posttransfection. Extracts were applied to glutathione columns, washed, eluted, and electrophoresed on denaturing gels. Filters were probed with anti-hemagglutinin (HA) antibodies to confirm that AT<sub>R</sub>:R<sub>ct</sub> specifically binds GABARAP. HA-GABARAP clearly associates with GST-AT<sub>R</sub>:R<sub>ct</sub> and is coeluted from the glutathione column.

**GABARAP Coimmunoprecipitates With AT<sub>R</sub> in Mammalian Cell Extracts**

CHO-K1 cells were transfected with pFlag/AT<sub>R</sub> and pCMV/HA/GABARAP (or empty vector [−]) or pCMV/HA/GABARAPL1 (or empty vector [−]) (Figure 2B). Twenty-four hours posttransfection, cell extracts were prepared and immunoprecipitated with anti-Flag antibody or preimmune serum (control) and protein A agarose. The immunoblot was probed with anti-HA antibody. The results indicate that the Flag/AT<sub>R</sub> interacts with GABARAP and with GABARAPL1 in CHO-K1 cells.

In parallel studies, the intermolecular complexes of HA/GABARAP:Flag/AT<sub>R</sub> and HA/GABARAPL1:Flag/AT<sub>R</sub> were immunoprecipitated with resin-bound anti-Flag antibod-
Figure 2. Interaction between GABARAP and AT1R. A, GST pull-down assay using the AT1RCT. Plasmids expressing the indicated proteins were cotransfected into CHO-K1 cells, and cell extracts were prepared 24 hours later. Five percent of the cell extract was reserved for expression analyses (input) and the remainder subjected to pull-down reactions. Proteins were separated on SDS-PAGE gels and detected by immunoblotting with the indicated antibodies. B through D, Immunoprecipitation with Flag-tagged full-length AT1R, CHO-K1 cells were transiently transfected with plasmids encoding the indicated proteins (+) or the respective empty vectors (−). B, Cell extracts were incubated with control (lgG) or anti-Flag antibody, followed by immunoprecipitation with protein A agarose. Immunoprecipitates were subjected to SDS-PAGE and transferred to poly(vinylidene difluoride) (PVDF) membrane, and the immunoblot was probed with anti-HA antibody to detect GABARAP or GABARAPL1. The antibody light chain (Ab LC) is indicated. C and D, Cell extracts were incubated with the indicated antibody resin. Immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF membrane, and immunoblots were probed with anti-HA antibodies. E, Direct interaction between GABARAP and AT1RCT using purified proteins. GST pull-down assays were performed as described in Materials and Methods using the indicated recombinant proteins. The eluates were subjected to SDS-PAGE and transferred to PVDF membrane. The membrane was probed with anti-GABARAP antibodies, stripped, and subsequently probed with antibody to GST. The GST-AT1RCT fusion is highly labile in Escherichia coli, even in BL21 derivatives (this strain used in this study) that lack the lon and ompT proteases. The primary cleavage site appears to be near the fusion junction within the AT1RCT sequence, resulting in the purification of two major species by affinity chromatography. The slower migration of the parental GST protein (lanes 3 and 4) relative to the cleaved GST (lanes 1 and 2) is attributable to the contribution of extra amino acids derived from the multiple cloning site downstream of the GST sequence.

GABARAP Interacts Directly With AT1RCT

GST pull-down assays using purified recombinant proteins were carried out to determine whether GABARAP binds directly to AT1RCT. As shown in Figure 2E, recombinant GABARAP is eluted from resin charged with the GST-AT1RCT fusion but not with GST alone (compare lanes 1 and 3), providing evidence for direct interaction between these proteins.

Bioluminescence Resonance Energy Transfer for GABARAP:AT1R Interactions

Bioluminescence resonance energy transfer (BRET) assays (Figure 3) indicate a significant intermolecular interaction between GABARAP and AT1R (pGFP2/GABARAP+patR/RLuc), P<0.001 versus patR/RLuc alone. (Note that all values have been corrected by subtracting the mock-transfected cell background at each emission wavelength, a function of the BRET program.) General negative controls include patR/RLuc transfected alone and cotransfected empty vectors (pGFP-C1+pRLuc-N1). Experiment-specific negative controls include mouse MAP1 light chain 3 (MAP1 LC3) as a GABARAP analog and the endothelin type A GPCR (ETR-A) as an AT1R correlate. The endothelin receptor is from the same GPCR family as the AT1R (Family 1, subgroup 1A) and, therefore, serves to show that GABARAP does not promiscuously interact with GPCRs. MAP1 LC3 is a MAP that binds to both MAP1 and MAP2 and, in this case, demonstrates that binding to the Ang receptor C terminus is not a general property of MAPs. Neither the general nor specific negative controls demonstrate significant BRET. The positive control, a commercial vector, pGFP2-RLuc, that encodes a cytomegalovirus (CMV)-regulated fusion protein of Renilla luciferase with GFP, has been shown to be expressed at high levels in a variety of cells and, indeed, shows very high energy transfer in our assay (P<0.001 versus patR/RLuc-transfected; P<0.001 versus pGFP2/...
The BRET\textsuperscript{2} assays verify and support our Y2H results, GST pull-down assays, and coimmunoprecipitation results. See the online data supplement (expanded Results section) for more information.

