

# Evidence for involvement of 3'-untranslated region in determining angiotensin II receptor coupling specificity to G-protein

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The mRNA 3'-untranslated region (3'-UTR) of many genes has been identified as an important regulator of the mRNA transcript itself as well as the translated product. Previously, we demonstrated that Chinese-hamster ovary-K1 cells stably expressing angiotensin receptor subtypes (AT<sub>1A</sub>) with and without 3'-UTR differed in AT<sub>1A</sub> mRNA content and its coupling with intracellular signalling pathways. Moreover, RNA mobility-shift assay and UV cross-linking studies using the AT<sub>1A</sub> 3'-UTR probe identified a major mRNA-binding protein complex of 55 kDa in Chinese-hamster ovary-K1 cells. In the present study, we have determined the functional significance of the native AT<sub>1A</sub> receptor 3'-UTR in rat liver epithelial (WB) cell lines by co-expressing the AT<sub>1A</sub> 3'-UTR sequence 'decoy' to compete with the native receptor 3'-UTR for its mRNA-binding proteins. PCR analysis using specific primers for the AT<sub>1A</sub> receptor and [<sup>125</sup>I]angiotensin II (AngII)-binding studies demonstrated the expression of the native AT<sub>1A</sub> receptors in WB ( $B_{\max} = 2.7$  pmol/mg of protein,  $K_d = 0.56$  nM). Northern-blot analysis showed a significant increase in native receptor mRNA expression in 3'-UTR decoy-expressing cells, confirming the role of 3'-UTR in mRNA destabilization. Compared with vehicle control, AngII induced DNA and protein synthesis in wild-type WB as measured by [<sup>3</sup>H]thymidine and [<sup>3</sup>H]leucine incorporation respectively. Activation of [<sup>3</sup>H]thymidine and [<sup>3</sup>H]leucine correlated with a sig-

nificant increase in cell number (cellular hyperplasia). In these cells, AngII stimulated GTPase activity by AT<sub>1</sub> receptor coupling with G-protein  $\alpha_i$ . We also delineated that functional coupling of AT<sub>1A</sub> receptor with G-protein  $\alpha_i$  is an essential mechanism for AngII-mediated cellular hyperplasia in WB by specifically blocking G-protein  $\alpha_i$  activation. In contrast with wild-type cells, stable expression of the 3'-UTR 'decoy' produced AngII-stimulated protein synthesis and cellular hypertrophy as demonstrated by a significant increase in [<sup>3</sup>H]leucine incorporation and no increase in [<sup>3</sup>H]thymidine incorporation and cell number. Furthermore, [<sup>125</sup>I]AngII cross-linking and immunoprecipitation studies using specific G-protein  $\alpha$  antibodies showed that in wild-type cells, the AT<sub>1A</sub> receptor coupled with G-protein  $\alpha_i$ , whereas in cells expressing the 3'-UTR 'decoy', the AT<sub>1A</sub> receptor coupled with G-protein  $\alpha_q$ . These findings indicate that the 3'-UTR-mediated changes in receptor function may be mediated in part by a switch from G-protein  $\alpha_i$  to G-protein  $\alpha_q$  coupling of the receptor. Our results suggest that the 3'-UTR-mediated post-transcriptional modification of the AT<sub>1A</sub> receptor is critical for regulating tissue-specific receptor functions.

**Key words:** angiotensin II, decoy, hyperplasia, hypertrophy, 3'-untranslated region.

## INTRODUCTION

The peptide hormone angiotensin II (AngII) mediates the physiological actions of the renin-angiotensin system, which includes blood pressure regulation, water and salt balance, neuro-modulation and cellular growth [1]. AngII actions are mediated by two major types of receptors, AT<sub>1</sub> and AT<sub>2</sub>, which are members of the seven transmembrane G-protein-coupled receptor (GPCR) superfamily [2]. cDNA cloning for the AT<sub>1</sub> in rodents has identified two isoforms called AT<sub>1A</sub> and AT<sub>1B</sub> [3]. AT<sub>1A</sub> appears predominant with respect to tissue distribution and biological function. AT<sub>1A</sub> receptors are expressed in a broad range of tissues, although at different levels. Stimulation of AT<sub>1A</sub> receptors results in developmental and tissue-specific activation of a number of signal-transduction pathways, including the stimulation of intracellular calcium, activation of multiple serine, threonine and tyrosine kinases and phosphatases [4]. AT<sub>1A</sub> receptors have also been suggested in AngII-mediated hyperplastic responses of cultured adrenocortical cells [5], liver epithelial cells [6] and cardiac fibroblasts [7] and in the hypertrophic responses of vascular smooth-muscle cells (VSMCs) [8] and proximal tubule cells [9]. Moreover, studies in several species

have shown developmental and tissue-specific expression of this receptor [1]. The molecular and biochemical mechanisms underlying these developmental and tissue-specific expression and coupling processes are not well understood.

Recent studies have identified a major role for the 3'-untranslated region (3'-UTR) of a number of mRNAs such as early response genes, structural and growth regulatory genes and germ-cell determinant genes in regulating complex cellular functions, presumably through recognition by specific nuclear and cytoplasmic mRNA-binding proteins [10]. The role of the 3'-UTR in altering mRNA stability is well established and for certain proteins (growth factors, hormone receptors, cytokines, transcription factors) is the primary regulator of their expression and function [11,12]. The mRNA 3'-UTR has been shown to regulate other cell functions as well. For example, a repressor element in the 3'-UTR of serine protease inhibitor 2.3 gene has been shown to regulate basal transcription as well as activation during inflammation [13]. Moreover, a 288 bp sequence in the 3'-UTR of  $\alpha_2C$  adrenergic receptor influences translational efficiency of the receptor and associated signalling events [14]. Similar translational control has been demonstrated for ornithine decarboxylase [15], phospholipase A2 [16], lipoprotein lipase [17]

Abbreviations used: AngII, angiotensin II; AT<sub>1A</sub> and AT<sub>1B</sub>, angiotensin receptor subtypes; GPCR, G-protein-coupled receptor; MEM, minimal essential medium; PTX, pertussis toxin; RAMP, receptor activity-modifying protein; RT, reverse transcriptase; SLO, streptolysin O; 3'-UTR, 3'-untranslated region; VSMC, vascular smooth-muscle cell; WB, rat liver epithelial cells.

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and 15-lipoxygenase [18]. mRNA localization facilitates cells to target proteins to specific intracellular compartments for appropriate protein folding and positioning for distinct functions. Several lines of evidence suggest that 3'-UTR-mediated mRNA transport is an important mode of localizing proteins within the cell and that it serves as a mechanism for establishing cellular asymmetry [19,20]. In addition, for troponin I, tropomyosin and  $\alpha$ -cardiac actin, the 3'-UTR has also been shown to control cell growth and differentiation. Probably, the mechanism of the 3'-UTR for such regulation is by an initial disruption of the cell cycle, leading to muscle-specific gene expression [21]. Alternatively, these 3'-UTRs can function as antisense messages and/or can regulate the transcription by competing with repressors or enhancers of other genes. Taken together, these results indicate that mRNA 3'-UTR is important in determining specific cellular functions by controlling the efficiency of mRNA transcription and protein translation, intracellular mRNA sorting and localization, and cell growth and differentiation.

