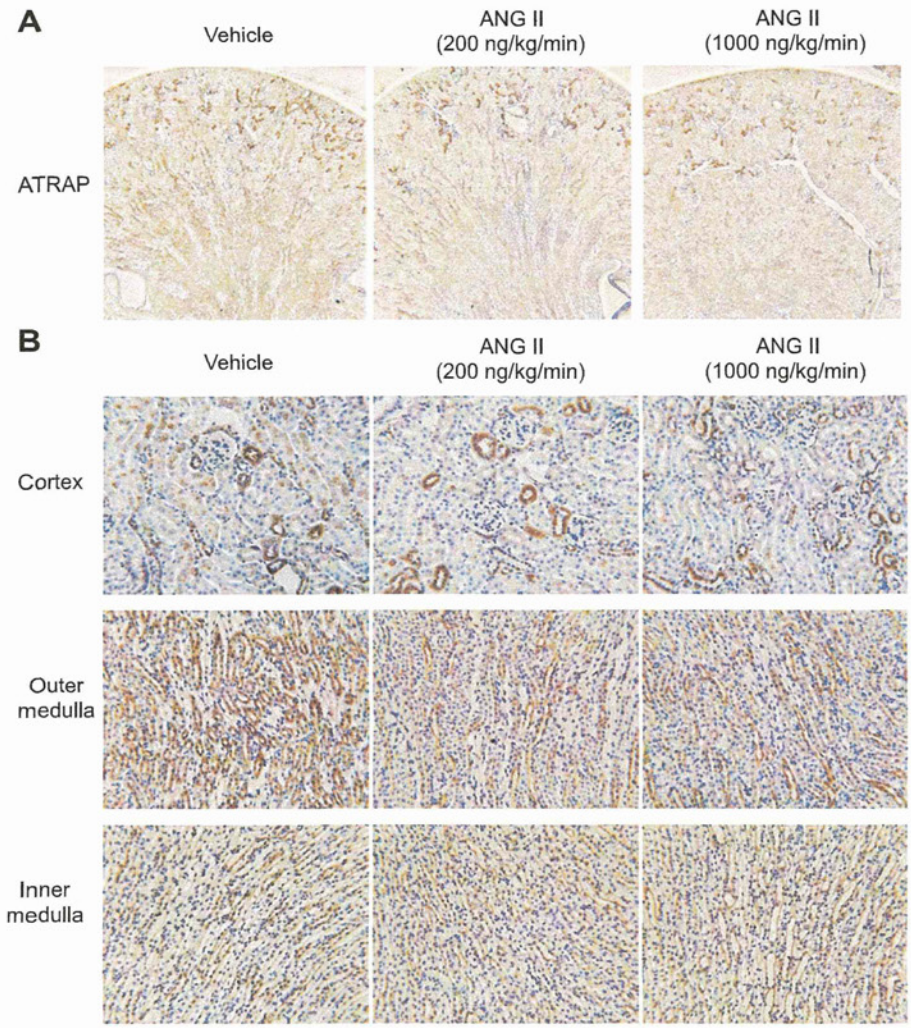

Fig. 4. Representative kidney sections showing the expression of total ATRAP protein in the kidney of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) for 14 days (*A*). Positive areas for ATRAP are evident as the brown dots in the sections. Higher magnification of the kidney sections show effects of continuous ANG II infusion on the immunohistochemical localization of ATRAP expression in the renal cortex, outer medulla, and inner medulla in mice treated with vehicle or ANG II (*B*). Original magnification: ×20 (*A*); ×200 (*B*).

dose in the ANG II-infused mice, without any significant change in the pattern of intrarenal distribution or levels of AT1R immunostaining (Fig. 6). Furthermore, the results of Western blot analysis using tissue extracts from the respective kidney regions confirmed the region-specific decrease in ATRAP protein expression in the outer medulla by chronic ANG II infusion (Fig. 7).

Effects of AT1R-specific blocker olmesartan on ANG II-mediated suppression of ATRAP expression in the kidney. We further examined whether the AT1R was responsible for the ANG II infusion-mediated intrarenal suppression of ATRAP expression using the AT1R-specific blocker olmesartan. Olmesartan treatment did not affect the BW gain in mice infused with the subpressor dose (200 ng·kg^{-1}·min^{-1}, % BW increase, 20.7 ± 1.7%, $n = 8$), but restored normal BW gain in the mice infused with the pressor dose (1,000 ng·kg^{-1}·min^{-1}) of ANG II (% BW increase, 18.1 ± 1.6%, $n = 8$, $P < 0.05$, ANG II 1,000 ng·kg^{-1}·min^{-1}+olmesartan vs. ANG II 1,000 ng·kg^{-1}·min^{-1}). Olmesartan treatment also inhibited the development of hypertension in the mice treated with the pressor dose (1,000 ng·kg^{-1}·min^{-1}) of ANG II (systolic blood pressure 104 ± 6 mmHg, $n = 6$, $P < 0.05$, ANG II 1,000 ng·kg^{-1}·min^{-1}+olmesartan vs. ANG II 1,000 ng·kg^{-1}·min^{-1}), while olmesartan did not affect systolic blood pressure in the mice infused with the subpressor dose (200 ng·kg^{-1}·min^{-1}, 101 ± 7 mmHg, $n = 6$). Furthermore, olmesartan treatment completely prevented the suppressive effects of either the presser or subpressor dose of ANG II on ATRAP protein expression in the kidney (Fig. 8). No significant changes were observed in AT1R protein expression in the kidney by olmesartan treatment.

Lack of any decrease in plasma membrane AT1R expression in the kidney by chronic ANG II infusion. The results in Fig. 2 show that ANG II stimulation led to a decrease in the levels of total ATRAP protein expression in the kidney, but not other tissues, including the testis. On the other hand, the total AT1R protein expression in all of the tissues examined was unchanged by ANG II treatment (Fig. 2). Thus, to examine whether ANG II-mediated suppression of intrarenal ATRAP expression affects cell surface AT1R expression in the kidney in response to ANG II stimulation, the plasma membrane fraction was specifically extracted from the kidney and testis, and the plasma membrane AT1R protein expression was analyzed.

In the testis, the ANG II infusion at the subpressor dose (200 ng·kg^{-1}·min^{-1}) tended to decrease the expression of the plasma membrane AT1R protein, and the pressor dose (1,000

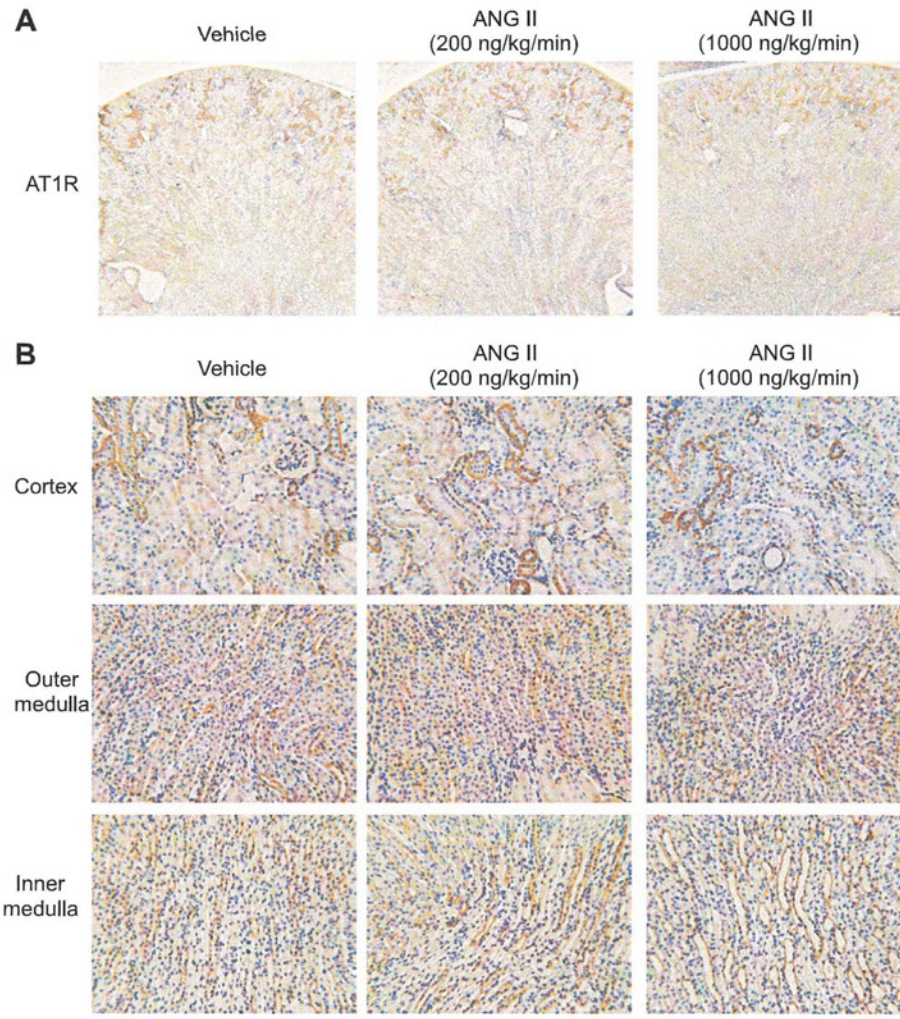

Fig. 5. Representative kidney sections showing the expression of the total AT1R protein in the kidney of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) for 14 days (*A*). Positive areas for the AT1R appear as the brown dots in the sections. Higher magnification of kidney sections showing the effects of continuous ANG II infusion on immunohistochemical localization of AT1R expression in the renal cortex, outer medulla, and inner medulla in mice treated with vehicle or ANG II (*B*). Original magnification: ×20 (*A*); ×200 (*B*).

ng·kg^{-1}·min^{-1}) significantly reduced the plasma membrane AT1R protein levels (Fig. 9*A*). Since olmesartan treatment completely prevented the ANG II-induced suppressive effects on the plasma membrane AT1R protein levels in the testis (Fig. 9*A*), these results indicated that ANG II stimulation promoted AT1R internalization. In the kidney, the plasma membrane AT1R protein levels for the subpressor and pressor doses in the ANG II-infused mice were comparable to those in the vehicle-infused mice and were not affected by olmesartan treatment (Fig. 9*B*).

Decrease in plasma membrane AT1R expression in the kidney of ATRAP transgenic mice. In terms of AT1R internalization in the kidney, although ANG II stimulation decreased the ATRAP protein level and olmesartan treatment recovered it to the baseline value (Fig. 8), the plasma membrane AT1R protein level was still unaltered (Fig. 9). We hypothesized that olmesartan-mediated recovery of the downregulated ATRAP expression back to the baseline level would be insufficient to promote AT1R internalization in the kidney and that an increased expression of renal ATRAP beyond the baseline level would promote AT1R internalization and decrease plasma membrane AT1R expression. Thus, to upregulate renal ATRAP expression, we newly generated ATRAP transgenic mice using HA-tagged mouse ATRAP cDNA subcloned into the pCAGGS expression vector to test these hypotheses (Fig. 10*A*) (28).

We used these ATRAP transgenic mice for the first time to analyze a putative function of ATRAP in vivo. Western blot analysis of ATRAP expression at the protein level revealed the highest renal expression level (= 3-fold) of ATRAP (HA-ATRAP) in line 19 (Tg19), among the three lines of ATRAP transgene positive (+) mice (Fig. 10*B*), and Tg19 was therefore used for further analysis. The results of real-time quantitative RT-PCR analysis also showed a 3.7-fold increase in the baseline renal ATRAP mRNA expression over littermate control mice (Wt) in the Tg19 mice (Fig. 10*C*). While the ATRAP (HA-ATRAP) protein expression in the kidney of Tg19 mice increased compared with Wt, the total kidney AT1R protein expression in Tg19 did not differ from that in Wt (Fig. 10*D*). On the other hand, the plasma membrane AT1R protein expression in the kidney of Tg19 was significantly decreased compared with Wt at baseline (Fig. 10*E*).

Promotion of AT1R internalization and inhibition of induced expression of angiotensinogen gene in response to ANG II in the kidney of ATRAP transgenic mice. With respect to the inhibitory effect of ANG II treatment on the renal ATRAP-to-

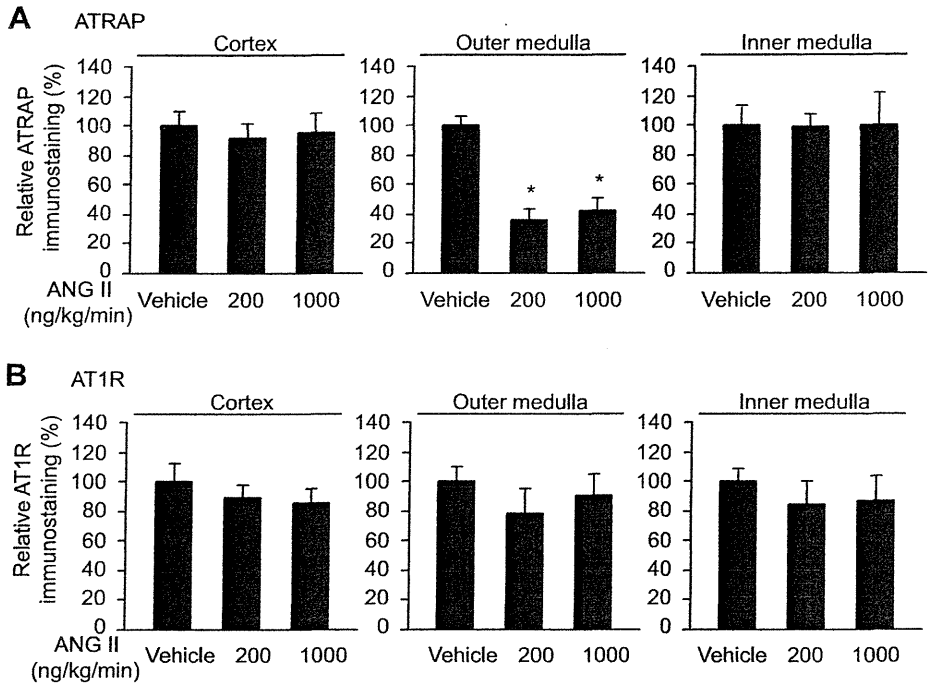

Fig. 6. Semiquantitative evaluation of the immunohistochemical analysis of ANG II-mediated effects on total ATRAP (*A*) and AT1R (*B*) protein expression in the renal cortex, outer medulla, and inner medulla in mice treated with vehicle or ANG II. The values were calculated relative to those obtained with extracts from mice infused with vehicle and are expressed as means ± SE. $*P < 0.05$ vs. vehicle.

AT1R ratio, while chronic ANG II infusion significantly decreased the ratio through a suppression of renal ATRAP expression in C57BL/6 wild-type mice (Fig. 2), ANG II treatment did not affect the ratio at all in Tg19 mice (Fig. 11*A*). Regarding AT1R internalization in the kidney, while the plasma membrane AT1R protein level was not affected by either chronic ANG II stimulation or olmesartan treatment in C57BL/6 wild-type mice (Figs. 8 and 9), it was significantly decreased by ANG II infusion in Tg19 (Fig. 11*B*), thereby indicating that enhancement of renal ATRAP expression beyond baseline promotes AT1R internalization.

