# Expression of ATP sensitive $K^+$ channel subunit Kir6.1 in rat kidney

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ATP-sensitive $K^+$ ($K_{ATP}$) channels in kidney are considered to play roles in regulating membrane potential during the change in intracellular ATP concentration. They are composed of channel subunits (Kir6.1, Kir6.2), which are members of the inwardly rectifying $K^+$ channel family, and sulphonylurea receptors (SUR1, SUR2A and SUR2B), which belong to the ATP-binding cassette superfamily. In the present study, we have investigated the expression and localization of Kir6.1 in rat kidney with Western blot analysis, immunohistochemistry, *in situ* hybridization histochemistry, and immunoelectron microscopy. Western blot analysis showed that Kir6.1 was expressed in the mitochondria and microsome fractions of rat kidney and very weakly in the membrane fractions. Immunohistochemistry revealed that Kir6.1 was widely distributed in renal tubular epithelial cells, glomerular mesangial cells, and smooth muscles of blood vessels. In immunoelectron microscopy, Kir6.1 is mainly localized in the mitochondria, endoplasmic reticulum (ER), and very weakly in cell membranes. Thus, Kir6.1 is contained in the kidney and may be a candidate of mitochondrial $K_{ATP}$ channels.

Key words: ATP-sensitive $K^+$ channel, Kir6.1, immunohistochemistry, *in situ* hybridization, immunoelectron microscopy, kidney, rat

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ATP-sensitive $K^+$ ($K_{ATP}$) channels, originally discovered by a patch-clamp technique in the cardiac muscle (Noma 1983), belong to the inwardly rectifying $K^+$ channel superfamily. These channels have ATP sensitivity and weak inward rectification capacity (Aguilar-Bryan *et al.* 1998). In 1995, a pore-forming subunit was cloned (Inagaki *et al.* 1995), and was ubiquitously localized in tissues and cells. Initially, the subunit was called $uK_{ATP}$-1, and later was named as Kir6.1. It was detected in liver and skeletal muscle (Suzuki *et al.* 1997), brain (Lacza *et al.* 2003a; Zhou *et al.* 1999), heart (Lacza *et al.* 2003b; Zhou *et al.* 2005), human embryo kidney (HEK) 293 cells (Ammala *et al.* 1996; Braun *et al.* 2002; Kondo *et al.* 1998; Satoh *et al.* 1998), and in kidney as well (Anzai *et al.* 1996; Braun *et al.* 2002; Brochiero *et al.* 2002). The functional $K_{ATP}$ channels need four pore-forming subunits of Kir6.x (Kir6.1 or Kir6.2) as well as four regulatory subunits of SURs (SUR1, SUR2A, or SUR2B) that form a hetero-octameric compound (Clement *et al.* 1997; Inagaki *et al.* 1996). In most excitable tissues $K_{ATP}$ channels are activated when cell metabolism is impaired; thereby the cell is clamped in the resting state which conserves ATP, with the benefit of keeping the structural integrity of the cell (Quast 1996).

Although several lines of evidence including patch clamp techniques (Wang *et al.* 1995), RT-PCR (Brochiero *et al.* 2002), Northern blot analysis (Inagaki *et al.* 1995), Western blot analysis (Braun *et al.* 2002) and immunohistochemistry (Anzai *et al.* 1996; Braun *et al.* 2002) clearly revealed that Kir6.1 localized in kidney, and Kir6.1 mRNA increased significantly after renal ischemia (Sgard *et al.* 2000), a conflicting report claimed that Kir6.1 protein was not expressed in rat kidney (Sun *et al.* 2004). Thereby, it is important to elucidate whether or not Kir6.1 is localized in the kidney. Confirmation of its localization will lead to improved understanding of its functions in renal tubules. The cellular and subcellular localization of Kir6.1 in renal tubular epithelial cells has not been established, although it was found in the mitochondria as well as in the surface plasma membrane of rat liver, skeletal muscle, brain neurons and glial cells, and cardiomyocytes (Lacza *et al.* 2003b; Singh *et al.* 2003; Suzuki *et al.* 1997; Zhou *et al.* 2005; Zhou *et al.* 1999).

The aim of the present study is to determine detailed information of Kir6.1 in renal tubular epithelial cells. Its expression was assessed by Western blot analyses with cellular fractions, its localization was observed by immunohistochemistry and *in situ* hybridization, and its subcellular localization was revealed by immunoelectron microscopy.

## Materials and Methods

### *Generation of anti-Kir6.1 antibody*

Rabbit anti-Kir6.1 antibody was raised against a synthetic 14 amino acid peptide, $NH_2$-(C)QFMT-PEGNQCPSES-OH, which corresponds to amino acid residues 411 to 424 of rat Kir6.1 (Gene No. D42145). The polyclonal peptide antibody production was processed according to Van Bueren *et al.* (1993), with some modifications. In brief, the synthetic peptide representative of rat Kir6.1 was coupled to the carrier protein keyhole limpet hemocyanin (KLH) via the N-terminal cysteine residue added to the peptide. Two Japanese white rabbits, 2.5-3.0 kg (Japan SLC, Hamamatsu, Japan) were injected with approximately 200 μg of peptide-KLH conjugate emulsified with an equal volume of Freund's complete adjuvant (Rockland Immunochemicals) at multiple intradermal sites, followed by 3 boosters once 2 weeks interval later by injection with the same dosage of the peptide conjugate emulsified in Freund's incomplete adjuvant (Rockland Immunochemicals, Gilbertsville, PA, USA) three time. The antiserum was harvested 1 week after the final injection. The antiserum was purified by immunoaffinity column chromatography before using for Western blot analysis and immunohistochemistry.

