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Rat Receptor-Activity-Modifying Proteins (RAMPs) for Adrenomedullin/CGRP Receptor: Cloning and Upregulation in Obstructive Nephropathy

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Adrenomedullin (AM) is a potent vasorelaxing peptide originally isolated pheochromocytoma. Recently, a family of receptor-activity-modifying proteins (RAMPs 1–3) were identified in humans. Associated with the calcitonin receptor-like receptor (CRLR), RAMP2 or RAMP3 may function as the AM receptor. Here we cloned rat RAMP family, analyzed their distribution in rat tissues, and examined regulation of their expression in the kidney using an obstructive nephropathy model. Northern blot analyses revealed that the RAMP family genes are expressed in various tissues with different tissue specificity; RAMP1 is abundantly expressed in the brain, fat, thymus, and spleen, RAMP2 in the lung, spleen, fat, and aorta, while RAMP3 is most abundant in the kidney and lung. After ureteral obstruction, RAMP1, RAMP2, and CRLR gene expressions in the obstructed kidney were markedly upregulated, whereas RAMP3 expression was unchanged. Thus, RAMPs are regulated differently in obstructive nephropathy, suggesting their distinct roles in renal pathophysiology.

Adrenomedullin (AM), a potent vasorelaxing and natriuretic peptide originally isolated from human pheochromocytoma, belongs to the calcitonin gene-related peptide (CGRP) family (1). AM is synthesized in various tissues including the adrenal gland, kidney, lung, and heart (2). Moreover, it is secreted from endothelial cells (3), and localized in the glomeruli and distal tubules in the kidney (4). We have previously reported that AM is secreted from cultured mesangial cells and elicits a potent anti-proliferative effect in an autocrine/paracrine manner (5). AM exerts its actions by stimulating cAMP production and nitric oxide release in target tissues (1, 6–8). These actions have been thought to be mediated via the receptors shared with CGRP and those specific for AM, but the precise molecular mechanisms of its receptor system have not been clearly understood yet.

Recently, a family of receptor-activity-modifying proteins (RAMP1, RAMP2, and RAMP3) with a single transmembrane domain have been identified in humans (9). Associated with the seven-transmembrane-domain calcitonin receptor-like receptor (CRLR) (10), it has been shown that RAMPs coexpressed with CRLR can function as either a CGRP receptor (CRLR/RAMP1) or an AM receptor (CRLR/RAMP2 or CRLR/RAMP3), depending on which RAMP is expressed (9, 11, 12). Subsequent reports have shown that, in studies using cultured cells, the expression of endogenous RAMPs may determine the specificity of the receptors for AM and CGRP (13–15). However, the expression of the RAMP family *in vivo* has so far been poorly characterized, and very little has been known about the regulation of the RAMP family *in vivo* or *in vitro*. In the present study, we have cloned cDNAs encoding the rat RAMP family and analyzed their distribution in normal rat tissues. Furthermore, to clarify the regulation of the RAMP family expression in the kidney, we investigated the changes in the gene expressions of the RAMP family together with those of CRLR and AM, in the renal fibrosis model following ureteral obstruction in rats.

MATERIALS AND METHODS

Tissue preparation, RNA extraction, and cDNA synthesis. All animal experiments were conducted in accordance with our institu-

Abbreviations used: RAMP, receptor-activity-modifying protein; AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; TGF-β, transforming growth factor-β.

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tional guidelines for animal research. The whole heart and kidney were obtained from an adult male Wistar rat. Total RNA was extracted by the acid guanidinium thiocyanate-phenol chloroform method (16) and poly $(A)^+$ RNA was purified using PolyATract (Promega, Madison, WI). cDNA was synthesized from 1 μg of poly(A)+ RNA using SuperScript II reverse transcriptase (Gibco BRL, Rockville, MD) (16) and used for reverse transcription-PCR.

Primer design and PCR-based cloning. The expressed sequence tag (EST) database searching by the nucleotide sequences of the human RAMP family (9) was performed in the dbEST database using the BLASTN program (17). Four partial EST sequences (GenBank Accession Nos: A012429, H33975, A012814, and AA388255) that exhibited high homology to the human RAMP family were identified from rat placenta, PC-12 cells and mouse embryo databases. The sequences were used for designing sense and antisense PCR primers to isolate clones encoding the rat RAMP family. The primers used were: RAMP1-S (5′-CGGCGGGCTCTGCTTGCCAT-3′), RAMP1-AS (5′-GGTTCCCCAATCCCCCATCA-3′); RAMP2-S (5′-TGGAGTACGAG-GCAGACAAGT-3′), RAMP2-AS (5′-AAAAG-ATGGCTGCTGAGAGGC-3′); RAMP3-S (5′-GTCTGGAAGTGGT-GCAACCTGT-3′), and RAMP3-AS (5′-CCACACCACCAG-GCCAG-CCAT-3′). The PCR products of the expected size were all subcloned into a pGEM-T vector (Promega) and nucleotide sequences of several clones were determined on both strands by the dideoxy chain-termination method using Dye Terminator cycle sequencing kit FS and 373B DNA sequencer (Applied Biosystems, Foster City, CA).

