Expression of MAK-V/Hunk in renal distal tubules and its possible involvement in proliferative suppression

Masashi Sakai,1,2 Kouichi Tamura,1 Yuko Tsurumi,1 Yutaka Tanaka,1 Yuichi Koide,1 Miyuki Matsuda,1 Tomoaki Ishigami,1 Machiko Yabana,1 Yasuo Tokita,2 Yukio Hiroi,3 Issei Komuro,4 and Satoshi Umemura1

1Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, Yokohama; 2Renal Division, Department of Medicine, Fujisawa Municipal Hospital, Fujisawa; 4Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan; and 3Vascular Medicine Research, Brigham and Women’s Hospital, Harvard Medical School, Cambridge, Massachusetts

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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. First published February 13, 2007; doi:10.1152/ajprenal.00451.2006.—MAK-V/Hunk is an SNF1-related serine/threonine kinase which was previously shown to be highly expressed in the mammary gland and central nervous system. In this study, we found MAK-V/Hunk is abundantly and specifically expressed in the thick ascending limbs and distal convoluted tubules (DCT) of the kidney from the embryonic stage to the adult stage. We demonstrated that dietary salt depletion significantly enhances renal MAK-V/Hunk mRNA levels compared with a normal-salt diet. To analyze the possible renal cellular function of this kinase, we employed mouse distal convoluted tubule (mDCT) cells. The results of reverse transcriptase-polymerase chain reaction and Western blot analysis revealed that MAK-V/Hunk is expressed endogenously in mDCT cells. Overexpression of MAK-V/Hunk by adenoviral gene transfer significantly inhibited the ANG II-induced stimulation of c-fos gene transcription and suppressed the ANG II-mediated increases in transforming growth factor-β production into the medium. This phenomenon was accompanied by inhibition of ANG II-induced activation of BrdU incorporation. On the other hand, the MAK-V/Hunk knockdown by siRNA activated the ANG II-induced c-fos gene expression. In the consecutive sections stained for MAK-V/Hunk and AT1 receptor, MAK-V/Hunk-immunopositive distal tubules expressed the AT1 receptor. This is the first report on the intrarenal localization of MAK-V/Hunk and its cellular function in renal tubular cells.

SNF1 protein kinase; angiotensin II; transforming growth factor-β; renal epithelial cell

PROTEIN KINASES ARE A KEY COMPONENT of many signaling pathways in eukaryotic cells, and the accumulated evidence shows them to be important regulators of such cell activities as proliferation, differentiation, and development. We previously performed a screen to identify protein kinases that were expressed in the early differential stage of mouse embryonic stem cells (ES cells), by using degenerative oligonucleotide primers specific to consensus sequences of a number of protein kinase domains (7). In this paper, we focused our studies on one positive clone with a predicted putative kinase domain structure, since sequence analysis revealed that this clone was a partial cDNA encoding hormonally upregulated Neu-associated kinase (MAK-V/Hunk) that was originally cloned by Gardner et al. (6) and Korobko et al. (18).

The MAK-V/Hunk protein has an NH2-terminal (N-terminal) catalytic domain typical of serine/threonine kinases and an SNF1-homology domain immediately after this catalytic domain. The protein also has a unique COOH-terminal (C-terminal) part without homology to any known protein. SNF1 family members have been implicated in the regulation of developmental processes including cell cycle control, establishment of cell polarity, and differentiation. For example, the MARK/Par-1 subgroup of the SNF-1-like protein has been shown to be involved in the establishment of anterior-posterior polarity in early embryonic development as well as the maintenance of epithelial cell polarity (11), and SNF-1 itself has been found to mediate cell-cycle arrest in response to starvation (30). In fact, the MAK-V/Hunk mRNA of Xenopus laevis is predominantly found on the animal hemisphere of the egg (22). Although MAK-V/Hunk mRNA has been reported to be expressed in a variety of tissues of the adult mouse, such as the ovary, thymus, lung, brain, breast, uterus, liver, and kidney in vivo (6), no specific cellular targets or specific sequences of molecular events involving MAK-V/Hunk in vitro have been identified to date. In the present study, we developed a polyclonal anti-MAK-V/Hunk antibody and focused our investigation on the renal distribution/localization and developmental expression of MAK-V/Hunk in vivo and the renal cellular function in vitro. In the course of these studies, we demonstrated that MAK-V/Hunk is abundantly expressed in the kidney with localization to the renal distal tubules in vivo and that MAK-V/Hunk is specifically involved in the inhibition of ANG II-induced proliferative responses of renal distal tubular cells.