### Influence of GABARAP Overexpression on AT\textsubscript{1}R Accumulation and Plasma Membrane Presentation

PC-12 neural cells were plated, treated with NGF (7S, 100 ng/mL) for 4 days (to enhance the neuronal differentiation)\textsuperscript{14,15} and then transfected with pAT\textsubscript{1}R/EYFP or pECFP/GABARAP+pAT\textsubscript{1}R/EYFP. Cells were evaluated at 24 and 48 hours posttransfection for cell surface expression of AT\textsubscript{1}R/EYFP using 3D deconvolution microscopy. Under our transfection conditions, AT\textsubscript{1}R/EYFP, as reported in our previous studies, is observed at the plasma membrane but is found predominantly in the secretory pathway (endoplasmic reticulum, Golgi, vesicles) (Figure 4A1 and 4A2). By 48 hours posttransfection, GABARAP overexpression increased AT\textsubscript{1}R cell surface expression 6.74-fold when standardized to the level of intracellular expression (P<0.005, n=3, 100 transfected cells per experiment) (Figure 4B through 4D). See the online data supplement (expanded Results section) for more details.

### Radioligand Binding Assays

CHO-K1 cells, which do not express detectable Ang receptor\textsuperscript{16–18} were stably transfected with AT\textsubscript{1}R/EYFP to obtain a working cell line with a defined Ang receptor. One high-level fluorescent clonal isolate was subsequently transfected with either pECFP/GABARAP or pECFP-C1 (control) and mixed (nonclonal) stable double transfectants were selected. In this manner, the AT\textsubscript{1}R uninduced baseline expression in both double-transfectant lines should be equal. Our studies show that expression of ECFP/GABARAP increases total binding 3.6-fold over the ECFP-C1 control (Figure 5). This increase is sensitive the AT\textsubscript{1}R blocker losartan but not to the AT\textsubscript{2}R blocker PD123319. GABARAP coexpression clearly increases not only the cell surface manifestation of AT\textsubscript{1}R/EYFP (Figure 4) but also the number of plasma membrane-associated Ang II binding sites.

### Immunoblot

By Western blot analysis, we observed a 7.7-fold AT\textsubscript{1}R protein increase (n=3, P<0.001) in COS-7 cells transfected with pCMV/myc/AT\textsubscript{1}R+pCMV/HA/GABARAP as compared with pCMV/myc/AT\textsubscript{1}R with either pCMV/Flag/SNAPAP (control) or control unmodified vectors (Figure 6). Therefore, GABARAP overexpression is shown to increase cell surface expression of AT\textsubscript{1}R in PC-12 cells.

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**Figure 4.** GABARAP promotes cell surface expression of the AT\textsubscript{1}R in PC-12 cells. Rat PC-12 cells were transfected with pAT\textsubscript{1}R/EYFP (green) (A1 and A2) or pAT\textsubscript{1}R/EYFP and pECFP/GABARAP (green and blue, respectively) (B through D) and imaged at 48 hours posttransfection. A, Note the little plasma membrane expression of AT\textsubscript{1}R/EYFP in the absence of GABARAP. B, Note the high expression of AT\textsubscript{1}R/EYFP and expression in long membrane processes (arrow); GABARAP is confined to the cytoplasm. C, Note the little expression of plasma membrane AT\textsubscript{1}R/EYFP in the cell which expresses only AT\textsubscript{1}R/EYFP compared with the cell expressing both AT\textsubscript{1}R/EYFP and ECFP/GABARAP. D, Composite is typical of a channel view series for 3I software. D1, Yellow filter image showing expression of AT\textsubscript{1}R/EYFP. D2, Cyan filter image showing expression of ECFP/GABARAP. D3, Merged images from D1 and D2. Arrowheads show concentrated plasma membrane AT\textsubscript{1}R.
GABARAP not only alters the distribution but also increases the steady-state AT1R protein level.

SNAPAP was included as a negative control in this study. We isolated SNAPAP as a potential AT1R binding partner in an initial screen of the Y2H library, but we were subsequently unable to confirm it by affinity pull-down or by coimmunoprecipitation assays. We later learned that SNAPAP (also called SNAPIN) is frequently isolated as a false-positive in initial Y2H assays performed in 2-hybrid service facilities (personal communication, Chandra Tucker, Duke Yeast Model Systems Genomics Group).

GABARAP Effects on Extracellular Signal-Regulated Kinase Activation

Ang II:AT1R-mediated extracellular signal-regulated kinase (ERK)1/2 phosphorylation stimulation is known to occur in many cell types and through several mechanisms.19–21 Because our imaging data and radioligand binding assays suggest that GABARAP increases plasma membrane accumulation of the AT1R, as well as ligand association, we asked whether GABARAP might also (indirectly through increasing plasma membrane accumulation of receptor) stimulate ERK phosphorylation (Figure 7A and 7B). Our data show that Ang II treatment of pAT1R/EYFP stably transfected CHO-K1 cells increases the ratio of phosphorylated to unphosphorylated ERK1/2 levels 2.7-fold over vehicle treatment (n=3, P=0.005). In comparison, Ang II treatment of pECFP/GABARAP, pAT1R/EYFP double-transfectants increases the ratio 5.5-fold (n=3, P=0.005). GABARAP overexpression, therefore, significantly augments accumulation of phospho-ERK1/2 levels (n=3, P<0.005), consistent with greater plasma membrane expression of functional AT1R in double-transfectants.