We have investigated the role of AT<sub>1A</sub> receptor 3'-UTR in mRNA expression and receptor function. The AT<sub>1A</sub> receptor protein is regulated by agonist-induced 3'-UTR-mediated mRNA destabilization in VSMCs [22]. Studies in our laboratory showed that the function of the 3'-UTR might extend beyond the control of mRNA destabilization. Compared with cells stably expressing the recombinant AT<sub>1A</sub> with 3'-UTR, AT<sub>1A</sub> without 3'-UTR differed in coupling with intracellular signalling pathways [23]. We also showed that a 55 kDa mRNA-binding protein specifically binds to the 3'-UTR of the AT<sub>1A</sub> receptor. To our knowledge, no other study has reported on *trans*-acting factors that interact with the 3'-UTR of seven transmembrane receptor mRNAs in determining the specificity for receptor function. In the present study, we have expressed the AT<sub>1A</sub> 3'-UTR as a decoy in rat liver epithelial (WB) cells to compete with the native receptor 3'-UTR for mRNA-binding protein(s). We performed a series of experiments to determine the functional role of 3'-UTR in altering the specificity for receptor G-protein coupling and receptor function. Our results show that in WB, AT<sub>1A</sub> couples with G-protein  $\alpha_i$  and causes hyperplasia, whereas in the presence of 3'-UTR 'decoy', AT<sub>1A</sub> couples with G-protein  $\alpha_q$  and causes hypertrophy. This observation suggests a role for the 3'-UTR in determining receptor G-protein specificity and alterations in cellular responses. The possible role of 3'-UTR binding protein(s) in the regulation of receptor G-protein coupling is also discussed.

## EXPERIMENTAL

### Materials

WB were kindly provided by Dr H. Shelton Earp (University of North Carolina, Chapel Hill, NC, U.S.A.). EXP3174 was provided by Merck Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). pRc/cytomegalovirus (CMV) vector was from Invitrogen (Carlsbad, CA, U.S.A.). Richter's improved minimal essential medium (MEM) was obtained from Irvine Scientific Co. (Santa Ana, CA, U.S.A.). Foetal bovine serum (FBS) was from Gemini Bio-Products, Inc. (Calabasas, CA, U.S.A.). LIPOFECTAMINE™ was from Life Technologies (Gaithersburg, MD, U.S.A.). AngII was from Sigma. *Thermus aquaticus* (*Taq*) polymerase and PCR reagents were from PerkinElmer (Norwalk, CT, U.S.A.). Restriction and DNA-modifying enzymes were from Promega (Madison, WI, U.S.A.). Oligonucleotide primers were obtained from Integrated DNA Technologies, Inc (Coralville, IA, U.S.A.). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). [<sup>125</sup>I]AngII, [ $\gamma$ -<sup>32</sup>P]GTP and [ $\alpha$ -<sup>35</sup>S]ATP were from DuPont–New England Nuclear (Boston, MA, U.S.A.). [<sup>3</sup>H]-

Thymidine and [<sup>3</sup>H]leucine were from Amersham (Arlington Heights, IL, U.S.A.). Electrophoresis reagents and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

### Cell culture and transfections

We have reported previously [24] the cloning and isolation of the rat AT<sub>1A</sub> receptor gene. To construct an expression vector with AT<sub>1A</sub> receptor 3'-UTR, a 848 bp fragment containing the 3'-UTR was PCR-amplified from the clone using a sense primer (5'-CAGGTTCAAAGCACACTGGC-3') and an antisense primer (5'-TACATTACAATAAA ATTACTTTATTAGAGG-3'). The amplified DNA was subcloned into the pRc/CMV vector. The authenticity and orientation of the construct was confirmed by sequencing with the sequenase® as described previously [25]. WB were cultured in Richter's improved MEM containing 0.01  $\mu$ M insulin, 50  $\mu$ g/ml gentamicin and 10% FBS. To isolate 3'-UTR 'decoy'-expressing stable cell lines, WB were grown (60% confluent) in 100 mm plate and transfected with 2  $\mu$ g DNA using the LIPOFECTAMINE™ method as described by the manufacturer. After 24 h, cells were exposed to a medium containing 600  $\mu$ g/ml G418 for 16 days to select 3'-UTR 'decoy'-expressing stable cells. To eliminate individual colony artifacts, colonies of cells resistant to G418 were pooled together for propagation. Cells were maintained under a selection pressure of 200  $\mu$ g/ml G418.

### Reverse transcriptase (RT)-PCR analysis

Expression of the native AT<sub>1A</sub> and 3'-UTR 'decoy' transcripts in WB was determined by RT-PCR. Total RNA was isolated from wild-type and 3'-UTR 'decoy'-transfected WB by the acid guanidinium thiocyanate-phenol-chloroform method, as described previously [25]. Total RNA (2  $\mu$ g) was annealed to 10 pmol of random hexamer oligonucleotide, and first-strand cDNA was synthesized using 20 units of SuperScript® RT (Life Technologies). The cDNA of 546 bp was then amplified using a sense primer 5'-GCCTTACCAAGAATATTCTGG-3' corresponding to the coding region and an antisense primer 5'-GGCTGCCCTGGCTTCTGTGTCAG-3' specific for the 3'-UTR of the AT<sub>1A</sub> receptor. The cDNA of 992 bp corresponding to the 3'-UTR 'decoy' or 144 bp corresponding to the polylinker region of the expression vector without the 3'-UTR was amplified using primers flanking the polylinker sequence of pRc/CMV expression vector. PCR was performed in a 50  $\mu$ l solution containing 2.5 units of *Taq* polymerase and subjected to 24 cycles of repeated denaturing (30 s at 95 °C) → annealing (30 s at 55 °C) → extension (60 s at 72 °C) in a DNA thermal cycler (Perkin-Elmer GeneAmp PCR system 9600). The amplified DNA fragments were separated and visualized on a 1% ethidium bromide agarose gel.

### Northern-blot analysis

Total RNA (10  $\mu$ g) from each cell line was separated by 1.2% agarose-gel electrophoresis in the presence of 6.5% (v/v) formaldehyde [23]. RNA was transferred to nitrocellulose membrane, UV cross-linked and prehybridized at 60 °C for 2 h in a buffer containing 0.5 M sodium phosphate (pH 7.5), 1 mM EDTA, 1% (w/v) BSA and 7% (w/v) SDS. A <sup>32</sup>P-labelled random primed probe corresponding to the 848 bp 3'-UTR fragment of the AT<sub>1A</sub> receptor was used to probe the membrane at 60 °C for 18 h. The membrane was washed twice with 2 × SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS at room temperature (23 °C) and twice with 1 × SSC at 60 °C. After

the wash, the blot was air-dried, the radioactivity was visualized by autoradiography and its intensity of density was quantified by image analysis.

