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FAT is a component of glomerular slit diaphragms

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FAT is a component of glomerular slit diaphragms.

Background. Slit diaphragms are intercellular junctions of podocytes of the renal glomerulus. The molecular composition of slit diaphragms is still elusive. Slit diaphragms are characterized by the presence of a wide intercellular space. The morphological feature is shared by desmosomes and adherens junctions, which contain members of the cadherin superfamily. Thus, we have hypothesized that some components of slit diaphragms belong to the cadherin superfamily. Consequently, we have isolated cDNA encoding FAT from reverse-transcribed (RT) glomerular cDNA by homology polymerase chain reaction (PCR) using primers based on conserved sequences in cadherin molecules. FAT is a novel member of the cadherin superfamily with 34 tandem cadherin-like extracellular repeats, and it closely resembles the *Drosophila* tumor suppressor *fat.*

Methods. Expression of FAT was examined in glomeruli of the adult rat kidney by the ribonuclease protection assay and in situ hybridization. To localize the FAT protein in podocytes minutely, we prepared affinity-purified antibody against FAT by immunizing rabbits against an oligopeptide corresponding to the C-terminal 20 amino acids.

Results. Expression of FAT mRNA was detected in total RNA from glomeruli. In situ hybridization revealed significant signals in podocytes. Western blot analysis using solubilized glomeruli showed a single band, in which the molecular weight was more than 500 kD. Immunostaining of cultured epithelial cells from rat kidney (NRK52E) revealed FAT accumulation in cell–cell contact sites. In the glomerulus, FAT staining was observed distinctly along glomerular capillary walls. Double-label immunostaining using monoclonal antibody against slit diaphragms (mAb 5-1-6) showed identical localization of anti-FAT antibody and mAb 5-1-6. Furthermore, the double-label immunogold technique with ultrathin cryosections demonstrated that gold particles for FAT cytoplasmic domain were located at the base of slit diaphragms labeled by mAb 5-1-6 and that the cytoplasmic domain of FAT colocalized with ZO-1, a cytoplasmic component associated with slit diaphragms.

Conclusion. The molecular structure of FAT and its colocalization with 5-1-6 antigen and ZO-1 indicate that FAT is a component of slit diaphragms.

Key words: renal glomerulus, podocytes, cadherin, nephrin, ZO-1, intercellular junction, cell permeability.

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Visceral epithelial cells of the renal glomerulus, or podocytes, are a highly specialized epithelium structurally adapted to facilitate bulk flow of the glomerular filtrate through the intercellular spaces or filtration slits. The foot processes of podocytes are mostly attached to one another by slit diaphragms or podocyte-specific intercellular junctions, which cover the outer aspect of the glomerular basement membrane as the terminal element in the filtration barrier. Monoclonal antibody (mAb) 5-1-6 reacts with an epitope on rat slit diaphragms and causes severe complement- and leukocyte-independent proteinuria when injected into rats [1]. These data strongly suggest that slit diaphragms contribute significantly to the hydraulic and macromolecular permeability properties of the glomerulus. Although the glomerular slit diaphragms are known to consist of zipper-like substructures in the normal rat kidney [2], the molecular composition of these slit diaphragms bridging the filtration slits and connecting the podocyte foot processes is still elusive. Recently, a mutation in the nephrin gene was identified as the cause of congenital nephrotic syndrome of the Finnish type [3]. Nephrin, a member of the immunoglobulin superfamily, is expressed exclusively in podocytes and is thought to be the major component of slit diaphragms [4].

Typical polarized epithelial cells possess characteristic tripartite junctional complexes: tight junctions, actin microfilament-anchoring adherens junctions, and intermediate filament-anchoring desmosomes [5]. Although podocytes lack such junctional complexes, slit diaphragms have been reported to share some properties with tight junctions. ZO-1, a protein on the cytoplasmic face of tight junctions, is also expressed on the cytoplasmic surface of podocyte foot processes at the point of insertion of slit diaphragms [6, 7]. Tight junctions serve as a fence

maintaining the polarized distribution of membrane proteins by restricting the intermixing of distinct apical and basolateral surface proteins [8]. Slit diaphragms, like tight junctions, retain a fence-like function, because a membrane glycoprotein of podocytes, podocalyxin, is confined to the apical cell surface above slit diaphragms [9].