Isolation of full-length cDNA clones. To obtain the full-length cDNA clones, rapid amplification of 5′- and 3′-cDNA ends (5′- and 3′-RACE) was performed (16) with Marathon cDNA amplification kit (Clontech, Palo Alto, CA) using rat heart cDNA ligated with the Marathon adaptor as template and nested PCR between adaptor primers (APs) and internal gene-specific primers (GSPs). The primers used were: AP1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′ for primary PCR), AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′ for nested PCR); RAMP2-GSP1 (5′-CACCACAAGCGTAACGAGGAAAGG-3′ for 5′-RACE primary), RAMP2-GSP2 (5′-TTCTGCCAAGGGATTTGGG-AAGC-3′ for 5′-RACE nested); RAMP3-GSP1 (5′-GCCTATGGATAC-CCGTGATAAAGC-3′ for 5′-RACE primary), RAMP3-GSP2 (5′-GGTTGGGCCAGTAGCAGCCCACG-3′ for 5′-RACE nested), RAMP3-GSP3 (5′-GGTGCAACCTGTCGGAGTTCATCG-3′ for 3′-RACE primary), and RAMP3-GSP4 (5′-CCCAGAGCTTTATCACGGGTATCC-3′ for 3′-RACE nested). PCR was performed using *LA Taq* polymerase (Takara Shuzo, Tokyo, Japan) according to the manufacturer's instructions. The RACE products were subcloned and sequenced as above.

Northern blot analysis. Northern blot analysis was performed as described (16) using 40 μg of total RNA extracted from various tissues of 10-week-old male Wistar rats. RNA was electrophoresed in 1.4% agarose gel and transferred to Biodyne nylon membranes (Pall BioSupport, East Hills, NY). Hybridization was performed at 42°C overnight with [^{32}P]dCTP-labeled cDNA probes for rat RAMP1 (nucleotides −19–476), RAMP2 (319–574), and RAMP3 (143–413) clones. The membranes were washed at 55°C in 1× SSC/0.1% SDS, and exposed to BAS-III imaging plate (Fuji, Kanagawa, Japan) for 6 h.

Unilateral ureteral obstruction (UUO). Male Wistar rats weighing 200 to 250 g were subjected to either unilateral ureteral obstruction (UUO) or sham operation (18). In UUO rats, the right ureter was ligated with 4-0 silk at two points through a midline abdominal incision under pentobarbital anesthesia and was cut between the ligatures to prevent retrograde infection. Rats were sacrificed 6 and 14 days after UUO or sham operation (n = 7 each), and both the obstructed kidney and the contralateral kidney were harvested. Northern blot analysis was performed using 40 μg of total RNA prepared from each kidney with [^{32}P]dCTP-labeled cDNA probes for the rat RAMP family, rat CRLR (nucleotides 2001–2668) (10), AM (19–591) (2), and transforming growth factor (TGF)-β1 (1142–1546) (18).

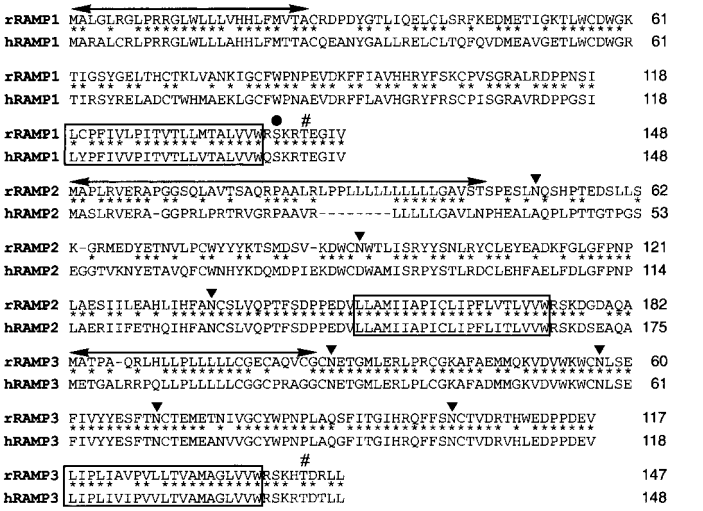

FIG. 1. Amino acid sequence alignment of rat RAMP (rRAMP) family compared with the human RAMP (hRAMP) family. The number on the right refers to the last amino acid on the line. Identical residues between rRAMP and hRAMP are indicated by asterisks. *Arrows,* putative signal sequences; *boxes,* putative transmembrane regions; *triangles,* potential *N*-glycosylation sites; *circle,* a potential protein kinase C phosphorylation site; *#,* potential protein kinase A phosphorylation sites.