MATERIALS AND METHODS

Materials. ANG II, endothelin-1, and aldosterone were purchased from Sigma. The AT1 receptor-specific antagonist telmisartan and AT2 receptor-specific antagonist PD123319 were supplied by Boehringer Ingelheim and Parke Davis, respectively.

Plasmid construction. We subcloned MAK-V/Hunk cDNA in pcDNA3.1 (+) (Invitrogen) and pSRK-HA (kindly provided by Dr. S...
Ohno, Department of Molecular Biology, Yokohama City University) to produce expression vectors for MAK-V/Hunk (pc-MAK-V/Hunk) and NH2-terminal HA-tagged MAK-V/Hunk (pHA-MAK-V/Hunk), respectively.

**Cell culture.** H9c2 cells were cultured as described previously (28). Mouse distal convoluted tubule cells (mDCT cells) were kindly provided by Dr. P. A. Friedman (University of Pittsburgh School of Medicine, Pittsburgh, PA). The cells had been previously isolated and functionally characterized as described previously (8–10, 20). Cells were grown on 100-mm dishes (Corning) in DMEM/HAM F-12 media (1:1; Sigma) supplemented with 5% heat-inactivated fetal calf serum (GIBCO), 0.5 μg/ml streptomycin, 0.5 μg/ml penicillin, and 1 μg/ml neomycin (GIBCO), in a humidified atmosphere of 5% CO2–95% air at 37°C.

**Animals.** Adult male C57BL/6J (8–12 wk), housed under a 12:12-h day-night cycle at a temperature of 25°C and given free access to tap water and fed a standard pellet diet, were used for the present study. For the salt depletion study, 8 wk-old mice were placed on a low-salt (0.02% NaCl, n = 4) or a normal-salt (0.3% NaCl, n = 4) diet for 2 wk, as described previously (31). Systolic blood pressure was measured by the tail cuff method (BP-monitor MK-2000; Muromachi Kikai). Following experimental treatment, the mice were anesthetized with 10% chloral hydrate in sodium chloride (0.02% NaCl, water and fed a standard pellet diet, were used for the present study. The experimental protocols were approved by the Yokohama City University School of Medicine Institutional Care and Use Committee.

**RNA isolation and Northern blot analysis.** Total RNA was isolated from snap-frozen tissue samples and cultured cells, using single-step method with acid guanidinium thiocyanate-phenol-chloroform extraction. Total RNA (30 μg) was size fractionated on agarose gels and transferred to nylon membranes (Amersham). The membranes were hybridized with 32P-labeled probes, corresponding to amino acid residues 182–249 of MAK-V/Hunk and 18S ribosomal RNA, respectively. The membrane was then washed and exposed to the imaging plate of FUJIX BIO-Imaging Analyzer BAS2500 (Fuji Photo Film) as described previously (26, 27).

**RT-PCR analysis.** RT-PCR was performed to examine the endogenous expression of MAK-V/Hunk mRNA in the renal cortex and medulla, essentially as described previously (26, 28, 31). Briefly, in vitro translated products or cellular extracts were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore), which were blocked with phosphate-buffered saline that contained 5% skim milk powder. The membranes were incubated with MAK-V/Hunk antibody diluted at 1:1,000. The membranes were washed and incubated with the goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences) diluted at 1:1,500 before exposure to the enhanced chemiluminescence (Amersham Biosciences) and placed on film.

**Preparation of recombinant adenoviral vectors and gene transfer.** Adenoviral vectors were prepared using cDNAs coding for MAK-V/Hunk (Ad.MAK-V/Hunk) and bacterial β-galactosidase (Ad.LacZ) commercially available system (Adeno X Expression System, Clontech) as described previously (28). The virus titers were determined by a plaque assay. Ad.MAK-V/Hunk or Ad.LacZ (5 × 10^9 pfu/ml) was transfected into cells. All experiments were performed 48 h after infection.