Since the body size and BW of Tg19 mice were not different from the Wt at baseline (data not shown), we finally examined the physiological effects of overexpression of ATRAP in Tg19 with respect to blood pressure, response to ANG II, and target organ effects. The systolic blood pressure of Tg19 mice was comparable with that of Wt at baseline, and chronic ANG II infusion significantly and similarly increased systolic blood pressure in Tg19 and Wt (Fig. 11*C*). However, while ANG II infusion in Wt increased the angiotensinogen mRNA expression level in the kidney by 2.25-fold, the mRNA upregulation in response to ANG II infusion was significantly inhibited in Tg19 (Fig. 11*D*). These results indicate that the renal enhancement of ATRAP expression inhibits the ANG II-mediated activation of renal angiotensinogen gene expression, most likely through a promotion of AT1R internalization in response to ANG II.

DISCUSSION

The present data show that either a subpressor or pressor infusion of ANG II in mice causes a significant suppression of intrarenal ATRAP expression and that this response is dependent on the activation of AT1R. The decrease in intrarenal ATRAP expression during continuous ANG II infusion was demonstrated at the mRNA level by quantitative real-time RT-PCR, and at the protein level by Western blotting, and was supported by immunohistochemistry. In addition, the ANG II-mediated decrease in renal ATRAP expression through AT1R signaling occurred concomitantly with the lack of ANG II-induced decrease in plasma membrane AT1R expression in the kidney. Furthermore, a transgenic model increase in renal ATRAP expression beyond baseline expression was accompanied by a reduction in plasma membrane AT1R expression in the kidney, and by the promotion of renal AT1R internalization and the inhibition of an increase in renal angiotensinogen gene expression in response to ANG II.

Several previous studies have reported that activation of the intrarenal renin-angiotensin system and the AT1R pathway plays an important role in the pathogenesis of hypertension and renal injury (19, 25, 33). With respect to the mechanisms involved in ANG II-induced hypertension, the AT1R-mediated enhancement of renal angiotensinogen, collecting duct renin, intrarenal ANG II levels, medullary oxidative stress, and the failure to downregulate renal AT1R expression levels are all reported to be involved in the sustained effects of continuous ANG II elevation on eliciting hypertension (12, 13, 20, 23, 31, 50). Because the biological actions of ANG II are influenced by the AT1R expression levels, and ANG II infusion in mice specifically lacking AT1R in the kidney failed to develop hypertension (6), investigation of the renal activity of AT1R signaling in ANG II-induced hypertension is important to elucidate the mechanisms responsible for the cardiovascular and renal functional changes observed in this hypertension model.

We previously cloned ATRAP as a novel molecule which interacts with AT1R and showed that ATRAP suppressed ANG II-induced hypertrophic and proliferative responses of cardiovascular cells by inducing a constitutive internalization

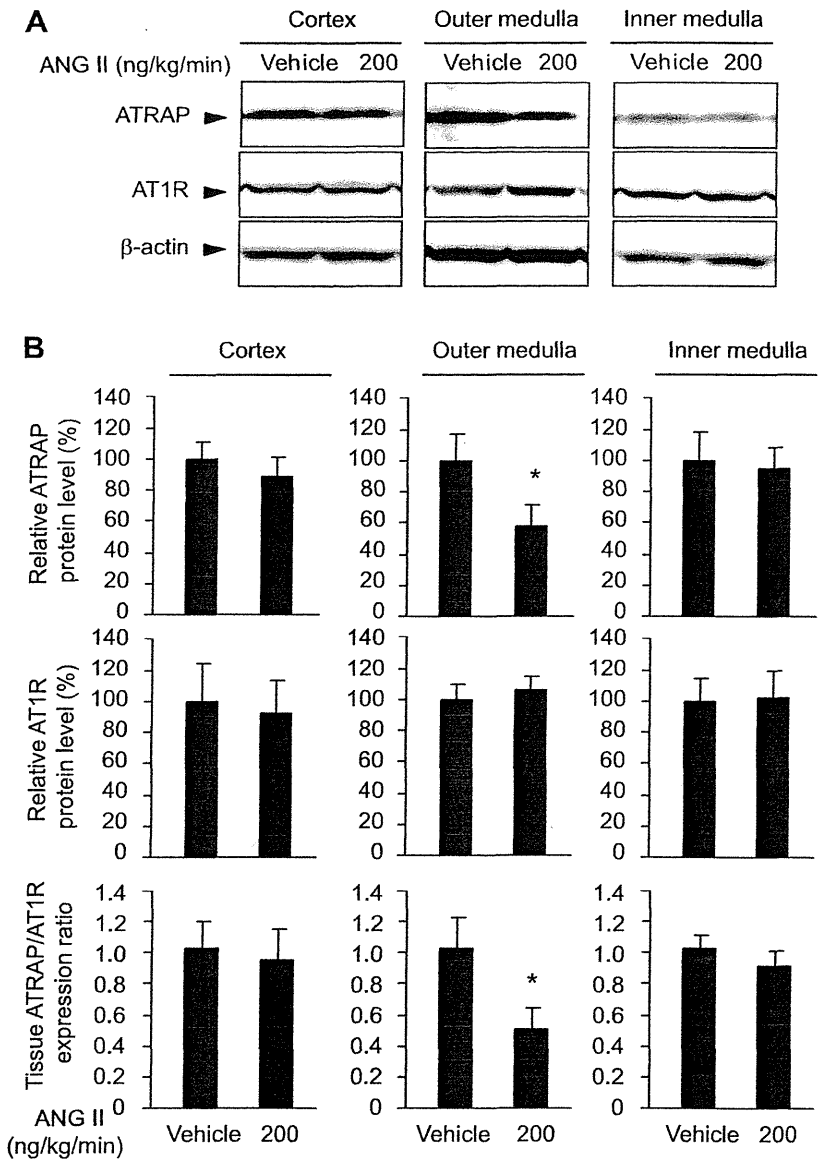

Fig. 7. Representative Western blots showing the effects of continuous ANG II infusion on the total protein expression of ATRAP and AT1R in the renal cortex, outer medulla, and inner medulla in mice infused with vehicle or ANG II (200 ng·kg^{-1}·min^{-1}) for 14 days (*A*). Measurement of the ATRAP-to-AT1R ratio was performed as described in METHODS (*B*). The values were calculated relative to those obtained with extracts from mice infused with vehicle and are expressed as means ± SE (n = 6/group). *$P < 0.05$ vs. vehicle.

of AT1R (22, 37). Thus a tissue-specific regulatory balancing of ATRAP and AT1R expression may be involved in the modulation of AT1R signaling in each tissue. We previously showed that ATRAP is expressed in a variety of mouse tissues, as is the AT1R, and that dietary salt intake modulates renal ATRAP expression (42). In this study, the expression of the hepatic and testicular ATRAP protein was not affected by continuous ANG II infusion. Although activation of the tissue renin-angiotensin system is important for the pathogenesis of hypertension and is associated with organ injury, the liver and testis are not target organs of hypertensive tissue injury. Our previous studies showed that the progression of hypertension did not affect hepatic angiotensinogen gene expression in genetically hypertensive rats, which is consistent with the results in the present study (38, 39).

In terms of the regulation of the intrarenal renin-angiotensin system by ANG II stimulation, previous studies by Navar and others (9, 20, 25, 27) established that ANG II is accumulated in the kidney of rats upon infusion, a response that is prevented by AT1R-specific blockers. Further evidence from experiments using rats suggests that AT1R-specific blockers decrease intrarenal ANG II levels by preventing AT1R-mediated uptake, as well as AT1R-mediated induction of intrarenal angiotensinogen, which is a substrate of ANG II (26). We previously showed that ATRAP is abundantly expressed and widely distributed along the renal tubules from Bowman's capsule to the inner medullary collecting ducts in mice (42). In this study, while continuous ANG II infusion did not have any apparent effects on renal total AT1R protein expression in C57BL/6 wild-type mice, which is consistent with previous reports using rats (12, 13), there was a significant decrease in renal ATRAP expression in ANG II-infused mice, and thereby a marked suppression of the renal expression ratio of ATRAP to AT1R at a subpressor dose of ANG II, even without an increase in blood pressure. This suppression of the renal ATRAP expression by ANG II is AT1R dependent, as it is prevented by treatment with olmesartan.

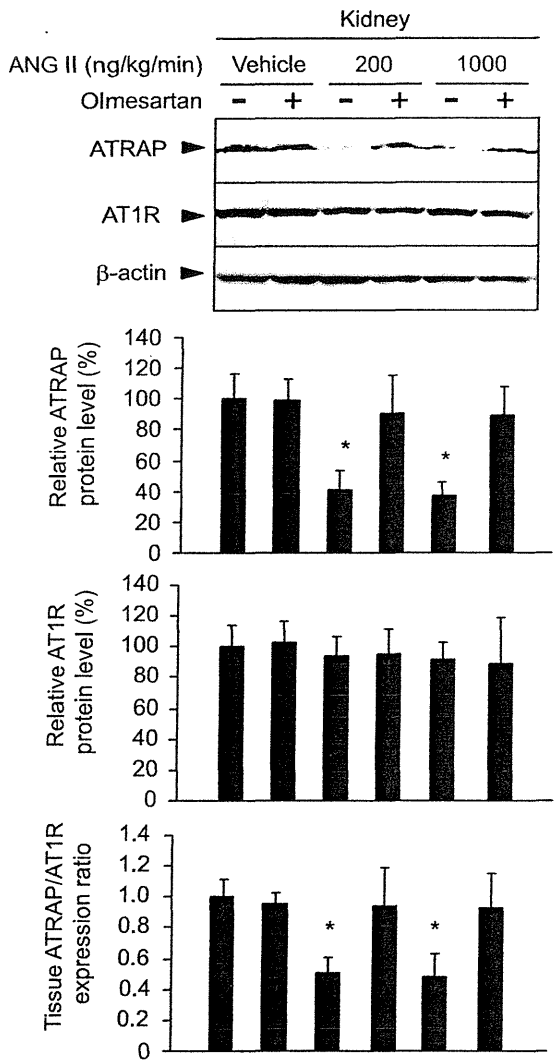

Fig. 8. Representative Western blots showing the effects of continuous ANG II infusion on the total protein expression of ATRAP and AT1R in the kidney of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) with or without olmesartan treatment (10 mg·kg^{-1}·day^{-1} in the drinking water) for 14 days. Measurement of the ATRAP-to-AT1R ratio was performed as described in METHODS. The values were calculated relative to those obtained with extracts from mice infused with vehicle without olmesartan and are expressed as means ± SE (n = 6/group). *P < 0.05 vs. vehicle without olmesartan.

Previous studies also showed that chronic ANG II stimulation in rats leads to the activation of the intrarenal renin-angiotensin system, with an augmentation of renal angiotensinogen expression (20), enhancement of oxidative stress through increases in NADPH oxidase activity (3, 4), and increases in sodium retention through an upregulation of α-ENaC expression (2). On the other hand, a previous study reported that the mouse kidney is relatively resistant to ANG II, including oxidative stress, compared with the rat kidney (45). In the present study, intrarenal angiotensinogen, NADPH oxidase, and α-ENaC mRNA expression was not significantly affected by ANG II infusion of either the subpressor (200 ng·kg^{-1}·min^{-1}) or pressor dose (1,000 ng·kg^{-1}·min^{-1}) for 2 wk, despite a decrease in renal ATRAP expression.

Thus we next employed a higher dose of ANG II (2,500 ng·kg^{-1}·min^{-1}) for 2 wk of treatment, which was recently shown to cause hypertension and renal injury even in mice (46), and showed that it did provoke progressive blood pressure increases and pathological renal responses, including elevated expression levels of renal angiotensinogen and α-ENaC genes, along with a concomitant decrease in renal ATRAP expression (Fig. 3). These observations suggest that a decrease in renal ATRAP expression might be a preceding renal marker of pathological responses to ANG II stimulation in vivo. Nevertheless, because ANG II infusion of the subpressor dose already exerted a down-regulatory effect on renal ATRAP expression without increases in the renal mRNA level of angiotensinogen and α-ENaC, there was a lack of any direct relationship between ATRAP and the expression of angio-

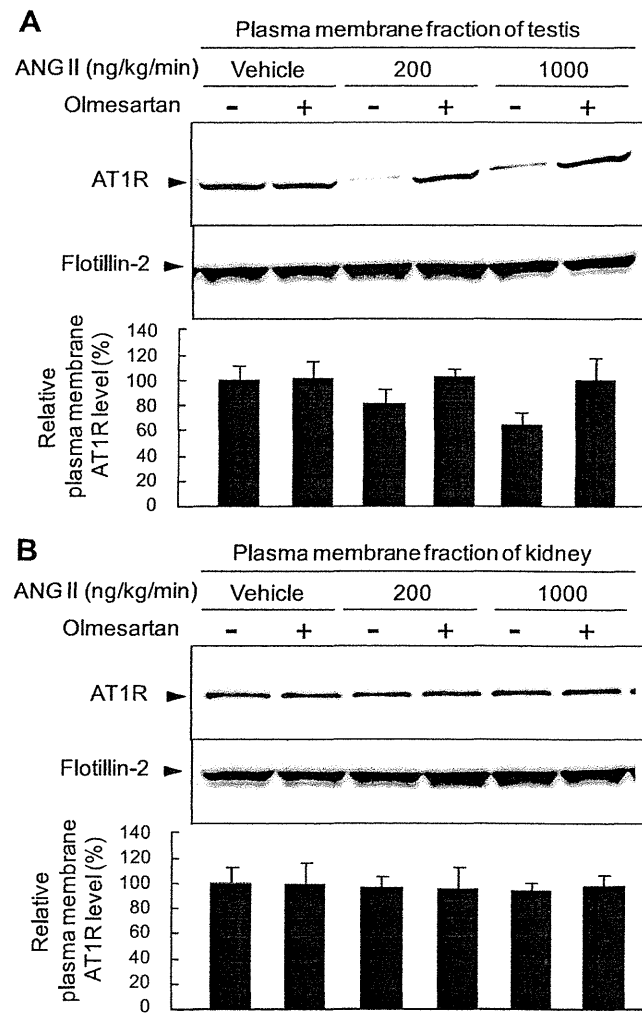

Fig. 9. Representative Western blots showing the effects of continuous ANG II infusion on the plasma membrane AT1R protein level in the tissues of mice infused with vehicle or ANG II (200 or 1,000 ng·kg^{-1}·min^{-1}) with or without olmesartan treatment (10 mg·kg^{-1}·day^{-1} in the drinking water) for 14 days [testis (*A*); kidney (*B*)]. Flotillin-2 is constitutively localized to the plasma membrane and is an internal control protein. The values were calculated relative to those using plasma membrane fractions from mice infused with vehicle without olmesartan and are expressed as means ± SE (n = 6/group). *P < 0.05 vs. vehicle without olmesartan.

