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C-Terminal Processing of GABARAP is Not Required for Trafficking of the Angiotensin II Type 1A Receptor

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Abstract

Objectives—GABARAP, a small (117 aa) trafficking protein, binds to the C-terminal, cytoplasmic domain of rat angiotensin type-1A receptor (AT_1R), the predominant effector of the octapeptide angiotensin II (Ang II) (Cook *et al*., Circ. Res. 2008;102:1539-47). The objectives of this study were to map the interaction domains of GABARAP and AT_1R, to determine the effect of GABARAP association on AT_1R signaling activity, and to determine the importance of post-translational processing of GABARAP on accumulation of AT_1R on the plasma membrane and its signaling function.

Results—Deletion analysis identified two regions within GABARAP necessary for interaction with AT1R in yeast two-hybrid assays: 1) a domain comprised of residues 32-51 that is nearly identical to that involved in binding and intracellular trafficking of the $GABA_A$ receptor and 2) a domain encompassing the C-terminal 21 aa. The GABARAP interaction domain of AT_1R was delimited to the 15 aa immediately downstream of the last membrane spanning region. Overexpression of GABARAP in rat adrenal pheochromocytoma PC-12 cells increased the cell surface expression of AT_1R and Ang II-dependent activation of the cAMP signaling pathway. Residues within AT_1R necessary for these responses were identified by mutational analysis. In PC-12 cells, GABARAP was constitutively and quantitatively cleaved at the C-terminus peptide bond and this cleavage was prevented by mutation of Gly^{116}. Wild-type GABARAP and the G116A mutant were, however, equally effective in stimulating AT_1R surface expression and signaling activity.

Conclusions—GABARAP and AT_1R interact through discrete domains and this association regulates the cell-surface accumulation and, consequently, ligand-induced function of the receptor. Unlike that observed with the $GABA_A$ receptor, this regulation is not dependent on C-terminal processing and modification of GABARAP.

Keywords

Protein binding; yeast two-hybrid; GPCR; cAMP signaling pathway; autophagy

Disclosures None

INTRODUCTION

Angiotensin II (Ang II), a physiologically active octapeptide in the renin-angiotensin system (RAS), plays a major role in the development of hypertension and functions as both an endocrine and intracrine factor [1-3]. The cellular and physiological effects of Ang II are mediated primarily through binding to its cognate receptors of which two types have been identified, AT_1R and AT_2R. AT_1R, the most prevalent and best characterized of the Ang II receptors, is a 7-transmembrane, G protein–coupled receptor (GPCR) with a short C-terminal cytoplasmic domain that is important for intracellular trafficking and targeting of the receptor, and also for activation of ligand-induced signaling pathways. These activities are presumably mediated in conjunction with accessory proteins that interact with this region of the receptor. Through yeast two-hybrid (Y2H) screening, we have recently identified one such accessory protein, GABARAP, that binds to the C-terminal domain of the rat AT_1R variant, $AT_{1A}R$ (hereafter referred to as AT_1R), and promotes accumulation of the receptor on the plasma membrane [4].

GABARAP was originally identified as a protein that binds to the γ2 subunit of the pentameric ionotropic $GABA_A$ receptor [5]. GABA, the major inhibitory neurotransmitter in the brain, acts through the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptor. Of these, GABARAP is known to bind only to the $GABA_A$ receptor. Follow-up studies demonstrated that GABARAP promotes trafficking of the $GABA_A$ receptor to the plasma membrane via microtubule tracks and affects both clustering and kinetic properties of the receptor (reviewed in [6]). Subsequent to the identification of γ2 as a binding partner, numerous GABARAP protein ligands have been identified including, among others, N-ethylmaleimide-sensitive factor (NSF), gephyrin, glutamate receptor interacting protein (GRIP), phospolipase C-related, catalytically inactive protein (PRIP) and the transferrin receptor (reviewed in [6]). Furthermore, GABARAP is detected in all tissues and cell lines tested [7-10], implying a broader, more generalized role in cellular protein trafficking well beyond the central nervous system.

GABARAP, a member of the microtubule-associated protein (MAP) family, is comprised of two structural domains: a small N-terminal domain (residues 1-26) that interacts with microtubules and a larger C-terminal domain (residues 27-117) with structural similarities to ubiquitin [11]. Interestingly, GABARAP and other MAP family members undergo a series of post-translational processing and conjugation events similar to ubiquitinylation. An early step in the modification process is cleavage of one or few amino acid residues at the C-terminus, an enzymatic reaction that is dependent on a conserved glycine residue immediately upstream of the cleaved peptide bond (*i.e*., residue 116 of GABARAP). Unlike activated ubiquitin, activated MAP proteins are ultimately attached not to other proteins but rather to the membrane lipid phoshphatidylethanolamine. Presumably, such a modification is an important requirement in the ability of GABARAP and other MAP proteins to facilitate transport of target proteins to and from different membrane compartments [6]. Indeed, Chen *et al*. [12] have recently reported that C-terminal cleavage and modification of GABARAP is required for optimal presentation of $GABA_A$ receptors on the plasma membrane and, concomitantly, promotion of GABA-evoked currents.

The present study was undertaken to further characterize the interaction between GABARAP and AT_1R. The salient results of this study are as follows: 1) the AT_1R- and $GABA_A$-interaction domains of GABARAP are nearly identical; 2) GABARAP enhances Ang II- and AT_1R-dependent activation of the cAMP signal transduction pathway in adrenal pheochromocytoma cells; 3) this enhancement is dependent on a short sequence motif, $F(X)_6LL$, previously described as an endoplasmic reticulum (ER) export signal in AT_1R and other GPCR proteins [13]; and 4) unlike the case for the $GABA_A$ receptor, stimulation of AT_1R accumulation on

the plasma membrane and activation of the cAMP pathway do not require cleavage and modification of GABARAP.

MATERIALS AND METHODS

Materials

Tissue culture media were from Invitrogen, Inc. and fetal bovine serum was obtained from Mediatech. Restriction endonucleases and other DNA modifying enzymes were purchased from either Life Technologies, Inc. or New England BioLabs. Oligonucleotides were synthesized by IDT, Inc. Reagents for luciferase assays were purchased from Sigma Chemical Co. Ang II peptide was purchased from Sigma-Aldrich and other peptides were synthesized by GenScript. Media and reagents for culturing yeast were obtained from Clontech, Inc. The following antibodies (source, catalog #, and dilution) were used in this study: Flag M2 mouse monoclonal (Sigma-Aldrich, F-3165, 1:2000); HA.11 mouse monoclonal clone 16B12 (Covance, MMS-101P, 1:5000); c-Myc mouse monoclonal clone 9B11 (Cell Signaling, #2362, 1:2000); anti-Gal4 DBD mouse monoclonal RK5C1 (Santa Cruz, sc-510, 1:2000); anti-Gal4 AD mouse monoclonal (Clontech, 5398-1, 1:2000); rabbit anti-GFP polyclonal (Santa Cruz Biotech, sc-8334, 1:5000); goat anti-GST polyclonal (Amersham, 27-4577-01, 1:10,000); All other chemicals were reagent grade.