### *Transfection and preparation of cells extract*

COS-7 cells were transfected with rat Kir6.1 in the mammalian expression vector pFLAG-CMV5a by lipofectamine method according to the manufacturer's instructions. Briefly, The PCR product, a full-length rat Kir6,1 cDNA was subcloned into BamHI site of pFLAG-CMV-5a vector (Sigma-Aldrich, St. Louis, MO, USA), designated as pFLAG-CMV-5a-rKir6.1. Twenty-four hours prior to transfection, COS-7 cells were plated at a density of $2.0\times10^6$ cells/100-mm cell-culture dish (TPP, Trasadingen, Switzerland) in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gemini Bioproducts Woodland, CA, USA). Cells were incubated at 37°C in 5% $CO_2$ with 95% air and transfected with 4 μg of pFLAG-CMV-5a-rKir6.1 plasmid vector using 15 μL of LipofectAMINE™ 2000 Reagent (Invitro-gen, Carlsbad, CA, USA).

The transfected cells were washed with PBS 48 or 72 h after transfection, suspended in 0.25 M sucrose/20 mM Tris-HCl buffer, pH 7.4, containing 4 mM EDTA, 1 mM EGTA and 1 mM DTT with complete protease inhibitor cocktail tablets (Roche, Basel, Switzerland) and sonicated for 5 min with 50% duty cycle on ice with an Ultrosonic Processor (Sonics & Materials inc., Newtown, Connecticut, USA). The cell lysate was clarified by centrifugation (1500 × g for 10 min at 4°C), stored at -80°C until use.

### *Preparation of cRNA probes*

Single strand riboprobes were labeled with digoxigenin prepared using Dig RNA Labeling Kit (Boehringer Mannheim, GmbH, Germany) according to the manufacturer's instructions. The template for synthesis of digoxigenin (DIG)-labeled RNA probes was generated by using PCR amplification from pGEM-rat-Kir6.1 plasmid. The primer sequences were 5′-TCCATCTTGATTCAGACCC TCCAAAAGAGTGAACTGTCGCACCAG-3′ and 5′-GGCGACAGGTCCGA TACTTCGATCACCAGAACTCA GCAAACTGTC-3′ corresponding to 1266-1310 and 1514-1558 of the rat Kir6.1 (D42145). PCR reaction was run with Taq polymerase (Takara, Otsu, Japan) for 34 cycles of 94°C × 30 sec denaturating, 42°C × 30 sec annealing, and 72°C × 1 min extension each, with a final extension of 7 min at 72°C. A 293-bp PCR product was obtained and subcloned into a TA cloning pCRII vector (Invitrogen, San Diego, CA, USA). The identity and orientation of the constructs were verified by sequence analysis (Takara, Otsu, Japan) and the product was identical to the published sequence of rat Kir6.1. After linearization of the construct with

*Bam*HI (Toyobo, Osaka, Japan), the antisense RNA probe was generated using T7 RNA polymerase (Takara, Otsu, Japan). To generate a sense RNA probe, the construct was linearized with *Eco*RI (Boehringer Mannheim, GmbH, Germany), and transcription was performed using SP6 RNA polymerase (Takara, Otsu, Japan).

### *Animals and tissue preparations*

Male Wistar rats (4 to 6 weeks) were used (Japanese SLC; Hamamatsu, Japan). The protocols for animal experimentation described herein were previously approved by the Animal Research Committee, Akita University; all subsequent animal experiments adhered to the *Guidelines for Animal Experimentation* of the University.

The rats were anesthetized through peritoneal injection of pentobarbital sodium (Abbott Lab.; Chicago, Illinois, USA) as 50 mg per kilogram body weight, and 4% paraformaldehyde (PFA) buffered at pH 7.4 by 0.1 M phosphate buffered saline (PBS) was perfused through the left ventricle at room temperature. The excised kidneys were cut into thin slices and placed into the same fixative at 4°C over night and subsequently transferred into 30% sucrose in PBS at 4°C. For *in situ* hybridization histochemistry, kidneys were quickly removed and immediately frozen on powdered dry ice. Cryosections for both groups were cut at a thickness of 8-10 μm and thaw-mounted on MAS-coated glass slides (Matsunami Glass Ind. Ltd.; Kishiwada, Japan).

### *Subcellular fractionations*

All procedures were carried out at 0-4°C. The kidneys were immediately excised from anesthetized rats and quickly washed with 0.9% NaCl solution. They were then cut into small pieces and homogenized with protein extraction buffer with 0.25 M sucrose in 50 mM Tris-HCl, pH7.4, containing proteinase inhibitor cocktail tablets (Roche Diagnostics GMbH, Mannheim, Germany). Subcellular fractions were extracted as described elsewhere (Itoh *et al.* 2002). Briefly, after centrifugation at 600 × *g* for 10 min, the precipitate was discarded, and the 600 g supernatant was centrifuged at 7000 × *g* for an additional 10 min. The 7000 *g* precipitate (P1) was re-dissolved in the buffer and centrifuged at 5000 × *g* for 10 min, and the 5000 *g* precipitate was used as the mitochondrial fraction. The 7000 g supernatant (S1) was centrifuged at 54,000 × *g* for 60 min, and the supernatant (S2) was centrifuged at 105,000 × *g* for an additional 60 min. The 54,000 g precipitate was used as the cell membrane fraction, the 105,000 *g* precipitate as the microsome fraction, and the supernatant as the cytoplasm fraction.