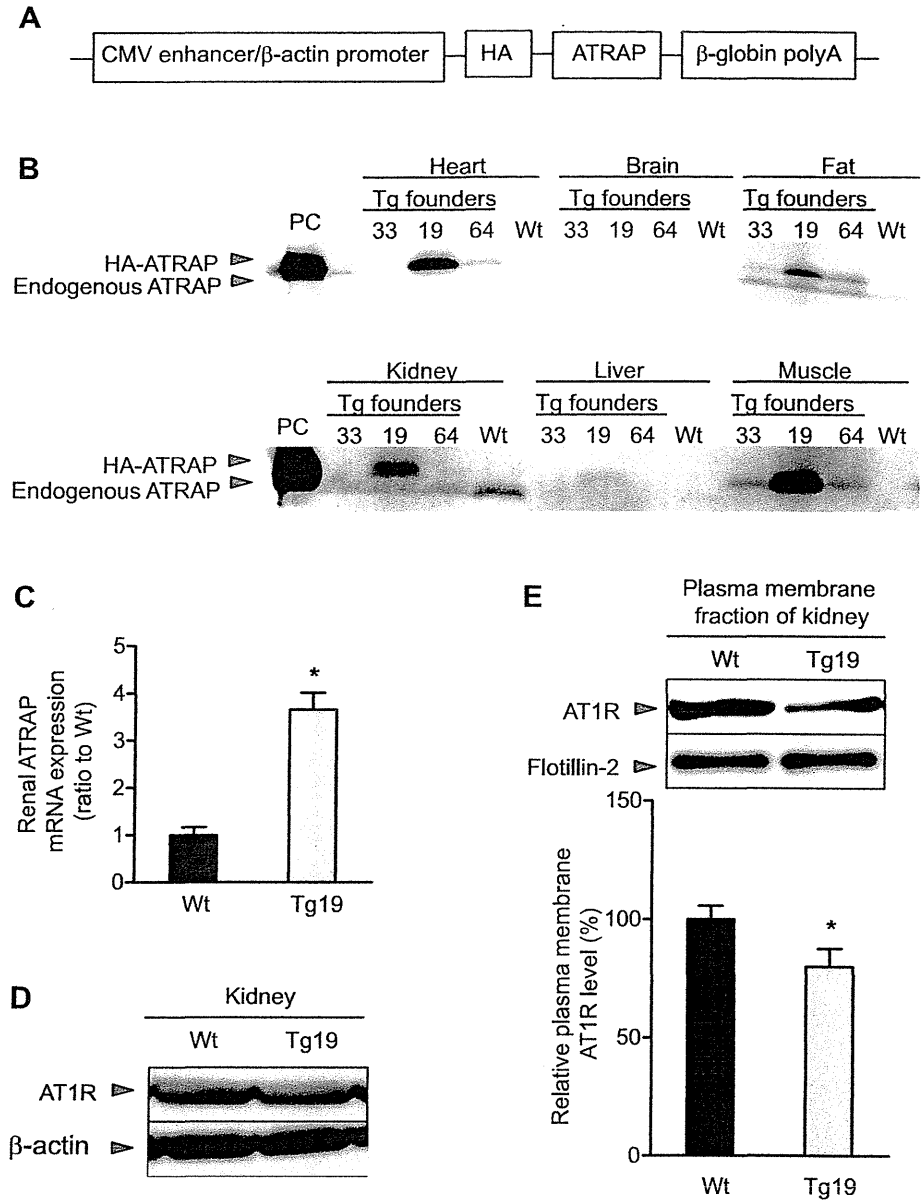

Fig. 10. Generation of ATRAP transgenic mice and decrease in the plasma membrane AT1R expression in the kidney. *A*: transgenic mice expressing ATRAP were generated on a C57BL/6J background with standard techniques. Briefly, the hemagglutinin (HA)-tagged mouse ATRAP cDNA was subcloned into the pCAGGS expression vector, which contained the cytomegalovirus (CMV) enhancer and chicken β-actin (CAG) promoter, and the resultant transgene construct was microinjected into the pronuclei of fertilized mouse embryos at the single-cell stage to generate transgenic mice (C57BL/6 strain). *B*: Western blot analysis of ATRAP expression at the protein level revealed the highest renal expression level (= 3-fold) of ATRAP (HA-ATRAP) in line 19 (Tg19), among the 3 lines of ATRAP transgene positive (+) mice obtained. Tg19 was used for further analysis in the present study. *C*: results of real-time quantitative RT-PCR analysis showed a 3.7-fold increase in the baseline renal ATRAP mRNA expression over littermate control mice (Wt) in ATRAP transgenic mice (Tg19). The values were calculated relative to those in kidneys from Wt and are expressed as means ± SE (n = 7/group). *$P < 0.05$ vs. Wt. *D*: results of Western blot analysis showed that the total kidney AT1R protein expression of Tg19 did not differ from that in Wt. *E*: results of Western blot analysis showed that the plasma membrane AT1R protein expression in the kidney of Tg19 was significantly decreased compared with Wt at baseline. The values were calculated relative to those from the plasma membrane fractions of Wt and are expressed as means ± SE (n = 9/group). *$P < 0.05$ vs. Wt.

tensinogen and α-ENaC genes in the kidney. Thus the results did not establish any causality or effect with respect to changes in renal ATRAP at this stage. Therefore, further investigation is needed to elucidate the exact molecular causal relationship between them.

The results of immunohistochemistry, including semiquantitative evaluation and Western blot analysis using the respective kidney regions, revealed a reduction of ATRAP expression in the outer medulla resulting from ANG II stimulation. Since previous studies showed that the outer medulla plays an important role in ANG II-mediated renal injury (23, 24), the suppression of renal ATRAP, particularly in the outer medullary region, may play a role in the renal pathological responses elicited by ANG II stimulation. While the intrarenal colocalization of ATRAP with AT1R suggests a functional role for ATRAP, it does not necessarily implicate ATRAP in electrolyte transport, renal injury, or hypertension, and the precise tubular function of ATRAP remains to be determined. The detailed molecular mechanism responsible for the tissue-specific AT1R-mediated suppression of ATRAP expression is still unclear. Further studies are necessary to determine the regulatory machinery of ATRAP gene transcription, including the transcription factors interacting with the promoter region of ATRAP gene and the functional effects of ATRAP on ANG II-mediated pathological responses using cultured renal tubular cells, and such studies are now underway.

Previous in vitro results suggested that ATRAP promotes AT1R internalization so as to inhibit AT1R signaling (37). In the present study, chronic ANG II infusion with either the a low or high dose caused significant suppression of endogenous ATRAP expression in the kidney, but not in the testis (Fig. 2).

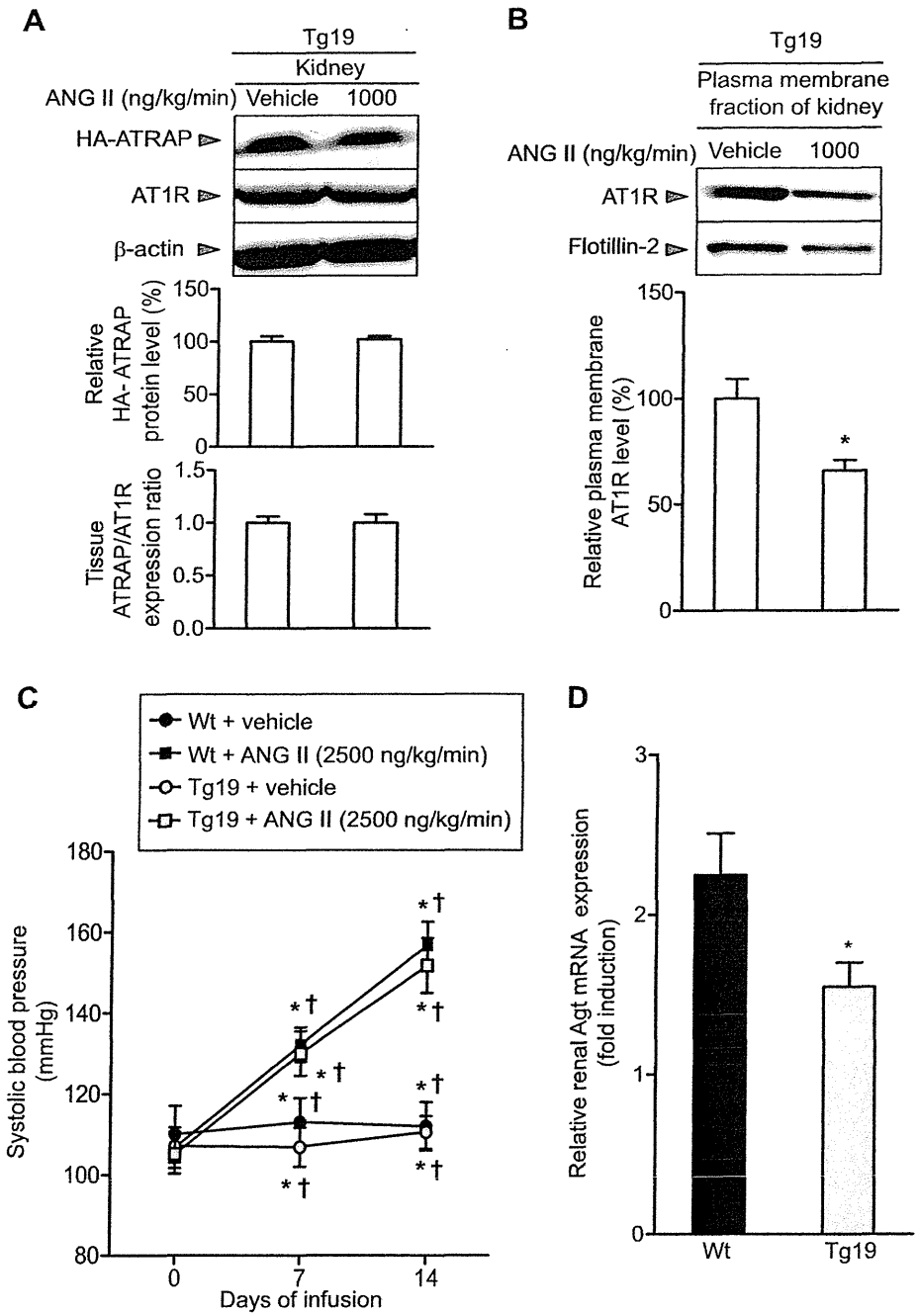

Fig. 11. Promotion of AT1R internalization and inhibition of induced expression of angiotensinogen gene in response to ANG II in the kidney of ATRAP transgenic mice. *A*: representative Western blots showing the effects of continuous ANG II infusion on the total protein expression of HA-ATRAP and AT1R in the kidney of ATRAP transgenic mice (Tg19) infused with vehicle or ANG II (1,000 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days. Measurement of the ATRAP-to-AT1R ratio was performed as described in METHODS, and the values were calculated relative to those in extracts from Tg19 infused with vehicle and are expressed as means ± SE (n = 6/group). *B*: representative Western blots showing the effects of ANG II infusion on the plasma membrane AT1R protein level in the kidney of Tg19 infused with vehicle or ANG II (1,000 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days. The values were calculated relative to those obtained with extracts from Tg19 infused with vehicle and are expressed as means ± SE (n = 6/group). *C*: effects of ANG II infusion on systolic blood pressure during the treatment period. Tg19 and littermate control mice (Wt) were infused with either vehicle or ANG II (2,500 $ng \cdot kg^{-1} \cdot min^{-1}$) for 14 days. The values of systolic blood pressure are expressed as means ± SE (n = 6/group). *$P < 0.05$ vs. vehicle. †$P < 0.05$ vs. *day 0*. *D*: effects of ANG II infusion on renal Agt mRNA expression in Wt and Tg19. Values are calculated as the fold-induction of those from extracts in the vehicle-infused mice and are expressed as means ± SE (n = 6/group). *$P < 0.05$ vs. Wt.

On the other hand, ANG II infusion did not alter plasma membrane AT1R expression in the kidney but significantly decreased it in the testis (Fig. 8), while total AT1R expression was not altered by ANG II in either of these tissues (Fig. 2). As a result, it is probable that testicular ATRAP promoted substantial ANG II-induced AT1R internalization, which was estimated by comparing the plasma membrane AT1R level with the total AT1R level.

In terms of AT1R internalization in the kidney, although ANG II downregulated ATRAP expression and olmesartan treatment recovered it to the baseline level (Fig. 8), the plasma membrane AT1R expression itself was not affected at all (Fig. 9), thereby still indicating the possibility that renal ATRAP exerts no effect on AT1R internalization in the kidney. We hypothesized that upregulation of renal ATRAP expression beyond baseline promotes AT1R internalization such that it is detected as a decrease in plasma membrane AT1R expression in the kidney without a change in the total AT1R protein level.

Thus, to examine the ATRAP-mediated effect on renal AT1R internalization by a different strategy in vivo, we produced ATRAP transgenic mice. The results demonstrated that enhancement of renal ATRAP expression in transgenic mice caused a decrease in the plasma membrane AT1R level, even at baseline without ANG II stimulation, irrespective of there

being no change in the total AT1R level in the kidney (Fig. 10). Furthermore, the results of ANG II infusion experiments showed that the plasma membrane AT1R level was further decreased by ANG II stimulation in the kidney of ATRAP transgenic mice (Fig. 11), which was accompanied by a decreased response of renal angiotensinogen mRNA expression to ANG II. This is in contrast to there being no change in the plasma membrane AT1R level by ANG II in the kidney of wild-type mice (Fig. 9). These results collectively suggest that enhancement of renal ATRAP expression beyond baseline promotes AT1R internalization in response to ANG II. A recent study by Oppermann et al. (29) also showed that a genetic deficiency of ATRAP in mice caused an enhanced surface expression of AT1R in the kidney, which is consistent with the results in this study.

The results of the present study show that continuous ANG II infusion decreased intrarenal ATRAP expression through ANG II-mediated AT1R signaling, particularly in the outer medulla, with the lack of any decrease in plasma membrane AT1R expression in the kidney in C57BL/6 wild-type mice. On the other hand, transgenic overexpression of ATRAP reduced the plasma membrane AT1R level in the kidney at baseline and further decreased the plasma membrane AT1R expression in response to ANG II stimulation, concomitant with the decreased ANG II-induced response of the angiotensinogen gene, despite there being no change in the total AT1R level.

Nevertheless, a limitation of the present study is that our findings strongly suggest that ATRAP at supraphysiological levels can alter the plasma membrane levels of AT1R at baseline and in response to ANG II stimulation, but with no detectable physiological effect on blood pressure. Target organ effects such as proteinuria and degree of renal damage by histological examination were not analyzed in this study. The only detectable consequence was a difference in angiotensinogen mRNA expression in response to ANG II stimulation, but it remains unclear whether this would translate to a difference in angiotensinogen protein levels.

In conclusion, these results suggest that AT1R and ATRAP modulate each other, at least in the kidney, and that activation of AT1R signaling has a dominant effect over endogenous renal ATRAP under chronic ANG II infusion, but that renal ATRAP activation by a transgenic model that increases ATRAP expression beyond baseline may cause a constitutive reduction of plasma membrane AT1R expression and a promotion of AT1R internalization in response to ANG II. Further studies to analyze downstream signaling events mediated by activation of AT1R under the condition of ATRAP overexpression are warranted to elucidate the detailed molecular mechanisms and pathophysiological significance of ATRAP-mediated inhibition of AT1R signaling in vivo.

ACKNOWLEDGMENTS

The authors thank Emi Maeda and Hiroko Morinaga for technical assistance and helpful discussion. The authors also thank Dr. Kevin Boru for English editing of this manuscript.