Plasmids

Yeast expression plasmids—Construction of plasmid pAT_1R_{CT}/GBKT7, a fusion between the C-terminal portion of the rat AT_1R (aa 306-359) and the Gal4 DNA-binding domain, has been described previously [4]. C-terminal deletion mutants of pAT_1R_{CT}/GBKT7 were cloned in an analogous manner using PCR amplification products (306-349, 306-339) or double-stranded oligonucleotides (306-329, 306-325, 306-325[F309A/L316A/L317A], 306-320). Plasmid pGABARAP/GADT7 expresses a fusion between the mouse GABARAP protein (aa 32-117) and the yeast Gal4 activation domain (AD) and was isolated in the yeast two-hybrid screening with pAT_1R_{CT}/GBKT7 [4]. N-terminal (42-117, 52-117) and C-terminal (32-107, 32-96) deletion mutants of GABARAP fused to the Gal4 AD were generated by cloning the appropriate PCR amplification products into plasmid pGADT7 (Clontech, Inc.).

Fluorescent protein expression plasmids—Plasmids pEYFP-N1, pECFP-C1, and pECFP-N1 were obtained from Clontech. Construction of pAT_1R/EYFP [14] containing the full-length rat AT_1R fused upstream of EYFP, and pECFP/GABARAP [4] containing the full-length mouse GABARAP fused downstream of ECFP, has been described previously. The plasmid encoding the $F(X)_6LL$ triple mutant of AT1R [F309A/L316A/L317A] fused to EYFP, pAT_1R/3M/EYFP, was constructed by cloning the appropriate overlap PCR mutagenesis fragment into pEYFPN1. The mouse GABARAP coding sequence was PCR amplified and cloned into pECFP-N1 to generate plasmid pGABARAP/ECFP with GABARAP fused upstream of ECFP. The G116A mutants of pECFP/GABARAP and pGABARAP/ECFP were cloned in an analogous manner after amplification of the mutant GABARAP fragments by overlap PCR. The full-length mouse Cox1 coding sequence was amplified by PCR and cloned into pECFP-N1 from to generate plasmid pCox1/ECFP with Cox1fused upstream of ECFP.

Other plasmids—The mammalian expression plasmid, pCMV/myc/AT_1R, contains the full-length rat AT_1R tagged at the N-terminus with the c-Myc epitope and its construction has been described [4]. Site-directed mutants of pCMV/myc/AT_1R were cloned in an analogous manner using mutant fragments generated by overlap PCR. Construction of pCMV/HA/GABARAP, expressing the full-length mouse GABARAP protein tagged at the N-terminus with the HA epitope, has been described [4]. Plasmids pCMV/HA/GABARAP (G116A) and pCMV/HA/GABARAP (ΔN31) were cloned in an analogous manner using insert fragments generated by

overlap or standard PCR reactions, respectively. Plasmid pCRE-luc in which expression of the firefly luciferase gene is under the control of the cAMP response element was obtained from Stratagene, Inc. Plasmid pCMV/β-gal, encoding the *E. coli* β-galactosidase gene, has been described [15]. The integrity of all clones generated using PCR amplification products was confirmed by DNA sequence analyses.

Yeast methodologies

Yeast strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 ::* $GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3, MEL1* $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2, URA3::*$MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*) was obtained from Clontech, Inc., and two-hybrid analysis and preparation of whole cell extracts were carried out according to the manufacturer's recommendations. Yeast growth efficiencies in liquid or solid selective media were measured and calculated according to published protocols [16,17]. Briefly, plasmids encoding a fusion between the test sequences and the Gal4 activation domain (AD) or the Gal4 DNA-binding domain (DBD) were co-transformed into AH109 cells and equal amount of cells were cultured on the following media: 1) media lacking the amino acids leucine and tryptophan to select for the presence of both plasmids; 2) media lacking leucine, tryptophan, and histidine to detect weak protein interaction; or 3) media lacking leucine, tryptophan, histidine, and adenine to detect strong interaction. Cells were cultured for 24-96 hours and quantified by counting colonies (plating efficiency on agar plates) or measuring absorbance at 600 nm (growth efficiency in liquid culture). Efficiencies were calculated as the ratio of growth in "interaction" media/growth in "plasmid selection" media.

Mammalian cell culture, transfection, and analysis

Rat adrenal pheochromocytoma PC-12 (ATCC CRL 1721) cells were cultured in a humidified atmosphere (95% air, 5% CO_2) at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS) and 50 ng/ml gentamicin. Transient transfection was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendation. For immunoblot analysis, cells were seeded (1×10^6/ 60 mm plate) and transfected 24 h later with 5 μg of the expression plasmid DNA. The transfection media was removed 6 h later and the cells were cultured for an additional 42 h in standard growth media. Harvesting of cells, extract preparation and immunoblot analysis were carried out as previously described [4]. For imaging, cells were seeded (1×10^6/plate) on 35 mm MatTek (Ashland MA) glass bottom culture dishes and transfected 24 h later (~70-80% confluence) with a DNA mixture consisting of 1 μg each of plasmids encoding fluorescent or control proteins. 3D deconvolution microscopy was carried out 24-48 h after transfection as previously described [4]. For reporter gene analysis, cells were seeded (1×10^5/well of 12-well plate) and transfected 24 h later with a DNA mixture consisting of (per well): 133 ng of pCRE-luc, 100 ng of pCMV/β-gal, 133 ng pCMV/myc/AT_1R, and 400 ng of pCMV/HA/GABARAP or the corresponding mutants or the empty vector. After 24 h, the media was replaced with fresh media containing 0.5 % FBS and the cells were cultured for an additional 24 h. Cells were treated for 5 h with vehicle or 100 nm Ang II in fresh culture media supplemented with 0.5% FBS and then harvested for enzyme assays. Preparation of cell extract and measurement of luciferase and β-galactosidase activities were carried out as previously described [15].

Statistics and Correlation Coefficients

Comparison of yeast growth efficiencies between wild-type and alanine-scanning mutants of AT_1R were carried out using the Student's unpaired *t*-test. 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

Identification of GABARAP residues necessary for interaction with the C-terminal domain of AT_1R (AT_1R_{CT}) using yeast 2-hybrid assays

The three-dimensional crystal structure indicates that GABARAP is composed of two domains: a small N-terminal domain (residues 1-26) that interacts with microtubules and a larger C-terminal domain (residues 27-117) with structural similarities to ubiquitin [11]. The N-terminal domain is not required for interaction with AT_1R_{CT} since all GABARAP clones isolated in the initial Y2H screen encoded the region from 32-117. A similar result was observed in the Y2H screen using the γ2 subunit of the $GABA_A$ receptor [5]. Deletion analysis in conjunction with Y2H assays identified residues 36-51 of GABARAP as critical for binding to the γ2 subunit [17,18]. A similar analysis was carried out to identify GABARAP residues necessary for interaction with AT_1R_{CT}. For this analysis, AT_1R_{CT} was fused in frame with the DNA-binding domain of Gal4 (Gal4-DBD) whereas GABARAP sequences were fused to the activation domain of Gal4 (Gal4-AD). The corresponding expression plasmids were co-transformed into yeast AH109 cells and equal amounts of cells were plated on solid media lacking leucine and tryptophan (to select for cells harboring both plasmids) or on restrictive media lacking leucine, tryptophan, histidine, and adenine (to test for protein interaction). As evidenced by robust growth on restrictive media, GABARAP(32-117), the original screening isolate, interacted strongly with AT_1R_{CT} (Fig. 1A, right panel). Deletion of the next 10 residues from the N-terminus significantly reduced protein interaction; GABARAP(42-117) exhibited an approximately 9-fold lower affinity for AT_1R_{CT} (Fig. 1B). Finally, deletion of an additional 10 residues [GABARAP(52-117)] completely abolished this interaction. These results demonstrate that essentially the same region of GABARAP is involved in interaction with both the γ2 subunit of the $GABA_A$ receptor and the AT_1R_{CT}.