### *Immunoblotting*

Proteins of harvest COS-7 cells transfected with pFLAG-CMV5a-rKir6.1 plasmid vector, COS-7 cells only, and whole rat kidney, heart, brain, liver, and testis, and subcellular fractions of kidney were denatured in a modified sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol blue and 10% 2-mercaptoethanol). Electrophoresis was performed on 10 or 12% SDS-polyacrylamide gel. The proteins were subsequently transferred onto a polyvinylidine difluoride (PVDF) membrane (NENTM Life Science, Boston, CA, USA) by using a semi-dry transfer unit (Hoefer TE70 series, Amersham Pharmacia Biotech) according to the manufacturer's instruction. The transferred PVDF membranes were then blocked with 5% Blot-QuickBlocker (Chemicon International, Inc.; Temecula, CA, USA) in PBS over night at room temperature. The PVDF membranes were incubated with rabbit anti-rat Kir6.1 antibody (Zhou *et al.* 2005) diluted to 1:1000, or mouse anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:1000 for 1 h at 37°C. After rinsing with PBS-T (PBS containing 0.1% Tween-20), they were then reacted to HRP-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) diluted to 1:3000, or HRP-conjugated sheep anti-mouse (Amersham Pharmacia Biotech) diluted to 1:6000 for 30 min at room temperature. Membrane was washed three times with PBS-T; bands were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions and exposed to X-OMAT™ film (Eastman Kodak, Rochester, NY, USA).

### *Immunohistochemistry*

Cryosections of kidney were kept in PBS containing 0.3% Tween-20 for 45 min. Prior to incubation with the first antibody, sections were treated with 0.3% $H_2O_2$/methanol solution and ABC blocking kit (Vector Lab., Inc.) to reduce endogenous peroxidase reaction as well as non-specific binding with avidin-biotin complex. After incubation with 5%

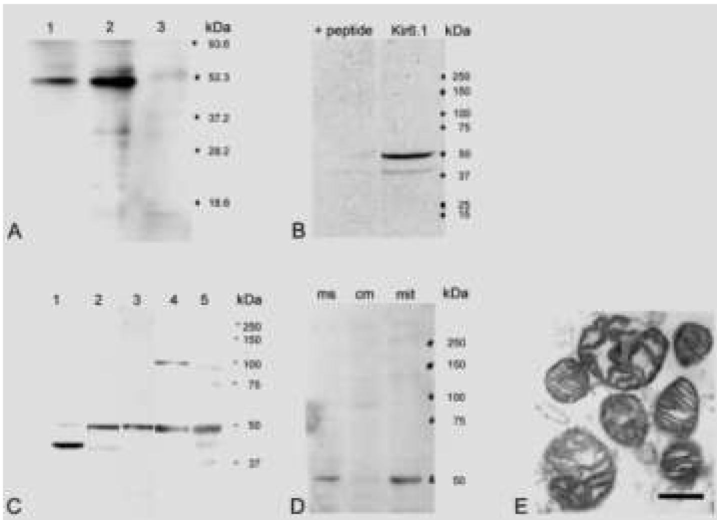


**Figure 1. Immunoblot analysis of Kir6.1 from COS-7 cells, rat kidney extracts and preparation of cellular fractions. (A) Anti-FLAG M2 antibody recognizes a 50 kDa band in the extract of COS-7 cells transfected with pFLAG-CMV5a-rKir6.1 plasmid vector (lane 1). Polyclonal anti-Kir6.1 antibody recognizes a prominent band (50 kDa) in the extract of COS-7 cells transfected with pFLAG-CMV5a-rKir6.1 plasmid vector (lane 2), but no remarkable band was detected in the extract of COS-7 cells only (lane 3). (B) Anti-Kir6.1 antibody recognizes a prominent band (50 kDa) and a faint small band below in kidney extracts (right lane, Kir6.1). Preabsorption with immunizing peptide antigen prevents the appearance of these bands (left lane, + peptide). (C) The anti-Kir6.1 antibody also expresses in all tissues examined such as heart (lane 1), kidney (lane 2), brain (lane 3), liver (lane 4) and testis (lane 5). (D) In the kidney cellular fractions, the anti-Kir6.1 antibody recognizes a prominent band in the mitochondrial fractions (mit), a weaker band in the microsome fractions (ms), and a faint band, in the cell membrane fractions (cm). (E) Electron micrograph shows the purity of the mitochondrial fractions. Scale bars: 500 nm.**

normal goat serum for 1 h, the sections were reacted with rabbit anti-Kir6.1 antibody at a dilution of 1:500 for 12 h at room temperature. The sections were then treated with biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories Inc., Burlingame, CA) at a dilution of 1:200 for 30 min, and then with ABC complex (Vectastain ABC kit, Vector Lab., Inc.) for 45 min according to the manufacturer's instructions. Reactivity was visualized by incubating the sections in 0.001~0.005% DAB (3,3'-diaminobenzidine tetrahydrochloride) reaction with 0.003% $H_2O_2$ and counterstained with methyl green. Between the above steps, each section was carefully rinsed 3 times with PBS containing 0.05% Tween-20, except between the normal goat serum and the first antibody.

### *Immunoelectron microscopy*

For immunoelectron microscopy, the sections that showed good immunoreaction to anti-Kir6.1 antibody mentioned above were post-fixed in 1% osmium tetroxide/PBS for 30 min, dehydrated in an ethanol series, and embedded in Quetol 812. Thin sections were cut and observed under an electron microscope without uranyl acetate and lead citrate staining.

### *In situ hybridization*

Fresh frozen sections of rat kidneys were fixed in 4% paraformaldehyde for 15 min and then digested with 10 μg/mL proteinase K at room temperature for 5 min. They were refixed in 4% paraformaldehyde for 10 min, treated with 0.2N HCl for 10 min for inactivation of internal alkaline phosphatase, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0 for 10 min. After dehydration with a graded series of ethanol and air drying, hybridization was performed at 50°C for 16 h under a parafilm coverslip with the hybridization buffer, which contained 50% deionized formamide, 10% dextran sulfate, 10 mM Tris HCl pH 7.6, 200 μg/mL salmon sperm DNA, 1 × Denhardt's solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, and 0.1-0.5 μg/slide cRNA probe. After rinsing and treatment with 50 μg/mL RNase at 37°C for 30 min, hybridized digoxigenin-labeled probes were detected by Nucleic Acid Detection Kit (Boehringer Mannheim, GmbH, Germany) according to the manufacturer's instructions. After color reaction, sections were rinsed with 10 mM Tris HCl pH 7.6 and 1.0 mM EDTA, post-fixed with 4% parafomaldehyde in PBS, rinsed with distilled water, and sealed without counter staining. For the control experiment, the same procedure was employed with a sense cRNA probe.

