# Role of C-terminal cytoplasmic domain of the AT2 receptor in ligand binding and signaling

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**Abstract** **A stop codon at position 322 was introduced to generate a truncated, C-terminal-deleted AT2 receptor. Expression studies in *Xenopus* oocytes showed that C-terminal-deleted AT2 had reduced affinity to [$^{125}$I]angiotensin II ($K_d$ = 1.7 nM) and enhanced binding of the AT2-specific peptidic ligand [$^{125}$I]CGP42112A ($K_d$ = 0.097 nM). AT2 activation by angiotensin II resulted in reduction of cGMP levels in oocytes and this reduction was further enhanced by C-terminal deletion, implying that the C-terminus may have a negative effect on the AT2-mediated cGMP reduction. Moreover, interaction of the AT2 with the ATP-binding domain of the human ErbB3 receptor in yeast two-hybrid assay was abolished by C-terminal deletion. In summary, the C-terminal cytoplasmic tail of AT2 modulates its ligand binding and signaling properties.** 

*Key words:* Angiotensin II; AT2 receptor; C-terminal cytoplasmic tail; cGMP; ErbB3; *Xenopus* oocyte

## 1. Introduction

Angiotensin II (Ang II) has long been recognized as an important component of the neuroendocrine control of cardiovascular function [1–3]. The two receptor subtypes of Ang II, AT1 and AT2, share structural similarity (both are proteins with seven transmembrane topology) and 34% homology at the amino acid level [4–7]. Most of the well-known physiological effects caused by Ang II, including vasoconstriction, water and sodium intake and induction of cell growth, are mediated via the receptor type AT1. The AT1 receptor is known to activate Gi and Gq proteins, Jak/STAT proteins and mitogen-activated protein kinases [4–12]. The C-terminal cytoplasmic tail of the AT1 is implicated in its ability to couple to Gi protein, phospholipase Cγ and Jak/STAT proteins [13–15]. The YIPP motif (amino acids 319–322) in the C-terminal cytoplasmic tail of the AT1 is shown to be essential for its interaction with Jak2. The nuclear localizing signal (NLS) of AT1 (KKFKK, amino acids 307–311) seems to be needed for nuclear translocation of this receptor upon Ang II activation [16,17]. Moreover, the STL motif (amino acids 335–337) is essential for the internalization of the AT1 receptor upon Ang II activation [18]. In summary, a substantial body of literature that sheds light on the structure–function relationship of the C-terminal cytoplasmic tail of the AT1 exists.

The AT2 receptor is a 363 amino acid protein and many of the amino acids that are functionally important for the AT1 receptor are conserved in AT2. Indeed, the roles of conserved residues such as Arg182 in the second extracellular loop, Asp297 in the third extracellular loop, Lys215 in the fifth transmembrane domain, and His273 and Asp279 in the sixth transmembrane domain in ligand binding to AT2 are well-established [19–24]. However, AT1 and AT2 differ in several aspects. While the mechanisms involved in AT1 internalization are well delineated, thus far there is no evidence to suggest that AT2 becomes internalized upon Ang II activation ([25] and references therein). The AT2 receptor is expressed at high levels, but in a transient manner, during fetal development and during the first few weeks after birth [26,27]. Although these observations imply an important role for the AT2 receptor in embryogenesis and in the development of specific neuronal pathways, the exact physiological role of this receptor is currently unclear. Several studies indicate an important role for AT2 in kidney and urinary tract development [28,29]. The adult tissues including kidney, uterus, pancreas, heart and adrenal medulla also have high level expression of this receptor [30,31]. In general, activation of AT2 by Ang II seems to inhibit the physiological effects resulting from the activation of AT1 by Ang II [32,33]. For example, while binding of Ang II to AT1 activates cell growth, AT2 activation by Ang II inhibits cell growth and induces apoptosis in many cell types. AT2 is also shown to directly interact with other membrane-bound proteins including AT1 and the human ErbB3 receptor [34–36]. We have shown previously that a truncated rat AT2 receptor that contained the amino acids 226–363 could interact with the ATP-binding domain of the human ErbB3 receptor in a yeast two-hybrid protein–protein interaction assay [36]. Since this region spanned the third intracellular loop and the C-terminal cytoplasmic tail of AT2, this result implied that the C-terminal cytoplasmic tail of AT2 may be directly involved in this interaction. As mentioned above, while the functional role of the C-terminal cytoplasmic tail of the AT1 receptor is well-established, currently not much information is available regarding the functional role of the C-terminal cytoplasmic tail of AT2. A comparison of the C-terminal cytoplasmic domain of AT1 and AT2 is given in Fig. 1. In an attempt to define the functional role of the C-terminal cytoplasmic tail of AT2, we have generated a truncated AT2 receptor in which a stop codon was introduced at position 322 (Fig. 2). The ligand-binding and signaling prop-