### Receptor binding studies

AngII binding studies were performed in triplicate on WB (with and without expressing the 3'-UTR 'decoy') in 12-well plates as described previously [24]. Briefly, cells were rinsed twice with PBS and incubated at 22 °C for 30 min with 0.05 nM [<sup>125</sup>I]AngII in binding buffer [50 mM Tris/HCl (pH 7.4), 120 mM NaCl, 4 mM KCl, 1 mM CaCl<sub>2</sub>, 10 µg/ml of bacitracin, 0.25% BSA and 2 mg/ml of dextrose]. Non-specifically bound AngII was removed by washing three times with the binding buffer (without BSA). Finally, cells were dissolved in 0.2 M NaOH, transferred to counting vials and radioactivity was determined using a Packard® auto-γ-scintillation spectrometer. Specific [<sup>125</sup>I]AngII binding was defined as that portion of the total binding displaced by 10 µM unlabelled AngII. At equilibrium, specific binding was more than 95% of the total binding. Competition binding studies were performed in the presence of 0.05 nM [<sup>125</sup>I]AngII and increasing concentrations (1 pM–10 µM) of unlabelled AngII or EXP3174 (AT<sub>1</sub> receptor antagonist) or PD 123177 (AT<sub>2</sub> receptor antagonist).

### <sup>3</sup>H incorporation and growth assay

To determine AngII-mediated growth response, DNA and protein synthesis were measured in WB. Measurement of [<sup>3</sup>H]-thymidine and [<sup>3</sup>H]leucine incorporation was performed on WB with and without 3'-UTR 'decoy' as described previously [23]. Briefly, 'decoy' transfected and non-transfected cells were cultured for 24 h in 12-well plates in Richter's improved MEM containing 10% FBS. The cells were serum-depleted for 24 h and incubated with AngII (100 nM) and other agents for 18 h. [<sup>3</sup>H]Thymidine or [<sup>3</sup>H]leucine was added during the final 2 h of incubation. Cells were washed three times with PBS and fixed with 10% trichloroacetic acid. After washing with 95% ethanol, insoluble material was solubilized with 0.2 M NaOH. Incorporation of the radioactivity was determined as trichloroacetic acid-precipitable material using a liquid-scintillation counter. To block functional coupling of AT<sub>1A</sub> receptor with G-protein α subunit, permeabilized cells were pretreated with specific G-protein α antibodies (1:50 dilution) for 20 min. In these studies, we used the following antibodies: G-protein αi (epitope corresponding to amino acids 325–344 near the C-terminus of rat G-protein αi, which is also within the highly conserved region of all members of G-protein αi family), G-protein αq (epitope corresponding to amino acids 341–359 mapping within the common C-terminal domain of G-protein αq and G-protein α11), and G-protein αs (epitope corresponding to amino acids 377–394 mapping within the C-terminal domain of rat G-protein αs). Specificity of each antibody was established by a blocking peptide corresponding to each epitope. To ensure reliable internalization of G-protein antibody, reversible cellular membrane permeabilization was performed by exposing cells to 0.2 unit/ml streptolysin 0 (SLO) for 20 min (time and dose had no detectable variation in cell viability and proliferation after treatment) [26,27]. The suitable SLO concentration was determined based on cell viability (over 95% viable cells after treatment) and normal cell growth. Viability of cells and variations in cell proliferation were determined by Trypan Blue exclusion assay [28] and [<sup>3</sup>H]thymidine incorporation assay respectively. Cells were exposed to AngII (100 nM) and [<sup>3</sup>H]thymidine incorporation, measured as described above. For cell proliferation assay, WB with and without 'decoy' expression were grown as mono-

layer and serum-starved for 24 h. This was considered day 0. Serum-depleted cells were exposed to AngII (1 µM), detached from the dish at 24 and 48 h after treatment and counted using a haemocytometer.

### GTPase activity analysis

Measurement of the GTPase activity of signal-transducing G-protein was performed in membranes prepared from WB [24]. Confluent cultures were washed twice with PBS, scraped into ice-cold buffer containing 50 mM Tris/HCl (pH 7.6), 1 mM EDTA and 1 mM PMSF. They were homogenized and centrifuged at 4000 g for 10 min. The supernatant was centrifuged at 48000 g for 20 min, and the membrane pellet was washed and resuspended in membrane storage buffer [10 mM Tris/HCl (pH 7.4)/1 mM EDTA/2 mM benzamidine/50 µM chlorpromazine/50 µM leupeptin/1 TIU (trypsin inhibitor unit)/ml soya bean trypsin inhibitor/0.25 TIU/ml aprotinin/10 µM PMSF]. Assay for GTPase activity was performed in triplicate [29] in a 100 µl reaction mixture containing 25 µl of membrane suspension (25 µg of protein), 50 µl of GTPase assay buffer [40 mM Tris/HCl (pH 7.4)/200 mM NaCl/4 mM EDTA/2 mM creatine/2 mM ouabain/10 mM MgCl<sub>2</sub>/1 µM GTP/50 units/ml creatine kinase] and 50000 c.p.m. [<sup>32</sup>P]GTP and incubated at 37 °C for 10 min. The reaction was terminated by transferring all the tubes to ice and agitating the tubes for 1 min. Then 900 µl of ice-cold 2% activated charcoal suspension was added and centrifuged at 1500 g for 10 min at 4 °C. The supernatant was counted in a liquid-scintillation counter for <sup>32</sup>P content.

### Receptor labelling and immunoprecipitation studies and Western-blot analysis

To determine the association of AT<sub>1A</sub> receptor and its signal-transducing G-protein, we performed ligand-mediated activation of the receptor, which was cross-linked [30] and immunoprecipitated with specific anti-G-protein antiserum [31,32]. [<sup>125</sup>I]AngII cross-linking studies were performed due to the unavailability of AT<sub>1A</sub> receptor-specific antibodies. Confluent cultures of WB with and without the 3'-UTR 'decoy' were washed twice with PBS and membranes prepared as described above. Equal amounts of membrane suspensions (200 µg of protein) were incubated with 0.05–0.1 nM [<sup>125</sup>I]AngII in a buffer containing 50 mM Hepes (pH 7.4), 125 mM NaCl, 6.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10 µg/ml bacitracin and 2 mg/ml dextrose for 30 min at room temperature (23 °C). AngII was cross-linked with 500 µM disuccinimidyl suberate for 30 min at room temperature (23 °C) [30]. The reaction was stopped by adding 50 mM Tris/HCl (pH 8.0) and incubated for an additional 15 min. Immunoprecipitation was performed by incubating with specific G-protein α antiserum (1:100 dilution) and protein A/G agarose overnight at 4 °C. Precipitates were washed three times with lysis buffer without Nonidet P40 and the radioactivity was counted in a γ-counter. To detect the expression of different G-protein α subunits in WB, Western-blot analysis was performed. Cell membranes were solubilized in lysis buffer containing 0.1% Tween 20, separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed with specific G-protein α antibodies with or without blocking peptides and enhanced chemiluminescence (ECL®) was used for secondary detection.