GRANTS

This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, and Culture, by a Health and Labor Sciences Research grant, and by grants from the Salt Science Research Foundation (1033), the Mitsubishi Pharma Research Foundation, and the Strategic Research Project of Yokohama City University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. **Azuma K, Tamura K, Shigenaga A, Wakui H, Masuda S, Tsurumi-Ikeya Y, Tanaka Y, Sakai M, Matsuda M, Hashimoto T, Ishigami T, Lopez-Ilasaca M, Umemura S.** Novel regulatory effect of angiotensin II type 1 receptor-interacting molecule on vascular smooth muscle cells. *Hypertension* 50: 926–932, 2007.
2. **Beutler KT, Masilamani S, Turban S, Nielsen J, Brooks HL, Ageloff S, Fenton RA, Packer RK, Knepper MA.** Long-term regulation of ENaC expression in kidney by angiotensin II. *Hypertension* 41: 1143–1150, 2003.
3. **Block K, Eid A, Griendling KK, Lee DY, Wittrant Y, Gorin Y.** Nox4 NAD(P)H oxidase mediates Src-dependent tyrosine phosphorylation of PDK-1 in response to angiotensin II: role in mesangial cell hypertrophy and fibronectin expression. *J Biol Chem* 283: 24061–24076, 2008.
4. **Chabrashvili T, Kitiyakara C, Blau J, Karber A, Aslam S, Welch WJ, Wilcox CS.** Effects of ANG II type 1 and 2 receptors on oxidative stress, renal NADPH oxidase, and SOD expression. *Am J Physiol Regul Integr Comp Physiol* 285: R117–R124, 2003.
5. **Coffman TM, Crowley SD.** Kidney in hypertension: guyton redux. *Hypertension* 51: 811–816, 2008.
6. **Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, Coffman TM.** Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci USA* 103: 17985–17990, 2006.
7. **Cui T, Nakagami H, Iwai M, Takeda Y, Shiuchi T, Tamura K, Daviet L, Horiuchi M.** ATRAP, novel AT1 receptor associated protein, enhances internalization of AT1 receptor and inhibits vascular smooth muscle cell growth. *Biochem Biophys Res Commun* 279: 938–941, 2000.
8. **Daviet L, Lehtonen JY, Tamura K, Griese DP, Horiuchi M, Dzau VJ.** Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J Biol Chem* 274: 17058–17062, 1999.
9. **Gonzalez-Villalobos RA, Satou R, Seth DM, Semprun-Prieto LC, Katsurada A, Kobori H, Navar LG.** Angiotensin-converting enzyme-derived angiotensin II formation during angiotensin II-induced hypertension. *Hypertension* 53: 351–355, 2009.
10. **Gonzalez-Villalobos RA, Seth DM, Satou R, Horton H, Ohashi N, Miyata K, Katsurada A, Tran DV, Kobori H, Navar LG.** Intrarenal angiotensin II and angiotensinogen augmentation in chronic angiotensin II-infused mice. *Am J Physiol Renal Physiol* 295: F772–F779, 2008.
11. **Guo S, Lopez-Ilasaca M, Dzau VJ.** Identification of calcium-modulating cyclophilin ligand (CAML) as transducer of angiotensin II-mediated nuclear factor of activated T cells (NFAT) activation. *J Biol Chem* 280: 12536–12541, 2005.
12. **Harrison-Bernard LM, El-Dahr SS, O'Leary DF, Navar LG.** Regulation of angiotensin II type 1 receptor mRNA and protein in angiotensin II-induced hypertension. *Hypertension* 33: 340–346, 1999.
13. **Harrison-Bernard LM, Zhuo J, Kobori H, Ohishi M, Navar LG.** Intrarenal AT_1 receptor and ACE binding in ANG II-induced hypertensive rats. *Am J Physiol Renal Physiol* 282: F19–F25, 2002.
14. **Hirose T, Satoh D, Kurihara H, Kusaka C, Hirose H, Akimoto K, Matsusaka T, Ichikawa I, Noda T, Ohno S.** An essential role of the universal polarity protein, aPKClambda, on the maintenance of podocyte slit diaphragms. *PLoS One* 4: e4194, 2009.
15. **Hong SW, Isono M, Chen S, Iglesias-De La Cruz MC, Han DC, Ziyadeh FN.** Increased glomerular and tubular expression of transforming growth factor-beta1, its type II receptor, and activation of the Smad signaling pathway in the db/db mouse. *Am J Pathol* 158: 1653–1663, 2001.
16. **Hunyady L, Bor M, Balla T, Catt KJ.** Identification of a cytoplasmic Ser-Thr-Leu motif that determines agonist-induced internalization of the AT1 angiotensin receptor. *J Biol Chem* 269: 31378–31382, 1994.
17. **Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, Katsuragi T.** Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^+/Ca^{2+} exchanger type-1 in vascular smooth muscle. *Nat Med* 10: 1193–1199, 2004.

18. **Kagiyama S, Matsumura K, Fukuhara M, Sakagami K, Fujii K, Iida M.** Aldosterone-and-salt-induced cardiac fibrosis is independent from angiotensin II type 1a receptor signaling in mice. *Hypertens Res* 30: 979–989, 2007.
19. **Kobori H, Nangaku M, Navar LG, Nishiyama A.** The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 59: 251–287, 2007.
20. **Kobori H, Prieto-Carrasquero MC, Ozawa Y, Navar LG.** AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension* 43: 1126–1132, 2004.
21. **Lopez-Ilasaca M, Liu X, Tamura K, Dzau VJ.** The angiotensin II type I receptor-associated protein, ATRAP, is a transmembrane protein and a modulator of angiotensin II signaling. *Mol Biol Cell* 14: 5038–5050, 2003.
22. **Mogi M, Iwai M, Horiuchi M.** Emerging concepts of regulation of angiotensin II receptors: new players and targets for traditional receptors. *Arterioscler Thromb Vasc Biol* 27: 2532–2539, 2007.
23. **Mori T, Cowley AW Jr.** Angiotensin II-NAD(P)H oxidase-stimulated superoxide modifies tubulovascular nitric oxide cross-talk in renal outer medulla. *Hypertension* 42: 588–593, 2003.
24. **Mori T, O'Connor PM, Abe M, Cowley AW Jr.** Enhanced superoxide production in renal outer medulla of Dahl salt-sensitive rats reduces nitric oxide tubular-vascular cross-talk. *Hypertension* 49: 1336–1341, 2007.
25. **Navar LG, Harrison-Bernard LM, Nishiyama A, Kobori H.** Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39: 316–322, 2002.
26. **Nishiyama A, Nakagawa T, Kobori H, Nagai Y, Okada N, Konishi Y, Morikawa T, Okumura M, Meda I, Kiyomoto H, Hosomi N, Mori T, Ito S, Imanishi M.** Strict angiotensin blockade prevents the augmentation of intrarenal angiotensin II and podocyte abnormalities in type 2 diabetic rats with microalbuminuria. *J Hypertens* 26: 1849–1859, 2008.
27. **Nishiyama A, Seth DM, Navar LG.** Angiotensin II type 1 receptor-mediated augmentation of renal interstitial fluid angiotensin II in angiotensin II-induced hypertension. *J Hypertens* 21: 1897–1903, 2003.
28. **Niwa H, Yamamura K, Miyazaki J.** Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–199, 1991.
29. **Oppermann M, Gess B, Schweda F, Castrop H.** Atrap deficiency increases arterial blood pressure and plasma volume. *J Am Soc Nephrol* 21: 468–477, 2010.
30. **Oshita A, Iwai M, Chen R, Ide A, Okumura M, Fukunaga S, Yoshii T, Mogi M, Higaki J, Horiuchi M.** Attenuation of inflammatory vascular remodeling by angiotensin II type 1 receptor-associated protein. *Hypertension* 48: 671–676, 2006.
31. **Prieto-Carrasquero MC, Kobori H, Ozawa Y, Gutierrez A, Seth D, Navar LG.** AT_1 receptor-mediated enhancement of collecting duct renin in angiotensin II-dependent hypertensive rats. *Am J Physiol Renal Physiol* 289: F632–F637, 2005.
32. **Reich HN, Oudit GY, Penninger JM, Scholey JW, Herzenberg AM.** Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. *Kidney Int* 74: 1610–1616, 2008.
33. **Rohrwasser A, Morgan T, Dillon HF, Zhao L, Callaway CW, Hillas E, Zhang S, Cheng T, Inagami T, Ward K, Terreros DA, Lalouel JM.** Elements of a paracrine tubular renin-angiotensin system along the entire nephron. *Hypertension* 34: 1265–1274, 1999.
34. **Sakai M, Tamura K, Tsurumi Y, Tanaka Y, Koide Y, Matsuda M, Ishigami T, Yabana M, Tokita Y, Hiroi Y, Komuro I, Umemura S.** Expression of MAK-V/Hunk in renal distal tubules and its possible involvement in proliferative suppression. *Am J Physiol Renal Physiol* 292: F1526–F1536, 2007.
35. **Shigenaga A, Tamura K, Wakui H, Masuda S, Azuma K, Tsurumi-Ikeya Y, Ozawa M, Mogi M, Matsuda M, Uchino K, Kimura K, Horiuchi M, Umemura S.** Effect of olmesartan on tissue expression balance between angiotensin II receptor and its inhibitory binding molecule. *Hypertension* 52: 672–678, 2008.
36. **Solis GP, Hoegg M, Munderloh C, Schrock Y, Malaga-Trillo E, Rivera-Milla E, Stuermer CA.** Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. *Biochem J* 403: 313–322, 2007.
37. **Tamura K, Tanaka Y, Tsurumi Y, Azuma K, Shigenaga A, Wakui H, Masuda S, Matsuda M.** The role of angiotensin AT1 receptor-associated protein in renin-angiotensin system regulation and function. *Curr Hypertens Rep* 9: 121–127, 2007.
38. **Tamura K, Umemura S, Nyui N, Yamakawa T, Yamaguchi S, Ishigami T, Tanaka S, Tanimoto K, Takagi N, Sekihara H, Murakami K, Ishii M.** Tissue-specific regulation of angiotensinogen gene expression in spontaneously hypertensive rats. *Hypertension* 27: 1216–1223, 1996.
39. **Tamura K, Umemura S, Yamakawa T, Nyui N, Hibi K, Watanabe Y, Ishigami T, Yabana M, Tanaka S, Sekihara H, Murakami K, Ishii M.** Modulation of tissue angiotensinogen gene expression in genetically obese hypertensive rats. *Am J Physiol Regul Integr Comp Physiol* 272: R1704–R1711, 1997.
40. **Tanaka Y, Tamura K, Koide Y, Sakai M, Tsurumi Y, Noda Y, Umemura M, Ishigami T, Uchino K, Kimura K, Horiuchi M, Umemura S.** The novel angiotensin II type 1 receptor (AT1R)-associated protein ATRAP downregulates AT1R and ameliorates cardiomyocyte hypertrophy. *FEBS Lett* 579: 1579–1586, 2005.
41. **Tang H, Guo DF, Porter JP, Wanaka Y, Inagami T.** Role of cytoplasmic tail of the type 1A angiotensin II receptor in agonist- and phorbol ester-induced desensitization. *Circ Res* 82: 523–531, 1998.
42. **Tsurumi Y, Tamura K, Tanaka Y, Koide Y, Sakai M, Yabana M, Noda Y, Hashimoto T, Kihara M, Hirawa N, Toya Y, Kiuchi Y, Iwai M, Horiuchi M, Umemura S.** Interacting molecule of AT1 receptor, ATRAP, is colocalized with AT1 receptor in the mouse renal tubules. *Kidney Int* 69: 488–494, 2006.
43. **Vila-Carriles WH, Kovacs GG, Jovov B, Zhou ZH, Pahwa AK, Colby G, Esimai O, Gillespie GY, Mapstone TB, Markert JM, Fuller CM, Bubien JK, Benos DJ.** Surface expression of ASIC2 inhibits the amiloride-sensitive current and migration of glioma cells. *J Biol Chem* 281: 19220–19232, 2006.
44. **Wakui H, Tamura K, Tanaka Y, Matsuda M, Bai Y, Dejima T, Masuda S, Shigenaga A, Maeda A, Mogi M, Ichihara N, Kobayashi Y, Hirawa N, Ishigami T, Toya Y, Yabana M, Horiuchi M, Minamisawa S, Umemura S.** Cardiac-specific activation of angiotensin II type 1 receptor-associated protein completely suppresses cardiac hypertrophy in chronic angiotensin II-infused mice. *Hypertension* 55: 1157–1164, 2010.
45. **Wesseling S, Ishola DA Jr, Joles JA, Bluyssen HA, Koomans HA, Braam B.** Resistance to oxidative stress by chronic infusion of angiotensin II in mouse kidney is not mediated by the AT_2 receptor. *Am J Physiol Renal Physiol* 288: F1191–F1200, 2005.
46. **Wolak T, Kim H, Ren Y, Kim J, Vaziri ND, Nicholas SB.** Osteopontin modulates angiotensin II-induced inflammation, oxidative stress, and fibrosis of the kidney. *Kidney Int* 76: 32–43, 2009.
47. **Ye M, Wysocki J, William J, Soler MJ, Cokic I, Batlle D.** Glomerular localization and expression of angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. *J Am Soc Nephrol* 17: 3067–3075, 2006.
48. **Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupershmidt S, Roden DM, Wagner T, Yatani A, Vatner DE, Vatner SF, Sadoshima J.** Cardiac-specific overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest* 115: 3045–3056, 2005.
49. **Zhuo JL, Imig JD, Hammond TG, Orengo S, Benes E, Navar LG.** Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: role of AT_1 receptor. *Hypertension* 39: 116–121, 2002.
50. **Zou LX, Imig JD, von Thun AM, Hymel A, Ono H, Navar LG.** Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. *Hypertension* 28: 669–677, 1996.

Am J Physiol Renal Physiol 299: F720–F731, 2010.
First published August 4, 2010; doi:10.1152/ajprenal.00667.2009.

Expression of angiotensin II type 1 receptor-interacting molecule in normal human kidney and IgA nephropathy

Shin-ichiro Masuda,[1]* Kouichi Tamura,[1]* Hiromichi Wakui,[1] Akinobu Maeda,[1] Toru Dejima,[1] Tomonori Hirose,[2] Masao Toyoda,[3] Koichi Azuma,[1] Masato Ohsawa,[1] Tomohiko Kanaoka,[1] Mai Yanagi,[1] Shin-ichiro Yoshida,[1] Hiroshi Mitsuhashi,[1] Miyuki Matsuda,[1] Tomoaki Ishigami,[1] Yoshiyuki Toya,[1] Daisuke Suzuki,[3] Yoji Nagashima,[4] and Satoshi Umemura[1]

[1]*Department of Medical Science and Cardiorenal Medicine,* [2]*Department of Molecular Biology, and* [4]*Department of Molecular Pathology, Yokohama City University Graduate School of Medicine, Yokohama; and* [3]*Division of Nephrology and Metabolism, Department of Internal Medicine, Tokai University School of Medicine, Isehara, Japan*

Submitted 23 November 2009; accepted in final form 30 July 2010

Masuda S, Tamura K, Wakui H, Maeda A, Dejima T, Hirose T, Toyoda M, Azuma K, Ohsawa M, Kanaoka T, Yanagi M, Yoshida S, Mitsuhashi H, Matsuda M, Ishigami T, Toya Y, Suzuki D, Nagashima Y, Umemura S. Expression of angiotensin II type 1 receptor-interacting molecule in normal human kidney and IgA nephropathy. *Am J Physiol Renal Physiol* 299: F720–F731, 2010. First published August 4, 2010; doi:10.1152/ajprenal.00667.2009.—The intrarenal renin-angiotensin system plays a crucial role in the regulation of renal circulation and sodium reabsorption through the activation of vascular, glomerular, and tubular angiotensin II type 1 (AT_1) receptor signaling. We previously cloned a molecule that specifically interacted with the murine AT_1 receptor to inhibit AT_1 receptor signaling, which we named ATRAP (for AT_1 receptor-associated protein). Since murine ATRAP was shown to be highly expressed in the kidney, in the present study we investigated expression and distribution of human ATRAP in normal kidney and renal biopsy specimens from patients with IgA nephropathy. In the normal human kidney, both ATRAP mRNA and protein were widely and abundantly distributed along the renal tubules from Bowman's capsule to the medullary collecting ducts. In all renal tubular epithelial cells, the ATRAP protein colocalized with the AT_1 receptor. In renal biopsy specimens with IgA nephropathy, a significant positive correlation between ATRAP and AT_1 receptor gene expression was observed. There was also a positive relationship between tubulointerstitial ATRAP expression and the estimated glomerular filtration rate in patients with IgA nephropathy. Furthermore, we examined the function of the tubular AT_1 receptor using an immortalized cell line of mouse distal convoluted tubule cells (mDCT) and found that overexpression of ATRAP by adenoviral gene transfer suppressed the angiotensin II-mediated increases in transforming growth factor-β production in mDCT cells. These findings suggest that ATRAP might play a role in balancing the renal renin-angiotensin system synergistically with the AT_1 receptor by counterregulatory effects in IgA nephropathy and propose an antagonistic effect of tubular ATRAP on AT_1 receptor signaling.

renin-angiotensin system; immunohistochemistry; renal biopsy

THE RENIN-ANGIOTENSIN SYSTEM plays a critical role in the regulation of blood pressure and the maintenance of water-electrolyte metabolism. It acts through the production of the bioactive molecule angiotensin II (ANG II), exerting direct effects on the constriction of blood vessels, tubular sodium reabsorption, and the release of aldosterone. In addition, activation of the renin-angiotensin system at local sites is involved in the pathogenesis of hypertension and related target organ damage, as well as the development of renal inflammatory and fibrotic disease. For these actions of ANG II at local tissue sites, the ANG II type 1 (AT_1) receptor is the main receptor subtype. It is distributed in a variety of tissues, including the heart, brain, artery, adrenal gland, and kidney.