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erties of this truncated AT2 were analyzed by expressing them in *Xenopus* oocytes. The ability of the truncated AT2 to interact with the human ErbB3 receptor was assessed using yeast two-hybrid assay. Our results indicate that the C-terminal cytoplasmic tail is important for both ligand binding and signaling by the AT2 receptor.

## 2. Materials and methods

### 2.1. Materials

Radiolabeled material for sequencing and binding studies ([$^{35}$S]dATP, [$^{125}$I]Ang II, [$^{125}$I]CGP42112A) and cGMP RIA kit were obtained from NEN Life Science Products (Boston, MA, USA). Oligonucleotides used for sequencing and mutagenesis were purchased from Gibco BRL Life Technologies (Gaithersburg, MD, USA). The other kits that were used in this study were Quick Change site-directed mutagenesis kit (Stratagene Products, La Jolla, CA, USA), DTth DNA polymerase sequencing kit (Clontech, Palo Alto, CA, USA) and Riboprobe Gemini in vitro transcription system (Promega, Madison, WI, USA). Female *Xenopus* were obtained from Nasco (Fort Atkinson, WI, USA).

### 2.2. Strains, plasmids and growth conditions

The growth conditions for *Escherichia coli* strains TG1 and HB101 and *Saccharomyces cerevisiae* strains CG1945, Y190 and HF7C were as described previously [36–38]. Ampicillin was used to a final concentration of 50 μg/ml and kanamycin was used to a final concentration of 25 μg/ml wherever selection was made.

### 2.3. Construction of C-terminal-deleted AT2

The C-terminal-deleted AT2 receptor was generated by using the Quick Change site-directed polymerase chain reaction mutagenesis kit. The oligonucleotide primers used to introduce a stop codon at position 322 of the open reading frame of AT2 were 5′-CCCTTCC-TGTATTGTTTCGTTTGAAACCGC-3′ and 5′-GCGGTTTCAAA-CGAAACAATACAGGAAGGG-3′ and the mutagenesis was carried out according to instructions provided with the kit. The parental DNA template was the pSP64 polyA plasmid carrying the DNA specifying open reading frame (ORF) of the rat AT2 [20]. The pSP64 polyA plasmid carrying the C-terminal-deleted AT2 DNA was designated pBG5001.