### Data analysis

Results are presented as means ± S.E.M. and the value of *P* < 0.05 was considered significant. Values were normalized to

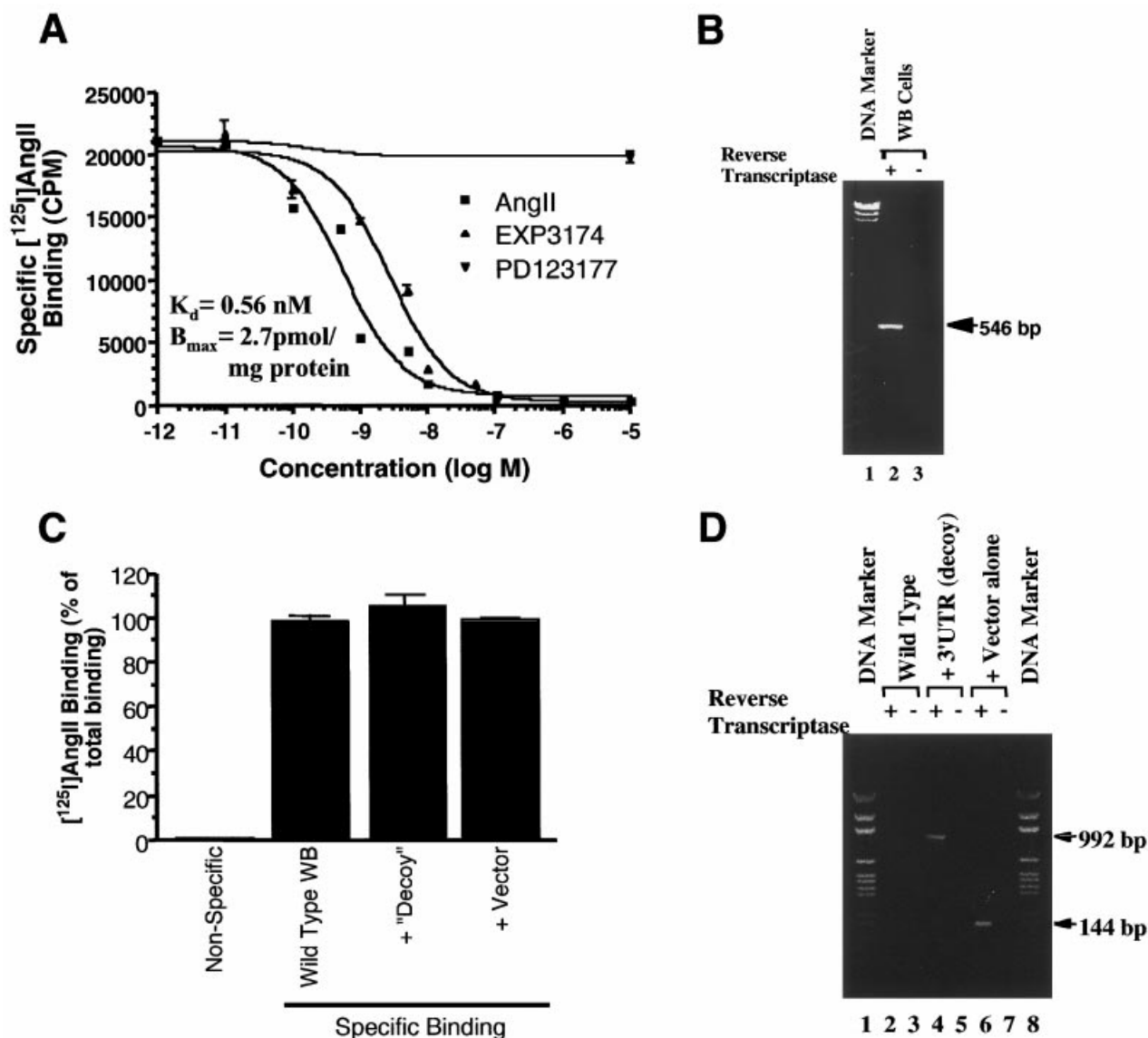
the amount of protein determined using Bio-Rad DC protein assay. Data were analysed using the software GraphPad Prism, and the binding constants were determined as described previously [24].

## RESULTS

### AT<sub>1A</sub> receptor and 3'-UTR 'decoy' expression in WB

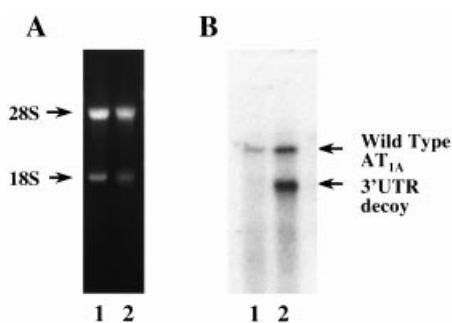
To determine a functional role for the 3'-UTR of the native AT<sub>1A</sub> receptor, we used a continuously passaged WB line [33]. Competition binding studies using AngII, EXP3174 (an AT<sub>1</sub> receptor antagonist) and PD123177 (an AT<sub>2</sub> receptor antagonist) were

performed in the presence of 0.05 nM [<sup>125</sup>I]AngII. The affinity of AngII ( $K_d = 0.56$  nM) and EXP3174 ( $K_d = 2.8$  nM) are within the range reported for angiotensin plasma membrane receptors (Figure 1A). No displacement was observed with AT<sub>2</sub> receptor antagonist PD123177. RT-PCR analysis using specific primers for the rat AT<sub>1A</sub> receptor amplified a single cDNA fragment of 546 bp, confirming the presence of AT<sub>1A</sub> mRNA transcript in WB (Figure 1B). In addition, [<sup>125</sup>I]AngII binding studies were performed to determine the level of native receptor protein expression before and after expressing the 3'-UTR 'decoy'. Compared with wild-type WB, 'decoy'-expressing and vector-alone-expressing cells showed no significant change in cell surface receptor expression as demonstrated by [<sup>125</sup>I]AngII-binding



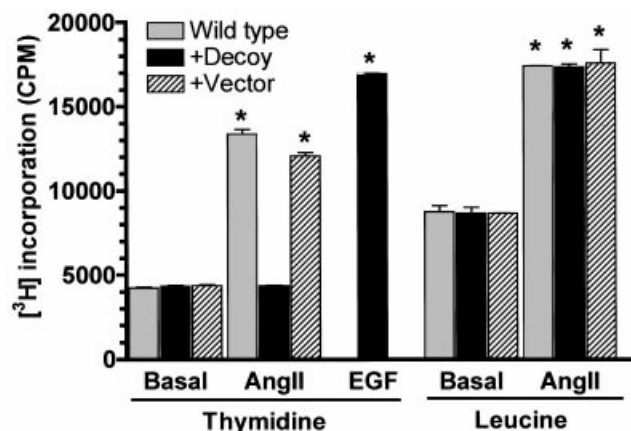
**Figure 1** Identification of the native AT<sub>1A</sub> receptor expression and co-expression of the 3'-UTR 'decoy' or vector in WB

(A) Competition curves showing ligand specificity for [<sup>125</sup>I]AngII binding to WB. Competition curves were generated for AngII (■), EXP3174 (▲) and PD123177 (▼). Each point is the mean ± S.E.M. of triplicate values. (B) RT-PCR analysis of mRNA isolated from WB using specific primers for the rat AT<sub>1A</sub> receptor. Arrow indicates the 546 bp cDNA band specific to the rat AT<sub>1A</sub> receptor. (C) Specific binding of [<sup>125</sup>I]AngII to wild-type and 3'-UTR 'decoy'- and vector-expressing WB. Data are expressed as percentage of total binding. (D) RT-PCR analysis using primers flanking the polylinker sequence of pRc/GMV vector to demonstrate the expression of the construct in WB. Arrows indicate the band for the 3'-UTR (992 bp) and the vector without the 3'-UTR (144 bp). mRNA-specific amplification was determined by the presence or absence of RT in the reaction.