Statistical analysis. Results are expressed as the mean ± SEM. Data were analyzed by ANOVA followed by Scheffe's test. P value $<$ 0.05 was considered statistically significant.

RESULTS

Cloning of the Rat RAMP Family

Figure 1 shows the deduced amino acid sequences of the rat RAMP family. Rat RAMP1 cDNA encodes a protein of 148 amino acid residues with 71% identity to human RAMP1; rat RAMP2 of 182 residues with 65% identity and rat RAMP3 of 147 residues with 85% identity to human RAMP2 and RAMP3, respectively. Hydropathy analysis predicted that the proteins possess two hydrophobic regions corresponding to amino-terminal signal sequences and transmembrane domains. The nucleotide sequences of rat RAMP1, RAMP2, and RAMP3 cDNAs have been deposited in the GenBank database with Accession Nos. AB030942, AB030943, and AB030944, respectively.

Northern Blot Analysis

Northern blot analyses (Fig. 2) revealed that the rat RAMP family genes are expressed in various tissues with different tissue specificity; RAMP1 mRNA was detected as a single 1.1-kb transcript in almost all tissues examined with abundant expression in the brain, epididymal fat, thymus, spleen, and aorta. RAMP2 mRNA was also widely detected as a 1.1-kb band with highest expression in the lung, followed by the spleen, fat, aorta, heart, and kidney. RAMP3

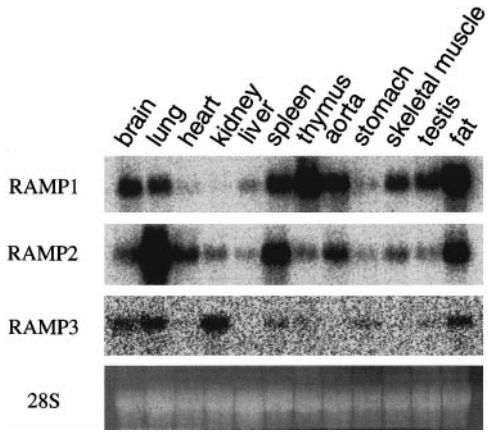

FIG. 2. Tissue distribution of the RAMP family mRNA expression in rats. Forty μg of total RNA was electrophoresed in each lane.

mRNA was expressed as a 1.3-kb band at the highest level in the kidney, and less abundantly in lung, fat, and brain. Thus, RAMPs 1, 2, and 3 have distinct tissue distributions.

Unilateral Ureteral Obstruction (UUO)

AM has potent vasodilatory and natriuretic actions in the kidney (4, 8), which is one of the main targets of AM. In order to explore the regulation of RAMPs in the kidney, we examined changes in their gene expressions in the renal fibrosis model. Figure 3 illustrates the expression of the RAMP family, CRLR, AM, and TGF-β1 genes 6 and 14 days after UUO. TGF-β1 mRNA was significantly augmented in the kidneys with ureteral obstruction as compared to control sham-operated kidneys or contralateral kidneys (Fig. 3A), as previously reported (18). The obstructed kidneys revealed a marked induction of RAMP1 mRNA from day 6 (6.2-fold of control) (Fig. 3, A and B), an early stage of interstitial fibrosis (18). This upregulation became more marked at day 14 (13-fold). RAMP2 and CRLR mRNA levels also significantly increased in the obstructed kidneys (3.2- and 3.6-fold, respectively, of control at day 14). In contrast, the RAMP3 mRNA level was unchanged, as was AM mRNA, which showed a tendency to decrease (0.9- and 0.8-fold of contralateral kidneys at day 6 and 14, respectively), but the changes were not statistically significant.

DISCUSSION

We obtained and sequenced full-length cDNA clones encoding the rat RAMP family. The rat RAMP family shows a high degree of overall homology (65–85% amino acid identity) to the human RAMP family (Fig. 1). Among the family members, however, rat RAMPs exhibit low amino acid homology (~30% identity) except for the common residues beyond the species, suggesting that each RAMP may have a distinct role in the regulation of AM/CGRP receptors, or of other unknown receptors.