### 2.4. In vitro transcription, expression in Xenopus oocytes, ligand-binding studies and cGMP assay

To generate cRNAs corresponding to the wild-type and mutant AT2 receptor genes, in vitro transcription of the pSP64 polyA vectors carrying the wild-type and mutant AT2 receptor genes was carried out using 'Riboprobe Gemini Systems'. Techniques for injection of cRNA were performed as described previously [20]. Oocytes were microinjected with 50 nl of cRNA solution at a concentration of 1 mg/ml and after 2 days were treated with collagenase (0.5 mg/ml) for 18 min [20]. The residual follicular cells were removed manually. It is known that some donor frogs yield oocytes that have Ang II receptors in their membrane [39]. Therefore, for these experiments, we selected oocytes that did not have endogenous Ang II receptors. This was done by screening the oocytes from each donor frog by performing binding experiments with [$^{125}$I]Ang II. During the 4 days before binding experiments, the oocytes were maintained in Barth's medium [39] with continuous renewal of the medium for every 4–6 h. For binding experiments, the oocytes were washed with Barth's medium before incubating in the pre-incubation buffer [20]. Under these experimental conditions, no evidence for endogenous Ang II-like peptides was observed. For cGMP assay, groups of five oocytes were used per sample. The experiment was done in triplicate and was repeated using samples from three different donors. The cells were exposed to 50 nM Ang II in pre-incubation buffer for 5 min. To achieve blocking of Ang II binding with PD123319, a 15 min pre-incubation in the presence of PD123319 was performed before the addition of Ang II. At the end of the treatment with Ang II, the fluid was removed and the cells were subjected to flash-freezing in liquid nitrogen. Cells were stored at −70°C until the radioimmunoassay was performed according to the instructions of the cGMP RIA kit purchased from NEN Life Science Products.

*2.4.1. Yeast two-hybrid assay.* The strategy used to construct a fusion protein in which the yeast GAL4 DNA-binding domain was fused to the C-terminal-deleted AT2, was similar to that described previously [36]. The oligonucleotide primers corresponding to the 5′ region of the rat AT2 ORF (5′-GAAGGACAACTTCAGTTTGC-3′) and the 3′ region of the C-terminal-deleted AT2 (5′-GCGG-TTTCAAACGAAACAATACAGGAAGGG-3′) were used to amplify the nucleotide sequence of the ORF of C-terminal-deleted rat AT2 receptor from plasmid pBG5001 which was cloned in pCR®2.1 initially. This fragment was then subcloned as an *Eco*RI fragment into the *Eco*RI-digested pGBKT7 to generate translational fusion of the C-terminal-deleted AT2 with the GAL4 DNA-binding domain and the sequence at the junction was verified by dideoxynucleotide sequencing. The plasmid pBGKT7 harboring GAL4 BD:C-terminal-deleted AT2 translation fusion was designated pBG5003. Plasmids pBG857 (in which the yeast GAL4 transcription activation domain was fused to the ATP-binding domain of the ErbB3 receptor) and pBG1101 (in which the truncated AT2 ($AT2_{226–363}$) was fused to Gal4 BD) were described previously [36]. The plasmid pBG1157 carried a translation fusion of the complete AT2 receptor with the GAL4 BD of pGBKT7. This was constructed using a strategy similar to that used for the construction of pBG877 [36]. The transformation of the *S. cerevisiae* strains CG1945 or HF7C with the 'bait' vector pBG5003 and prey plasmid pBG857, and analysis of interaction between the C-terminal-deleted AT2 and the ATP-binding domain of the ErbB3 by yeast two-hybrid assay was carried out using methods described previously [36].

## 3. Results and discussion

### 3.1. Ligand-binding properties of the C-terminal-deleted AT2

To determine whether deleting the C-terminal cytoplasmic domain of the rat AT2 receptor influences the ligand-binding properties of AT2, we microinjected equal amounts of cRNA corresponding to wild-type AT2 or C-terminal-deleted AT2 into *Xenopus laevis* oocytes. The ability of these receptors to bind the peptidic ligands [$^{125}$I]Ang II and [$^{125}$I]CGP42112A (AT2-specific ligand) and the non-peptidic AT2-specific ligand PD123319 was evaluated by binding assays. Collagenase treatment followed by manual removal of any residual follicular layer was carried out prior to ligand-binding experiments. Previously we have shown that rat AT2 receptors expressed in *Xenopus* oocytes seem to have retained all the binding properties that are reported for them when they are expressed in mammalian cells [20]. The C-terminal-deleted AT2 retained its ability to bind [$^{125}$I]Ang II, [$^{125}$I]CGP42112A and PD123319 (Fig. 3), but demonstrated differences in its affinity to the peptidic ligands when compared to the affinity of the wild-type AT2 to these ligands. The C-terminal-deleted AT2 showed increased affinity to [$^{125}$I]CGP42112A ($K_d$ = 0.097 nM) and decreased affinity to [$^{125}$I]Ang II ($K_d$ = 1.7 nM) (Fig. 3 and Table 1). These results indicated that the C-terminal cytoplasmic domain of the AT2 plays a role in deter-