Proliferation Assays

Because Ang II has been shown to mediate proliferation of many cell types through the AT1R19,22,23 and our data indicate that GABARAP increases cell surface expression of the AT1R, we measured and compared growth of CHO-K1-pAT1R/EYFP-pECFP/GABARAP versus CHO-K1-pAT1R/EYFP-pECFP-C1 in the presence of Ang II, losartan (AT1R blocker), and/or PD123319 (AT-R blocker) as in Cook et al.19
Ang II increases both 5-bromodeoxyuridine incorporation into DNA and cell counts (3.5- and 3.3-fold, respectively) in the AT1R engineered CHO-K1 cell line in a manner that is sensitive to losartan but refractory to PD123319 (Figure 7C and 7D). Furthermore, Ang II promotes cell proliferation to a greater extent (approximately 2-fold greater) in the AT1R/GABARAP as compared with the AT1R recombinant cell line, consistent with higher plasma membrane expression of functional AT1R in these cells.

Small Interfering RNA–Mediated Knockdown of GABARAP Reduces Cell Surface Expression of Recombinant AT1R/EYFP

The ability of GABARAP complementary small interfering (si)RNA oligonucleotides to reduce GABARAP protein was tested using hybrid complexes (siGABARAP-1, siGABARAP-2, and siGABARAP-3) made complementary to 3 phylogenetically conserved regions of the mRNA. CHO-K1 cells stably transfected with pECFP/GABARAP and pDsRed2-Nuc were transiently transfected with siRNA duplexes. Negative control (Silencer #1 from Ambion), and scrambled siGABARAP-1 negative control caused no diminution in ECFP/GABARAP compared with mock-transfected cells (Figure 8, I). However, siGABARAP-1, siGABARAP-2, and siGABARAP-3 duplex RNAs all significantly reduced ECFP/GABARAP accumulation, as determined by both deconvolution image analysis (85% to 93% reduction, P<0.001 versus mock, n=3 experiments) and Western blot analyses (Figure 8, II) (standardized 5.3-fold reduction of ECFP/GABARAP levels in siRNA-treated cells [P<0.001 versus mock, n=3 experiments]). Neither negative control RNAs nor experimental siRNAs significantly affected DsRed2-Nuc nuclear fluorescence.

The GABARAP siRNA duplexes were subsequently applied to CHO-K1 cells stably transfected with pAT1R/EYFP and pDsRed2-Nuc to determine whether a reduction in native GABARAP might also diminish fluorescent AT1R plasma membrane accumulation. GABARAP siRNAs reduced AT1R/EYFP and ECFP/GABARAP accumulation, as determined by both deconvolution image analysis (85% to 93% reduction, P<0.001 vs corresponding vehicle, n=3 experiments) and Western blot analyses (Figure 8, II) (standardized 5.3-fold reduction of ECFP/GABARAP levels in siRNA-treated cells [P<0.001 versus mock, n=3 experiments]). Neither negative control RNAs nor experimental siRNAs significantly affected DsRed2-Nuc nuclear fluorescence.

Figure 7. Effect of GABARAP on AT1R-mediated ERK1/2 activation and cell proliferation. A, CHO-K1 stable double transfectants were serum-starved for 24 hours and then treated with Ang II (10⁻⁷ mol/L) or vehicle for 10 minutes. Whole cell extracts were subjected to SDS-PAGE and immunoblotting with anti-phospho-ERK1/2 antibodies. The blot was stripped and reprobed with antibody to determine total ERK1/2 levels. B, The ratio of the corresponding phosphorylated and unphosphorylated bands for each treatment was quantified by densitometry (n=3). 1Significantly different from corresponding vehicle at the P<0.001 level, 2significantly different from Ang II treatment of alternate cell line at the P<0.001 level. C and D, Growth measurements for CHO-K1 stable transfectants treated with Ang II, losartan (Los), and/or PD123319 (PD), all at 10⁻⁷ mol/L (n=4 experiments [6 replicates per experiment]).
EYFP steady-state levels an average of 43% (P<0.005 versus scrambled negative control [siGABARAPC-1], n=3 experiments/hybrid complex, 150 cells evaluated/experiment) (Figure 8, III) and with 84% reduction in plasma membrane-associated yellow fluorescence (P<0.01 versus scrambled negative control; SlideBook 4.2 software, “Masks” to quantify regions of interest). Immunoblot analyses were also performed to verify the quantitative image data (Figure 8, IV). GABARAP siRNA transfection (for any of the 3 hybrid complexes) reduced AT1R/EYFP expression an average of 4.2-fold as compared with transfection with scrambled control siRNA (P<0.005, n=3 experiments).

Discussion

In this report, we show, by 3D deconvolution imaging and radioligand binding, that GABARAP, a MAP involved in GABA_β receptor trafficking, increases plasma membrane accumulation of the AT1R. We further show that the increase in cell surface AT1R correlates, in response to Ang II, with increased phospho-ERK1/2 accumulation and enhanced proliferation. We further demonstrate association of these 2 proteins by yeast complementation, GST affinity pull-down, coimmunoprecipitation assays, and BRET assays. In addition, GABARAP-targeted siRNAs effectively reduce cell surface accumulation of fluorescent AT1R, demonstrating the importance of endogenous GABARAP for efficient plasma membrane-directed transport.

Using a similar approach, Dzau and colleagues have discovered that the AT1R binds to a transmembrane protein, ATRAP (Ang II type 1 receptor-associated protein), a 162-aa 3-transmembrane protein that modulates Ang II signaling. ATRAP appears to generally downregulate Ang II–mediated AT1R function, reducing inositol lipid levels, decreasing Ang II–mediated c-fos transcription, and reducing cell proliferation. ATRAP was isolated from a mouse kidney yeast library using the AT1AR amino acids 297 to 359 as bait. In contrast, we did not recover ATRAP from a mouse brain yeast library. ATRAP mRNA does appear to be ubiquitous, but it is apparently present at very low levels in brain compared with the levels in kidney, testis, or heart, perhaps accounting for our failure to isolate this message. ATRAP binds to the AT1R through the ATRAP C-terminal amino acids 110 to 122. AT1R binds to ATRAP through the AT1R terminal 20 amino acids (339 to 359).