To determine if other regions of GABARAP can affect association with AT_1R_{CT}, a similar analysis was carried out using C-terminal deletion mutants (Fig. 2). Deletion of the 10 terminal residues [GABARAP(32-107)] resulted in reduced cell growth under restrictive conditions (Fig. 2A, panels 3 & 4) although this reduction was not observed under less stringent conditions (*i.e.*, in the absence of leucine, tryptophan and histidine; Fig. 2A, panel 2). Quantitative Y2H analysis indicated that the (32-107) mutant interacted with AT_1R_{CT} at approximately 55% efficacy compared to the parent construct (32-117) (Fig. 2B). Deletion of an additional 11 amino acid residues (32-96) completely abolished interaction. The different Gal4-GABARAP fusion proteins were expressed at similar steady-state levels (Figs. 1B [inset] and 2C) precluding this as an explanation for the differences in the levels of cell growth/protein interaction. While this analysis implicates specific residues or domains of GABARAP as necessary for interaction with AT_1R_{CT}, we cannot preclude the possibility that the deletions alter the globular structure of GABARP and that this conformational alternation is the proximate cause for the lack of interaction.

Identification of AT_1R_{CT} residues necessary for interaction with GABARAP using yeast 2-hybrid assays

Studies complementary to those described above were carried out to identify AT_1R_{CT} residues necessary for interaction with GABARAP. Systematic C-terminal deletion identified the minimum GABARAP-interacting domain of AT_1R_{CT} as the membrane-proximate region comprised of residues 306-320 [GKKFKKYFLQLLKYI] (Fig. 3).

GABARAP stimulates AT_1R-dependent, Ang II-mediated activation of the camp pathway

In order to better understand the importance of GABARAP-AT_1R interaction, we examined the effect of GABARAP on the signal transduction function of AT_1R. Ang II, upon binding to AT_1R, activates multiple signaling cascades, including the cAMP pathway as we have previously documented [14,19]. Rat adrenal pheochromocytoma PC-12 cells were used in these experiments because they contain undetectable levels of immuno-reactive GABARAP (data not shown) and because they exhibit good expression and constitutive C-terminal processing of exogenous GABARAP [12]. PC12 cells were transfected with an AT_1R expression plasmid and a plasmid encoding the luciferase reporter gene under the control of the cAMP response element (pCRE-Luc). Treatment of these cells with 100 nM Ang II for 5 hours increased luciferase activity by an average of 26.6-fold (Fig. 4). No induction was observed in cells lacking AT_1R (data not shown). Co-expression of GABARAP had no significant effect on luciferase activity in the absence of Ang II treatment (Fig. 4A, WT) but enhanced Ang II-dependent activity by 2.4-fold (Fig. 4B, WT). These results are consistent with our recent observations that GABARAP increases the cell surface accumulation of AT_1R as judged by [^{125}I]-Ang II binding [4].

Duvernay *et al*. [13] have previously described a sequence motif, $F(X)_6LL$ (where X is any residue and L is either leucine or isoleucine), that is conserved in the cytoplasmic tails of multiple GPCRs. Furthermore, the corresponding elements in the α-2B-adrenergic receptor and AT_1R are necessary for transport of these proteins from the ER to the cell surface. Because this sequence element (residues 309-317) is contained within the region of AT_1R required for GABARAP binding, we speculated that the effect of GABARAP on Ang II-mediated cAMP signaling may be mediated through this motif. This idea was evaluated by testing the signaling activity of several AT_1R mutants. As shown in Fig. 4B, compared to the wild-type receptor, the F309A mutant was only minimally responsive to GABARAP (1.3- vs. 2.4-fold stimulation) and the L316A and L317A mutants were completely unresponsive. In the absence of GABARAP, the single mutants exhibited Ang II-dependent signaling activity comparable to wild-type AT_1R, but the activity of the triple mutant was significantly diminished (9.9- vs. 26.6-fold stimulation). No statistically significant differences in Ang II-independent luciferase activities were observed for any of the AT_1R proteins in the absence or presence of GABARAP (Fig 4A) and the AT_1R mutants were expressed at levels similar to or greater than wild-type AT_1R as judged by immunoblotting of total cellular extracts (data not shown). Our working model is that GABARAP directly interacts with AT_1R to promote trafficking of the receptor to the cell surface; increased AII-mediated signaling activity would then be a consequence of a greater abundance of the receptor on the plasma membrane [4]. This hypothesis, in conjunction with the results provided above, would suggest that GABARAP does not bind (or binds with reduced affinity) to the $F(X)_6LL$ mutants of AT_1R and thus does not promote plasma membrane accumulation of the mutant receptors. This idea is corroborated with the lack of interaction between GABARAP and the $F(X)_6LL$ triple mutant in yeast two-hybrid assays (Fig. 4C).

C-terminal cleavage of GABARAP in PC-12 cells

Chen *et al*. [12] have reported that C-terminal cleavage and modification of GABARAP is required for stimulation of plasma membrane localization of the $GABA_A$ receptor. We, therefore, investigated whether this processing is also required for GABARAP-mediated trafficking of AT_1R. To determine the extent of GABARAP cleavage in PC12 cells, GABARAP and the G116A mutant were fused to the Flag or c-*myc* epitope tags at the N- and C-terminus, respectively (Fig. 5A). Additionally, ECFP was fused in-frame to GABARAP and GABARAP(G116A), either at the N-terminus (ECFP-GABARAP) or at the C-terminus (GABARAP-ECFP) (Fig. 5B). These expression plasmids were transfected into PC12 cells and the quantity and molecular mass of GABARAP fusion proteins were assayed by

immunoblotting of whole cell extracts using antibodies directed against the epitope tags. When using the C-terminal tag antibody, anti-c-*myc*, no wild-type GABARAP was observed whereas the G116A mutant was readily detected at the expected molecular mass (Fig. 5A). These results are consistent with cleavage of the C-terminus, a process dependent on a glycine residue at position 116. As expected, the N-terminal tag antibody, anti-Flag, detected both the wild-type and mutant GABARAP species, and the latter exhibited a slightly faster migration rate consistent with cleavage of the C-terminus. Similar results were obtained when the ECFP protein was used as a tag. When placed at the C-terminus of GABARAP, a cleavage product consistent with the molecular mass of ECFP was readily detected (Fig. 5B, compare lanes 1 and 6). This cleavage did not occur with the G116A fusion (Lane 2). As expected, cleavage could not be discerned when the ECFP moiety was placed upstream of GABARAP (Lanes 3 & 4). A Cox1-ECFP fusion was used as a negative control and did not exhibit the type of cleavage observed with GABARAP-ECFP. Collectively, these results indicate that GABARAP is quantitatively cleaved at the C-terminus in PC-12 cells, that this cleavage requires a glycine residue at position 116, and that the nature and size of the sequences downstream of this residue are not critical for cleavage.

C-terminal cleavage is not required for GABARAP-mediated cell surface localization of AT_1R

We have recently shown GABARAP enhances cell surface localization of AT_1R [4]. To determine if cleavage and modification of GABARAP are necessary for this response, PC-12 cells were transfected with an expression plasmid encoding an AT_1R-EYFP chimera in the absence or presence of plasmids expressing either ECFP-GABARAP or ECFP-GABARAP (G116A). Cells were evaluated at timed intervals after transfection for cell surface accumulation of AT_1R-EYFP by 3D deconvolution microscopy. In the absence of GABARAP proteins, AT_1R-EYFP was observed at low levels on the plasma membrane and the predominant fraction was found in the secretory pathway (*i.e.*, ER, Golgi, vesicles) at all times tested (Fig. 6A & F). In comparison, cells expressing both AT_1R-EYFP and ECFP-GABARAP exhibited a 6-fold greater surface accumulation of AT_1R-EYFP at 24 hours posttransfection (Fig. 6C & F). Interestingly, a similar level of stimulation was observed with co-expression of ECFP-GABARAP(G116A) (Fig. 6E & F), suggesting that cleavage and modification of GABARAP is not essential for trafficking AT_1R to the plasma membrane. No significant differences between GABARAP and GABARAP(G116A) were observed at any of the time points examined.