**In vitro kinase assay.** In vitro kinase assay was performed essentially as described previously (6). Briefly, cellular extracts prepared from the transfected cells by Ad.MAK-V/Hunk or Ad.LacZ were solubilized in 50 mM Tris–HCl (pH 7.5), 140 mM NaCl, 1 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, and 1 μg of aprotinin/ml (buffer A) in the presence of 1% Triton X-100 and 1 μCi/mg histone H1 for 45 min at 37°C. The samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and were exposed to the imaging plate of FUJIX BIO-Imaging Analyzer BAS2500 (Fuji Photo Film) as described previously (26, 27).

**Transcriptional c-fos promoter assay.** For transcriptional fos promoter assay, the c-fos luciferase reporter genes (p2FTL, 1 μg) were transfected into mDCT cells using Lipofectamine 2000 as described...
previously (12, 28). The c-fos luciferase reporter gene consists of two copies of the c-fos-regulated enhancer element (−357 to −276) containing a serum response element (SRE), the herpes simplex virus thymidine kinase gene promoter, and the luciferase gene (23). To normalize the transfection efficiency, we employed a dual reporter assay system, in which the pRL-SV40 plasmid (Promega), containing the sea pansy luciferase gene under the control of the SV40 early enhancer/promoter, was cotransfected as an internal control. The transfected cells were incubated with serum-free medium for 4 h. The cells were further incubated in the presence or absence of ANG II (100 nM) or endothelin-1 (10 nM) for 4 h and lysed for luciferase assay.

ELISA of transforming growth factor-β. ELISA was performed to examine the effect of MAK-V/Hunk on ANG II-mediated transforming growth factor-β (TGF-β) secretion from mDCT cells. mDCT cells were inoculated in the 96-well tray at the concentration 3 × 10^5/ml, incubated overnight, and infected with Ad.LacZ or Ad.MAK-V/Hunk, and then rested in a serum-free medium for 24 h. The cells were further incubated in the presence or absence of ANG II (100 nM) or aldosterone (1 μM) for 36 h. Total TGF-β released into the media was measured by an ELISA system (Promega).

Bromodeoxyuridine incorporation assay. The activity of DNA synthesis was evaluated using a bromodeoxyuridine (BrdU) Labeling and detection kit (Boehringer Mannheim). The mDCT cells were incubated overnight, and infected with Ad.LacZ or Ad.MAK-V/Hunk, and then further incubated in the presence or absence of ANG II (100 nM) or endothelin-1 (10 nM) for 48 h. BrdU labeling was performed during the last 4 h. The incorporated BrdU in place of thymidine into the DNA of proliferating cells was assayed by incubation with an anti-BrdU antibody, detected by a subsequent enzyme reaction and quantified spectrophotometrically at 405 nm. The statistical analysis was performed using the unpaired t-test. A P value of <0.05 was considered to be statistically significant.

RNA interference. The RNA interference experiment was performed essentially as described previously (12). Briefly, small interference RNA (siRNA) with 25 nucleotides was synthesized from Invitrogen Technology (Stealth RNAi). To knockdown the endogenous MAK-V/Hunk expression, the sense oligonucleotide is 5'-AGG AGU ACG AGA AUU UGC U-3', and the antisense oligonucleotide is 5'-AGC AAA UUC UCU AUC UUC AAG U CU C-3'. As a negative control, the sense oligonucleotide 5'-AGG AGU UUA GAG AGA AUU UGC U-3', and the antisense oligonucleotide 5'-AGC AAA UUC UCU AUC UUC AAG UCU C-3' were synthesized. The annealed double-strand siRNA (20 nM) was introduced into mouse distal convoluted tubule (mDCT) cells with the help of Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection nuclear extracts were prepared and subjected to Western blot analysis.

Statistical analysis. Values are expressed as means ± SE in the text and figures. The data were analyzed using ANOVA. If a statistically significant effect was found, a post hoc analysis was performed to detect the difference between the groups. Values of P < 0.05 were considered statistically significant.