The AT_1 receptor is a member of the G protein-coupled receptor superfamily, having a seven-transmembrane spanning structure which activates G proteins through the third intracellular loop and the intracellular carboxyl-terminal (C-terminal) tail of the receptor (23, 24). The C-terminal cytoplasmic domain of the AT_1 receptor is involved in the control of AT_1 receptor internalization and plays an important role in linking receptor-mediated signal transduction to the specific biological responses to ANG II, such as hypertension, cardiovascular remodeling, and renal injury (12, 21). To further investigate the pathophysiological molecular mechanisms of AT_1 receptor signaling, we performed a yeast two-hybrid system screening of a murine kidney cDNA library. We identified the molecule angiotensin II type 1 receptor-associated protein (ATRAP), which interacts specifically with the C-terminal cytoplasmic domain of the AT_1 receptor (4). Murine ATRAP is predicted to have a three-transmembrane domain structure and is localized to the plasma membrane and intracellular trafficking vesicles in cultured cells (1, 18, 33). Functionally, several in vitro studies have shown that overexpression of murine ATRAP induces the internalization of the AT_1 receptor and decreases both the generation of inositol lipids and the cellular hypertrophic and proliferative responses, suggesting that ATRAP may be a negative regulator of AT_1 receptor signaling (1, 18, 33).

With respect to a human homolog of murine ATRAP, a previous study reported the cloning of human ATRAP cDNA, with its DNA and amino acid sequences 85 and 75% identical to the murine ATRAP gene, respectively (37). Human ATRAP was also shown to specifically interact with the C terminal of the human AT_1 receptor in vitro (37). To date, almost all of the studies on the physiological roles of the AT_1 receptor in the kidney have been performed in rat or mouse models, and there have been few studies on the distribution and function of the AT_1 receptor in the human kidney (15). A few previous studies showed that AT_1 receptor immunohistochemical staining was broadly observed in the blood vessels, renal tubules, and glomeruli in the normal human kidney and disclosed a

* S.-I. Masuda and K. Tamura contributed equally to this work.

Address for reprint requests and other correspondence: K. Tamura, Dept. of Medical Science and Cardiorenal Medicine, Yokohama City Univ. Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan (e-mail: tamukou@med.yokohama-cu.ac.jp).

marginal increase in immunostaining for the AT_1 receptor in renal tubules in biopsy specimens from patients with IgA nephropathy (3, 5, 22).

Furthermore, at present there is no information available concerning the distribution of ATRAP in the normal and diseased human kidneys. Thus, to explore the possible pathophysiological role of ATRAP in human kidney disease, it is important to examine the expression of ATRAP in human renal tissues. Therefore, in the present study we examined the precise localization of renal human ATRAP expression at the mRNA and protein level by in situ hybridization and immunohistochemistry to determine whether the ATRAP protein coincided with the AT_1 receptor in the normal human kidney. Furthermore, we evaluated the relationship between ATRAP and AT_1 receptor protein expression, as well as the relationship between clinical variables and renal tissue expression of ATRAP and AT_1 receptor proteins in biopsy specimens from patients with IgA nephropathy.

METHODS

Materials. ANG II was purchased from Sigma. The AT_1 receptor-specific blocker candesartan (CV11974) and ANG II type 2 receptor (AT_2 receptor)-specific blocker PD123319 were kindly supplied by Takeda Pharmaceutical Company and the Parke-Davis division of Pfizer, respectively.

Cell culture and transient transfection. The immortalized cell line H9c2, which expresses the endogenous AT_1 receptor, was cultured as described previously (33). The human ATRAP cDNA expression plasmid (PCMV6XL5), the NH_2-terminal heme agglutinin (HA) epitope-tagged murine ATRAP in pcDNA3 (pc-HA-mATRAP), or the empty vector pCAGGS plasmid was transiently transfected into H9c2 cells according to the Lipofectamine 2000 protocol (Invitrogen) (4). Forty-eight hours after the transfection, whole cellular extracts were prepared from the transfected cells (18).

Mouse distal convoluted tubule (mDCT) cells were kindly provided by Dr. Peter A. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA). The cells had been previously isolated and functionally characterized as described (9). Cells were grown on 100-mm dishes (Corning) in DMEM/HAM F-12 media (1:1, Sigma-Aldrich) supplemented with 5% heat-inactivated fetal calf serum (MBL), 2 mM L-glutamine (GIBCO), 0.5 mg/ml streptomycin, 0.5 mg/ml penicillin, and 1 mg/ml neomycin (GIBCO), in a humidified atmosphere of 5% CO_2-95% air. For the stimulation with ANG II, mDCT cells were subcultured in 6-cm-diameter dishes (1.5×10^5/ml), incubated overnight, and further incubated in serum-free medium for 24 h.

Preparation of normal human renal tissue and biopsy specimens with IgA nephropathy. Normal, uninvolved human male renal tissue ($n = 1$) obtained from resected renal carcinoma kidneys was perfusion-fixed with 4% paraformaldehyde and used for in situ hybridization and immunohistochemistry. Needle renal biopsy specimens were also obtained from 22 patients with IgA nephropathy and were used for immunohistochemistry. The diagnosis of IgA nephropathy was

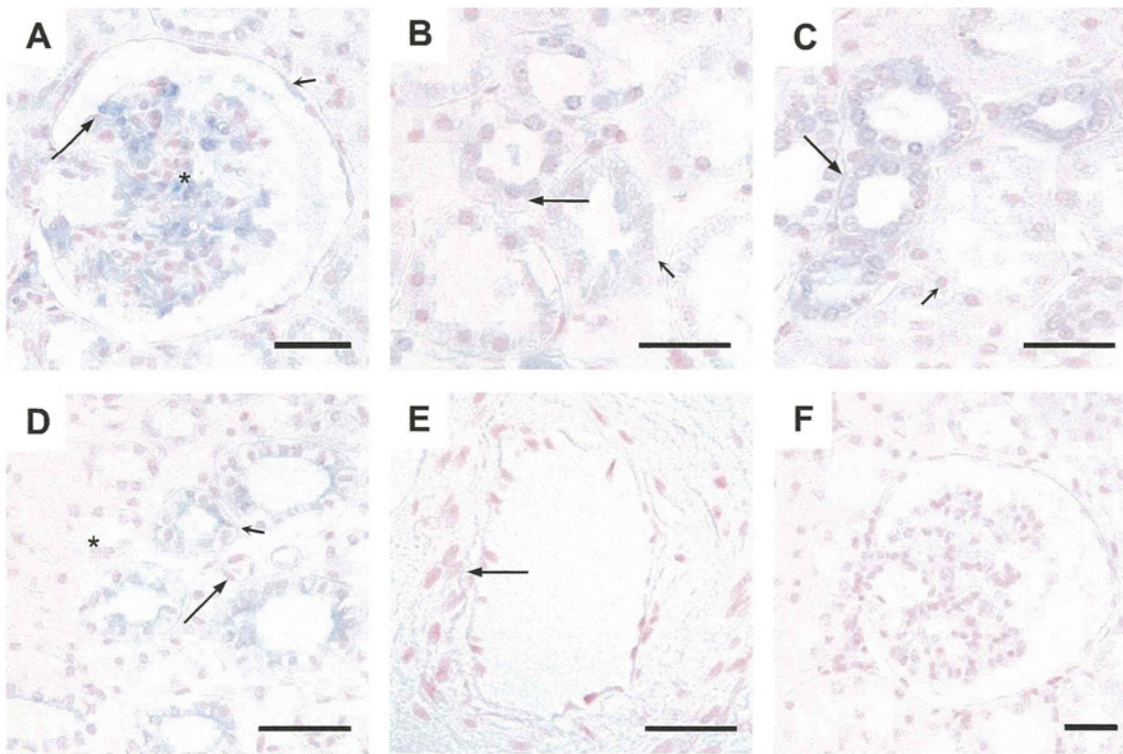

Fig. 1. Localization of ANG II type 1 (AT_1) receptor-associated protein (ATRAP) mRNA in a normal human kidney by in situ hybridization. *A*: glomerular section showing human ATRAP mRNA (positive staining blue) in Bowman's capsule (short arrow), podocytes (long arrow), and mesangial cells (asterisk). *B*: cortical section showing ATRAP in the proximal convoluted tubule (PCT; short arrow) and distal convoluted tubule (DCT; long arrow). *C*: cortical section showing ATRAP in the proximal straight tubule (PST; short arrow) and cortical collecting duct (CCD; long arrow). *D*: medullary section showing ATRAP in the medullary collecting duct (MCD; short arrow), thin limb (TL; long arrow), and thick ascending limb of Henle's loop (TAL; asterisk). *E*: interlobular artery showing weak ATRAP mRNA staining. *F*: consecutive section of that in *A* hybridized with the ATRAP sense probe as a negative control. ATRAP mRNA-positive staining is shown in blue, nuclear staining in pink, and overlapped staining in purple. Original magnification ×400. Bars = 50 μm.

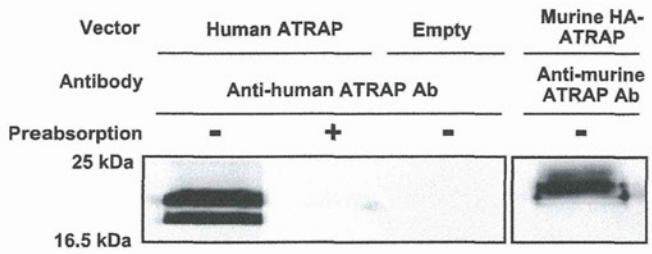

Fig. 2. Specificity of purified polyclonal anti-human ATRAP antibody. H9c2 cells were transfected with the human ATRAP cDNA expression plasmid (Human ATRAP) or an empty pCAGGS vector (Empty), and Western blot analysis was performed using an anti-human ATRAP antibody (Anti-human ATRAP Ab). The specificity of the anti-human ATRAP antibody was confirmed by preabsorption of the anti-human ATRAP antibody with the competing antigenic peptide (preabsorption +). As a reference, H9c2 cells were also transfected with the NH_2-terminal heme agglutinin (HA) epitope-tagged murine ATRAP in pcDNA3 (Murine HA-ATRAP), and Western blot analysis was performed using the anti-murine ATRAP antibody (Anti-murine ATRAP Ab).

confirmed by pathological evaluation of renal biopsy specimens, such as light microscopy, electron microscopy, and immunofluorescence staining. No patients received steroids or immunosuppressive drugs before renal biopsy. The following clinical parameters were examined at the time of needle renal biopsy: age, gender, body mass index, blood pressure, serum creatinine, total protein, urinary protein, and estimated glomerular filtration ratio (eGFR). We calculated eGFR with an application of a revised equation for the Japanese population: eGFR ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$) = $194 \times$ serum $creatinine^{-1.094} \times age^{-0.287} \times 0.739$ (if female) (19). The study protocol was approved by the Human Ethics Review Committee of Yokohama City University Graduate School of Medicine, and written informed consent was obtained from each patient.

In situ hybridization. In situ hybridization was performed as described previously (34). After fixation, the normal human renal tissue ($n = 1$) was embedded in paraffin and sectioned. The riboprobes were generated complementary to the human ATRAP cDNA fragments (981 bases, +132 to +1112). Digoxygenin-labeled antisense and sense probes were prepared by the transcription of linearized human ATRAP cDNA in a pcDNA3.1 vector (Invitrogen) using a digoxygenin RNA labeling kit (Roche, Basel, Switzerland). Hybridized digoxygenin-labeled probes were detected using anti-digoxygenin-alkaline phosphatase conjugate. The probe was visualized using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate.

Production of rabbit polyclonal anti-human ATRAP antibody. A 16-amino acid synthetic peptide corresponding to amino acids 144–158 of the C-terminal tail of human ATRAP was produced using standard solid-phase peptide synthesis techniques. Analysis using the BLAST computer program showed no significant overlap of the immunizing peptide with any known eukaryotic protein. The peptide was purified, conjugated, and injected five times intradermally into rabbits at 2-wk intervals for the production of the polyclonal antiserum. The rabbits developed enzyme-linked immunosorbent assay titers >1:128,000 before exsanguination. The selectivity of the antiserum was validated by the recognition of the PCMV6XL5-transfected H9c2 cells by Western blot analysis. Anti-ATRAP polyclonal antibodies were affinity-purified. This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the animal studies committee of Yokohama City University.

SDS-PAGE and immunoblotting. Whole cellular extracts (20 μg/lane) from H9c2 cells were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with either *1*) human ATRAP antibody diluted to 1:1,000; *2*) human ATRAP antibody preabsorbed with a 10-fold excess of the peptide used to generate the antibody; or *3*) murine ATRAP antibody (34). Sites of antibody-antigen reaction were visu-

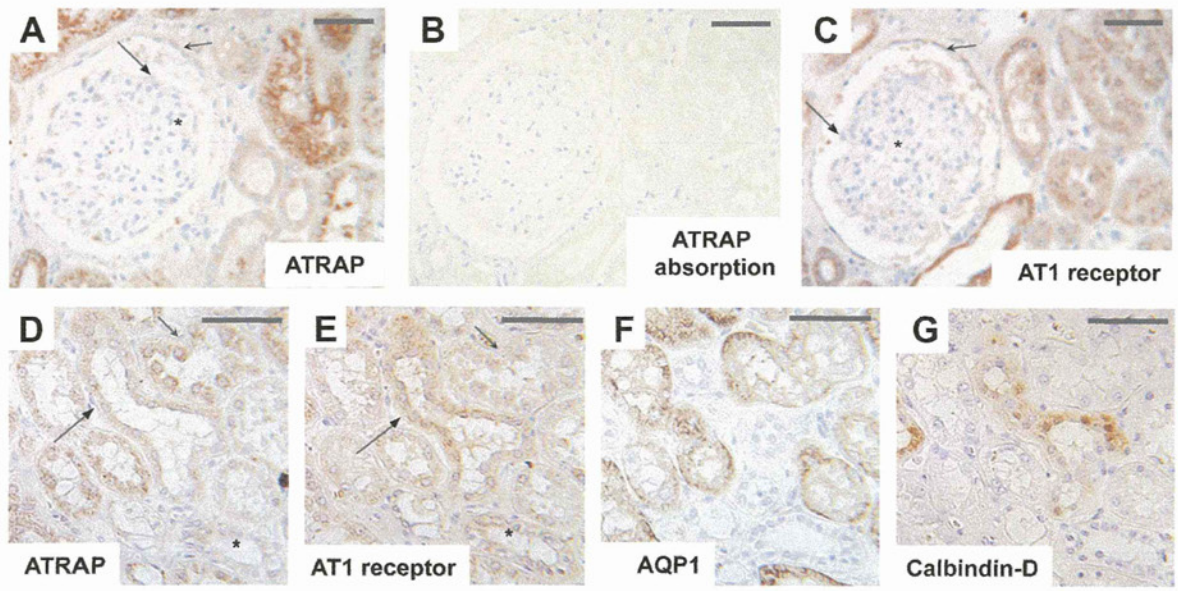

Fig. 3. Localization of ATRAP and AT_1 receptor proteins in the cortical region of a normal human kidney by immunohistochemistry. *A*: glomerular section showing human ATRAP in Bowman's capsule (short arrow), podocytes (long arrow), and mesangial cells (asterisk). *B*: consecutive section stained with ATRAP antibody preabsorbed with a 10-fold excess of immunizing peptide as a negative control. *C*: consecutive section showing human AT_1 receptor in Bowman's capsule (short arrow), podocytes (long arrow), and mesangial cells (asterisk). Consecutive tubular sections show human ATRAP (*D*) and the AT_1 receptor (*E*) in the PCT (short arrow), DCT (long arrow), and CCD (asterisk). The consecutive sections were also stained with a polyclonal antibody against aquaporin-1 (AQP1; *F*), a specific marker of proximal tubules, and a monoclonal antibody against calbindin-D (*G*), a specific marker of DCT and connecting tubule (CNT). Both the ATRAP- and AT_1 receptor protein-positive staining patterns are in brown. Original magnification ×400. Bars = 50 μm.

alized by enhanced chemiluminescence (Amersham Biosciences). The images were analyzed with an LAS-3000 imaging system (Fuji Film, Tokyo, Japan).