By further example, using rat AT1R amino acids 295 to 359 as bait to screen a mouse 10 day embryo library, Guo et al isolated ARAP1 (type I Ang II receptor-associated protein 1), a ubiquitous 493-aa protein that interacts (in rather broad terms) with residues 319 to 359 of the AT1R. ARAP appears to enhance receptor recycling to the plasma membrane and

Figure 8. Effects of GABARAP-targeted siRNAs on AT1R expression. GABARAP siRNAs reduce expression of ECFP/GABARAP in stably transfected CHO-K1 cells (I and II). I, Cells stably transfected with pECFP/GABARAP and pDsRed2-Nuc were transfected with vehicle (A), Silencer #1 (B), scrambled negative control siGABARAPC-1 (C), siGABARAP-1 (D), siGABARAP-2 (E), or siGABARAP-3 (F). siRNA oligonucleotides reduce cyan fluorescence accumulation 89% (P<0.005 vs siGABARAPC-1), with no effect on nuclear red fluorescence. II, Cell extracts were collected 48 hours posttransfection and analyzed by immunoblot. Histone H1 is a negative control to which experimental values are normalized. GABARAP siRNA duplexes reduce ECFP/GABARAP protein accumulation 5.3-fold (P<0.001 vs siGABARAPC-1). III and IV, RNA interference–mediated GABARAP inhibition reduces plasma membrane expression of AT1R/EYFP in stably transfected CHO-K1 cells. III, Cells stably transfected with pAT1R/EYFP were subsequently transiently transfected with vehicle (A), Silencer #1 (B), scrambled negative control siGABARAPC-1 (C), siGABARAP-1 (D), siGABARAP-2 (E), or siGABARAP-3 (F). GABARAP siRNAs reduce plasma membrane expression of AT1R/EYFP by 84% (P<0.01 vs siGABARAPC-1) and intracellular accumulation by 43% (P<0.001 vs siGABARAPC-1). IV, Cell extracts were collected 48 hours posttransfection and analyzed by immunoblot. GABARAP siRNAs reduce AT1R/EYFP protein accumulation by an average of 4.2-fold (P<0.005 vs siGABARAPC-1).
receptor resensitization to a second Ang II stimulus. Transgenic mice overexpressing ARAP1 in kidney proximal tubule cells demonstrate high blood pressure and kidney hypertrophy. Therefore, both ATRAP and ARAP1 bind to the distal portion of the AT1R C terminus and mediate quite different effects; ATRAP attenuates the Ang II–mediated AT1R downstream effects, whereas ARAP1 potentiates Ang II effects by upregulating the receptor level. Both of these also appear to interact with the distal portion of the AT1R C terminus. In contrast, our preliminary studies (data not shown) suggest that the binding site for GABARAP is in the membrane proximal region of the AT1R cytoplasmic C terminus (proximal to residue 316). Both ARAP1 and GABARAP interactions with AT1R appear to serve, in a similar capacity, to upregulate the renin–Ang system.

GABARAP family members appear to be highly connected with binding partners that include NSF (N-ethylmaleimide-sensitive factor),27,28 tubulin (and microtubules),29,30 transferrin receptor,31 GABAAR,32–34 AT1R, DXD 47 (an RNA helicase),35 GRIP1 (an adapter and steering protein),36 gephrin,37,38 ULK1 (a neuronal serine/threonine kinase involved in axonal elongation),39 and p130 (inositol triphosphate binding protein).40 GABARAP has, in fact, been referred to as a “multifunctional adapter molecule” because of the myriad of binding partners associated with it.30 The fact that GABARAP is involved in trafficking of both the pentameric ionotropic GABAAR and the 7-transmembrane GPCR AT1R suggests that these receptors could be cotransported/coregulated and that conditions that favor upregulation of one may also favor upregulation of the other. This remains to be tested. GABARAP is a ubiquitous protein though it is primarily known for its role in trafficking of the GABAAR in the central nervous system. Our studies indicate that interaction of AT1R with GABARAP occurs in nonneuronal cells, although the relative importance of GABARAP for AT1R trafficking in neural and nonneural tissue is yet unknown.

Two primary motor proteins control microtubule-based protein transport. Anterograde trafficking (toward the plasma membrane) involves the motor protein kinesin, whereas retrograde movement involves dynein. Kinesin is an ATP-binding protein that “walks” along the length of the microtubule with ATP hydrolysis occurring at each successive 8-nm step. In contrast, GABARAP is not a nucleotide-binding motor protein and does not directly convey the AT1R or GABAAR-containing vesicles to the plasma membrane. Nonetheless, GABARAP increases the steady-state level of these proteins at the plasma membrane. How might GABARAP enhance plasma membrane accumulation of proteins like GABAAR and AT1R?