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AT1 306 GKKFKKYF LQLLKYIPPK  AKSHSSLSTK MSTLSYRPSD NMSSSAKKPA SCFEVE 359
AT2 322 GNRFQQKL RSVFRVPITW  LQGKRETMSC RKSSSLREMD TFVS             363
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Fig. 1. Comparison of the C-terminal cytoplasmic regions of the Ang II receptors AT1 and AT2. The NLS of AT1 (KKFKK, amino acids 307–311) is marked in a rectangular box. The YIPP motif (amino acids 319–322) that interacts with Jak2 is marked by an overline. The STL motif (amino acids 335–337) is shown in a circle.

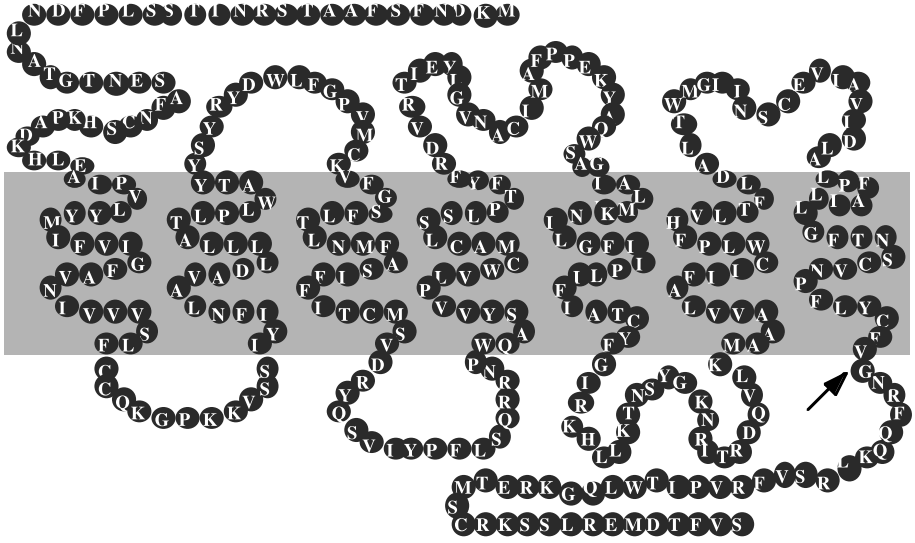

Fig. 2. Location of the stop codon that terminates the translation of AT2 to generate C-terminal-deleted AT2. Predicted organization of the rat AT2 receptor is shown. Location of the Gly322 (GGA) that was converted to the stop codon (TGA) is marked by the arrow.

mining the extent of affinity of this receptor to different peptidic ligands. No significant differences were observed between the extent of blocking by PD123319 (at concentrations 0.1 nM, 10 nM and 1 μM) of the binding of [$^{125}$I]CGP42112A (at 1.0 nM) to the wild-type AT2 receptor and to the C-terminal-deleted AT2. Therefore, the C-terminal deletion did not seem to alter (either increase or decrease) the ability of PD123319 to bind the AT2. Previously we have shown that even the mutations in the fifth transmembrane domain (Lys215Glu and Lys215Gln) that abolished the ability of the receptor to bind Ang II and reduced the ability of the receptor to bind CGP42112A did not affect the ability of the receptor to bind PD123319 [20]. These results imply that there are significant differences between the binding requirements for the non-peptidic ligand PD123319 and the peptidic ligands Ang II and CGP42112A.