RESULTS

Expression of MAK-V/Hunk mRNA in mouse tissues. We previously isolated protein kinases from early differential stages of mouse ES cells by using degenerative oligonucleotide primers specific to the consensus sequences of a great number of protein kinases (7). PCR products from each cDNA source were subcloned individually and screened by DNA sequencing. One of these encompassed the putative kinase domain of a serine/threonine kinase MAK-V/Hunk corresponding to amino acid residues 182–249 (6, 18), which we named the MAK-V/Hunk probe. We first examined the endogenous expression of MAK-V/Hunk mRNA in adult C57BL/6J mouse tissues. The result of Northern blot analysis using the MAK-V/Hunk probe showed that MAK-V/Hunk mRNA was expressed much more abundantly in the kidney, brain, and lung, and only faintly in adipose tissue (Fig. 1A). This result is essentially consistent with a previous report of Gardner et al. (6) describing a tissue distribution of MAK-V/Hunk in FVB mouse. RT-PCR analysis also revealed endogenous expression of MAK-V/Hunk mRNA in the kidney cortex as well as the medulla (Fig. 1B).

Isolation of cDNA clones encoding MAK-V/Hunk. To obtain the cDNA clone covering the entire MAK-V/Hunk coding region, we performed plaque-lifting hybridization screening of a mouse kidney cDNA library (CLONTECH Laboratories) using a [32P]dCTP-labeled random-primed MAK-V/Hunk probe (TakaRa Biomedicals). The longest clone was completely DNA sequenced to ensure that no nucleotide substitutions had occurred (data not shown), and we successfully obtained a cDNA covering the entire MAK-V/Hunk protein coding region.

The results of an in vitro transcription and translation reaction performed in the presence of [35S]methionine showed a prominent band of 80 kDa, which was consistent with the predicted molecular weight (79.6) of MAK-V/Hunk, thereby supporting that the MAK-V/Hunk cDNA cloned in this study covered the entire MAK-V/Hunk protein coding region (Fig. 1C).

Production and validation of the specificity of the antibody for MAK-V/Hunk. We next generated a rabbit anti-MAK-V/Hunk polyclonal antibody. Western blot analysis of HA epitope-tagged MAK-V/Hunk-transfected H9c2 cells revealed that the MAK-V/Hunk antibody as well as the anti-HA antibody recognized the apparent molecular mass of the major bands ~80 kDa, which was consistent with the results of in vitro transcription and translation reaction (Fig. 1D, lanes 2 and 3). The result also revealed the presence of two bands of ~80 kDa and suggested that this doublet represents a product of posttranslational modification. These bands were not observed when empty vector pHA-transfected H9c2 cells were used instead of MAK-V/Hunk-transfected cells or when the MAK-V/Hunk antibody was preabsorbed with a 10-fold excess of the peptide used to generate the antibody (Fig. 1D, lanes 1 and 4). These data indicate that the MAK-V/Hunk antibody is able to recognize the MAK-V/Hunk protein specifically.

Immunohistochemistry for MAK-V/Hunk protein in the mouse adult kidney. Since the result of Northern blot analysis revealed that the MAK-V/Hunk gene was highly expressed in the kidney, we determined the distribution of the MAK-V/Hunk protein in kidney sections from the normal adult mouse by immunohistochemistry. We found the protein translational sites of MAK-V/Hunk to be localized to the cortex and outer medulla of the kidney, but not to the region of the inner medulla and papilla (Fig. 2A). To identify the definite sites of MAK-V/Hunk immunostaining, consecutive sections were stained for MAK-V/Hunk and markers specific for the tubular segments. We used a polyclonal antibody against Tamm-Horsfall protein that is specifically expressed in the thick ascending limbs (TAL) and found that MAK-V/Hunk immunostaining was detected in the TAL but not in the collecting ducts (Fig. 2B, MAK-V/Hunk; Fig. 2C, Tamm-Horsfall pro-
tein). We also used a monoclonal antibody against calbindin-D, a calcium-binding protein expressed primarily in the DCTs and the connecting tubules (CNT) (19, 32), and the immunoreactivity of the MAK-V/Hunk protein was observed specifically in the DCT and CNT (Fig. 2D, MAK-V/Hunk; Fig. 2E, calbindin-D).