The tissue distribution studies reveal that the RAMP family genes are expressed in the majority of tissues examined with various degrees of expression with different tissue specificity (Fig. 2). This finding is consistent with the wide distribution of AM/CGRP receptors in rat tissues revealed by binding studies (19). RAMP3 mRNA exhibited a less abundant expression, suggesting that rat RAMP1 and RAMP2 may have a broader physiological role in AM/CGRP signaling in various tissues, while RAMP3 may play a role in a limited number of tissues such as the kidney and lung. Inter-

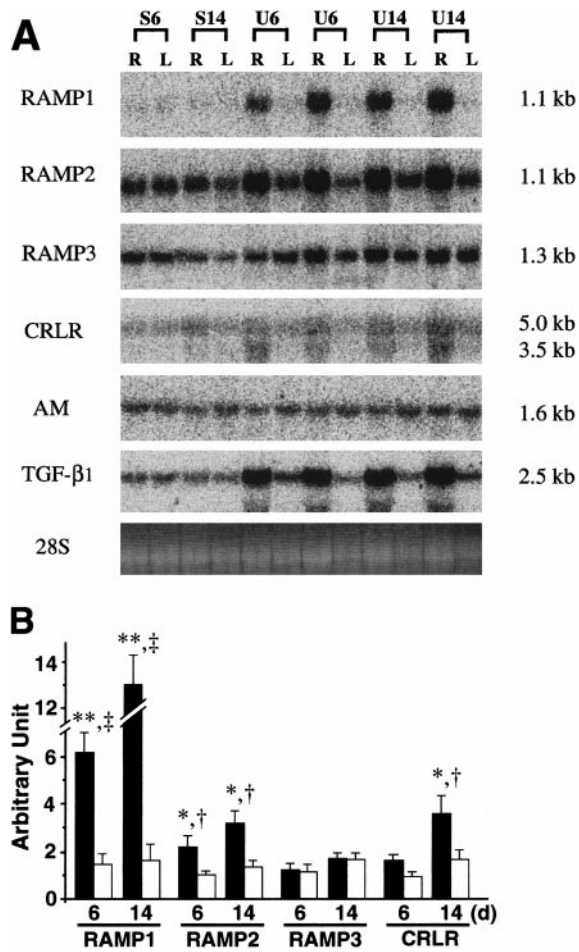

FIG. 3. (A) Changes of mRNA expressions of the RAMP family in the kidney together with those of CRLR, AM, and TGF-β1 after unilateral ureteral obstruction (UUO). Forty μg of total RNA from the whole kidney was electrophoresed in each lane. Representative results are shown. S, sham-operated rat; U, UUO rat; 6, day 6; 14, day 14; R, right obstructed kidney, L, left contralateral kidney. (B) The relative mRNA levels of RAMPs and CRLR normalized with 28S ribosomal RNA in the obstructed (*closed bars*) and contralateral (*open bars*) kidney at day 6 and day 14. Mean levels of the right kidney from control sham-operated rats are regarded as 1.0 arbitrary unit. n = 7; *$P < 0.05$, **$P < 0.01$ vs. sham operation; †$P < 0.05$, ‡$P < 0.01$ vs. contralateral kidney.

estingly, all the RAMP family are expressed abundantly in the fat tissue. The role of AM and CGRP in the fat tissue is currently unclear. Whether AM/CGRP or other ligands for receptors that could be regulated by RAMPs have important roles in adipocyte function should await further clarification. When compared to human tissues (9), cardiac expression of RAMP1 and RAMP3 mRNAs was much less abundant in rats. The kidney expresses RAMP2 and RAMP3, suggesting that both could potentially constitute a part of the renal AM receptor. Lower baseline expression of RAMP1 in rat kidney might explain a relatively lower potency of CGRP than AM in rat glomeruli (20) and renal tubules (21).

The present study demonstrates that the RAMP1 gene expression in rat kidney is markedly upregulated during obstructive nephropathy (Fig. 3). The gene expression RAMP2 was also significantly augmented, whereas that of RAMP3 was unchanged. These results suggest that the RAMP family genes are differently regulated during the progression of renal fibrosis. The CRLR mRNA was also significantly upregulated. These alterations appear to be ligand-independent, since there was no significant change in the AM gene expression (Fig. 3A). We and others have already proposed possible protective roles of AM against endothelial, mesangial or renal tubular injury in an autocrine/paracrine manner (5, 22–24). Upregulation of these genes in the obstructed kidney, therefore, may favor the protective response against proliferative and/or fibrotic changes in the obstructed kidney. This hypothesis is currently under investigation in detail in our laboratory.

The physiological role of the RAMP family is still not clearly defined. The reported competitive interactions of RAMP1 and RAMP2 with CRLR in cDNA-transfected cells (14) suggest that the ligand selectivity of AM/CGRP receptors may be determined by the regulated expression of each of the RAMP family. Of note, the RAMP family genes are expressed more abundantly than the CRLR gene (12, and Fig. 3A), suggesting that RAMPs could regulate receptors other than CRLR. Recently, it has been reported that RAMP1 and RAMP3 cotransfected with a calcitonin receptor generate a high-affinity amylin receptor (25, 26).

In conclusion, the present study describes the cloning and expression of the rat RAMP family. Furthermore, we show that RAMPs are regulated differently in the obstructive nephropathy model, suggesting their distinct roles in renal pathophysiology.

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