For the AT1R, this appears to be, in part, attributable to accumulation of a higher level of receptor as compared with total cell protein in the presence of GABARAP. However, in addition, the trafficking process is altered as established by the change in the distribution of fluorescent AT1R when expressed with GABARAP. The relative AT1R cell surface to-intracellular (secretory pathway) distribution is altered in PC-12 cells overexpressing GABARAP (a cytoplasmic protein). Clearly, GABARAP overexpression causes relatively more AT1R accumulation at the plasma membrane compared with the internal compartments. This observation, coupled with the knowledge that GABARAP binds tubulin and microtubules, suggests that GABARAP enhances the transport process. Because GABARAP is not a motor protein and does not actively move vesicular cargo to the plasma membrane, how might it enhance transport? One possibility is that GABARAP increases the processivity of plasma membrane trafficking. A kinesin molecule, on average, completes a 1-μm run length before dissociating from a microtubule.41 Often, cargos must be moved considerably further than 1 μm to reach their destination. Therefore, vesicular cargos must be passed from one to another microtubule-engaged kinesin complex to continue processive movement to the plasma membrane. GABARAP could function, therefore, as an accessory protein, binding both vesicle-associated AT1R and microtubules, so that AT1R containing vesicles are retained on the microtubules, on motor protein dissociation, until the vesicle can be recovered by a second kinesin complex. In this model, GABARAP would bind both vesicle-associated AT1R and a microtubule and act as a “rear wheel” stabilizing the kinesin–vesicle complex on the microtubule as it moves the cargo forward. In fact, there does exist evidence to suggest that motors may use secondary binding sites to aid in processivity.42

Given the central role of the AT1R in blood pressure homeostasis and cardiovascular disease, regulation of proteins that are involved in trafficking of this receptor and mechanisms by which these proteins interact with the receptor and modify its accumulation and function are of critical importance. We are the first to report an interaction between the AT1 receptor and the trafficking protein GABARAP. This novel finding introduces new areas of investigation in the renin–Ang system and in basic mechanisms of Ang II–mediated growth and blood pressure control and offers a new potential therapeutic target for pharmacological intervention.

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Disclosures

None.

References


The Trafficking Protein GABARAP Binds to and Enhances Plasma Membrane Expression and Function of the Angiotensin II Type 1 Receptor
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The trafficking protein, GABARAP, binds to and enhances plasma-membrane expression and function of the angiotensin AT$_1$ receptor (Online Supplement)

Materials and Methods

Yeast two-hybrid analysis

An adult mouse brain cDNA library cloned into a GAL4 activation domain vector (pGADT7-Rec) and transformed into yeast strain Y187 was obtained from Clontech (catalog #638863) and used for yeast two-hybrid screening as recommended by the manufacturer.

The AT$_1$R C-terminus was ligated in-frame into pGBKT7 in order to produce a Gal4-DBD:AT$_1$R C-terminus fusion protein. The Y2H target library consisted of mouse brain sequences ligated into pGADT7-Rec to produce Gal4-AD fusion sequences (library from Clontech). [DBD = DNA-binding domain; AD = activation domain].

Cell lines/Transfections

PC-12 (ATCC CRL 1721) is a clonal cell line derived from a rat adrenal pheochromocytoma and is frequently used as a model system for neurobiological studies.$^{1,2}$ Cells of this line respond reversibly to nerve growth factor (NGF) by induction of the neuronal phenotype and synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine. PC-12 cells were maintained in RPMI 1640 medium with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS) and 50 μg/ml gentamicin, and cultured in a humidified atmosphere (95% air, 5% CO$_2$) at 37°C. Cells were transiently transfected (for 3 h) with SuperFect (Qiagen) according to the manufacturer protocol. For imaging, cells were seeded at 10$^6$/35 mm MatTek (Ashland MA) glass bottom culture dishes and transfected 24 h later (70-80% confluence) with 1 μg each of
plasmids encoding fluorescent or control proteins. In some studies, cells were treated with NGF (100 ng/ml, Sigma, NGF-7S) for 48-to-96 h prior to imaging to increase differentiation.

COS-7 (ATCC CRL-1651) African green monkey kidney and Chinese hamster ovary (CHO-K1, ATCC CCL-61) were cultured in DMEM containing 0.45% glucose, 10% FBS, and 50 μg/ml gentamicin and subcultured every 3-4 days. Transient transfection of COS-7 and CHO-K1 cells was carried out with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendation. Unless otherwise indicated, cells were seeded (2 x 10^5/35 mm plate) and transfected 20 h later with a total of 500 ng of the appropriate plasmid DNA mixture. The transfection media were removed 24 h post-transfection and the cells were collected for analyses.

Stable cell lines were isolated according to Cook et al. Note that CHO-K1 cells were first transfected with \( pAT_1R/EYFP \) and one high expressing cell line (as determined by western blot and fluorescence microscopy) was isolated and subsequently transfected with either \( pECFP-C1 \) or \( pECFP/GABARAP \). 48 h post-transfection, selection was applied using 400 ug/ml G418. Mixed populations (nonclonal) of double transfectants were selected and used in further studies. Recombinant \( AT_1R \) baseline, therefore, should be the same in each of the mixed stable double transfectant populations.

**Antibodies**

The following antibodies or related reagents were used in this study: Flag M2 mouse monoclonal (Sigma, F-3165); HA.11 mouse monoclonal clone 16B12 (Covance, MMS-101P); c-Myc mouse monoclonal clone 9B11 (Cell Signaling, #2362); pre-immune mouse IgG (Sigma, A-6715); Flag M2-agarose conjugate (Sigma, A-2220); c-Myc-agarose conjugate (Sigma, A-7470); pre-
immune mouse IgG-agarose conjugate (Sigma, A-0919); sheep anti-mouse IgG HRP conjugated (Amersham, NXA931) and protein A-sepharose conjugate (Pharmacia, 17-0780-01). Mouse monoclonal anti-histone H1 antibodies (sc-8030) and rabbit polyclonal against full-length GFP (sc-8334) are from Santa Cruz.