Significant staining was not found in the glomeruli, the proximal tubules, the collecting ducts, or in the vasculature, including the arcuate artery, interlobular arteries, and arterioles (data not shown). No immunoreactivity of the MAK-V/Hunk protein was found in the negative control sections that were consecutive sections incubated with preabsorption of the anti-MAK-V/Hunk antibody along with the competing antigenic peptide and incubated with nonimmune rabbit IgG (data not shown). These results demonstrated a specific expression of MAK-V/Hunk in the TAL, DCT, and CNT in the adult mouse kidney.

**Immunohistochemistry for MAK-V/Hunk protein in the mouse embryonic kidney.** Since a previous study using in situ hybridization technique detected MAK-V/Hunk mRNA in the embryonic kidney (E13.5 and E18.5) (6), we examined whether the MAK-V/Hunk protein was expressed in the developing kidney. We determined the distribution of MAK-V/Hunk protein in kidney sections from the normal mouse embryo (E15.5) by immunohistochemistry. A high level of MAK-V/Hunk immunostaining was detected specifically in the distal tubules, which was confirmed by a serial section stained with anti-Tamm-Horsfall antibody (Fig. 3A, MAK-V/Hunk; Fig. 3B, Tamm-Horsfall protein).

**Effect of dietary salt depletion on mRNA expression of MAK-V/Hunk in the mouse kidney.** The above results demonstrated the restricted expression sites of MAK-V/Hunk in distal tubules in the kidney. Because tubular salt reabsorption is a major functional role of renal distal tubules, we investigated the effect of dietary salt depletion on MAK-V/Hunk expression. Mice were fed a low- or normal-salt diet for 2 wk. The mean body weight of the mice fed a low-salt diet (25.7 ± 1.3 g, n = 4) was not significantly different from that of the mice fed a normal-salt diet (26.4 ± 0.7 g, n = 4) at the end of the experiment. Systolic blood pressure was not significantly changed by a low-salt diet (129.3 ± 9.6 mmHg, n = 4) compared with a normal-salt diet (116.0 ± 6.2 mmHg, n = 4). Renal expression of MAK-V/Hunk and AT_1 receptor mRNA in the mice consuming a low- or normal-salt diet was determined by real-time quantitative PCR analysis. Salt depletion led to a 32% increase in the renal expression of the MAK-V/Hunk mRNA compared with a normal-salt diet (Fig. 4A), while the renal expression of AT_1 receptor mRNA was decreased by 27% by a low-salt diet (Fig. 4B).

**Endogenous expression of MAK-V/Hunk in mouse renal distal tubular cells.** Because the results of in vivo study demonstrated that renal MAK-V/Hunk is expressed specifically in the distal tubules in the kidney, we used mDCT cells...
for in vitro study (4). These cells have been shown to have the phenotype of a polarized tight junction epithelium with morphological and functional features retained from the parental distal convoluted tubule cells (9, 10). The mDCT cells express the endogenous MAK-V/Hunk mRNA as detected by RT-PCR (Fig. 5A). Western blot analysis using the anti-MAK-V/Hunk antibody also recognized the endogenous MAK-V/Hunk protein of 80 kDa in mDCT cells (Fig. 5B). These results demonstrate that the MAK-V/Hunk protein is endogenously expressed in mDCT cells.