Immunohistochemistry. Immunohistochemistry was performed essentially as described previously (13, 34). After fixation, the renal tissues were embedded in paraffin, and sectioned at 4-μm thickness. The sections were treated for 60 min with 10% normal goat serum in phosphate-buffered saline and blocked for endogenous biotin activity using an Avidin/Biotin Blocking kit (Vector Laboratories). For the study of ATRAP, the sections were incubated with one of the following: *1*) ATRAP antibody diluted at 1:100; *2*) ATRAP antibody preabsorbed with a 10-fold excess of the peptide used to generate the antibody; or *3*) nonimmune rabbit IgG. For the study of AT_1 receptor, the sections were incubated with *1*) AT_1 receptor antibody [AT_1 (N-10), sc-1173, Santa Cruz Biotechnology, Santa Cruz, CA] diluted at 1:100; or *2*) nonimmune rabbit IgG. The characterization and specificity of the anti-AT_1 receptor antibody was described previously (34). For the study of specific nephron markers, the sections were incubated with either *1*) aquaporin-1 antibody (ab9566, Abcam) diluted at 1:100; *2*) calbindin D-28K antibody (C9848, Sigma-Aldrich) diluted at 1:3,000; *3*) Tamm-Horsfall glycoprotein antibody (sc-20631, Santa Cruz Biotechnology) diluted at 1:100; or *4*) nonimmune rabbit IgG. The sections were incubated for 60 min with biotinylated goat anti-rabbit IgG or biotinylated goat anti-murine IgG (SAB-PO kit, Nichirei, Tokyo, Japan), blocked for endogenous peroxidase activity by incubation with 0.3% H_2O_2 for 20 min, treated for 30 min with the streptavidin and biotinylated peroxidase (DAKO, Heidelberg, Germany), and then exposed to diaminobenzidine. The sections were counterstained with hematoxylin, dehydrated, and mounted.

Continuous high-resolution and high-magnification immunohistochemical images were obtained by using a BZ-9000 (KEYENCE, Osaka, Japan), and immunoreactivity was semiquantitatively evaluated in a blind manner. Examination was performed using a microscope with ×400 magnification (Olympus, Tokyo, Japan) and an integrated digital camera system (Olympus). Image Pro-plus computer

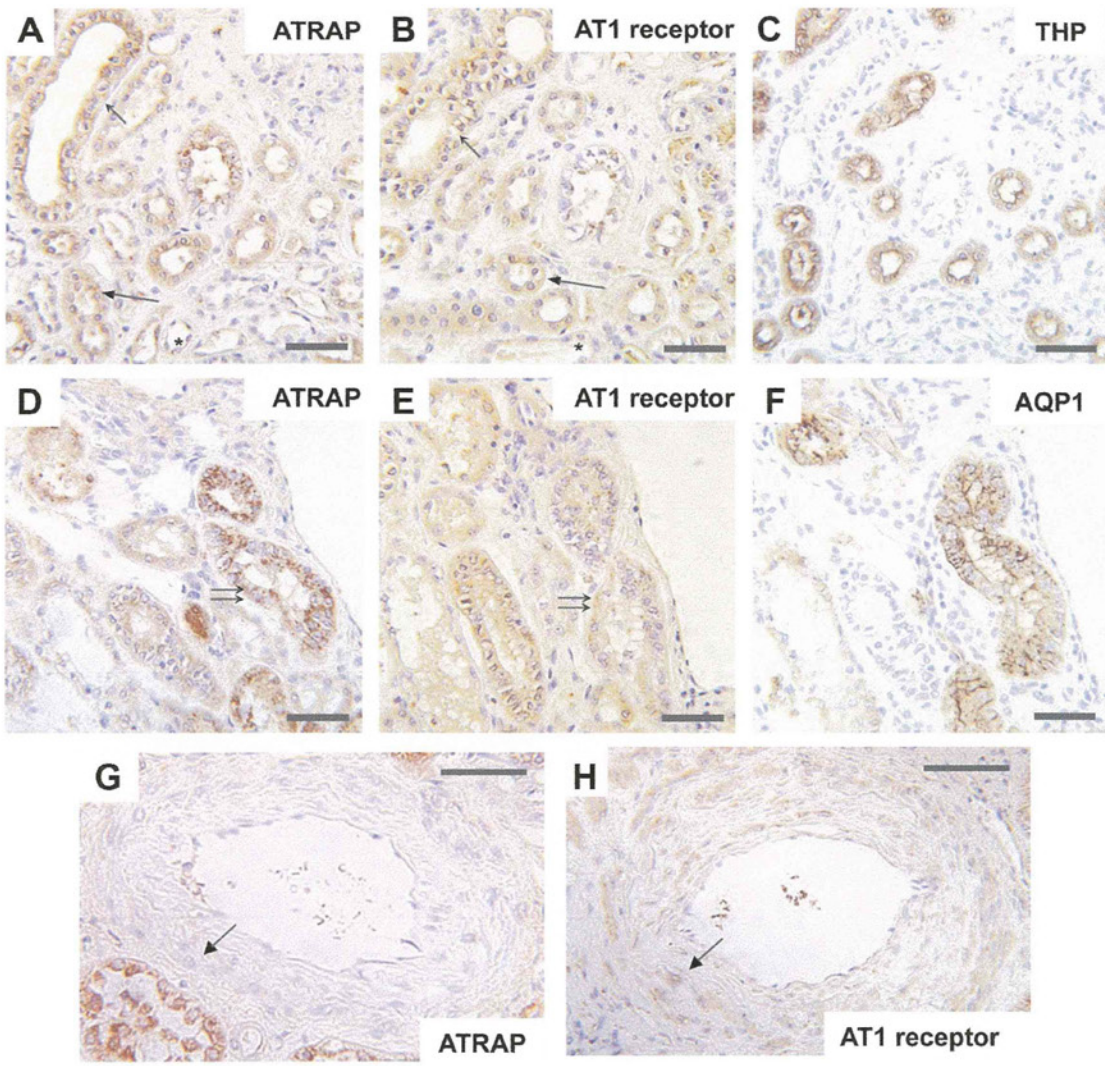

Fig. 4. Localization of ATRAP and AT_1 receptor proteins in the medullary region and the vasculature of a normal human kidney by immunohistochemistry. Consecutive medullary sections (*A–C* and *D–F*) show human ATRAP (*A* and *D*; positive staining brown) and AT_1 receptor (*B* and *E*; positive staining brown) in the MCD (short arrow), TAL (long arrow), TL (asterisk), and PST (double arrow). The consecutive sections were also stained for a polyclonal antibody against Tamm-Horsfall protein (THP; *C*), a TAL-specific marker, and a polyclonal antibody against AQP1 (*F*), a specific marker of the proximal tubules. Consecutive sections of the interlobular artery exhibit very weak human ATRAP immunostaining (*G*) and moderate AT_1 receptor staining (*H*) in the vascular smooth muscle cells. Original magnification ×400. Bars = 50 μm.

image-analysis software (Media Cybernetics, Bethesda, MD) was used to analyze the brown staining pixel density and to quantify the protein levels, as described previously (6, 10, 14, 28, 39).

To further assess the colocalization of the AT_1 and ATRAP proteins, 4-μm-thick mirror image paraffin sections were subjected to immunohistochemistry. Mirror image immunohistochemical staining was performed as described above. To demonstrate colocalization in the identical nephron section, ATRAP was detected by peroxidase-3-amino-9-ethylcarbazole and AT_1 receptor by alkaline phosphatase-nitroblue tetrazolium.

Preparation of recombinant adenoviral vectors and gene transfer. Adenoviral vectors were prepared using cDNAs coding for the NH_2-terminal HA epitope-tagged ATRAP (Ad.HA-ATRAP) and bacterial β-galactosidase (Ad.LacZ) using a commercially available system (Adeno X Expression System, Clontech), and the virus titer was determined with a plaque assay (33). For the adenoviral gene transfer experiments, mDCT cells were subcultured in 6 cm-diameter dishes (5×10^4/ml), incubated overnight, infected with recombinant adenovirus (Ad.HA-ATRAP or Ad.LacZ) at 50 multiplicity of infection for 24 h, and further incubated in a serum-free medium for an additional 24 h. Then, the cells were treated with ANG II (10^{-6} M) for the indicated time, and subsequently harvested for analysis.

ELISA of transforming growth factor-β. ELISA using conditioned medium derived from the cultured dishes was performed to examine the effect of candesartan, PD123319, or ATRAP on ANG II-mediated transforming growth factor-β (TGF-β) secretion from mDCT cells, essentially as described previously (30). The total TGF-β released into the media was determined with an ELISA system by Immuno Mini NJ-2300 (Nalge Nunc International) and Quantikine TGF-β1 (MB100B, R&D Systems), according to the manufacturer's instructions.

Statistical analysis. Data are expressed as means ± SE. Statistical significance was determined using an unpaired Student's *t*-test, with $P < 0.05$ being considered statistically significant.

RESULTS

Distribution of ATRAP mRNA in normal human kidney. We first examined the expression and distribution of ATRAP mRNA in normal human renal tissue sections from a patient with renal cell carcinoma without other obvious chronic kidney disease by in situ hybridization using a human ATRAP antisense cRNA probe. In the glomerular region, expression of human ATRAP mRNA was observed in Bowman's capsule, podocytes, and mesangial cells (Fig. 1*A*). In the cortical tubular region, a high level of ATRAP mRNA was observed in the proximal convoluted tubules (PCT), DCT (Fig. 1*B*), proximal straight tubules (PST), and cortical collecting ducts (CCD) (Fig. 1*C*). In the medullary region, positive staining was observed in the medullary collecting ducts (MCD), while a lower level of ATRAP mRNA was observed in the thin limbs (TL) and thick ascending limbs (TAL) (Fig. 1*D*). The ATRAP mRNA staining was weak in the vascular smooth muscle cells of the vasculature, including the interlobular arteries (Fig. 1*E*). No labeling of human ATRAP mRNA was found in the negative control sections that were hybridized with the sense probe (Fig. 1*F* which is the consecutive section of Fig. 1*A*).

Production of anti-human ATRAP antibody and validation of specificity. We developed a specific polyclonal antibody against human ATRAP. Western blot analysis of human ATRAP cDNA-transfected H9c2 cells revealed that the antiserum for human ATRAP recognized a prominent band of 17 kDa, which was consistent with the predicted molecular weight of human ATRAP, and also with the murine ATRAP band (= 18 kDa) derived from murine ATRAP cDNA-transfected cells, which was detected by an anti-murine ATRAP antibody (Fig. 2). This band was not observed when the antiserum was replaced with the preimmune serum (data not shown), or when empty vector pCAGGS-transfected H9c2 cells were used instead of human ATRAP-transfected cells (Fig. 2). These data indicate that the anti-human ATRAP antibody is able to recognize the human ATRAP protein specifically.

Distribution of ATRAP protein in normal human kidney. We next examined the expression and distribution of ATRAP protein in the normal human kidney of the same patient, who had been analyzed for the distribution of ATRAP mRNA expression, by immunohistochemistry using the anti-human ATRAP antibody. In the renal cortex, a relatively high level of ATRAP immunoreactivity was observed in the tubular nephron segments (Fig. 3*A*), and a lower level of ATRAP immunostaining was detected in Bowman's capsule, podocytes, and mesangial cells in the glomerulus (Fig. 3*A*). No significant immunoreactivity of the ATRAP protein was found in the negative control sections incubated with preabsorption of the anti-human ATRAP antibody along with the competing antigenic peptide (Fig. 3*B*) and incubated with nonimmune rabbit IgG (data not shown).

To definitively identify the ATRAP immunostaining sites in the tubular segments, consecutive sections were stained for ATRAP and markers specific for the tubular segments. We used a polyclonal antibody against aquaporin-1, which is specifically expressed in the proximal tubules (20, 25), a monoclonal antibody against calbindin-D, a calcium-binding protein expressed primarily in the DCT and connecting tubules (CNT) (17, 35), and a polyclonal antibody against the Tamm-Horsfall protein, which is specifically expressed in the TAL. In the renal cortex, a relatively high level of ATRAP immunostaining was detected in the PCT and DCT and a lower level was observed in the CCD (Fig. 3*D*). In the renal medulla, a relatively high level of ATRAP immunoreactivity was observed in the PST (Fig. 4, *A* and *D*), with a lower level detected in the MCD and TAL, and a weak level in the TL (Fig. 4*A*). In

Table 1. *Summary of distribution of ATRAP and AT_1 receptor in normal human kidney tissue*

	ATRAP		AT_1 Receptor
	mRNA	Protein	Protein
Glomerulus			
Podocyte	+	±	±
Mesangial cell	+	±	±
Renal tubule			
Bowman's capsule	+	±	++
PCT	+	++	++
PST	+	++	++
DCT	+	++	++
CCD	+	±	++
TAL	±	+	++
TL	±	±	±
MCD	+	+	++
Vasculature (interlobular artery, arteriole)			
Vascular smooth muscle cell	±	±	+
Vascular endothelial cell	−	−	−

ATRAP, ANG II type 1 receptor (AT_1)-associated protein; PCT, proximal convoluted tubule; PST, proximal straight tubule; DCT, distal convoluted tubule; CCD, cortical collecting duct; TAL, thick ascending limb of Henle's loop; TL, thin limb; MCD, medullary collecting duct.