Plasmids

**pAT_1R_CT/GBKT7.** The C-terminus of the rat AT1_a receptor was PCR amplified from pAT_1R/EYFP using upstream primer, 5’-
ACGTACGGATCCATGGGAAGAAATTTAAAAAGTAT-3’ and downstream primer 5’-
ACGTACCTGCAGGCTCCACCTCAAAACAAGACGC-3’. The resulting 162 bp product was digested with BamHI and PstI, purified and ligated in-frame and downstream of the Gal4 DNA-binding domain in the yeast expression vector pGBK7 (Clontech).

**pAT_1R/RLuc.** The full-length rat AT1a receptor coding sequence was PCR amplified from pAT_1R/EYFP using upstream primer (AT1-U) 5’-
GATCGAAAGCTTGCCACCATGGCCCTTAACTCTTCTGCT-3’ and downstream primer (AT1-D) 5’- CGAGACCGAGGATCCTGCTCCACCTCAAAACAAGACGC -3’, digested with HindIII/BamHI, and ligated in-frame into HindIII/BamHI-digested pRLuc-N1 (Perkin-Elmer). AT1R is upstream of Renilla luciferase in the recombinant protein produced by this plasmid.

**pECFP/GABARAP.** The full-length mouse GABARAP coding sequence was PCR amplified from pCMV/GABARAP/SPORT6 (image clone MGC-6656) using upstream primer mmGABARAP-U1 (5’-ACGTACAAGCTTGGATGTTGTGCTGTTAAGAG-3’) and downstream primer mmGABARAP-D1 (5’-
ACGTAGGGCCCGTCACAGACCATAAGACGCTTTCATC-3’) digested with HindIII/Apal, and ligated in-frame into HindIII/Apal-digested pECFP-C1.

**pGFP²/GABARAP.** The full-length mouse GABARAP coding sequence was PCR amplified from pECFP/GABARAP using upstream primer mmGABARAP-U1 and downstream primer mmGABARAP-D1, digested with HindIII/Apal and ligated in-frame into HindIII/Apal-digested pGFP²-C1 (Perkin-Elmer). GABARAP is downstream of GFP² in the recombinant protein produced by this plasmid.

**pCMV/myc/AT1R.** The full length rat AT₁a receptor coding sequence was PCR amplified from pAT₁R/EYFP using upstream primer, 5’ – TACGATGTCGACCATGGCCCTTAACTCTCTTGCT-3’ and downstream primer, 5’ – GACGATGGTACCCTCACTCCACCTCAAACAAGA-3’. The resulting 1 kbp product was digested with KpnI and SalI, purified and ligated in-frame to the c-myc tag in KpnI/SalI-digested pCMV/myc (Clontech).

**pGST/AT1RCT.** The C-terminus of the rat AT₁a receptor coding sequence was PCR amplified from pCMV/myc/AT₁R using upstream primer, 5 – CCCATCGGATCTGCATAGCGTATTTTAAC-3’ and downstream primer, 5’ – GCTTTGATCCACTCCACTCCACTCAAACA-3’. The resulting 200 bp product was digested with BamHI and SalI, purified and ligated in-frame downstream of the glutathione S-transferase (GST) cDNA in the vector pEFGST (also digested with BamHI/SalI). In this plasmid, expression of the GST/AT₁R_CT fusion is under control of the elongation factor-1 alpha promoter.

**pCMV/HA/GABARAP.** The full length mouse GABARAP coding sequence was PCR amplified from pCMV/GABARAP/SPORT6 (image clone MGC-6656) using upstream primer, 5’ –
CCAGCTGTCGACCATGAAGTTCTGTTCTACAAAGAG-3’ and downstream primer, 5’ – AGTCTAGGTACCCTCACAGACCATAGACGCTTTC-3’. The resulting 379 bp product was digested with KpnI and SalI, purified and ligated in-frame to the HA tag in KpnI/SalI-digested pCMV/HA (Clontech).

**pCMV/HA/GABARAPL1.** The full length mouse GABARAP coding sequence was PCR amplified from pCMV/GABARAPL1/SPORT6 (image Clone ID 3585132) using upstream primer, 5’– ACGTACAGATCTCTATGAAGTTCCAGTATAAGGAG-3’ and downstream primer, 5’-ACGTACGGTACCCTTCATTTTCCATAGACACTTTC-3’. The resulting 350 bp product was digested with BglII and KpnI, purified and ligated in-frame to the HA tag in BglII/KpnI-digested pCMV/HA (Clontech).

**pFLAG/AT1R.** The full length rat AT1a receptor coding sequence was isolated by digesting pECFP/AT1R/EYFP with HindIII (blunt-ended) and BglII. The resulting 1100 bp fragment was ligated in-frame to HindIII (blunt-ended)/BglII digested p3XFlag-myc-CMV-26 (Sigma-Aldrich).

**pCMV/FLAG/SNAPAP.** The full length mouse Snapap coding sequence was PCR amplified from pCMV/SNAPAP/SPORT6 (image clone 6511395) using upstream primer, 5’- TAATTAGAATTCCATGGCCGCGGCTGGTTCC-3’ and downstream primer, 5’– GCGGCCTCTCATTATTTGCTTGGAGAACC-3’. The resulting 400 bp product was digested with EcoRI and XbaI, purified and ligated in-frame to EcoRI/XbaI digested p3XFlag-myc-CMV-26 (Sigma-Aldrich).