In the absence of GABARAP, steady-state cellular distribution of the $F(X)_6LL$ triple mutant, AT_1R/3M-EYFP, was similar to that of wild-type AT_1R-EYFP (compare Figs. 6A & B). Unlike that observed for AT_1R-EYFP, GABARAP did not promote cell-surface accumulation of the mutant protein (Fig. 6D & F). This observation is consistent with the inability of GABARAP to interact with the $F(X)_6LL$ mutant in yeast two-hybrid assays (Fig. 4C) or stimulate its signaling activity in PC12 cells (Fig. 4B).

C-terminal cleavage of GABARAP is not required for enhancement of the signaling function of AT_1R

Our hypothesis is that the GABARAP-dependent enhancement of CRE signaling described above is a manifestation of increased cell-surface expression of AT_1R. If this is the case, then we would predict that cleavage and modification of GABARAP also is not required for AT_1R-dependent CRE activation. Consistent with this prediction, no significant differences were observed between the effects of wild-type GABARAP and the GABARAP(G116A) mutant in stimulation of CRE-dependent luciferase activity (Fig. 7). By comparison, a mutant lacking microtubule-binding activity, GABARAP(32-117), but still retaining the ability to bind

to AT_1R, was ineffective in this response, highlighting the importance of this domain in the trafficking function of GABARAP.

DISCUSSION

In a yeast two-hybrid screen, we recently identified GABARAP as an AT_1R-binding protein. Multiple *in vitro* and *in vivo* analyses confirmed this interaction in mammalian cells and also demonstrated that GABARAP promotes trafficking of AT_1R to the plasma membrane. We hypothesize that GABARAP may be an important determinant of the cellular and physiological effects of the renin-angiotensin system, in particular of Ang II-dependent regulation of blood pressure and cell growth [4]. In this report, we have extended our understanding of the interplay between GABARAP and AT_1R by identifying sequences necessary for protein interaction, assessing the importance of these sequences in AT_1R signaling activity, and demonstrating that C-terminal processing of GABARAP is not required for trafficking of AT_1R to the plasma membrane and manifestation of ligand-induced signaling function.

Two sequence motifs of GABARAP, both within the ubiquitin-like domain, are necessary for interaction with AT_1R as judged by Y2H analyses. One region is delimited by amino acid residues 32-51, whereas the second domain is defined by the C-terminal 21 amino acids. The internal sequence, interestingly, encompasses the site (aa 36-51) necessary for binding to γ2 subunit of the $GABA_A$ receptor [17]. The latter was identified by a similar approach and the results closely parallel that observed here with AT_1R - namely that GABARAP(41-117) exhibits partial binding activity towards γ2, and GABARAP(52-117) is completely inactive. More recently, GABARAP was identified as a binding partner of another membrane protein, the Na^+-dependent P_i-cotransporter, NaPi-Ha, and the interaction domain was de-limited to the region between amino acids 37 and 68 [20]. Furthermore, based on results from phage display experiments, Willbold and colleagues have identified several proteins including calreticulin [21], clathrin heavy chain [22], and Nix [23] as protein ligands of GABARAP. The minimum GABARAP-binding sequences within these proteins have similar, although not identical, primary structures and peptides corresponding to these sequences apparently bind to the same area on the GABARAP surface as determined by NMR spectroscopy. The residues forming this binding surface are found in several clusters located throughout the GABARAP protein. One prominent cluster is located between residues 44 and 56 and another includes amino acids 101-104. The requirement of certain residues within these patches for protein binding is consistent with the results described herein – namely that either N-terminal deletion to residue 52, or C- terminal deletion to residue 96, abolishes interaction between GABARAP and AT_1R_{CT}. Based on these findings, it is tempting to speculate that AT_1R binds to GABARAP via the same interface described for calreticulin, clathrin heavy chain, and Nix. However, as pointed out by others [24-26], given the compact three-dimensional structure of GABARAP, deletion mutants may not fully replicate the stable globular fold of the native protein. Therefore, fine–mapping of the AT_1R:GABARAP interface will require the use of additional strategies including an analysis of site-directed mutants.

The C-terminal cytoplasmic tail of AT_1R is immediately downstream of the 7^{th} transmembrane segment and spans the sequence from aa 306-359. The GABARAP-binding domain was de-limited to the membrane proximal region between residues 306-320, the sequence of which is completely conserved among all known mammalian $AT_{1A}Rs$ with the exception of a conservative substitution of an arginine residue for lysine at position 311 in the human receptor. Short discrete regions have been identified as targets of GABARAP and related proteins in several binding partners, but a consensus recognition sequence based on all these sites is not readily obvious. Thielmann *et al.* [27] have suggested that a tryptophan residue is a critical determinant of the ligand specificity of GABARAP. Indeed, the GABARAP-binding sites within calreticulin, clathrin heavy chain, and Nix, all contain a tryptophan residue that is critical

for protein-protein interaction [21-23]. A single tryptophan residue is also present in the binding site of γ2 and the putative interaction domain of NaPi-Ha. However, deletion of a region of NaPi-Ha containing this residue does not prevent binding of GABARAP [20]. Furthermore, the cytoplasmic tail of AT_1R does not contain a single tryptophan residue nor does the C-terminal tail of the human κ opioid receptor, which also interacts with GABARAP and the close family member, GABARAPL1 [28]. It is conceivable that for some binding sites, other aromatic residues such as phenylalanine or tyrosine may functionally substitute for tryptophan. Consistent with this supposition, the region between residues 306 to 320 of AT_1R_{CT} contains four such amino acids, one of which (Phe^{309}) is at least partly necessary for AT_1R_{CT}-GABARAP interaction and GABARAP-dependent AT_1R cell surface accumulation and signaling activity.

In addition to Phe^{309}, two other residues within AT_1R_{CT}, Leu^{316} and Leu^{317}, were identified as critical for binding to GABARAP and for GABARAP-mediated stimulation of receptor distribution and function. These residues were specifically tested because of the previous identification of a sequence motif, $F(X)_6LL$, that is necessary for exit of AT_1R and the α_{2B}-adrenergic receptor from the endoplasmic reticulum and eventual transport to the plasma membrane [13]. Interestingly, this motif is conserved in the C-terminii of multiple GPCRs, implying a common molecular mechanism for ER export. Rab1 GTPase has been identified as a rate-limiting factor for the $F(X)_6LL$-dependent transport of AT_1R from the ER through the Golgi to the cell surface, although this dependency appears to be manifested after exit of the receptor from the ER [29,30]. The results provided here strongly suggest that GABARAP is another critical modulator of this pathway.