**Figure 2** Northern-blot analysis of wild-type and 3'-UTR 'decoy'-expressing WB

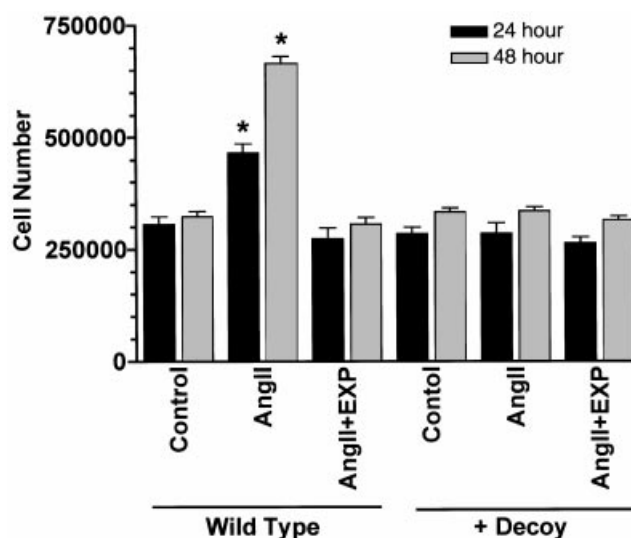
Total RNA (10  $\mu$ g) from each cell line was separated by gel electrophoresis, blotted and probed, as described in the Experimental section. Lane 1, RNA from wild-type cells; lane 2, RNA from 3'-UTR 'decoy'-transfected cells. (A) Ethidium bromide-stained gel, (B) hybridization blot showing native receptor and 3'-UTR 'decoy' mRNA expression.



**Figure 3** AngII-mediated changes in [ $^3$ H]thymidine and [ $^3$ H]leucine incorporation in wild-type and 3'-UTR 'decoy'-expressing WB

Wild-type and 3'-UTR 'decoy' and vector (pRc/CMV) stably expressing WB were exposed to AngII (100 nM) for 18 h. [ $^3$ H]Thymidine or [ $^3$ H]leucine was added to the medium during the last 2 h of incubation. [ $^3$ H] incorporation was determined as described in the Experimental section. Epidermal growth factor (EGF; 200 ng) was used as a positive control for [ $^3$ H]thymidine incorporation in 3'-UTR 'decoy'-expressing cells. Data are expressed as the means  $\pm$  S.E.M. of triplicate values. \* $P$  < 0.001 versus basal.

studies (Figure 1C). We also performed RT-PCR using primers corresponding to the polylinker of the expression vector pRc/CMV (in the presence or absence of RT) to confirm the expression of 3'-UTR 'decoy' transcripts in WB. In Figure 1(D), lane 4 shows the expression of the 3'-UTR 'decoy' and lane 6 shows the expression of the expression vector without 3'-UTR 'decoy', confirming the expression of both constructs in WB. Northern-blot analysis (Figure 2B) revealed that expression of the native AT<sub>1A</sub> receptor is significantly increased (2.7-fold) in 3'-UTR decoy-expressing cells compared with wild-type WB. This observation is consistent with our previous studies and studies from other laboratories showing the role of AT<sub>1A</sub> 3'-UTR in mRNA degradation [22,23]. The level of 3'-UTR decoy mRNA shows 1.78-fold higher expression than the native receptor in transfected cells and 3.7-fold higher expression than the native receptor in non-transfected cells.



**Figure 4** Effect of AngII on cell proliferation in wild-type and 3'-UTR 'decoy'-expressing WB

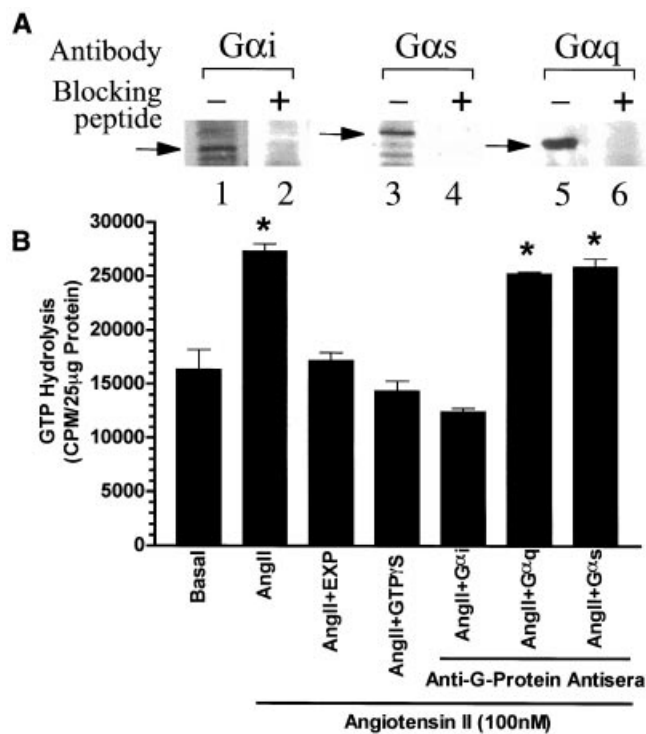
Wild-type and 3'-UTR 'decoy'-expressing WB were serum-depleted for 24 h, exposed to AngII (1  $\mu$ M) or AngII + EXP3174 (10  $\mu$ M) for 24 or 48 h. Cell number was determined as described in the Experimental section. Data are expressed as the means  $\pm$  S.E.M. of triplicate values and are representative of three experiments performed in triplicate. \* $P$  < 0.01 versus control.

#### Agonist-mediated changes in the growth of WB with or without 3'-UTR 'decoy' expression

AngII has been shown to be a hyperplastic agent in some cells and a hypertrophic agent in others, suggesting tissue-specific regulation of AngII receptor function. Therefore we performed [ $^3$ H]thymidine (as an indicator of DNA synthesis) and [ $^3$ H]leucine (as an indicator of protein synthesis) incorporation in WB with or without 3'-UTR 'decoy' expression. As shown in Figure 3, AngII (100 nM) stimulated [ $^3$ H]thymidine and [ $^3$ H]leucine incorporation in WB, whereas no significant stimulation of [ $^3$ H]thymidine was observed in 3'-UTR 'decoy'-expressing WB. However, 3'-UTR 'decoy' had no effect on the AngII-mediated stimulation of [ $^3$ H]leucine incorporation. In cells transfected with expression vector alone, AngII stimulated both [ $^3$ H]thymidine and [ $^3$ H]leucine incorporation. As a positive control (for DNA synthesis), epidermal growth factor (200 ng/ml) stimulated [ $^3$ H]thymidine incorporation in 3'-UTR 'decoy'-expressing WB, confirming the specificity for the AT<sub>1A</sub> 3'-UTR 'decoy' on homologous receptor. Furthermore, a direct analysis of AngII-mediated cell division was performed by counting the cell number of wild-type and 3'-UTR 'decoy'-expressing WB at 24 and 48 h after AngII exposure. Figure 4 shows that in WB, AngII (1  $\mu$ M) stimulated cell division with a doubling time of 46 h. This was blocked by EXP3174, the receptor antagonist. Compared with wild-type WB (in serum-free medium), 3'-UTR 'decoy'-expressing cells showed no significant increase in cell number at 24 or 48 h after AngII exposure.