### *Electron microscopy of the mitochondrial fractions*

In order to confirm the purity of the mitochondrial fractions used in the immunoblotting, pellets obtained from the 5000 g precipitate were fixed in 2% glutaraldehyde for 2 h, followed by 1% $OsO_4$ for 2 h. Between the above steps, the pellets were rinsed carefully, then dehydrated with a graded acetone series and embedded in Quetol 812. Thin sections were cut and directly examined under an electron microscope.

## Results

The anti-Kir6.1 antibody recognized a prominent 50 kDa protein band and a weak small band below in the extracts of COS-7 cells transfected with rat Kir6.1 cDNA (Figure 1A, line2) but no remarkable signal was detected in extracts of COS-7 cells only (Figure 1A, lane 3). The extracts of COS-7 cells transfected with rat Kir6.1 cDNA was also detected by mouse anti-FLAG M2 antibody (Figure 1A, lane 1). This result indicated that the cellular transfection was successfully and the specificity of anti-Kir6.1 antibody is qualifed. In rat kidney extract the anti-Kir6.1 antibody recognized a prominent 50 kDa band and a weak small band below (Figure 1B, right lane). The specificity of binding to Kir6.1 was further confirmed by the fact that the detection signal was completely removed by pre-absorption with the immunizing peptide antigen (Figure 1B, left lane). This antibody recognizes a 50 kDa band in all examined tissues such as kidney, brain liver, and testis except heart which showed a ~ 43 kDa band, and a weak 50 kDa band (Figure 1C).

In kidney cellular fractions, Kir6.1 was expressed prominently in the mitochondrial fraction (Figure 1D, mit), weakly in the microsomal fraction (Figure 1D, ms), and very weakly, if at all, in the cell membrane fraction (Figure 1D, cm).

The purity of mitochondrial fractions was confirmed by electron microscopy. In the electron micrograph, most of the visual field was covered with intact mitochondria (Figure 1E).

By immunohistochemistry, Kir6.1 protein was widely distributed in the renal cortex and medulla (Figure 2A). It was expressed in podocytes, mesangial cells of glomerulus, and epithelial cells of renal tubules (Figure 2B, C). Some of the moderate immunoreactivity with Kir6.1 was observed as fine granular or punctate reaction products in the cytoplasm of proximal tubules and distal tubules (Figure 2B). It was expressed only weakly in the basolateral membrane or apical membrane. The immunoreactivity was also expressed in collecting ducts in the medulla (Figure 2D). Immunoreactivity was also expressed in the smooth muscles and endothelium of blood vessels within the kidney (Figure 2E). No significant immunoreactivity could be observed in the rat kidney when anti-Kir6.1 antibody was pre-absorbed with the immunizing peptide antigen (Figure 2F).

By *in situ* hybridization histochemistry, the localization of Kir6.1 mRNA in the kidney was detected with antisense cRNA probes as purple deposition of

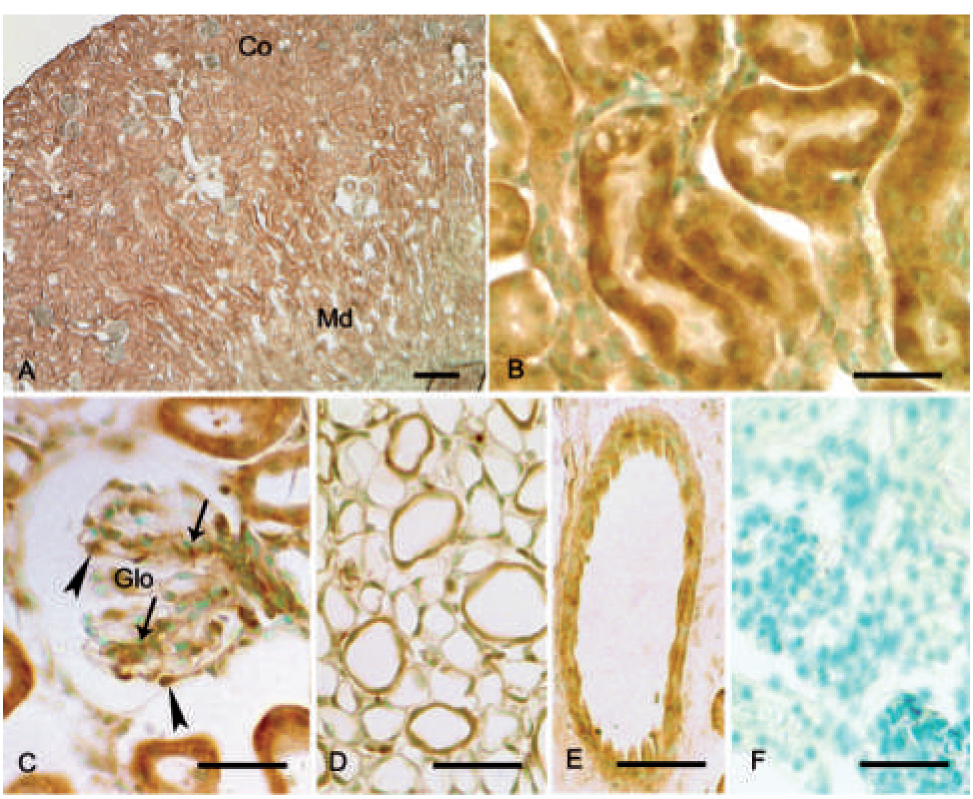


**Figure 2. Immunohistochemistry on cryosections of rat kidney showing expression of Kir6.1 protein. (A) Immuno-reactivity with anti-Kir6.1 is seen widely in the renal cortex (Co) and medulla (Md). (B) Proximal tubules in renal cortex show Kir6.1 immunoreactivity localized in the cytoplasm as granular or punctate products. (C) In the renal cortex, Kir6.1 is expressed in mesangial cells (arrows) and podocytes (arrowheads) of the glomerulus (Glo). (D) In the renal medulla Kir6.1 is also localized in the collecting ducts. (E) Smooth muscles and endothelium of blood vessels show immunoreactivity to Kir6.1. (F) No significant immunoreactivity of Kir6.1 is seen following pre-absorption with immunizing peptide antigen. Scale bars: A = 100 μm; B, C, D = 20 μm; E, F = 50 μm.**