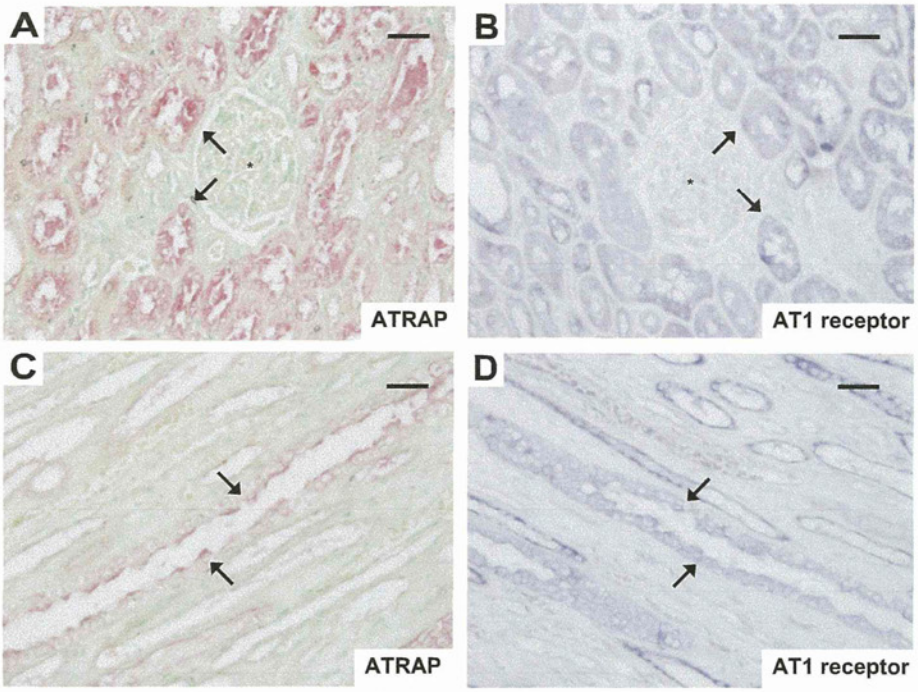

Fig. 5. Mirror-image analysis of ATRAP and AT_1 receptor proteins in a normal human kidney by immunohistochemistry. Mirror sections of the renal cortex (*A* and *B*) and medulla (*C* and *D*) show substantial colocalization of the human ATRAP (*A* and *C*; positive staining red) and AT_1 receptor (*B* and *D*; positive staining purple) proteins in the glomerulus of the renal cortex (asterisk) and in the renal tubules of both the renal cortex and medulla (arrow). Original magnification ×132. Bars = 50 μm.

vascular smooth muscle cells, including the interlobular arteries, ATRAP immunostaining was very weak (Fig. 4*G*).

Colocalization of ATRAP with the AT_1 receptor in human normal kidney. We also examined the intrarenal distribution of human AT_1 receptor protein by immunohistochemistry using the anti-AT_1 receptor antibody. The results of immunohistochemistry using the AT_1 receptor antibody revealed intense staining in Bowman's capsule (Fig. 3*C*), PCT, PST, DCT, CCD, TAL, and MCD (Fig. 3*E* and Fig. 4, *B* and *E*), and moderate staining in the vascular smooth muscle cells (of the vasculature), including the interlobular arteries (Fig. 4*H*). A lower level of AT_1 receptor immunostaining was also detected

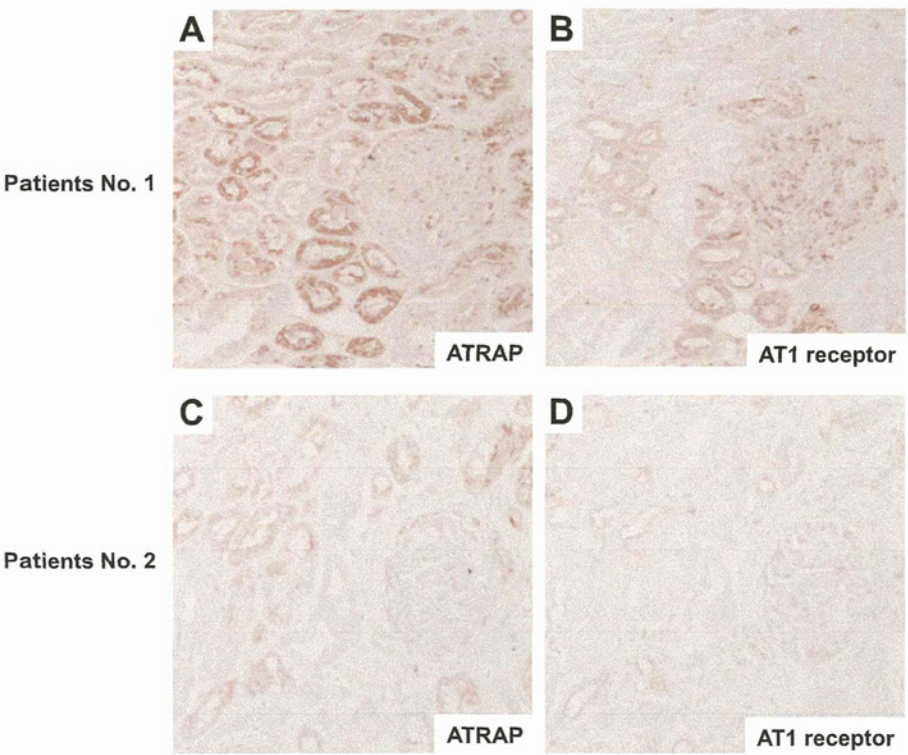

Fig. 6. Representative immunostaining of ATRAP and AT_1 receptor in needle renal biopsy specimens of IgA nephropathy. Consecutive sections from *patient 1* show a relatively high expression of ATRAP (*A*; positive staining brown) and AT_1 receptor (*B*; positive staining brown), while those from *patient 2* show a relatively low expression of ATRAP (*C*) and AT_1 receptor (*D*). Original magnification ×200. Bars = 50 μm.

in podocytes and mesangial cells in the glomeruli (Fig. 3*C*) and TL (Fig. 4*B*). In the consecutive sections stained for the ATRAP and AT_1 receptor, all of the ATRAP-immunopositive tubular nephron segments expressed the AT_1 receptor (Fig. 3, *D* and *E*, Fig. 4, *A*, *B*, *D*, and *E*; summarized in Table 1). The results of further immunohistochemical analysis of mirror sections which were stained for ATRAP and the AT_1 receptor also disclosed a significant colocalization of the proteins in the glomerulus and especially in the tubular nephron segments (Fig. 5).

Relationship between ATRAP and AT_1 receptor expression in human needle renal biopsy specimens of IgA nephropathy. The intrarenal expression and distribution of the ATRAP and AT_1 receptor genes were evaluated on needle renal biopsy specimens from 22 patients with IgA nephropathy by immunohistochemistry (Fig. 6). Since the densities of ATRAP and AT_1 receptor immunostaining were extremely high and likely to lie outside the range limit in 1 patient (data not shown), we excluded the data of this patient from further analysis and evaluated a potential correlation between ATRAP and AT_1 receptor gene expression patterns in the 21 other patients with IgA nephropathy. This relationship is thought to be of interest because ATRAP and the AT_1 receptor have opposite effects mediated by ANG II, i.e., opposite effects with regard to renal tissue damage.

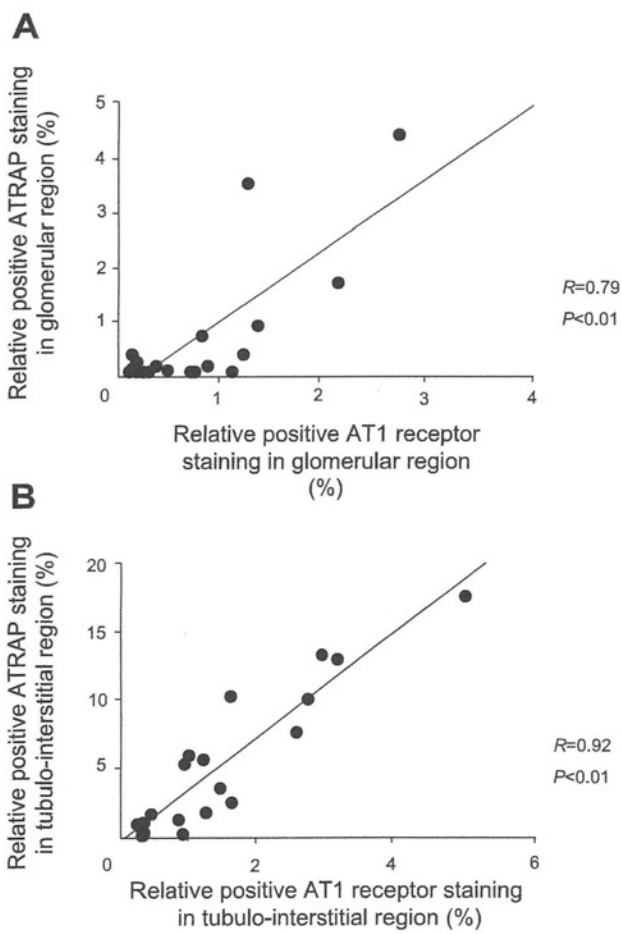

Fig. 7. Relationship between ATRAP and AT_1 receptor immunostaining levels in glomerular (*A*) and tubulointerstitial (*B*) regions of needle renal biopsy specimens of IgA nephropathy. Semiquantitative image analysis of immunostaining for ATRAP and AT_1 receptor in needle renal biopsy specimens of patients with IgA nephropathy ($n = 21$) was performed using Image Pro-plus computer software.

The results showed a positive relationship between ATRAP and AT_1 receptor immunostaining in the glomerular region ($r = 0.79$, $P < 0.01$) (Fig. 7*A*). There was also a significant correlation in the tubulointerstitial region ($r = 0.92$, $P < 0.01$) of the renal tissues with IgA nephropathy (Fig. 7*B*). We further examined a possible sex difference in the relationships between ATRAP and AT_1 receptor immunostaining. In male patients with IgA nephropathy ($n = 12$), although the relationship between ATRAP and AT_1 receptor immunostaining did not reach significance in the glomerular region ($r = 0.60$, $P = 0.09$) (Fig. 8*A*), there was a significant relationship in the tubulointerstitial region ($r = 0.82$, $P < 0.01$) (Fig. 8*B*). On the other hand, there were significant correlations between ATRAP and AT_1 receptor immunostaining in both the glomerular ($r = 0.90$, $P < 0.01$) (Fig. 8*C*) and tubulointerstitial ($r = 0.94$, $P < 0.01$) (Fig. 8*D*) regions in female patients with IgA nephropathy ($n = 9$).

Clinical variables and ATRAP and AT_1 receptor expression in human needle renal biopsy specimens of IgA nephropathy. Finally, we examined the relationship between clinical variables and renal ATRAP and AT_1 receptor immunostaining. The clinical parameters examined included age, body mass index, mean blood pressure, serum creatinine, total protein, urinary protein, and eGFR (Table 2). There was a significant positive relationship between eGFR and tubulointerstitial ATRAP immunostaining ($r = 0.44$, $P = 0.046$) in the needle renal biopsy specimens of IgA nephropathy (Fig. 9*D*). However, no significant correlation was observed between any other variables and the staining of the two proteins (Fig. 9, *A–C*; data not shown).

Endogenous expression of ATRAP and AT_1 receptor in renal distal tubular cells. The results of previous studies as well as the present study showed that renal ATRAP as well as the AT_1 receptor is expressed abundantly in the distal and proximal tubules in the mouse and human kidney (34). Thus, to examine the functional role of tubular ATRAP, we used mDCT cells (8). These cells have been shown to have the phenotype of a polarized tight junction epithelium with morphological and functional features retained from the parental cells (8). The mDCT cells expressed the endogenous AT_1 receptor and ATRAP mRNA, as detected by RT-PCR (Fig. 10*A*). Western blot analysis also recognized the endogenous AT_1 receptor and ATRAP protein in mDCT cells, respectively (Fig. 10*B*). These results demonstrate that the ATRAP and AT_1 receptor proteins are endogenously expressed in mDCT cells.

Effects of ANG II and adenoviral transfer of recombinant ATRAP on TGF-β secretion from renal distal tubular cells. ANG II has been implicated in the development of renal pathological processes due to its induction of the synthesis of the profibrotic cytokine TGF-β in the renal tubules (2, 11), which is associated with subsequent alterations in cell growth and matrix production (29, 38). In mDCT cells, activation of TGF-β secretion was dependent on the concentration of ANG II (Fig. 11*A*), and there was a time-dependent increase in TGF-β secretion from mDCT cells into the medium induced by ANG II treatment (Fig. 11*B*). While pretreatment of mDCT cells with candesartan (10^{-5} M) abolished the stimulatory

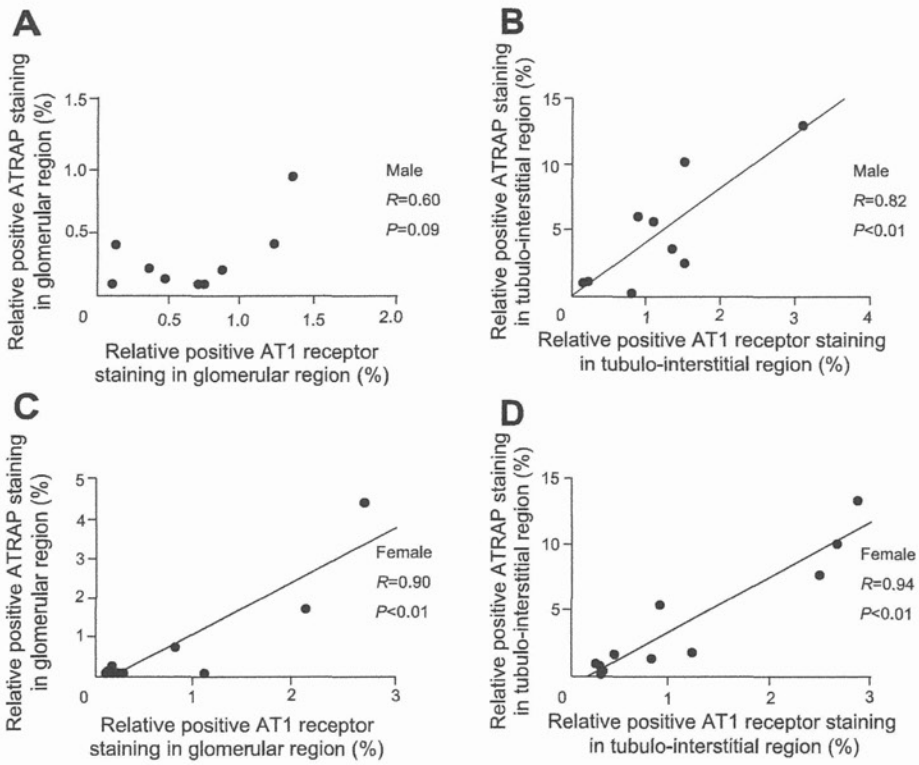

Fig. 8. Sex differences in relationship between ATRAP and AT_1 receptor immunostaining levels in glomerular (*A* and *C*) and tubulointerstitial (*B* and *D*) regions of needle renal biopsy specimens of IgA nephropathy. Semiquantitative image analysis of immunostaining for ATRAP and AT_1 receptor in needle renal biopsy specimens was performed, respectively, in male (*A* and *B*; $n = 12$) and female (*C* and *D*; $n = 9$) patients with IgA nephropathy using Image Pro-plus computer software.

effect of ANG II (10^{-6} M) on TGF-β secretion into the medium, preincubation of cells with PD123319 (10^{-5} M) did not affect the response to ANG II (Fig. 11*C*). These results indicate that the endogenously expressed AT_1 receptor is responsible for the ANG II-mediated activation of TGF-β production in mDCT cells.