In addition to, or in conjunction with, its role in intracellular trafficking of cell surface proteins, GABARAP is also a substrate for an ubiquitin-like conjugation system that is a molecular hallmark of autophagy, the bulk degradation of proteins and organelles by the lysosomal/vacuolar system (reviewed in [31]). In yeast, the Apg8 conjugation system is activated when the Apg8 modifier protein is cleaved at its C-terminal arginine residue by the cysteine protease Apg4, exposing a conserved glycine residue. Apg8 is subsequently activated by Apg7 and transferred to the Apg3 protein, the E1- and E2-like counterparts, respectively, of the ubiquitinylation pathway. In the final step, Apg8 is conjugated to membrane phosphoethanolamine by an E3-like activity that has not been fully characterized but may reside in a product of the second (Apg12) conjugation system [32]. Autophagy is a highly conserved process in eukaryotic cells and most of the mammalian components of the Apg8 and Apg12 conjugation systems have been identified. Microtubule-associated protein 1 light chain 3 (LC3) is the most prominent Apg8 homolog in mammals but GABARAP and GABARAP-like protein 2 are also authentic modifiers of the Apg8 conjugation system.

As shown here, in PC-12 cells, exogenous GABARAP is constitutively and quantitatively cleaved at the C-terminal peptide bond, consistent with the observation that this processing occurs shortly after translation [33]. Also, as expected, mutation of Gly^{116} effectively prevents cleavage of the protein. When overexpressed in PC-12 cells, GABARAP and GABARAP (G116A) were equally effective in promoting plasma membrane accumulation of AT_1R and subsequent signaling activity. These results are in marked contrast to those of Chen *et al.* [12] who used a similar approach to investigate the linkage between the trafficking and modification activities of GABARAP. In hippocampal neurons, exogenous GABARAP was efficiently and constitutively cleaved at the C-terminus and this cleavage was prevented by mutation of Gly^{116} to alanine. Overexpression of GABARAP, but not of GABARAP(G116A), increased cell surface expression of the γ2 subunit. Furthermore, GABARAP but not the G116A mutant, stimulated GABA-induced currents in *X. laevis* oocytes expressing $GABA_A$ receptor subunits.

The reason for the divergent effects of GABARAP(G116A) on AT_1R and γ2 trafficking is not known. It is instructive to point out that phospholipid-conjugated GABARAP (GABARAP-PL), which would be identified as a faster migrating band (by ~ 2-3 kDa) on SDS-PAGE [7], was not detected under our experimental conditions. Similarly, Tanida *et al.* [7] did not observe endogenous GABARAP-PL in mouse brain, liver or kidney, even after starvation-induced autophagy. In contrast, the conjugated form of the LC3 was readily observed in all tissues tested. Furthermore, very little to no endogenous GABARAP-PL was detected in several human and mouse cell lines under basal condition. The absence or low abundance of GABARAP-PL in these cells may reflect weak conjugating activity towards GABARAP, an overly active de-conjugating activity, and/or rapid turnover of the conjugated protein. Regardless of the mechanisms involved, these results suggest that, under steady-state conditions, a large fraction of the endogenous GABARAP in many cell types exists in the C-terminally cleaved but unmodified form. Therefore, it is conceivable that both lipid-conjugated and unconjugated GABARAP participate in protein trafficking, possibly at different stages of the process, in association with different protein complexes or membrane compartments, and/or in a target protein-specific manner. While conjugation to phospholipids is expected to support GABARAP's vesicular transport activities, it is noteworthy that even unconjugated GABARAP associates with membranes compartments [33]. If lipid conjugation of GABARAP is not necessary for transport of AT_1R, then it is not unreasonable to expect GABARAP (G116A) to facilitate this trafficking as effectively as the C-terminal processed, wild-type GABARAP, considering that these proteins differ by only two residues.

In summary, we have localized the GABARAP binding domain of AT_1R to a short region within the cytoplasmic tail just downstream of the last membrane spanning region and identified the $F(X)_6LL$ ER export motif within this domain as a GABARAP-responsive sequence. The juxtaposition of the binding domain next to the cellular membrane would appear to be an ideal location for GABARAP - a protein with a propensity to covalently attach to membrane lipids – in carrying out its vesicular trafficking function. Nevertheless, our findings indicate that such covalent modification is not essential for GABARAP to promote transport of AT_1R to the cell surface. We cannot, however, preclude the possibility of GABARAP associating with the membrane by other mechanisms during this transport process.

Acknowledgments

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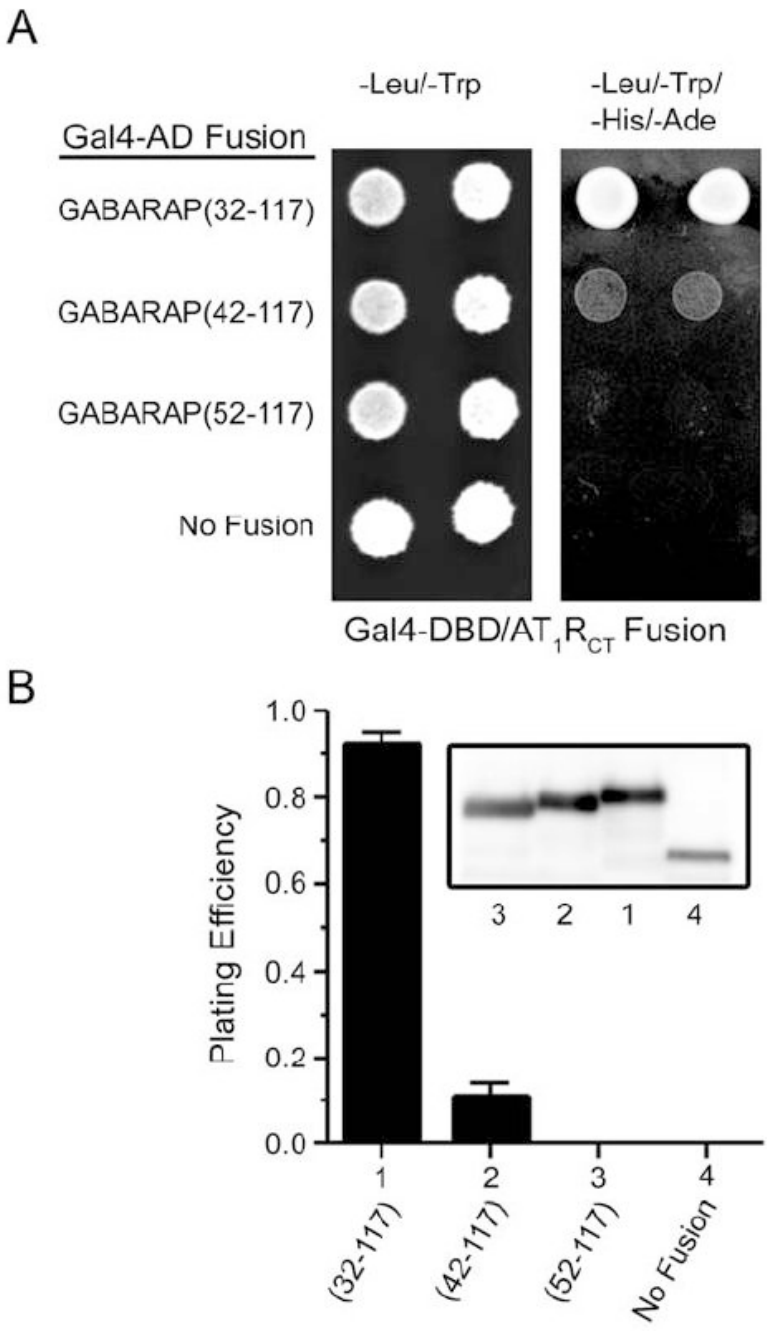

Fig. 1. Effect of N-terminal deletion of GABARAP sequences on interaction with AT_1R_{CT}

Plasmids expressing Gal4-DBD/AT_1R_{CT} and Gal4-AD/GABARAP(32-117) or corresponding N-terminal deletion mutants were co-transformed into yeast AH109 cells. Protein-protein interaction was assessed by cell spotting (A) or plating efficiency (B) as described in Materials and Methods. Relative protein levels were measured by immunoblotting of total yeast protein extracts (B inset).