reaction products of alkaline phosphatase with nitroblue tetrazolium chloride (NBT). The Kir6.1 mRNA was widely expressed in the renal cortex (Figure 3A) and the medulla (Figure 3B) as well as blood vessels (Figure 3C), as observed as the distribution of Kir6.1 protein. In the renal cortex, the glomerulus (Glo) expresses Kir6.1 at a weak level in the cells located within the Bowman's capsule (Figure 3A). The proximal convoluted tubule (PCT), distal convoluted tubule (DCT), and collecting ducts express Kir6.1 moderately (Figure 3A, B). Sections treated with sense probes expressed no significant reactions (Figure 3D).

Under electron microscope, the punctate immunoreaction products for Kir6.1 observed by light microscopy were localized in the mitochondria in the renal tubular epithelial cells (Figure 4A, B). Some endoplasmic reticulum and small vesicles, as well as microvilli, were also observed to be immunopositive in the apical portion of epithelial cells (Figure 4A, B).

## Discussion

Among various $K^+$ channels, Kir6.1 is distinct because of its ATP sensitivity and its ubiquitous distribution in various cells and organs including kidney as detected with Northern blot and PCR analysis (Inagaki *et al.* 1995; Brochiero *et al.* 2002). A new polyclonal anti-Kir6.1 antibody was applied to investigate the detailed distribution of Kir6.1 protein in rat kidney. The specificity of the antibody was assessed by analyzing cells transfected with the corresponding gene and Western blot analysis. This antibody recognized a prominent 50 kDa band and a faint small band in COS-7 cells transfected with Kir6.1 and kidney extraction. The immunopositive bands were eliminated after preabsorption with immunizing peptide antigen, further proved the specificity of the new anti-Kir6.1 antibody. The Kir6.1 protein was widely expressed in renal tubules both in cortex and medulla, which was confirmed by *in situ* hybridization with Kir6.1 cRNA probe. In addition, Kir6.1 was also expressed