**Effect of adenoviral transfer of recombinant MAK-V/Hunk on renal distal tubular cells.** To explore the possible cellular function of MAK-V/Hunk, we employed an adenoviral gene transfer of MAK-V/Hunk into mDCT cells. mDCT cells were infected by an adenoviral vector containing MAK-V/Hunk cDNA (Ad.MAK-V/Hunk) or control bacterial β-galactosidase cDNA (Ad.LacZ). We examined kinase activity associated with the MAK-V/Hunk gene product. The level of MAK-V/Hunk expression was significantly increased in Ad.MAK-V/Hunk-infected mDCT cells as shown by the result of Western blot analysis (Fig. 6A). To demonstrate that MAK-V/Hunk protein levels were correlated with kinase activity, an in vitro kinase assay was performed. The polyclonal anti-MAK-V/Hunk antibody was used to immunoprecipitate MAK-V/Hunk from cellular extracts prepared from Ad.MAK-V/Hunk-infected mDCT cells. The resulting immunoprecipitates were incubated with [γ-32P]ATP and histone H1 as a substrate. The MAK-V/Hunk-associated phosphotransferase ac-
Activity was significantly greater in immunoprecipitates prepared from Ad.MAK-V/Hunk-infected mDCT cells compared with Ad.LacZ-infected mDCT cells (Fig. 6B). These findings demonstrated that the MAK-V/Hunk cDNA clone isolated encodes a functional protein kinase and that the kinase activity is substantially increased by overexpression of MAK-V/Hunk. MAK-V/Hunk belongs to a new branch of the SNF1 family (1). The SNF1 family of kinases has been implicated in the regulation of intracellular mediators of responses related to cell growth and differentiation. ANG II is another well-known activator of these signaling pathways, and ANG II is capable of inducing the synthesis of the profibrotic cytokine TGF-β in renal tubules, associated with subsequent alterations in cell growth and matrix production (21, 35). Thus, to investigate a possible role of MAK-V/Hunk in the functional modulation of distal tubular cells, we examined the effects of MAK-V/Hunk on the downstream effectors of the AT1 receptor-signaling pathway in mDCT cells by performing adenoviral transfer of recombinant MAK-V/Hunk. We performed c-fos transcriptional assay, ELISA of TGF-β, and BrdU incorporation assay.

In mDCT cells infected by Ad.LacZ, treatment with ANG II (100 nM) induced the c-fos reporter gene by 1.9-fold (Fig. 6C). In contrast, pretreatment with an AT1 receptor-specific antagonist telmisartan (1 μM), but not an AT2 receptor-specific antagonist PD123319 (10 μM), completely inhibited ANG II-mediated activation of c-fos reporter gene expression. Interestingly, overexpression of MAK-V/Hunk also significantly decreased the ANG II-induced activation of c-fos gene tran-

Fig. 4. Effects of dietary salt depletion on MAK-V/Hunk and AT1 receptor mRNA expression in the mouse kidney. Real-time PCR analysis shows relative MAK-V/Hunk and AT1 receptor mRNA levels in the kidney of mice fed a low-salt diet (LS) or a normal-salt diet (NS; MAK-V/Hunk; A and AT1 receptor; B). Results are expressed as means ± SE (n = 4 in each group).

Fig. 3. Immunohistochemical localization of MAK-V/Hunk expression in the embryonic mouse kidney. Consecutive embryonic (E15.5) kidney sections stained for MAK-V/Hunk and Tamm-Horsfall protein show specific MAK-V/Hunk expression in the distal tubules (MAK-V/Hunk; A and Tamm-Horsfall protein; B). Original magnification: ×400.
Furthermore, knockdown of endogenous MAK-V/Hunk expression sensitized the stimulatory effect of ANG II (100 nM) on c-fos gene transcription (Fig. 7B). These results indicate that MAK-V/Hunk is involved in the suppression of the ANG II-induced proliferative activity of the renal distal tubular cells.

Colocalization of ATRAP with AT₁ receptor in the mouse adult kidney. Finally, to examine a possible colocalization of MAK-V/Hunk and AT₁ receptor in the kidney, immunohistochemistry using the MAK-V/Hunk antibody and the AT₁ receptor antibody within kidney sections from normal adult mouse was performed. In the consecutive sections stained for MAK-V/Hunk and AT₁ receptor in the renal cortex, MAK-V/Hunk-immunopositive tubules, which were mainly DCT, expressed the AT₁ receptor, and the immunoreactivity of both MAK-V/Hunk and AT₁ receptor was intense on the basal side of the distal tubules (Fig. 8).

DISCUSSION

A novel mammalian SNF1-related protein kinase, MAK-V/Hunk, was originally cloned by Gardner et al. and Korobko et al. (2, 5, 6, 18) as a kinase rich in the mouse mammary gland with regulated expression during pregnancy-induced changes in the mammary gland. Gardner et al. (5, 6) reported that MAK-V/Hunk is expressed in subsets of cells within several tissues including the brain, lung, ovary, thymus, kidney, duodenum, and uterus in addition to the breast, and showed that MAK-V/Hunk expression is developmentally and tissue specific.