**pGEX-3T/AT1RCT.** The sequence of the rat AT1a receptor cDNA encoding amino acids 289-359 was amplified by PCR using upstream primer, 5’ - CCCATCGGATCCTGCATAGCGTATTTT AAC - 3’, and downstream primer 5’ - GCTTTGGTCGACTCACTCCACCTCAAACAC - 3’. The resulting 231 bp product was digested with BamHI and SalI, purified and ligated to BamHI/
SalI–digested pGEX-3T (Amersham). GST and GST-AT₁aR proteins were expressed and purified as previously described.⁵

**pET30b/GABARAP-His.** The complete coding region of mouse GABARAP was PCR amplified from pSport6/GABARAP (I.M.A.G.E. #3497484, ATCC# MGC-6656) using upstream primer, 5' - CTCGGGGCATATGAAGTTCGTGTACAAAGAG - 3', and downstream primer 5' - GTGGTGCTCGAGCACGACCATAGACGCTTTTC - 3'. The resulting 400 bp product was digested with *NdeI* and *XhoI*, purified, and ligated to *NdeI/XhoI*–digested pET30b (Novagen). His-tagged GABARAP was expressed and purified using procedures previously described.⁶

**GST pull-down (GPD) assays**

Transfected CHO-K1 cells were collected by scraping in PBS followed by centrifugation. Cell pellets were lysed in 550 µl of cold GPD Lysis Buffer (50 mM Tris-HCl, pH 7.5, 1.0% Triton X-100, 2 mM EDTA, 2 mM EGTA) supplemented with 20 mM NaF, 1 mM DTT and 1 mM PMSF and clarified by centrifugation. A 50 µl aliquot was reserved for determining protein input while 250 µl aliquots of cell lysates were incubated with 25 µl bed volume of glutathione-sepharose beads (Pharmacia; pre-equilibrated in GPD Lysis Buffer) for 2 h at 4°C with gentle rotation. The resin was subsequently washed 5 times with 20 resin volumes of GPD Lysis Buffer. Resin-bound proteins were eluted by incubation in 20 µl of GT Elution Buffer (GPD Lysis Buffer supplemented with 20 mm glutathione and 5 mM DTT) at room temp for 20 min and analyzed by immunoblotting as previously described.⁷

**GST pull-down assays using purified proteins**
Five µg of purified GST or GST-AT₁AR_{CT} proteins, diluted in 100 µl of TEN100 Buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 100 mM NaCl), was mixed with 40 µl of a 50% GT-sepharose suspension (GE Healthcare; pre-equilibrated in TEN100) and incubated overnight at 4°C with gentle rotation. After 3 washings with 200 µl of TEN100, the resin was blocked by incubation with BSA (100 µl of a 1 mg/ml solution in TEN100) at 4°C for 2 h. The resin was washed as described above, resuspended in 100 µl of TEN100 or buffer containing 5 µg of His-tagged GABARAP, and incubated at 4°C for 2 h with gentle rotation. The resin was subsequently washed in 500 µl of TEN100 (3x) and the bound proteins were eluted by incubation in 20 µl of GT Elution Buffer (TEN100, pH 8.0, supplemented with 20 mM Glutathione and 5 mM DTT) at room temperature for 30 minutes. The eluates were analyzed by SDS-PAGE and immunoblotting.

Co-immunoprecipitation assays

Cell extracts from transfected CHO-K1 cells were prepared as described for GPD assays except that DTT was omitted from the lysis buffer. A 500 µl portion of the cell lysates was incubated with 10 µl bed volume of an antibody-resin conjugate (Method 1) or 3 µg of the soluble antibody (Method II) for 2.5 h at 4°C with gentle rotation. The samples from Method II were subsequently incubated with Protein A sepharose beads (25 µl bed volume) for an additional 2 h at 4°C with gentle rotation. The resins were washed 4 times with 40 resin volumes of GPD Lysis Buffer. Immunocomplexes were dissociated from the resin by incubation in 25 µl of 2x LDS sample buffer (Invitrogen) at 100°C for 5 min and analyzed by immunoblotting as previously described.
BRET$^2$ assays

In BRET$^2$ (bioluminescence resonance energy transfer), a *Renilla* luciferase (RLuc) is used as a donor protein while a green fluorescence protein (GFP$^2$) is the acceptor protein. In the presence of the substrate DeepBlueC (Perkin Elmer), RLuc emits blue light at 395-410 nm. If the GFP$^2$ is in close proximity to RLuc via biomolecular interactions, GFP$^2$ will absorb the light and reemit green light at 510-515 nm. The BRET$^2$ value as presented is the ratio of green light signal over the blue light signal$^8$ or

$$\frac{\text{emission at } 515 \text{ nm} - \text{emission of mock transfected cells}}{\text{emission at } 410 \text{ nm} - \text{emission of mock transfected cells}}$$

PC12 cells were plated in 100 mm dishes at approx. 50% confluence (~3 x 10$^6$ cells) and, on the following day, transfected with Superfect according to manufacturer protocol; 10 μg of each GFP$^2$ vector and 1 μg of each RLuc vector was used (optimum ratio as determined in pilot studies). At 48 h post-transfection, cells were examined by fluorescence microscopy to confirm transfection efficiencies of 80% or more. Cells were harvested, centrifuged at 800 rpm for 5 min and resuspended at a density of 2 x 10$^6$ cells/ml in BRET$^2$ buffer. Cells were aliquoted (6 wells/transfection, 60,000 cells/well) into white, opaque CulturPlate-96 microplates (Perkin-Elmer). Assays were performed using a BMG Labtech POLARStar Optima plate reader with simultaneous dual-emission detectors and Optima evaluation software for BRET assays. The experiment was repeated three times.

3D deconvolution microscopy

Deconvolution microscopy was performed using a Zeiss Axiovert 200M microscope, xenon light source with automated Z-axis and appropriate filters. Constrained iterative and nearest neighbors algorithms were performed using Slidebook 4.2 software (Intelligent Imaging Innovations,
Denver CO). All images were captured in 3D at 0.5 um steps through the Z axis (~10-15 Z-axis planes captured per image) and deconvolved to render confocal images.