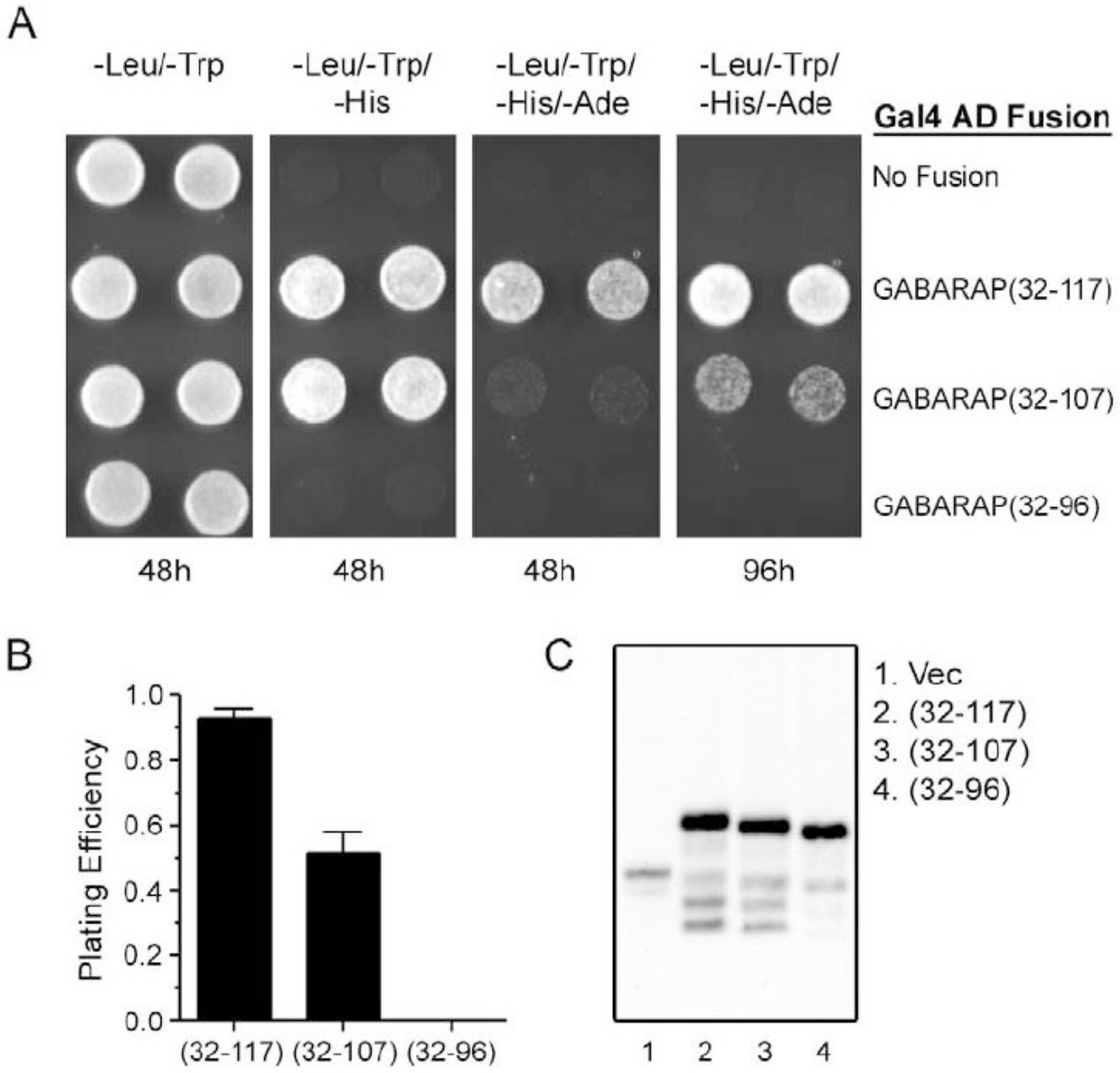

Fig. 2. Effect of C-terminal deletion of GABARAP sequences on interaction with AT_1R_{CT}

Plasmids expressing Gal4-DBD/AT_1R_{CT} and Gal4-AD/GABARAP(32-117) or corresponding C-terminal deletion mutants were co-transformed into yeast cells and interaction was assessed by yeast spotting (A) or yeast plating efficiency (B). Relative protein levels were measured by immunoblotting of total yeast protein extracts (C).

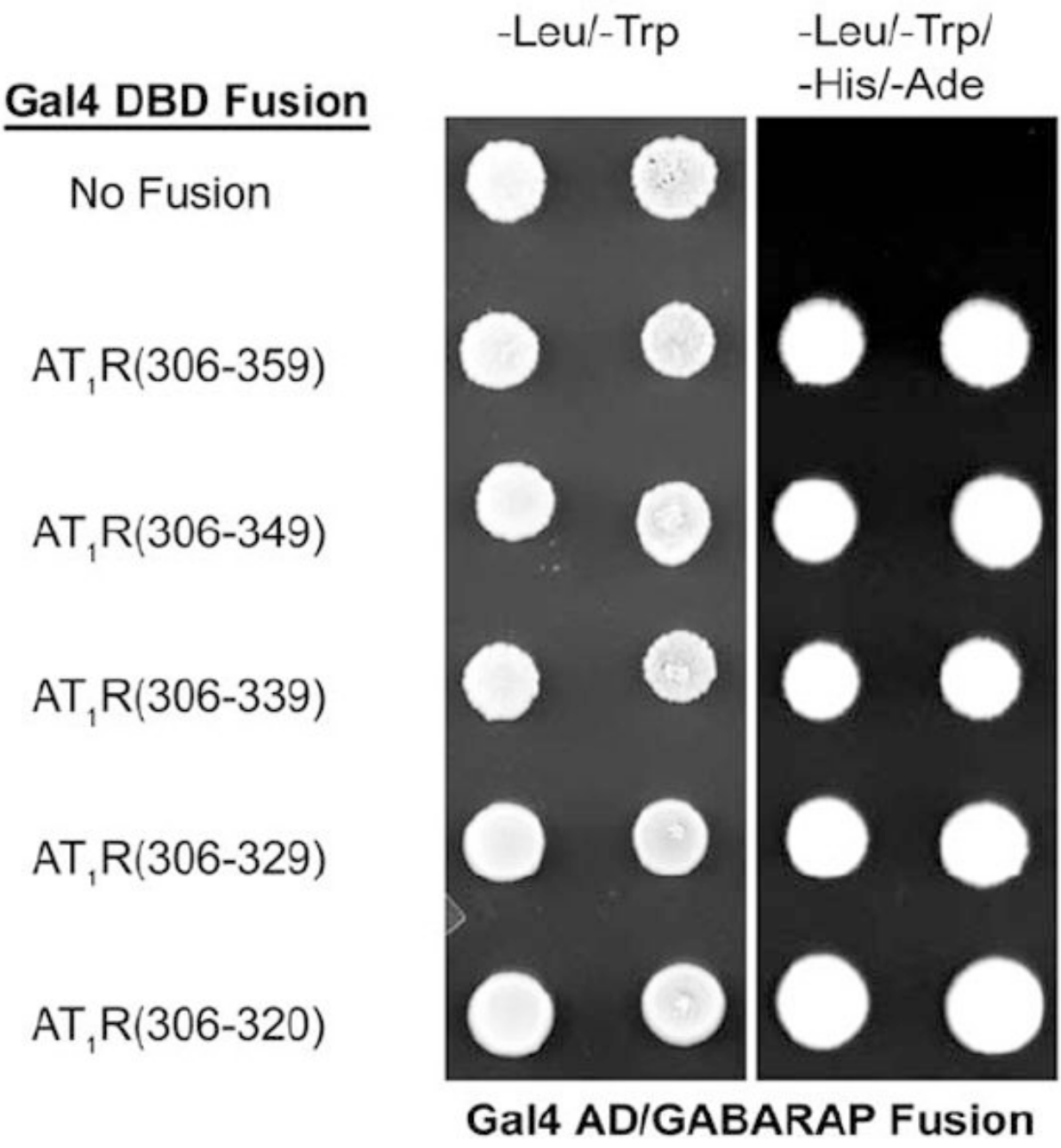

Fig. 3. The membrane-proximate region of AT_1R_{CT}, residues 306-320, is sufficient for interaction with GABARAP

Plasmids expressing Gal4-AD/GABARAP and Gal4-DBD fusion of AT_1R_{CT} or deletion mutants were co-transformed into yeast and interaction was assessed by yeast two-hybrid assays.