In the present study, we focused on the characterization of renal MAK-V/Hunk in vivo and in vitro. We identified the fact that MAK-V/Hunk is highly expressed in the kidney and its expression level is increased by salt restriction. In the kidney, MAK-V/Hunk expression is restricted to the distal tubules, and specifically, the endogenous expression of MAK-V/Hunk was demonstrated in mDCT cells derived from mouse distal renal tubular cells (4). The mDCT cells have been shown to have the phenotype of a polarized tight junction epithelium with morphologic and functional features retained from the parental distal convoluted tubule cells (9, 10). Moreover, the results of functional assays using mDCT cells infected by the adenoviral MAK-V/Hunk expression vector and transcriptional c-fos promoter assay using mDCT cells transiently transfected with MAK-V/Hunk siRNA indicate that MAK-V/Hunk plays a role in the regulation of cellular proliferative activity in response to ANG II, a potent stimulator of renal remodeling and fibrotic signaling (34, 36).

There has been no previous report that examines the spatial and temporal distribution of MAK-V/Hunk in the kidney in detail. By immunohistochemistry, we demonstrated that the MAK-V/Hunk protein was specifically expressed in the TAL, DCT, and CNT but not in other nephron segments, the glomerulus, or vessels of the kidney. This is the first report to demonstrate the restricted distribution of MAK-V/Hunk protein in the distal nephron segments in embryonic kidney as well as adult kidney. This finding is essentially consistent with the previous study performed by in situ hybridization, which showed that the expression of MAK-V/Hunk mRNA is generally restricted to a subset of cells within particular compartments (6). Furthermore, the finding that the MAK-V/Hunk protein is highly and specifically expressed in the distal tubules...
of the embryonic kidney suggests that this kinase may have a role in the development of the renal distal tubules. Since the consecutive renal sections were stained for MAK-V/Hunk and markers of nephron segments (Tamm-Horsfall protein and calbindin-D) to examine the nephron segment-specific expression of MAK-V/Hunk, limitations of the present study include the lack of immunofluorescent colocalization analysis with double staining of MAK-V/Hunk and these specific markers using the multiple fluorolabeling method and confocal laser microscopy.

The restricted expression pattern of the MAK-V/Hunk protein in the kidney might be explained by the mechanism of transcriptional regulation. For example, the promoter regions of ksp-cadherin and thiazide-sensitive Na-Cl cotransporter

of the embryonic kidney suggests that this kinase may have a role in the development of the renal distal tubules. Since the consecutive renal sections were stained for MAK-V/Hunk and markers of nephron segments (Tamm-Horsfall protein and calbindin-D) to examine the nephron segment-specific expression of MAK-V/Hunk, limitations of the present study include the lack of immunofluorescent colocalization analysis with double staining of MAK-V/Hunk and these specific markers using the multiple fluorolabeling method and confocal laser microscopy.

The restricted expression pattern of the MAK-V/Hunk protein in the kidney might be explained by the mechanism of transcriptional regulation. For example, the promoter regions of ksp-cadherin and thiazide-sensitive Na-Cl cotransporter
contain consensus binding motifs for activator protein-2 (AP-2) and DCT-specific transcription factor hepatocyte nuclear factor-3/folk head homolog-3 (HFH-3) that confer specific expression in renal distal tubular epithelial cells (14, 29, 33). Since we have found that the promoter region (from \(-1.0\) kb to \(+1\) of the transcriptional start site) of MAK-V/Hunk also contains several putative binding sites for AP-2 and HFH-3 (data not shown), it is possible that these sites are involved in the specific expression of MAK-V/Hunk in the renal distal tubules.