**Angiotensin II radioligand binding assay**

CHO-K1 cells were stably transfected with *pAT1R/EYFP* and a high expressing clonal line was isolated. This line was subsequently transfected with or *pECFP/GABARAP* or *pECFP-C1*. Double transfectants were plated at 1.25 x 10⁵ cells/well in 24 well plates and permitted to attach overnight. [¹²⁵I]Tyr4-Angiotensin II (human) (2200 Ci/mmol) was diluted into HEPES buffer (10mM HEPES acid, 130 mM NaCl, 1.5 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂·6H₂O, pH = 2) with 2% BSA. Varying amounts (0 – 1000 nmol/l) of cold Ang II (Sigma, A9525) were added to 0.5 uCi/ml radioactive Ang II-containing buffer and the mixture incubated with cells at room temperature for 60 min with occasional gentle swirling. Cells were washed three-times with ice-cold PBS, the lysate collected in lysis buffer (0.25 M NaOH, 0.05% SDS) and analyzed in a Wizard2 Perkin-Elmer gamma counter.

**ERK1/2 assays**

CHO-K1 cells (transfected lines described above) were serum-starved (24 h) in 0.5% FBS and then treated with Ang II (10⁻⁷ mol/L) for durations ranging from 5-to-60 min in order to determine the optimum stimulation time (determined to be 10 min, data not shown). ERK1/2 immunoblots were performed as in Cook et al.⁹

**Cell proliferation assays**
Cell growth measurements and BrdU labeling and staining were performed according to Cook et al.\textsuperscript{4, 9}

**RNA interference**

siRNA targets were selected and applied according to the methods of the Tuschl laboratory\textsuperscript{10-12} (see also, [www.rockefeller.edu/labheads/tuschl/siran.html](http://www.rockefeller.edu/labheads/tuschl/siran.html) for protocols). We specifically chose regions of the GABARAP mRNA sequence that are completely conserved from mouse-to-human to ensure that the target gene in CHO-K1 cells could be silenced. The hybrid oligonucleotide complexes include siGABARAP-1, siGABARAP-2, siGABARAP-3 and scrambled negative control siGABARAPC-1 of (sense strand) sequences 5’-

\begin{align*}
    &\text{CAAUUGUCAUCCACCCACCACtt-3’}, \\
    &\text{5’-UGUCAUCCACCCACGtt-3’}, \\
    &\text{5’-CACCAUGAAGAAGACUUCUtt-3’} \text{ and } \\
    &\text{5’-CAACAUUGUCCACCCGtt-3’},
\end{align*}

respectively.

The siRNA oligonucleotides were custom synthesized, PAGE-purified, annealed and provided as lyophilized powders by Ambion. Silencer Negative Control #1 siRNA (Ambion), also included as a negative control, is a generic 19 bp sequence with 3’-dT overhangs and no significant homology to known gene sequences in mouse, rat and human. It is reported to have no effects upon expression of a number of tested genes. \textit{pECFP/GABARAP, pDsRed2/Nuc} stably-transfected cells or \textit{pATjR/EYFP, pDsRed2/Nuc} stably-transfected cells were transiently transfected with siRNA complexes (final concentration 0.2 \textmu M) using Oligofectamine (Invitrogen) according to vendor recommendations. Cells were evaluated at 48 h post-transfection by deconvolution microscopy.

**Statistics and Correlation Coefficients**
Pearson correlation coefficients were determined using Slidebook 4.2 software. Images were deconvolved, segment masks applied for yellow, cyan and red fluorescence, intensities and cross channel statistical analyses performed, and correlation coefficients calculated.

Groups were compared using a one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post-hoc test.

**Results**

**Influence of GABARAP over-expression on AT₁R accumulation and plasma membrane presentation**

GABARAP is ubiquitous at the tissue level; see also GenAtlas (www.genatlas.org: Expression/Subcellular Localization profile). However, the function of GABARAP as a trafficking partner has been studied primarily in neural cells and frequently in PC-12 cells. Because PC-12 cells are often used as a model system for neurobiological studies, we have observed and captured the effects of GABARAP on AT₁R trafficking, by deconvolution microscopy, in these cells.

**Bioluminescence Resonance Energy Transfer (BRET²) for GABARAP:AT₁R interactions**

BRET, a quantitative method for assaying protein interactions, uses a bioluminescent luciferase that is genetically fused to one candidate protein, and a green fluorescent protein mutant fused to another protein of interest. Interactions between the two fusion proteins can bring the luciferase and green fluorescent protein close enough for resonance energy transfer to occur, thus shifting the wavelength of the emission. See Results, in manuscript, for experimental data.
References


Supplemental Figure

Figure 1 supplement. GABARAP(X) clones recovered from Y2H screen. GABARAP and GABARAPL1 are shown aligned. Boxed area indicates the protein region encoded by the largest recovered Y2H clone. Note that none of the rescued clones encodes the tubulin-binding amino-terminus (1-27).

Full-length GABARAP(X) clones were not detected in our Y2H assay, presumably due to their binding to tubulin and hence to microtubules which would obstruct entry of the Gal4-AD-GABARAP(X) into the nucleus. The Y2H assay is designed to identify cytoplasmic targets that are readily shuttled to the nucleus; nuclear manifestation is prerequisite for Y2H selection. Our findings are also similar to those published by Chen; the K-opioid receptor C-terminus effectively captured only GABARAPL1 clones lacking the microtubule-binding domain-encoding region.