#### Expression of different G-protein $\alpha$ subunits and AngII-mediated GTPase activity in WB

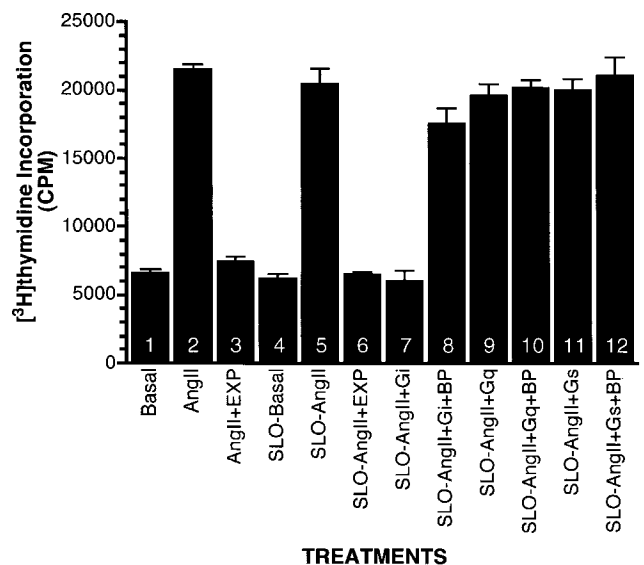
To determine the expression of different G-protein  $\alpha$  subunits in WB, immunoblot analysis was performed using specific anti-



**Figure 5** AngII-mediated increase in GTPase activity in WB

WB were grown to 90% confluency and membranes were isolated as described in the Experimental section. (A) Western-blot analysis using cell membranes. Membranes were solubilized in lysis buffer containing 0.1% Tween 20, separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed with specific anti-G-protein  $\alpha$  antibodies without (lanes 1, 3 and 5) or with (lanes 2, 4 and 6) blocking peptides, and enhanced chemiluminescence (ECL<sup>®</sup>) was used for secondary detection. (B) Isolated membranes (25  $\mu$ g) were incubated in the presence of [ $\gamma$ -<sup>32</sup>P]GTP with or without AngII (100 nM), AngII + EXP3174 (1  $\mu$ M), AngII + GTP guanosine-5'-0-(3-thiotriphosphate) (10  $\mu$ M), AngII + G-protein  $\alpha$ i antibody (1:100 dilution), AngII + G-protein  $\alpha$ q antibody (1:100 dilution) and AngII + G-protein  $\alpha$ s antibody (1:100 dilution). <sup>32</sup>P content was determined in a liquid-scintillation counter. Data are expressed as the means  $\pm$  S.E.M. of triplicate values and are representative of three experiments performed in triplicate. \* $P$  < 0.001 versus basal.

bodies raised against G-protein  $\alpha$ i, G-protein  $\alpha$ q and G-protein  $\alpha$ s subunits (Figure 5A). The G-protein  $\alpha$ i antibody recognized a 42 kDa protein (lane 1), the G-protein  $\alpha$ q antibody recognized a 42 kDa protein (lane 5) and the G-protein  $\alpha$ s antibody recognized a 47 kDa protein (lane 3) in isolated membranes of WB. To determine the possible involvement of G-protein(s) in AT<sub>1A</sub> receptor signal transduction, GTPase activity was measured in membranes of WB (Figure 5B). Non-specific hydrolysis was determined by incubating the membranes in the presence of a non-hydrolysable analogue of GTP guanosine-5'-0-(3-thiotriphosphate). AngII (100 nM) stimulated the GTPase activity in WB. Pretreatment of membranes with AT<sub>1</sub> receptor antagonist EXP3174 completely inhibited AngII-induced GTPase activity. AngII-induced activation of GTPase activity was reduced to basal level when the membranes were co-incubated with G-protein  $\alpha$ i specific antibody, whereas no change in GTPase activity was observed in the presence of G-protein  $\alpha$ q or G-protein  $\alpha$ s antibody, suggesting that in WB AT<sub>1A</sub> receptor couples through G-protein  $\alpha$ i subunit to signal-transduction pathways.



**Figure 6** AngII-mediated [<sup>3</sup>H]thymidine incorporation in WB in the presence of G-protein  $\alpha$  antibodies

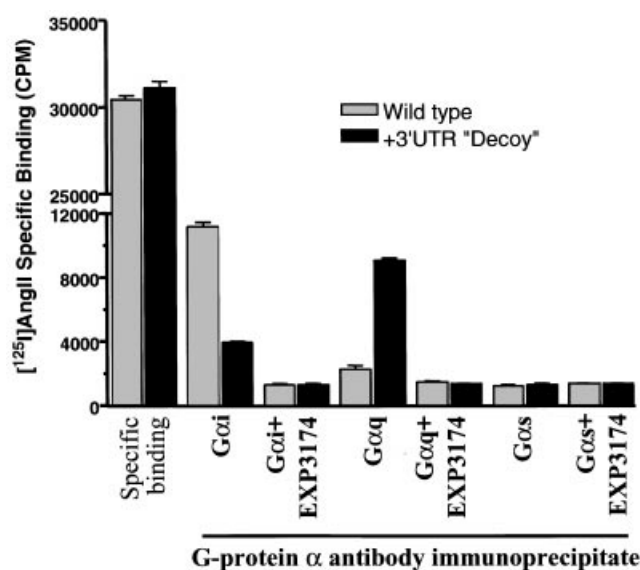
WB were serum-depleted for 24 h and exposed to AngII (100 nM) with or without specific G-protein antibodies (G-protein  $\alpha$ i,  $\alpha$ q and  $\alpha$ s) in the presence or absence of the blocking (immunizing) peptide (BP). Reversible cellular membrane permeabilization was achieved by exposing cells to 0.2 unit/ml SLO for 20 min to introduce antibodies into cells. [<sup>3</sup>H]Thymidine incorporation was measured as described in the Experimental section. Data are expressed as the means  $\pm$  S.E.M. of triplicate values and are representative of three experiments performed in triplicate.