To investigate the possible role of ATRAP in the functional modulation of distal tubular cells, we examined the effects of overexpression of ATRAP on the downstream effectors of the AT_1 receptor-signaling pathway in mDCT cells by performing adenoviral transfer of recombinant ATRAP. The mDCT cells were infected with an adenoviral vector containing ATRAP cDNA (Ad.HA-ATRAP) or control bacterial β-galactosidase cDNA (Ad.LacZ), and an ELISA of TGF-β was performed. Although ANG II (10^{-6} M) treatment of mDCT cells infected with Ad.LacZ increased the secretion of TGF-β protein into the medium, mDCT cells infected with Ad.HA-ATRAP exhibited an inhibition of the ANG II-induced enhancement (Fig. 11*D*), thereby indicating that ATRAP suppressed the ANG II-mediated activation of the downstream effectors of AT_1 receptor signaling in mDCT cells.

Table 2. *Clinical parameters in patients with IgA nephropathy at the time of needle renal biopsy*

Age, yr	37.4 ± 2.5
Body mass index, kg/m^2	22.8 ± 0.7
Mean BP, mmHg	95.3 ± 3.6
Serum creatinine, mg/dl	0.93 ± 0.06
Serum total protein, g/dl	6.8 ± 0.1
Urinary protein, g/g creatinine	0.97 ± 0.19
eGFR, ml·$^{-1}$·1.73 m^{-2}	66.7 ± 4.2

Values are means ± SE; $n = 21$, 12 men and 9 women. eGFR, estimated glomerular filtration rate.

DISCUSSION

The renin-angiotensin system is a well-coordinated hormonal cascade which critically regulates cardiovascular and renal homeostasis by maintaining fluid and electrolyte balance. In addition, stimulation of the renin-angiotensin system, particularly activation of AT_1 receptor signaling at local tissue sites, plays an important role in the pathophysiology of cardiovascular and renal disease. We previously cloned ATRAP as a novel molecule which interacts with the AT_1 receptor and showed that ATRAP suppressed ANG II-induced pathological responses by inducing constitutive AT_1 receptor internalization (4, 18). Thus ATRAP is suggested to be a potent counterregulator of the AT_1 receptor. We previously showed that ATRAP is expressed in a variety of murine tissues, as is the AT_1 receptor (34). Because activation of the AT_1 receptor at local tissue sites is thought to be a pivotal step in cardiovascular and renal injury, ATRAP has emerged as a potential inhibitor of tissue AT_1 receptor activation (24, 32).

There are previous reports on the human intrarenal renin-angiotensin system showing abundant expression of the human AT_1 receptor in the glomeruli, tubules, and vasculature and its upregulation in the renal tubules in IgA nephropathy (3, 5, 22). However, to date there has been no report on renal expression and distribution of ATRAP other than in the murine kidney (34). The role and significance of ATRAP in the regulation of the renin-angiotensin system in human renal tissue have yet to be elucidated. Moreover, the clinical and pathological factors modulating the expression of ATRAP in the human kidney

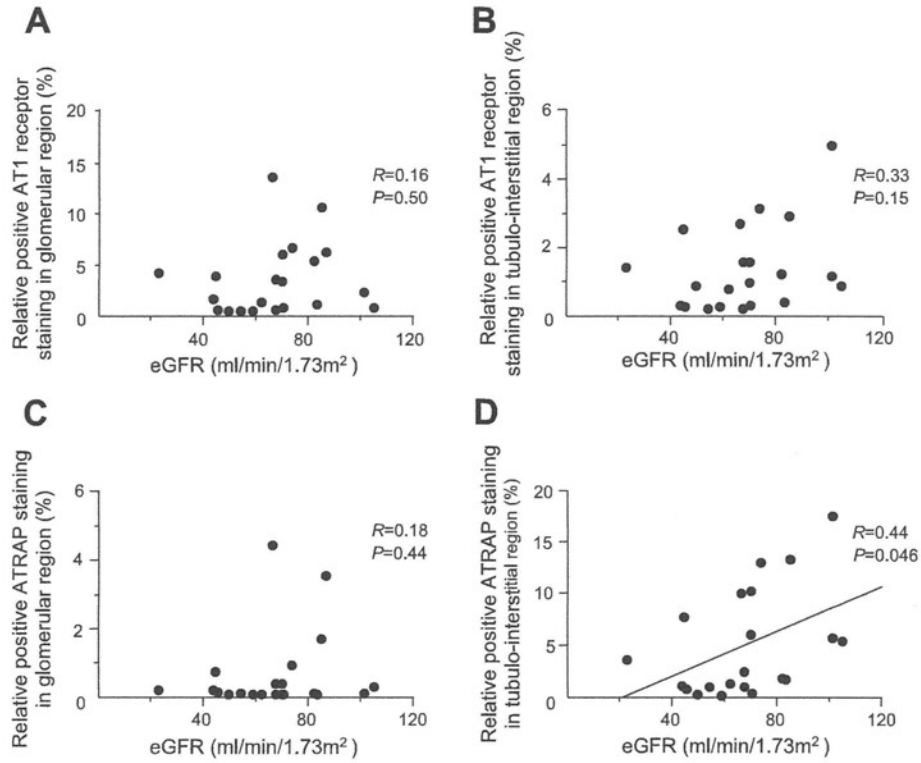

Fig. 9. Relationship between estimated glomerular filtration rate (eGFR) and immunostaining levels of ATRAP and AT_1 receptor in glomerular (*A* and *C*) and tubulointerstitial (*B* and *D*) regions of needle renal biopsy specimens from patients with IgA nephropathy. Semiquantitative image analysis of immunostaining for ATRAP and AT_1 receptor in renal biopsy specimens of patients with IgA nephropathy ($n = 21$) was performed using Image Pro-plus computer software. The clinical parameters examined included age, body mass index, mean blood pressure, serum creatinine, total protein, urinary protein, and eGFR. No significant correlation was observed between any of the clinical variables other than eGFR and the expression of the 2 proteins.

remain to be determined. Along these lines, a polyclonal anti-human ATRAP antibody was developed proven to be useful for the analysis of ATRAP protein expression in human tissue. Employing in situ hybridization and immunohistochemistry for ATRAP within human kidney sections, we showed for the first time that renal ATRAP expression, at both the mRNA and protein level, was widely and abundantly distributed in the renal tubules from Bowman's capsule to the MCD, with the relative abundance similar in each nephron segment. Furthermore, expression of ATRAP mRNA and protein was also detected in the glomerulus and vasculature, although the signals for both were comparatively weak in these areas.

With respect to the intrarenal distribution of the AT_1 receptor, specific immunoreactivity was observed in the vasculature and along the nephron segments, which was consistent with the findings of a previous study performed using normal kidney sections (22). From the immunohistochemical results of the present study, all ATRAP-immunopositive tubules from Bowman's capsule to the MCD expressed the AT_1 receptor in the consecutive sections which also stained for ATRAP. Thus the results of the present study demonstrate that human ATRAP is expressed along almost the entire nephron segment, as is the AT_1 receptor, with ATRAP abundantly expressed in the renal tubules. However, there was some dissociation between the two proteins in the pattern of intrarenal distribution. In particular, at the protein level, renal AT_1 receptor expression was more abundant than ATRAP expression in the vasculature. Previous studies have indicated that pathological vascular remodeling of the small renal arteries is critically involved in the development of hypertension and renal damage (7, 16, 31). They have also suggested that the protective effects of renin-angiotensin system inhibitors on the renal vasculature are, at least in part, responsible for prevention and amelioration of hypertension and renal injury (7, 16, 31).

On the other hand, the renal tubulointerstitial AT_1 receptor is also reported to play an important role in the regulation of sodium handling and epithelial-mesenchymal transition, as well as the pathogenesis of hypertension and renal fibrosis (15, 26, 27, 29). The results of the present study showed abundant renal tubular ATRAP expression in addition to tubular AT_1 receptor expression, while ATRAP expression in the vasculature was low. Interestingly, a significant correlation was detected between ATRAP and AT_1 receptor expression in the tubulointerstitial region, mainly in the tubular segments and not the vasculature, of the human needle renal biopsy specimens from patients with IgA nephropathy (Figs. 7 and 8). A previous study reported a similar synergistic expression of angiotensin-converting enzyme (ACE) and ACE2, a counterregulator of ACE in generating ANG II as one of the organ-protective factors in human renal tissue (36). Thus we consider this finding to be important in the effort to elucidate the mechanisms of the renal interplay between the AT_1 receptor and ATRAP and for establishing the role of renal ATRAP as a counterregulator of the AT_1 receptor.

In particular, the interpretation of the results showing a positive but not negative correlation between ATRAP and the AT_1 receptor in the diseased kidney might be controversial. The present results suggest that ATRAP protein expression does not actively alter the status of the renal renin-angiotensin system. We speculate that AT_1 receptor and ATRAP protein expression may be regulated in a coordinated manner, or synergistically, which is mediated via the local activation of AT_1 receptor signaling. A notable point here is that not only the AT_1 receptor, but also ATRAP, may be responsible for

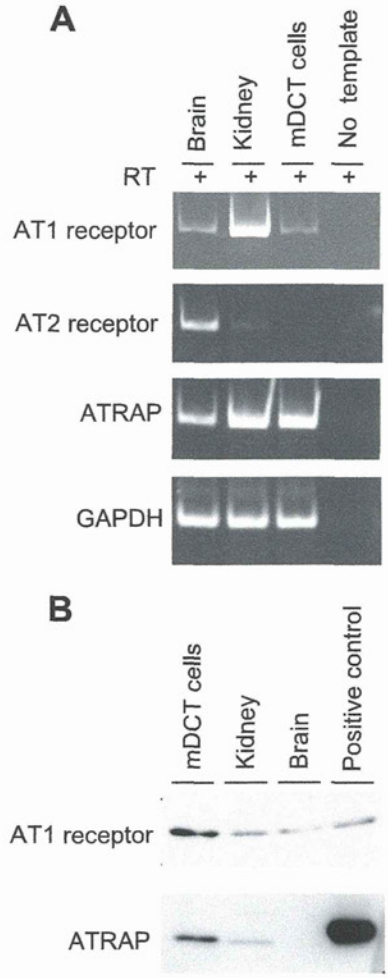

Fig. 10. Expression of endogenous AT_1 receptor and ATRAP in renal distal tubular cells. *A*: representative RT-PCR analysis of mDCT cells using the AT_1 receptor-, AT_2 receptor-, and ATRAP-specific primers. The negative control, consisting of RT-PCR reactions lacking a template (no template), are also shown. *B*: representative Western blot analysis of endogenous AT_1 receptor and ATRAP proteins with anti-AT_1 receptor and anti-ATRAP polyclonal antibodies, respectively, in extracts from mouse tissues and mDCT cells. The positive control using cellular extracts from H9c2 cells transiently transfected with the HA-tagged ATRAP in pcDNA3 (positive control) is also shown.

determination of the renal activity of AT_1 receptor signaling at the local tissue sites, with a coordinated expression of ATRAP exerting a countereffect against AT_1 receptor activity by constitutive stimulation of AT_1 receptor internalization. It is suggested that the role of ATRAP must be taken into consideration for the pathogenesis of renal tissue injury.

However, since the present study is a cross-sectional study examining the relationship between ATRAP and the AT_1 receptor in IgA nephropathy, but not a prospective study to examine effects of intervention to affect AT_1 receptor signaling, the exact mechanism for this regulation is not clear. We were not able to determine whether the AT_1 receptor influences ATRAP expression in the kidney at this stage. Additional studies with repeated biopsies and intervention in AT_1 receptor signaling such as by means of AT_1 receptor-specific blockers are needed to elucidate the mechanism of the relationship between ATRAP and the AT_1 receptor in the kidney of IgA nephropathy.

We also tested the possibility that the clinical characteristics are substantially related to renal ATRAP expression. Indeed, semiquantitative analysis of the relationship between the clin-

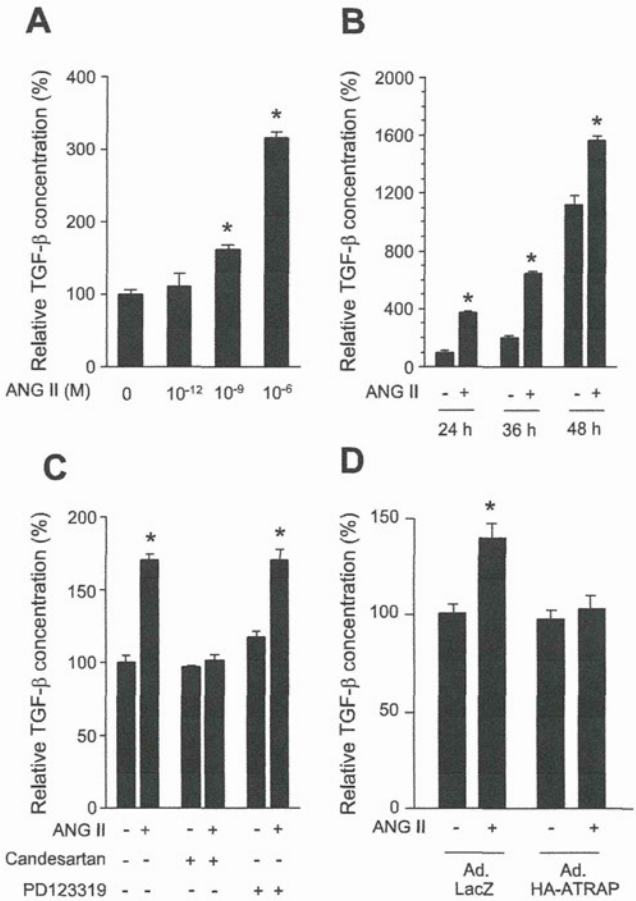

Fig. 11. Effects of ANG II and adenoviral transfer of recombinant ATRAP on transforming growth factor (TGF)-β secretion from renal distal tubular cells. (*A*) ELISA showing the relative TGF-β concentrations in the cultured medium of mouse (m) DCT cells stimulated with vehicle (0 M ANG II) or ANG II at the indicated concentrations (10^{-12} M~10^{-6} M) for 36 h. Values are calculated relative to those achieved with extracts from mDCT cells stimulated with vehicle and are expressed as means ± SE ($n = 7$ in each group). *$P < 0.05$ vs. 0 M ANG II. *B*: ELISA showing relative TGF-β concentrations in cultured medium of mDCT cells stimulated with vehicle (ANG II, −) or ANG II at 10^{-6} M for the indicated time (24 h~48 h). The values are calculated relative to those achieved with extracts from mDCT cells stimulated with vehicle (ANG II, −) for 24 h and are expressed as means ± SE ($n = 7$ in each group). *$P < 0.05$ vs. ANG II, −. *C*: ELISA showing relative TGF-β protein levels in cultured medium of mDCT cells pretreated with an AT_1 receptor-specific blocker (candesartan; 10^{-5} M) or AT_2 receptor-specific blocker (PD123319; 10^{-5} M) followed by stimulation with vehicle (ANG II, −) or ANG II at 10^{-6} M (ANG II, +) for 36 h. Values are calculated relative to those achieved with extracts from mDCT cells stimulated with vehicle without AT_1 receptor- or AT_2 receptor-specific blockers and are expressed as means ± SE ($n = 7$ in each group). *$P < 0.05$ vs. ANG II, −. *D*: ELISA showing relative TGF-β protein levels in cultured medium of mDCT cells infected with the ATRAP adenoviral vector (Ad.HA-ATRAP) or LacZ adenoviral vector (Ad.LacZ). Forty-eight hours after infection, the cells were stimulated with vehicle (ANG II, −) or ANG II at 10^{-6} M (ANG II, +) for 36 h. The values are calculated relative to those achieved with extracts from mDCT cells infected with Ad.LacZ and stimulated with vehicle and are expressed as means ± SE ($n = 9$ in each group). *$P < 0.05$ vs. ANG II.