Accumulated evidence demonstrated that the distal nephron including TAL and DCT is important for the regulation of the handling of sodium (16). In addition, a previous study showed that the AMP-activated protein kinase (AMPK), another SNF-1 family kinase, is also expressed in the renal distal tubules and that dietary salt intake regulates the activity of AMPK in the kidney (3). In this study, we found that the renal expression of MAK-V/Hunk is increased by dietary salt restriction. Dietary salt depletion is known to increase the components of the renin-angiotensin system in the kidney, to activate the circulating renin-angiotensin system (15, 17), and to decrease the expression of renal AT1 receptor mRNA (24, 25). The result from the present study indicates that MAK-V/Hunk and the AT1 receptor are regulated in opposite directions by the conditions that alter the activity of the renin-angiotensin system in vivo and suggests that tubular Na\(^+\) and/or Cl\(^-\) concentrations may modulate the expression of MAK-V/Hunk in the renal distal tubules and that physiological and pathological stimuli may affect renal MAK-V/Hunk expression.

From the immunohistochemical results of the present study, MAK-V/Hunk-immunopositive distal tubules expressed the AT1 receptor in the consecutive sections stained for MAK-V/Hunk and the AT1 receptor, thereby demonstrating a substantial colocalization of both proteins in the renal distal tubules.

Fig. 7. Effects of knockdown of endogenous MAK-V/Hunk by RNA interference on c-fos transcriptional activity of renal distal tubular cells. A: representative Western blot analysis showing the expression of the endogenous MAK-V/Hunk protein in mDCT cells transfected with control siRNA (Control-si) or MAK-V/Hunk siRNA (MAK-V/Hunk-si), with the expression of the housekeeping gene, \(\beta\)-actin, also presented for comparison. Untreated, no transfection. B: relative c-fos promoter activities in mDCT cells transiently transfected with the c-fos luciferase reporter gene (p2FTL), pRL-SV40, and control siRNA (Control-si) or MAK-V/Hunk siRNA (MAK-V/Hunk-si) were measured by luciferase assay. Relative c-fos luciferase activity is expressed as means \(\pm\) SE (\(n = 4\) in each group).

Fig. 8. Immunohistochemical colocalization of MAK-V/Hunk and AT1 receptor in the adult mouse kidney. The consecutive sections stained for MAK-V/Hunk and AT1 receptor in the mouse renal cortex show a substantial colocalization of both proteins in the renal distal tubules, mainly in the distal convoluted tubules (arrowheads; MAK-V/Hunk; A and AT1 receptor; B). Original magnification: \(\times 200\) for A and B.
Further molecular screens for both MAK-V/Hunk and AT₁ receptor will reveal the existence of additional partners for these molecules that may act cooperatively or independently both in time and in a specific cellular location in the kidney. These questions are interesting, and our laboratory is actively characterizing these interactions.

MAK-V/Hunk belongs to a new branch of the SNF1 family (1). The SNF1 family of kinases has been implicated in the regulation of developmental processes including cell cycle control, establishment of cell polarity, and differentiation. In the current study, gain-of-function and loss-of-function approaches demonstrated that MAK-V/Hunk negatively regulates cellular proliferative activity in distal tubular cells. Overexpression of MAK-V/Hunk by adenoviral gene transfer in mDCT cells blocked the ANG II-induced increases in c-fos transcriptional activity, TGF-β production, and BrdU incorporation, but did not affect the stimulatory effects by endothelin-1 or aldosterone. These results indicate that the MAK-V/Hunk overexpression specifically suppressed AT₁ receptor signaling in distal tubular cells. A previous study showed that the overexpression of MAK-V/Hunk in the mammary epithelium of MMTV-MAK-V/Hunk transgenic mice results in decreased proliferation of alveolar epithelial cells during pregnancy (5), consistently with our results. Conversely, the MAK-V/Hunk knockdown by siRNA increased the stimulatory effect of ANG II on the c-fos transcriptional activity.

The precise functions of MAK-V/Hunk in the renal distal tubular cells remain to be determined, but the results do imply that MAK-V/Hunk could well have a role in both the developmental and physiological functioning of the distal nephron via a modulation of cellular proliferative activity. Further studies will be needed to elucidate the molecular mechanism of MAK-V/Hunk-mediated inhibition of ANG II signaling in renal pathophysiolo, and these will be undertaken in due course. In conclusion, this study demonstrates the abundant and specific expression of MAK-V/Hunk and distribution in the kidney. MAK-V/Hunk is specifically and highly expressed in the distal tubules of the embryonic and adult kidney, and this is the first report characterizing the cellular localization of MAK-V/Hunk in renal tubular cells.

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