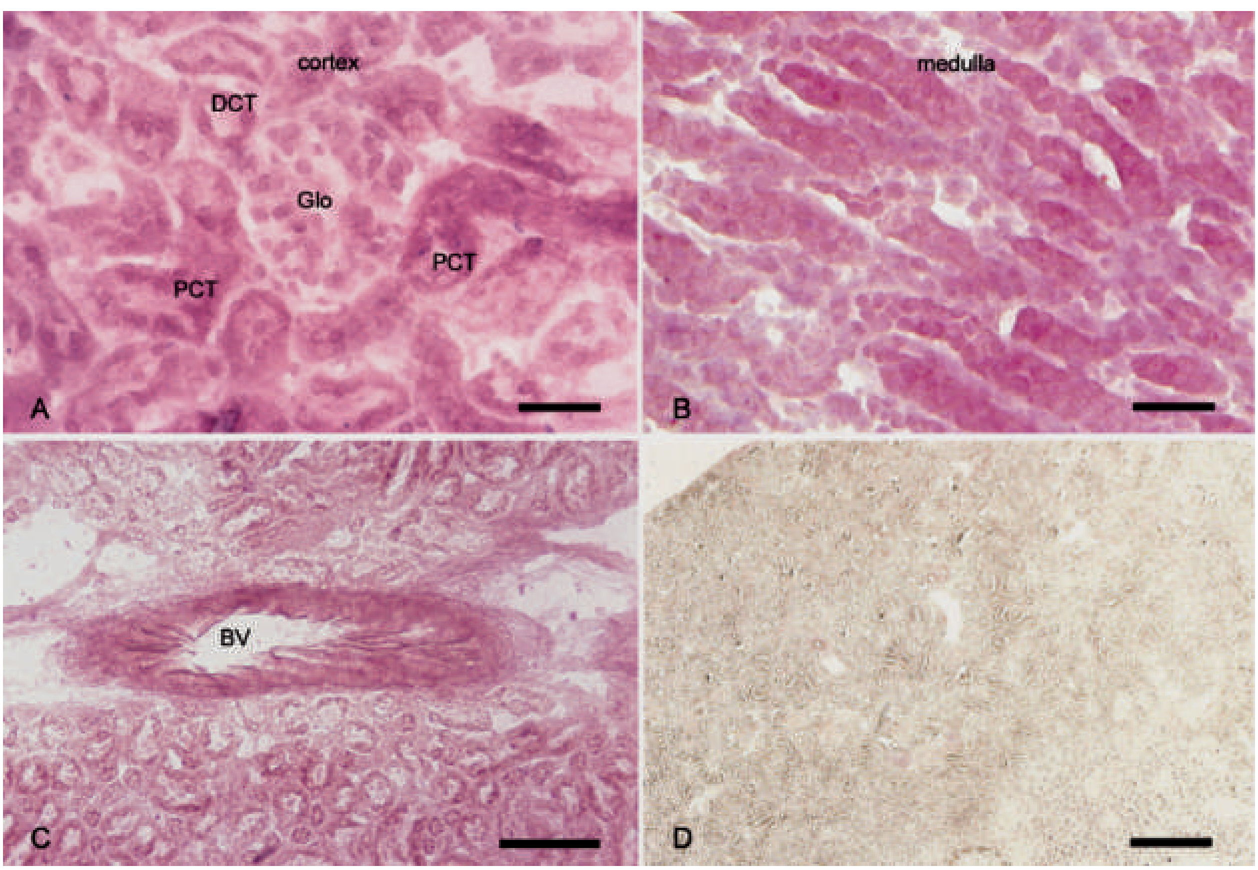


**Figure 3. *In situ* hybridization histochemistry of Kir6.1 in rat kidney detected by an antisense cRNA probe. Purple deposition indicates localized Kir6.1 mRNA. (A) Kir6.1 mRNA was clearly shown in the glomerulus (Glo), proximal convoluted tubules (PCT), and distal convoluted tubules (DCT) of the renal cortex. (B) Rat renal medulla also shows Kir6.1 mRNA. (C) The Kir6.1 mRNA was also expressed in blood vessels (BV). (D) A sense probe did not detect any signal for Kir6.1 mRNA. Scale bars: A, B = 20 µm; C = 40 µm; D = 200 µm.**

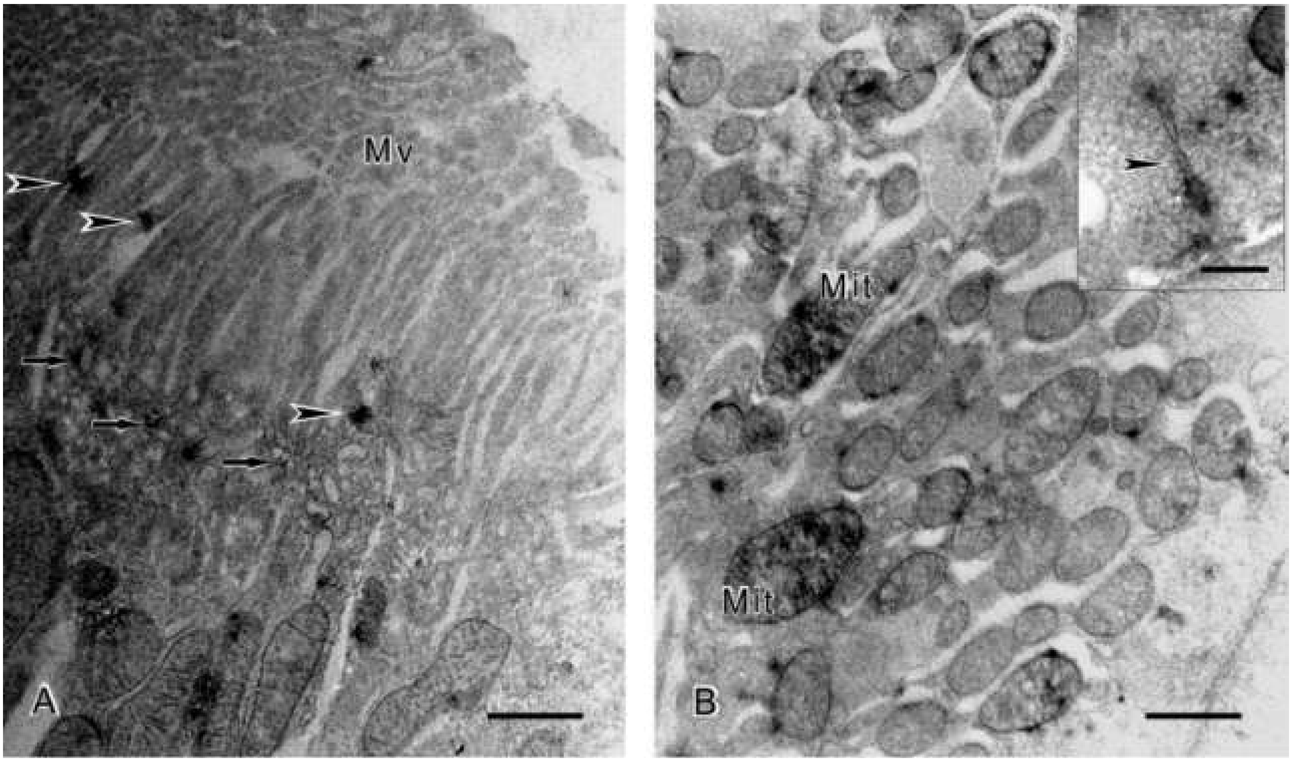


**Figure 4. Immunoelectron micrographs showing Kir6.1 protein in subcellular structures of rat proximal tubule. (A) Apical portion of proximal tubule with long, regularly oriented, and closely packed microvilli (Mv). Kir6.1 can be observed in the microvilli (arrowheads) and small vesicles and apical canaliculi (arrows). (B) In the cytoplasm of proximal tubule, Kir6.1 is localized in mitochondria (Mit) and in endoplasmic reticulum (inset, arrowhead). Scale bars: A, B = 1 μm; inset = 500 nm.**

in all tissues examined herein, such as heart, brain, liver and testis. The size differences of detected signals between heart (about 43 kDa) and other tissues (about 50 kDa) may be due to the post translational modifications in different tissues.

The results of the present study demonstrated that Kir6.1 protein not only localizes in the cell membrane but also in the mitochondrial and microsomal fractions of the kidney. The Kir6.1 is widely distributed in various renal tubular segments including glomerulus, proximal and distal tubules and collecting ducts by immunohistochemistry and *in situ* hybridization histochemistry. The subcellular localization of Kir6.1 is revealed in the mitochondrial and ER, and in the cell membrane by immunoelectron microscopy.