#### Determination of G-protein $\alpha$ subunit specificity for AngII-mediated growth in WB

We hypothesized that AngII-mediated increase in cellular hyperplasia in WB was due to functional coupling of the AT<sub>1A</sub> receptor to G-protein  $\alpha$ i subunit. We therefore pre-exposed cells with G-protein  $\alpha$ i antibody to impede receptor G-protein  $\alpha$ i coupling and AngII (100 nM)-mediated [<sup>3</sup>H]thymidine incorporation was measured (Figure 6). Antibody was introduced into cells by reversible permeabilization with SLO. In WB unexposed to G-protein  $\alpha$ i antibody, AngII stimulated a 3-fold increase in [<sup>3</sup>H]thymidine incorporation and this effect was blocked by EXP3174. No change in AngII-induced [<sup>3</sup>H]thymidine incorporation was observed in cells before or after SLO permeabilization, suggesting that permeabilization did not alter the normal growth properties of the WB (cf. lanes 1–3 with lanes 4–6). AngII-mediated increase in [<sup>3</sup>H]thymidine incorporation was completely inhibited in WB pretreated with G-protein  $\alpha$ i antibody (1:50 dilution) for 30 min, and it was reversed when the antibody was preincubated with the immunizing (blocking) peptide. In these cells, preincubation with G-protein  $\alpha$ q or G-protein  $\alpha$ s antibody did not inhibit AngII-mediated increase in [<sup>3</sup>H]thymidine incorporation. These results demonstrate that AngII-induced DNA synthesis (mitogenesis) in WB is through activation of G-protein  $\alpha$ i subunit.

#### 3'-UTR 'decoy'-mediated alterations in receptor G-protein coupling in WB

Since AT<sub>1A</sub> receptor induces DNA synthesis through G-protein  $\alpha$ i coupling, we questioned whether the observed changes in cell



**Figure 7** Effect of 3'-UTR 'decoy' on AngII-induced receptor G-protein coupling in WB

Wild-type and 3'-UTR 'decoy'-expressing stable WB were grown to semiconfluency in 100 mm plates. Membranes were isolated and [ $^{125}$ I]AngII binding, cross-linking and immunoprecipitation using specific G-protein  $\alpha$  antibodies were performed as described in the Experimental section. Immunoprecipitates were washed three times with cell lysis buffer and the radioactivity counted in a  $\gamma$ -counter. Data are expressed as the means  $\pm$  S.E.M. of triplicate values and are representative of three experiments performed in triplicate.

growth (hyperplasia versus hypertrophy) in 3'-UTR 'decoy'-expressing WB were mediated by alterations in receptor G-protein coupling. To test this hypothesis, we determined the role of 3'-UTR in AngII-mediated physical coupling of the AT<sub>1A</sub> receptor with a specific G-protein  $\alpha$  subunit in WB. We cross-linked the receptor in wild-type and 3'-UTR 'decoy'-expressing WB after agonist [ $^{125}$ I]AngII or [ $^{125}$ I]AngII plus EXP3174 exposure, and immunoprecipitated with specific G-protein  $\alpha$  subunit antibodies. As shown in Figure 7, AngII stimulated physical coupling of AT<sub>1A</sub> receptor to G-protein  $\alpha$ i in wild-type WB, as demonstrated by immunoprecipitation of [ $^{125}$ I]AngII cross-linked receptor by G-protein  $\alpha$ i antibody. This physical coupling was blocked by co-incubation of cells with EXP3174. No significant incorporation of  $^{125}$ I label in the immunoprecipitate by G-protein  $\alpha$ q or G-protein  $\alpha$ s antibody was observed. However, when the cells were expressed with 3'-UTR 'decoy', the AT<sub>1A</sub> receptor coupling switched from G-protein  $\alpha$ i to G-protein  $\alpha$ q and the receptor antagonist EXP3174 blocked the G-protein  $\alpha$ q coupling. These results suggest that the 3'-UTR may play a role in determining the specificity of AT<sub>1A</sub> receptor G-protein coupling and thereby function in WB.

## DISCUSSION

In our previous study [23], we demonstrated that Chinese-hamster ovary-K1 cells stably expressing AT<sub>1A</sub> receptor with and without its 3'-UTR couple differentially to intracellular signalling pathways. We have also shown by mobility-shift assays and UV cross-linking studies that at least one specific mRNA-binding protein of 55 kDa interacts with the receptor 3'-UTR. Our results suggested that the 3'-UTR of the AT<sub>1A</sub> receptor could control specific AngII-mediated cellular responses, perhaps through selective recognition of its 3'-UTR by specific mRNA-

binding protein(s). These findings raised two important questions: (1) What is the relevance of these observations to native AT<sub>1A</sub>, since in every tissue, native receptor expresses mRNA transcripts with the 3'-UTR, and (2) what are the physiological manifestations of these observations? The present study was designed to address these intriguing questions. We chose a continuously passaged WB line [33], which expresses native AT<sub>1A</sub>, as evidenced by [ $^{125}$ I]AngII-binding and RT-PCR analysis. Tissue distribution studies for the mRNA-binding protein(s) showed that rat liver tissue as well as the WB (results not shown) express the 55 kDa mRNA-binding protein, which specifically binds to the 3'-UTR of AT<sub>1A</sub> receptor. To investigate the functional significance of the 3'-UTR of AT<sub>1A</sub>, we evaluated the effects of expression of the 3'-UTR 'decoy' with native AT<sub>1A</sub> receptor by competing with the native receptor 3'-UTR for its mRNA-binding protein. Recent studies have demonstrated that co-expression of the regulatory region as minigenes with its native homologous transcripts resulted in specific antagonism or alteration of its function [34].

We performed initial studies to determine the normal response of the native AT<sub>1A</sub> in WB and showed that the native receptor stimulates cell proliferation by coupling specifically to the G-protein  $\alpha$ i subunit. AT<sub>1A</sub> is a member of the seven transmembrane GPCR superfamily. Binding of ligand to receptor (with seven transmembrane spanning domains) initiates cellular signalling first by direct coupling with a specific G-protein to form a ligand-receptor-G-protein complex, resulting in the exchange of GTP for GDP on the G-protein  $\alpha$  subunit. Although similar G-protein  $\alpha$  subunits express in most cells/tissues, AT<sub>1A</sub> receptor G-protein coupling is cell/tissue-specific [1,4]. For example, in liver, AngII couples with a pertussis toxin (PTX)-sensitive G-protein, whereas in VSMCs it does not. Previous studies utilizing indirect methods such as PTX sensitivity have suggested G-protein  $\alpha$ i coupling in AT<sub>1A</sub>-mediated signalling, but to our knowledge there is no direct evidence of specific G-protein  $\alpha$  involvement in AngII-mediated cell growth. There is evidence that antibodies raised against the C-terminal part of the G-protein  $\alpha$  subunit can effectively block G-protein-mediated receptor functions [29]. We used three different G-protein  $\alpha$  antibodies raised against the C-terminal region to identify the specific G-protein  $\alpha$  subunit activated by the AT<sub>1A</sub> receptor and determine AngII-mediated cell growth as an end point in WB. These antibodies specifically recognized three major classes of G-protein  $\alpha$  subunits: G-protein  $\alpha$ i, G-protein  $\alpha$ q and G-protein  $\alpha$ s. Our results show that in WB, AngII stimulated DNA and protein synthesis and increased cell number (hyperplasia) by activating AT<sub>1A</sub> receptors. The fact that G-protein  $\alpha$ i-specific antibody blocked AngII-mediated DNA synthesis in WB indicates that AT<sub>1A</sub> receptor coupling with G-protein  $\alpha$ i is an essential step in AngII-induced cellular hyperplasia. Similar results were also observed with PTX treatment (results not shown). Because we used G-protein  $\alpha$ i antibody, which recognizes multiple subtypes of G $\alpha$ i proteins, it is necessary to determine which G-protein  $\alpha$ i subtype specifically interacts with the AT<sub>1A</sub> receptor.