### 3.2. *cGMP levels of oocytes expressing the wild-type and C-terminal-deleted AT2*

It has been known for some time that activation of the AT2 by Ang II results in altering the cGMP levels in different cell types [32]. In neonatal neuronal cells it was shown that activation of the AT2 by Ang II resulted in decreasing cGMP levels [40]. AT2 receptor activation is also known to reduce basal cGMP levels in the neointima of rat aorta after balloon injury [41]. In PC12W cells it was shown that AT2 receptor activation by Ang II results in the inhibition of a particulate guanylate cyclase and concomitant reduction of cGMP levels [42]. In contrast, in differentiating NG108-15 cells and neuroblastoma neuro-2A cells, the AT2 activation by Ang II resulted in activation of a soluble guanylyl cyclase via nitric oxide synthesis [43,44]. Thus the ability of the AT2 to alter cGMP levels is different in different cell types.

Recently, the renin–angiotensin system has evolved as one of the many significant contributing factors in the endocrine control of ovarian function along with insulin-like growth factors and binding proteins [45–50]. It was shown that addition of the AT2 antagonist PD123319 to the perfusate used for in vitro perfusion of the rabbit ovaries inhibited gonadotropin-induced ovulation and oocyte maturation in a dose-dependent manner. Increases in Ang II levels in the ovary in response to gonadotropin exposure are also reported. Taken together, these results suggest that AT2 activation is one of the contributing factors for oocyte maturation. However, how AT2 activation by Ang II contributes to the intracellular changes in oocyte that lead to oocyte maturation is unclear. Interestingly, a decrease in cGMP levels is known to accompany spontaneous maturation of rat oocytes and microinjection of cGMP inhibits this spontaneous maturation [51]. Since the development of *Xenopus* oocytes parallels the development of mammalian oocytes and also since the AT2 is capable of reducing cGMP levels in some cell types, we analyzed whether rat AT2 activation affected the cGMP levels of *Xenopus* oocytes. As shown in Fig. 4, when oocytes were not injected with any cRNA and exposed to Ang II, the levels of cGMP for a group of five oocytes was about 1.5 pmol. This was comparable to the levels of cGMP for a group of oocytes that were not exposed to Ang II (Fig. 4). These uninjected oocytes did

Table 1
Binding parameters of [$^{125}$I]Ang II and [$^{125}$I]CGP42112A for oocytes injected with the wild-type AT2 receptor and the C-terminal-deleted AT2

| Receptor type | [$^{125}$I]Ang II | | [$^{125}$I]CGP42112A | |
|---|---|---|---|---|
| | $K_d$ (nM) | $B_{max}$ (fmol/oocyte) | $K_d$ (nM) | $B_{max}$ (fmol/oocyte) |
| Wild-type AT2 | 0.15 | 4.97 | 0.12 | 5.03 |
| C-terminal-deleted AT2 | 1.7 | 4.89 | 0.097 | 5.05 |

Ligand-binding experiments were carried out using [$^{125}$I]Ang II or [$^{125}$I]CGP42112A on oocytes expressing AT2 or C-terminal-deleted AT2 at concentrations ranging from 0.05 to 2 nM. Four to six oocytes from at least two different donors were used to obtain each data point to generate saturation isotherms for the specific binding of these ligands to the oocytes. Scatchard plot analysis was carried out to determine the dissociation constants.