In spite of these characteristics common to tight junctions, slit diaphragms are morphologically different from tight junctions, in which adjacent plasma membranes are in close contact without an intercellular space. Slit diaphragms are characterized by their spanning a wide intercellular space, that is, the filtration slits between adjacent foot processes of podocytes [2]. The morphological feature is common to desmosomes and adherens junctions rather than to tight junctions [5]. Because desmosomes and adherens junctions contain members of the cadherin superfamily as cell adhesion molecules, we have hypothesized that some components of slit diaphragms belong to the cadherin superfamily.

Cadherins are a group of cell adhesion proteins that mediate homophilic Ca^{2+}-dependent cell–cell adhesion [10]. Recent studies have revealed that a variety of cadherin-related proteins are expressed in different tissues of various organisms, and it is evident that these proteins constitute a large cadherin superfamily [11]. Members of the cadherin superfamily include the classic cadherins, desmosomal cadherins, protocadherins, and the *Drosophila fat* protein [12] and its mammalian homologue FAT [13, 14]. All of them are characterized by unique extracellular domains composed of multiple repeats of a cadherin-specific motif [11].

In the present study, we have isolated cDNA encoding FAT from reverse-transcribed (RT) glomerular cDNA by homology polymerase chain reaction (PCR) using primers based on conserved sequences in cadherin molecules. Extracellular domain of FAT contains 34 tandem cadherin-alike repeats, five epidermal growth factor (EGF)-like repeats, and a laminin A-G domain. Originally, FAT was found as a tumor suppressor gene called *fat* in *Drosophila* [12]. Its recessive mutation in the gene leads to hyperplastic overgrowth of the imaginal discs, indicating that the *fat* gene product controls cell proliferation and morphogenesis in the imaginal discs in a contact-dependent manner. The vertebrate homologue FAT has been identified in humans and rats [13, 14], and the amino acid sequence between the species is highly conserved (87.9%) throughout the entire molecule [14]. In situ hybridization has revealed widespread expression of FAT in many fetal tissues, including the kidney. FAT is predominantly expressed in epithelial cell layers and in the central nervous system with some endothelial and smooth muscle cells. In contrast to the fetal tissues, FAT signals are low or disappear in most adult tissues, suggesting that its expression may be developmentally regulated in these tissues [13, 14]. Our results clearly show that even in the adult, FAT expression is widely distributed in the kidney, especially in podocytes, indicating that FAT is a component of slit diaphragms.

METHODS

Animals and cultured cells

Wistar-Kyoto (WKY) and Wistar rats were purchased from Charles River Japan (Atsugi, Japan) and were used in these experiments at the ages of 8 to 12 weeks. Rat kidney epithelial cell line (NRK52E:ATCC CRL-1571) was cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 μ/mL penicillin and 100 μg/mL streptomycin.

Total RNA preparation

Glomeruli were isolated from the renal cortex fraction by a sieving method. The isolated glomeruli, renal cortices, medullae, or NRK52E cells were homogenized in TRIzol (GIBCO BRL, Grand Island, NY, USA) with a sonicator, and total cellular RNA was extracted from these samples.

Polymerase chain reaction

To search for novel cadherins, a PCR was performed on the cDNA preparation from rat decapsulated glomeruli [15] as a template using two degenerate oligonucleoside primers. These primers were designed on the highly conserved extracellular domain of known cadherins as previously described by Sano et al [16]. PCR was performed for 40 cycles consisting of denaturation at 95°C for one minute, annealing at 50°C for two minutes, and then polymerization at 72°C for two minutes. PCR products were size fractionated by agarose gel electrophoresis, and components of approximately 450 and 130 bp were extracted, ligated into pGEM-T vector (Promega Corp., Madison, WI, USA), cloned, and sequenced by an automated DNA sequencer (ABI 373A; Perkin-Elmer Japan, Urayasu, Japan). Some of the clones contained cDNA encoding rat FAT. They corresponded to 7511 to 7969 (457 bp), 10,061 to 10,513 (452 bp), and 10,391 to 10,513 (123 bp) of rat FAT (AF100960) [12].