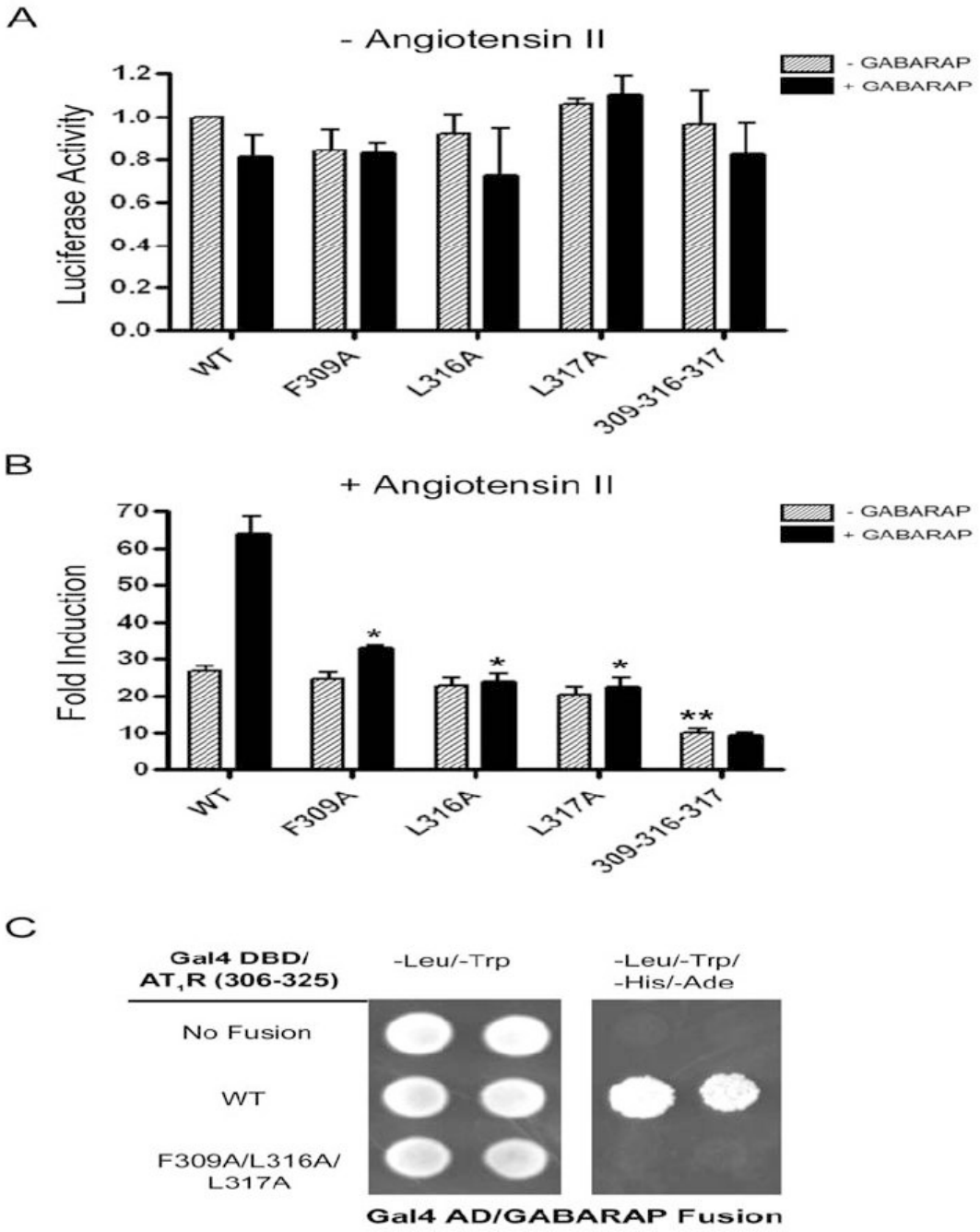

Fig. 4. GABARAP enhances Ang II-mediated activation of the cAMP response pathway in PC12 cells and this enhancement is dependent on the F(x)6LL motif of AT_1R

Transfection, treatment and enzyme assays were carried out as described in Materials and Methods. The AT_1R mutants are indicated and normalized luciferase activities, without (A) and with (B) Ang II treatment, are presented (Avg. ± SE, n = 5; **, $p < 0.01$ vs. wild-type AT_1R in the absence of GABARAP; *, $p < 0.01$ vs. wild-type AT_1R in the presence of GABARAP). Protein-protein interaction was assessed by yeast cell spotting (C) as described in Materials and Methods.