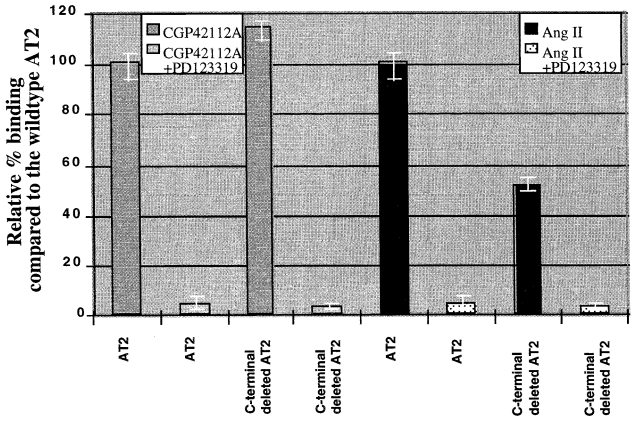


Fig. 3. Ligand-binding properties of the *Xenopus* oocytes expressing the wild-type AT2 receptor and the C-terminal-deleted AT2. The specific bindings of [$^{125}$I]Ang II and [$^{125}$I]CGP42112A (ligands at a concentration of 0.5 nM) to the oocytes expressing wild-type and mutated receptors are compared. Binding experiments were conducted for a period of 1 h according to the procedures described previously [20]. Results are shown as relative percentages of the binding of the appropriate ligand to the oocytes expressing wild-type AT2 receptor. Receptor expression was quantitated by binding studies using four to six oocytes from at least three donors (a total of at least 12 oocytes). Three different cRNA preparations were used in these experiments. The standard errors are marked in white lines. Student's *t*-test was performed to determine the significance levels for the above data sets. It was found that the extent of binding of oocytes expressing C-terminal-deleted AT2 to [$^{125}$I]Ang II (reduced affinity) or [$^{125}$I]CGP42112A (increased affinity) was significantly different from that of the wild-type since $P < 0.001$ for each data group.

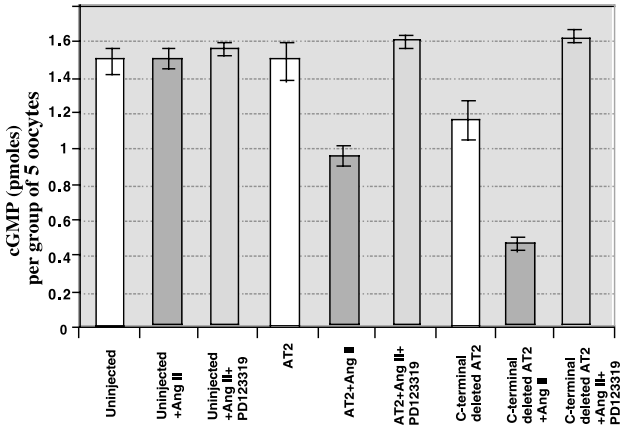


Fig. 4. cGMP levels of the *Xenopus* oocytes expressing the wild-type AT2 receptor and the C-terminal-deleted AT2 when activated by Ang II. Groups of five oocytes (uninjected or expressing the AT2 or C-terminal-deleted AT2) were exposed to 50 nM Ang II in the pre-incubation buffer for 30 s. When Ang II binding was blocked with PD123319, a pre-incubation in the presence of 1 μM PD123319 was performed before exposure to Ang II. cGMP measurements were carried out as described in Section 2. Results shown are derived from experiments using triplicate samples from three different donors. The standard errors for each data set are marked in black lines. Student's *t*-test showed that *P* is less than 0.001 for the differences observed between the cGMP levels of (a) uninjected oocytes exposed to Ang II versus the oocytes expressing AT2 and exposed to Ang II, (b) uninjected oocytes versus the oocytes expressing the C-terminal-deleted AT2, (c) uninjected oocytes exposed to Ang II versus the oocytes expressing the C-terminal-deleted AT2 and exposed to Ang II, and (d) oocytes expressing AT2 and exposed to Ang II versus oocytes expressing the C-terminal-deleted AT2 and exposed to Ang II.