Previously, $K_{ATP}$ channels were shown to be expressed in the mitoplasts of the inner membrane of liver mitochondria (Inoue *et al.* 1991). Since then, two conflicting views have been proposed. Kir6.1 was localized in the mitochondria in skeletal muscles (Suzuki *et al.* 1997) and in neurons and glial cells of rat brain (Lacza *et al.* 2003a; Zhou *et al.* 1999), in the isolated mitochondrial fraction of mouse cardiomyocytes (Lacza *et al.* 2003b), and in the isolated ventricular myocytes (Singh *et al.* 2003), but some reports have claimed that there was no Kir6.1 in mitochondria with gene transfer technique (Seharaseyon *et al.* 2000) and immunoblotting analysis for cellular fractions (Kuniyasu *et al.* 2003). Focusing on these conflicting results, we recently observed that Kir6.1 was expressed in mitochondria in rat cardiomyocytes by immunoblotting analysis in cellular fractions and immunoelectron microscopy (Zhou *et al.* 2005). With the same antibody, Kir6.1 was also shown to be expressed in kidney cell fractions including mitochondria. Thus, Kir6.1 not only localizes in the mitochondria of cardiomyocytes but also in the mitochondria of renal tubular epithelial cells, regardless of the lower expression level of the Kir6.1 mRNA in kidney (Inagaki *et al.* 1995).

Recently, a new report claimed that Kir6.1 was not expressed in rat kidney by Western blotting (Sun *et al.* 2004). These investigators explained that the translation of Kir6.1 was limited in kidney, but it may be up-regulated by hypoxic or ischemic stresses (Sun *et al.* 2004). We think that the negative reaction of kidney to anti-Kir6.1 antibody in these investigators's report may be due to the quantity of material applied for immunoblot, since the actin band detected in spleen was very heavy while those in liver and kidney were very light. If the applied concentration of sample was increased, the signal would be detectable in kidney by their anti-Kir6.1 antibody.

The observation of Kir6.1 in the kidney mitochondria in the present study could not deny its expression in the cell membrane, even though the detectable immunoreactivity is low. Previous studies with patch clamp technique had shown that ATP sensitive $K^+$ channel was localized in the basolateral membrane of rabbit proximal convoluted tubule and the cortical thick ascending limb (Beck *et al.* 1993; Hurst *et al.* 1993; Hurst *et al.* 1992; Tsuchiya *et al.* 1992), and in the apical membrane of rat cortical collecting tubule and rabbit thick ascending limb of Henle's loop (Wang *et al.* 1990a; Wang *et al.* 1990b). These channels play important roles in kidney as 1) maintenance of cell-negative potential, 2) $K^+$ recycling at the cell membrane, 3) $K^+$ secretion and 4) cell volume regulation (Kawahara and Anzai 1997). It is well established that the Kir6.1 may not co-localize with $Na^+/K^+$ ATPase but will be regulated by a common cellular mechanism, which coordinates the epithelial $Na^+$ transport and the basolateral $Na^+/K^+$ ATPase activity.

There is now convincing evidence that the mitochondrial volume is regulated by the balance between mitochondrial $K_{ATP}$ channel (mito$K_{ATP}$) and the $K^+/H^+$ antiport, because the $K^+$ cycle across the inner membrane consists of $K^+$ influx through the mito$K_{ATP}$ and electroneutral $K^+$ efflux by $K^+/H^+$ antiporter. The steady-state volume of the mitochondrial matrix is therefore maintained by the secondary active, energy consuming process of a $K^+/H^+$ antiporter (Garlid 1994), and mitochondrial $K^+$ cycle, while in turn, volume appeared to play a key role in regulating cellular bioenergetics, such as activity of the electron transport chain (Garlid 1996). Thus, our finding of an intracellular localization of Kir6.1 protein in mitochondria may support the hypothesis that mito$K_{ATP}$ may play a key signal role in providing an energy during epithelial ion transportation.

In conclusion, Kir6.1 was verified to exist in the kidney in various renal tubular epithelial cells, mesangial cells and podocytes of glomeruli, and endothelium and smooth muscles of renal blood vessels. The subcellular localization was mainly in the mitochondria. Although only weak immunoreaction was observed in the cell membrane of renal epithelial cells in this study, the membrane $K_{ATP}$ channel plays roles in the connection of membrane potential and cell metabolism no matter how low its level of expression. The Kir6.1 may be a molecular member of membrane $K_{ATP}$ channel in kidney, and it may also be a candidate for a mitochondrial $K_{ATP}$ channel as well.

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## References

Aguilar-Bryan L, Clement JPt, Gonzalez G, Kunjilwar K, Babenko A, Bryan J. Toward understanding the assembly and structure of $K_{ATP}$ channels. Physiol Rev 1998; 78: 227-45.

Ammala C, Moorhouse A, Ashcroft FM. The sulphonylurea receptor confers diazoxide sensitivity on the inwardly rectifying $K^+$ channel Kir6.1 expressed in human embryonic kidney cells. J Physiol 1996; 494:709-14.

Anzai N, Kawahara K, Sakai T, Komatsu Y, Inagaki N, Seino S. Localization of the inward rectifier subunit uKATP-1 (Kir6.1) in adult rat kidney. J Am Soc Nephrol 1996; 7:1275.

Beck JS, Hurst AM, Lapointe JY, Laprade R. Regulation of basolateral K channels in proximal tubule studied during continuous microperfusion. Am J Physiol 1993; 264: F496-501.

Braun GS, Veh RW, Segerer S, Horster MF, Huber SM. Developmental expression and functional significance of Kir channel subunits in ureteric bud and nephron epithelia. Pflugers Arch 2002; 445: 321-30.

Brochiero E, Wallendorf B, Gagnon D, Laprade R, Lapointe JY. Cloning of rabbit Kir6.1, SUR2A, and SUR2B: possible candidates for a renal $K_{ATP}$ channel. Am J Physiol Renal Physiol 2002; 282:F289-300.

Clement JPt, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L, et al. Association and stoichiometry of K(ATP) channel subunits. Neuron 1997; 18: 827-38.

Garlid KD. Cation transport in mitochondria--the potassium cycle. Biochim Biophys Acta 1996; 1275: 123-6.

Garlid KD. Mitochondrial cation transport: a progress report. J Bioenerg Biomembr 1994; 26: 537-42.