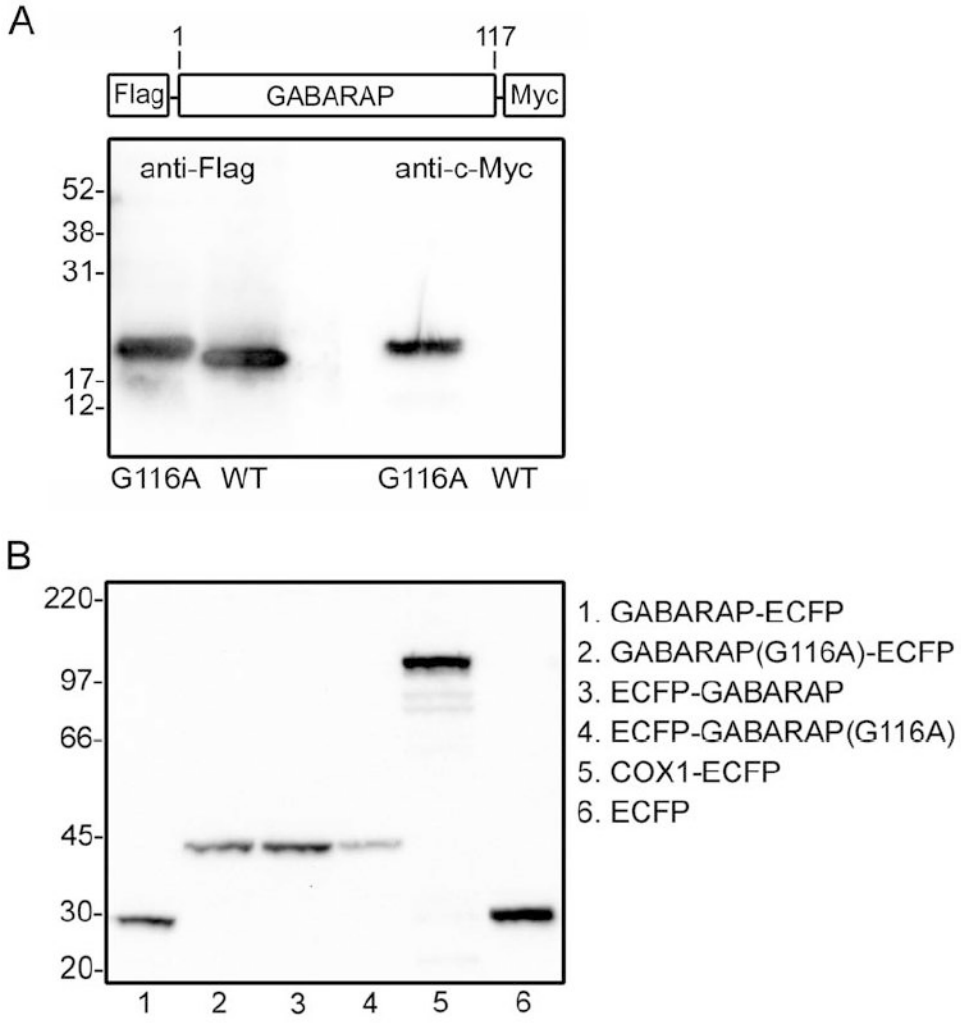

Fig. 5. The C-terminus of GABARAP is constitutively cleaved in PC12 cells

PC12 cells were transfected with plasmids expressing the indicated proteins and harvested 48 h post-transfection. Total cellular proteins were separated by SDS-PAGE, transferred to membranes, and subjected to immunoblotting with anti-Flag or anti-c-Myc antibodies (A) or anti-GFP antibodies (B).

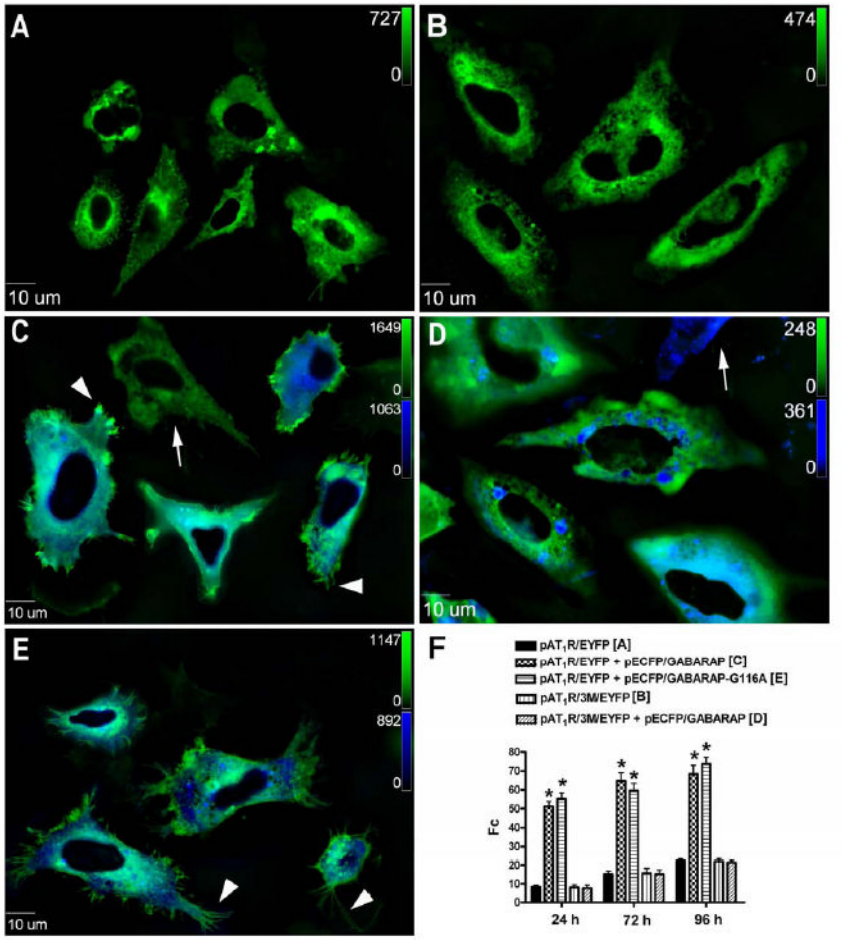

Fig. 6. GABARAP and mutant GABARAP(G116A) similarly promote cell surface accumulation of AT_1R

PC-12 cells were transfected with (A) *$pAT_1R/EYFP$*, (B) *$pAT_1R/3M/EYFP$*, (C) *$pAT_1R/EYFP$ + pECFP/GABARAP*, (D) *$pAT_1R/3M/EYFP$ + pECFP/GABARAP*, or (E) *$pAT_1R/EYFP$ + pECFP/GABARAP(G116A)*, and imaged at 48 h post-transfection using deconvolution microscopy (63X obj). White arrows indicate cells that express only AT_1R-EYFP or ECFP-GABARAP among other double-transfectants. Cell membrane distribution of AT_1R-EYFP was determined by measuring circumferential fluorescence intensity (Fc) using Slidebook 4.2 quantitative imaging. White arrowheads mark plasma membrane extensions and processes with high expression of AT_1R-EYFP. Fc is plotted as a function of time following transfection (F); Avg. ± SE, n = 3, 100 transfected cells per experiment; *, $p < 0.01$ vs. pAT_1R/EYFP at each time point.

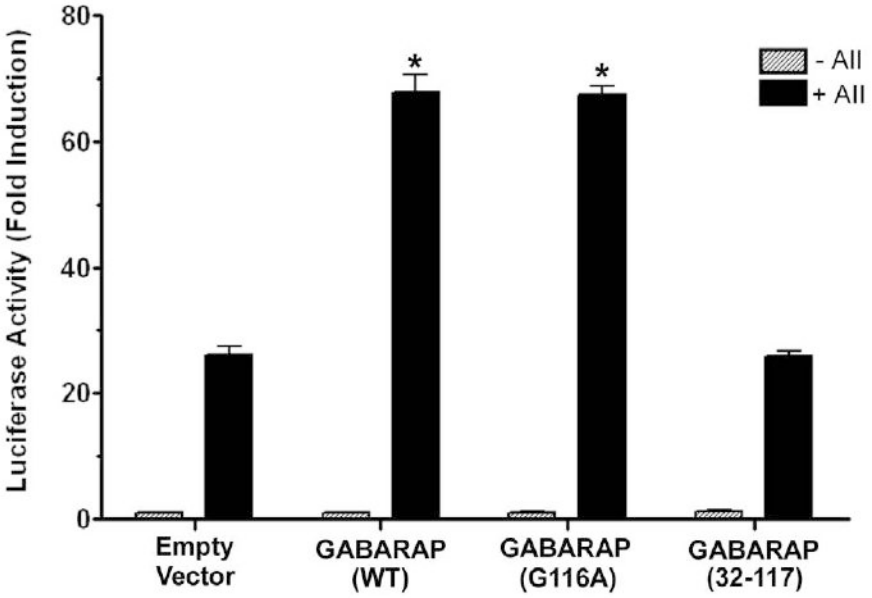

Fig. 7. GABARAP and mutant GABARAP(G116A) similarly enhance angiotensin II-mediated activation of the cAMP response pathway in PC12 cells

Transfection, treatment and enzyme assays were carried out as described in Methods. The GABARAP mutants are indicated and normalized luciferase activities, without (A) and with (B) Ang II treatment, are presented (Avg. ± SE, n = 4; *, $p < 0.01$ vs. activity in the presence of AII and the absence of GABARAP protein).