not show any detectable binding to [$^{125}$I]Ang II, suggesting that they did not have any native receptors. When the oocytes were injected with AT2 and exposed to Ang II, the levels of cGMP for a group of five oocytes was reduced to 0.95 pmol (Fig. 4). However, when a 15 min pre-treatment with PD123319 was carried out before exposure to Ang II, the oocytes expressing AT2 showed increased levels of cGMP (1.59 pmol), which was comparable to that found in the case of uninjected oocytes (1.5 pmol) (Fig. 4). The decrease in cGMP levels of the oocytes expressing C-terminal-deleted AT2 was even more pronounced (Fig. 4). The oocytes expressing the C-terminal-deleted AT2 showed reduced cGMP levels even in the absence of Ang II exposure when compared to the oocytes expressing the AT2 receptor that were not exposed to Ang II (Fig. 4). This reduction in cGMP levels in the oocytes expressing the C-terminal-deleted AT2 was further enhanced by Ang II activation. The levels of cGMP in these cells were diminished to 0.45 pmol (for a group of five oocytes), whereas this reduction was abolished by treatment with PD123319 (Fig. 4). Thus rat AT2 receptor activated by Ang II seems to be capable of reducing cGMP levels in *Xenopus* oocytes. Moreover, removing the C-terminal cytoplasmic domain of AT2 did not abolish, but potentiated this function of the receptor. The observation that even in the absence of Ang

Table 2
C-terminal-deleted AT2 does not interact with the ATP-binding domain of the ErbB3 receptor as determined by the growth characteristics and β-galactosidase activity of the yeast strain HF7C carrying the 'bait' plasmids and 'prey' plasmids

| Plasmid 1: GAL4 BD translationally fused to | Plasmid 2: GAL4 AD translationally fused to | Growth on $Trp^-Leu^-$ SD plates | Growth on $Trp^-Leu^-$ $His^-$ $3AT^+$ SD plates | β-Galactosidase activity | Interacting peptides |
|---|---|---|---|---|---|
| pVA3-1 (GAL4 BD:p53) | pTD1-1 (GAL4 AD:SV 40 large T antigen) | +++ | +++ | +++ | p53:T antigen, +ve control |
| pBG1157 (GAL4 BD:AT2 receptor) | pBG857 (GAL4 AD:ErbB3$_{668-782}$) | +++ | ++ | ++ | AT2 receptor: ErbB3$_{668-782}$ |
| pBG1101 (GAL4BD:AT2$_{226-363}$) | pBG857 (GAL4 AD:ErbB3$_{668-782}$) | +++ | ++ | ++ | AT2$_{226-363}$: ErbB3$_{668-782}$ |
| pBG5003 (GAL4 BD: C-terminal-deleted AT2) | none | (no growth on $Trp^-$ $His^-$ $3AT^+$ SD | − | − | C-terminal-deleted AT2 receptor alone |
| pBG5003 (GAL4 BD: C-terminal-deleted AT2) | pGAD 424 (GAL4 AD only) | +++ | − | − | C-terminal-deleted AT2: GAL4 AD |
| pBG5003 (GAL4 BD: C-terminal-deleted AT2) | pBG857 (GAL4 AD:ErbB3$_{668-782}$) | +++ | − | − | C-terminal-deleted AT2: ErbB3$_{668-782}$ |

+++ indicates positive and − indicates negative.

II activation the C-terminal-deleted rat AT2 receptor was capable of reducing cGMP levels in *Xenopus* oocytes, and that Ang II activation further enhanced this reduction, implied that the C-terminal cytoplasmic domain of AT2 has a negative regulatory role on the AT2-mediated cGMP reduction. This sharp decrease in the cGMP levels of oocytes expressing either AT2 or C-terminal-deleted AT2 and activated by Ang II could be observed even when the incubation in the presence of Ang II was carried out for 1 min. However, when the incubation was continued for 5 min or more, this effect was not observed (data not shown).