AngII has been shown to couple with growth by stimulating hyperplastic and/or hypertrophic response in a tissue-specific manner. In cardiomyocytes [35], VSMCs [8] and proximal tubule epithelial cells [9], activation of AT<sub>1A</sub> receptors induces hypertrophic responses, whereas in cardiac fibroblasts [7], adrenal [4] and liver cells [1], AT<sub>1A</sub> receptors induce hyperplastic responses. The signalling mechanism(s) by which GPCRs stimulate hypertrophic or hyperplastic responses is not fully understood. This is particularly true for AngII receptors, which couple positively or negatively with DNA synthesis in a tissue-specific manner. We showed that in WB, AngII-mediated hyperplastic

responses were altered to hypertrophic responses when the cells expressed 3'-UTR 'decoy' to block mRNA-binding protein interaction with the native AT<sub>1A</sub> 3'-UTR. In 3'-UTR 'decoy'-expressing cells, the receptor coupling with G-protein  $\alpha$  subunit also changed from G-protein  $\alpha_i$  to G-protein  $\alpha_q$ . Our results suggest that the 3'-UTR of the AT<sub>1A</sub> receptor has a role in cell growth (hyperplastic/hypertrophic) by allowing the receptor to couple with a specific G-protein  $\alpha$  subunit. In wild-type WB, AT<sub>1A</sub> coupling with G-protein  $\alpha_i$  subunit (alternatively known as inhibitory G-protein) results in increases in DNA synthesis possibly by inhibiting cAMP. This result compliments our previous observation in which we showed that Chinese-hamster ovary-K1 cells expressing the AT<sub>1A</sub> receptor without its 3'-UTR stimulated cAMP and inhibited DNA synthesis [36]. Furthermore, it has been reported that in VSMCs, stable expression of a constitutively active G $\alpha_16$  (G-protein  $\alpha_q$  family protein) mimicked the hypertrophic effects of vasoconstrictors, suggesting that G-protein  $\alpha_q$  coupling is an important step for vasoconstrictor-mediated hypertrophic response [37]. Taken together, the results suggest that the 3'-UTR of the AT<sub>1A</sub> receptor [by interacting with specific mRNA-binding protein(s)] play an important role in determining receptor G-protein coupling specificity and thereby altering cellular growth responses. Genetic complementation studies revealed specific regulatory roles for 3'-UTR of troponin I, tropomyosin and  $\alpha$ -cardiac actin in growth and differentiation [21]. Studies have shown functional correlation between 3'-UTR of many mRNAs and RNA-binding proteins. One possible explanation for these results is that tissues in which the mRNA-binding protein is present may allow the receptor into a conformation specific for G-protein  $\alpha_i$  coupling. Alternatively, in the absence of mRNA-binding protein the same receptors may be in an alternate conformation, specific for G-protein  $\alpha_q$  coupling. The mechanism(s) by which specific confirmation of the receptor determines G-protein coupling is unknown. Burns et al. [38] demonstrated that mRNA editing of the serotonin-2C receptor resulted in G-protein-specific alterations in serotonergic signal transduction and suggested that post-transcriptional modification may be critical for regulating different cellular functions mediated by other GPCRs by altering the efficacy of the interaction between the receptor and their G-proteins. It has been shown that protein kinase A-mediated conformational change in  $\beta_2$ -adrenergic receptor alters receptor coupling from G-protein  $\alpha_s$  to G-protein  $\alpha_i$  and can activate different signalling events [39]. This kind of alteration in G-protein  $\alpha$  subunit coupling is relevant to the interpretation of the tissue-specific G-protein coupling of native AT<sub>1A</sub> receptors.

Although we showed that the 3'-UTR is essential for determining AT<sub>1A</sub> receptor G-protein coupling specificity, how it promotes such regulation at the mRNA level is not known. One of the key roles for the 3'-UTR is intracellular localization and retention of specific mRNAs for precise post-translational modifications. In *Drosophila* eggs, correct localization of many mRNAs and their proteins are necessary to establish the polarity and subsequent segmentation of the embryo [40]. Two well-studied examples are oskar mRNA localization at the posterior end and bicoid mRNA at the anterior end of the embryo. The 3'-UTR of each of these mRNAs is sufficient for correct mRNA localization. Specific mRNA localization is not a characteristic of germline cells alone. Actin, an important structural component of all eukaryotic cells, uses its mRNA 3'-UTR for directing its transcripts to appropriate cell compartments for proper translation [10,20]. The proposed model for mRNA localization is that these RNAs contain *cis*-acting signal sequences recognized by RNA-binding proteins that function either in the transport of mRNAs from their site of synthesis to appropriate subcellular

locations or by anchoring the mRNA at translational sites. Localization by 3'-UTR may be necessary for correct chaperoning and functioning of the translated protein. McLatchie et al. [41] reported the identification of a new family of single-transmembrane domain proteins called receptor activity-modifying proteins (RAMPs). There are at least three proteins that have been cloned. These RAMPs are expressed in a cell/tissue-specific manner and control the transport and glycosylation of GPCRs such as calcitonin-receptor-like receptors. They play the role of chaperons for folding (specific conformation) of the receptor and translocation to the plasma membrane. Different RAMPs glycosylate the receptor differentially, allowing the GPCR to change its responsiveness, suggesting that RAMP mediates alterations in G-protein coupling and function. Although the function is not known, studies (Western-blot analysis) have demonstrated that the AT<sub>1A</sub> receptor is glycosylated, resulting in a significant increase in the molecular mass of the receptor protein [4]. We propose that in the presence of mRNA-binding protein, 3'-UTR may mediate targeting of the AT<sub>1A</sub> mRNA to a specific intracellular compartment for translation and subsequent specific RAMP-mediated translocation to the plasma membrane. However, in the absence of mRNA-binding protein, alternatively localized mRNA translation may allow for a different RAMP-mediated receptor translocation and, as a consequence, the receptor will couple with a different G-protein. Studies have demonstrated subcellular localization of transmembrane receptors, G-proteins and effectors within individual cells as potential mechanism for determining the specificity of receptor G-protein coupling [42]. The 3'-UTR of the AT<sub>1A</sub> receptor contains mRNA localization motifs similar to what has been described for actin mRNA [43]. The sequence has 100% identity with the two flanking regions (required) with an overall homology of 76%. The functional importance of this site in the AT<sub>1A</sub> 3'-UTR needs to be elucidated.

In summary, AngII induces cell proliferation in WB, mediated through specific G-protein  $\alpha_i$  coupling. 3'-UTR-mediated alterations in G-protein  $\alpha$  subunit coupling of the AT<sub>1A</sub> receptor results in functional switching of hyperplastic responses to hypertrophic responses, suggesting that the 3'-UTR of the AT<sub>1A</sub> receptor is important in determining the specificity of G-protein coupling and receptor-mediated cellular functions. However, it is not clear how the 3'-UTR of the mRNA alters G-protein specificity of its receptor protein. Further experiments will be needed to define the mechanism of such interaction. Isolation and functional characterization of the 55 kDa mRNA-binding protein will be necessary to determine the mechanisms by which 3'-UTR regulates receptor G-protein coupling in a tissue-specific manner.

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