Hurst AM, Beck JS, Laprade R, Lapointe JY. $Na^+$ pump inhibition downregulates an ATP-sensitive $K^+$ channel in rabbit proximal convoluted tubule. Am J Physiol 1993; 264:F760-4.

Hurst AM, Duplain M, Lapointe JY. Basolateral membrane potassium channels in rabbit cortical thick ascending limb. Am J Physiol 1992; 263: F262-7.

Inagaki N, Gonoi T, Clement JP, Wang CZ, Aguilar-Bryan L, Bryan J, et al. A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive $K^+$ channels. Neuron 1996; 16: 1011-7.

Inagaki N, Tsuura Y, Namba N, Masuda K, Gonoi T, Horie M, et al. Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. J Biol Chem 1995; 270: 5691-4.

Inoue I, Nagase H, Kishi K, Higuti T. ATP-sensitive $K^+$ channel in the mitochondrial inner membrane. Nature 1991; 352: 244-7.

Itoh H, Komatsuda A, Ohtani H, Wakui H, Imai H, Sawada K, et al. Mammalian HSP60 is quickly sorted into the mitochondria under conditions of dehydration. Eur J Biochem 2002; 269: 5931-8.

Kawahara K, Anzai N. Potassium transport and potassium channels in the kidney tubules. Jpn J Physiol 1997; 47: 1-10.

Kondo C, Repunte VP, Satoh E, Yamada M, Horio Y, Matsuzawa Y, et al. Chimeras of Kir6.1 and Kir6.2 reveal structural elements involved in spontaneous opening and unitary conductance of the ATP-sensitive K+ channels. Receptors Channels 1998; 6: 129-40.

Kuniyasu A, Kaneko K, Kawahara K, Nakayama H. Molecular assembly and subcellular distribution of ATP-sensitive potassium channel proteins in rat hearts. FEBS Lett 2003; 552: 259-63.

Lacza Z, Snipes JA, Kis B, Szabo C, Grover G, Busija DW. Investigation of the subunit composition and the pharmacology of the mitochondrial ATP-dependent $K^+$ channel in the brain. Brain Res 2003a; 994: 27-36.

Lacza Z, Snipes JA, Miller AW, Szabo C, Grover G, Busija DW. Heart mitochondria contain functional ATP-dependent $K^+$ channels. J Mol Cell Cardiol 2003b; 35: 1339-47.

Noma A. ATP-regulated $K^+$ channels in cardiac muscle. Nature 1983; 305:147-8.

Quast U. ATP-sensitive $K^+$ channels in the kidney. Naunyn Schmiedebergs Arch Pharmacol 1996; 354:213-25.

Satoh E, Yamada M, Kondo C, Repunte VP, Horio Y, Iijima T, et al. Intracellular nucleotide-mediated gating of SUR/Kir6.0 complex potassium channels expressed in a mammalian cell line and its modification by pinacidil. J Physiol 1998; 511:663-74.

Seharaseyon J, Ohler A, Sasaki N, Fraser H, Sato T, Johns DC, et al. Molecular composition of mitochondrial ATP-sensitive potassium channels probed by viral Kir gene transfer. J Mol Cell Cardiol 2000; 32: 1923-30.

Sgard F, Faure C, Drieu la Rochelle C, Graham D, O'Connor SE, Janiak P, et al. Regulation of ATP-sensitive potassium channel mRNA expression in rat kidney following ischemic injury. Biochem Biophys Res Commun 2000; 269: 618-22.

Singh H, Hudman D, Lawrence CL, Rainbow RD, Lodwick D, Norman RI. Distribution of Kir6.0 and SUR2 ATP-sensitive potassium channel subunits in isolated ventricular myocytes. J Mol Cell Cardiol 2003; 35:445-59.

Sun X, Cao K, Yang G, Huang Y, Hanna ST, Wang R. Selective expression of Kir6.1 protein in different vascular and non-vascular tissues. Biochem Pharmacol 2004; 67: 147-56.

Suzuki M, Kotake K, Fujikura K, Inagaki N, Suzuki T, Gonoi T, et al. Kir6.1: a possible subunit of ATP-sensitive $K^+$ channels in mitochondria. Biochem Biophys Res Commun 1997;241: 693-7.

Tsuchiya K, Wang W, Giebisch G, Welling PA. ATP is a coupling modulator of parallel Na,K-ATPase-K-channel activity in the renal proximal tubule. Proc Natl Acad Sci USA 1992; 89: 6418-22.

Van Bueren AM, Moholt-Siebert M, Begley DE, McCall AL. An immunization method for generation of high affinity antisera against glucose transporters useful in immunohistochemistry. Biochem Biophys Res Commun 1993; 197: 1492-8.

Wang T, Wang WH, Klein-Robbenhaar G, Giebisch G. Effects of a novel KATP channel blocker on renal tubule function and K channel activity. J Pharmacol Exp Ther 1995; 273: 1382-9.

Wang WH, Schwab A, Giebisch G. Regulation of small-conductance $K^+$ channel in apical membrane of rat cortical collecting tubule. Am J Physiol 1990a; 259: F494-502.

Wang WH, White S, Geibel J, Giebisch G. A potassium channel in the apical membrane of rabbit thick ascending limb of Henle's loop. Am J Physiol 1990b; 258: F244-53.

Zhou M, Tanaka O, Sekiguchi M, He HJ, Yasuoka Y, Itoh H, et al. ATP-sensitive $K^+$-channel Subunits on the Mitochondria and Endoplasmic Reticulum of Rat Cardiomyocytes. J Histochem Cytochem 2005; 53: 1491-500.

Zhou M, Tanaka O, Sekiguchi M, Sakabe K, Anzai M, Izumida I, et al. Localization of the ATP-sensitive potassium channel subunit (Kir6. 1/uK(ATP)-1) in rat brain. Brain Res Mol Brain Res 1999; 74:15-25.