### 3.3. Role of C-terminus of the AT2 in the interaction between the AT2 and the ErbB3

We have shown previously that the region spanning the ATP-binding domain of the human ErbB3 receptor interacts with the AT2 receptor in a yeast two-hybrid assay [36]. We have also shown that a truncated AT2 receptor (spanning amino acids 226–363) that contains the third intracellular loop and the C-terminal cytoplasmic region of the AT2 is sufficient for this interaction [36]. To determine whether the C-terminal cytoplasmic region of the AT2 is essential for this interaction, we generated a fusion protein in which GAL4 BD was fused to the C-terminal-deleted AT2 (pBG5003). The bait plasmid pBG5003 and prey plasmid pBG857 were introduced into yeast strains CG1945 and HF7C and the transformants were selected on $Trp^-Leu^-$ SD plates. The ability of these colonies to grow on $Trp^-Leu^-$ $His^-$ $3AT^+$ SD plates and express β-galactosidase activity was compared with the ability of the yeast colonies carrying plasmid combinations of pBG1157 (specifies GAL4 BD:complete AT2 fusion protein) and pBG857, or pBG1101 (specifies GAL4 BD:$AT2_{226-363}$ fusion protein) and pBG857, to do the same functions. As shown in Table 2, the yeast transformants of the strain HF7C that express the GAL4 BD:complete AT2 fusion protein and GAL4 AD:$ErbB3_{668-78)}$ fusion protein (due to the presence of pBG1157 and pBG857) were able to grow on $Trp^-Leu^-$ $His^-$ $3AT^+$ SD plates and show β-galactosidase activity. Similarly, the yeast transformants of HF7C that express GAL4BD:$AT2_{226-363}$ fusion protein and GAL4 AD:$ErbB3_{668-782}$ fusion protein (due to the presence of pBG1101 and pBG857) were also able to grow on $Trp^-Leu^-$ $His^-$ $3AT^+$ SD plates and show β-galactosidase activity (Table 2). However, the yeast transformants of HF7C that express GAL4 BD:C-terminal-deleted AT2 fusion protein and GAL4 AD:$ErbB3_{668-782}$ fusion protein (due to the presence of pBG5003 and pBG857) showed highly reduced growth on $Trp^-Leu^-$ $His^-$ $3AT^+$ SD plates and did not exhibit any detectable β-galactosidase activity (Table 2). Similar results were obtained when the yeast transformants of strain CG1945 with the above-mentioned plasmid combinations were grown on $Trp^-Leu^-$ $His^-$ $3AT^+$ SD plates. These results suggested that the C-terminal cytoplasmic domain of the AT2 is needed for optimal interaction between the AT2 and the $ErbB3_{668-782}$ in a yeast two-hybrid assay.

In summary, our results show that the C-terminal cytoplasmic domain of the AT2 plays a significant role in both ligand-binding and signaling properties of this receptor. Deleting the C-terminal cytoplasmic domain reduced high-affinity binding of AT2 to Ang II, whereas it improved the high-affinity binding of AT2 to CGP42112A. We and others have shown that the requirements for high-affinity binding forms of AT2 to different peptidic ligands are different [19–24]. This result is consistent with this observation and indicates that the C-terminus of AT2 modulates the high-affinity form of the receptor. We have also shown that in *Xenopus* oocytes, activation of the rat AT2 can result in reduction of basal cGMP levels. In rat oocytes, reduction in cGMP levels is a step that is shown to contribute to oocyte maturation [51]. As mentioned before, elevating cGMP levels in rat oocytes inhibit oocyte maturation [51]. The observation that in developing, stage V *Xenopus* oocytes, rat AT2 receptor reduces cGMP levels suggests a possible signaling mechanism by which rat AT2 may assist oocyte maturation. The observation that deleting the C-terminal cytoplasmic domain improves the ability of AT2 to reduce cGMP levels further implies that this region may control the ability of AT2 to activate the signaling pathway leading to the reduction of cGMP levels. Finally, we also show that the optimal interaction of AT2 with the ATP-binding domain of the ErbB3 in yeast two-hybrid assay requires the C-terminal cytoplasmic domain.

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