Ribonuclease protection assay

The ribonuclease protection assay was carried out as described previously [17–19]. ^{32}P-labeled cRNA probes for FAT mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized by in vitro transcription, using the linearized plasmids inserted with FAT cDNA (452 bp, range 10,061 to 10,513) and GAPDH cDNA (114 bp, 673 to 787) as a housekeeping gene [14]. Ten micrograms of total RNA were hybridized with the cRNA at 45°C overnight. The unhybridized probes were digested with ribonuclease A (1.2 μg/mL) and ribonuclease T1 (120 U/mL) for 60 minutes at 30°C,

and the ribonucleases were digested with proteinase K (0.45 μg/mL) at 37°C for 30 minutes. After phenol/chloroform extraction, the hybridized probes were precipitated with ethanol and were heat denatured for electrophoresis on 6% polyacrylamide gels. The dried gels were exposed to x-ray films at −70°C to detect FAT mRNA expression.

In situ hybridization

In situ hybridization was done by a modified procedure as described previously [20]. In brief, digoxigenin-labeled antisense and sense cRNA probes (antisense: 452 bp, 10,061 to 10,513 and 457 bp, 7511 to 7969; sense: 452 bp, 10,061 to 10,513) were synthesized by in vitro transcription using the linearized templates. Rat kidneys were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for five hours at 4°C, immersed in 20% sucrose-PBS solution at 4°C for overnight, and frozen in *n*-hexane at −70°C. Frozen sections of the kidneys on poly-L-lysine–coated glass slides were 4% paraformaldehyde in PBS, treated with 0.5 μg/mL proteinase K for 15 minutes at room temperature, and hybridized with the digoxigenin-labeled probes overnight at 50 to 55°C. After washing in 3 × standard saline citrate (SSC), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.05% Triton X-100, the sections were treated with 20 μg/mL ribonuclease A in 10 mmol/L Tris-HCl (pH 7.6) for 30 minutes at room temperature, followed by washing in 3 × SSC, 1 mmol/L EDTA, 0.05% Triton X-100. For immunodetection of digoxigenin-labeled probes, sections were incubated with alkaline phosphatase-conjugated sheep antidigoxigenin antibody at a dilution of 1:500 for two hours at room temperature. Visualization was performed using nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate.

Antibodies

To prepare anti-FAT antibody, an oligopeptide corresponding to near the C-terminal 20 amino acids of rat FAT (YESGDDGHFEEVTIPPLDSQ) with an added cysteine at the N-terminus was synthesized and conjugated with keyhole limpet hemocyanin as a carrier protein. The conjugate of 1 mg was emulsified with complete Freund's adjuvant and injected subcutaneously into New Zealand White rabbits several times. Anti-FAT antibody was affinity purified by using a FAT synthetic peptide-conjugated column. The reactivity of the antibody was confirmed by immunohistochemistry and Western blotting with the use of recombinant protein expressed by expression vector (pTrcHis Vectors; Invitrogen Co., Carlsbad, CA, USA) including the cDNA (538 bp, 13,471 to 14,008) of rat FAT. The specificity of the immunostaining of tissues was verified by blocking of the staining after absorption of the antibody with the synthetic peptide. For absorption, 6 μg of the synthetic peptide (more than 10 times excess to IgG at molar ratio) were mixed with 1 mL of the affinity-purified anti-FAT antibody (0.04 mg IgG/mL) for 16 hours at 4°C.

Murine mAb 5-1-6 was prepared as previously described [1]. TRITC-conjugated phalloidin was purchased from Sigma Chemical Company (St. Louis, MO, USA). Anti-megalin antibody was a kind gift from Dr. A. Saito (Niigata University, Niigata, Japan).

Western blotting

Rat isolated glomeruli and cortex of kidney (0.3 g) were homogenized in 3 mL of 8 mol/L urea buffer (50 mmol/L Tris-HCl, pH 8.0, 1 mmol/L dithiothreitol, and 1 mmol/L EDTA), diluted in the 2 × Laemmli sample buffer, and boiled for five minutes. A total of 30 μL of each sample was separated by electrophoresis on 5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membrane. The membranes were preincubated overnight with blocking buffer (10% no-fat milk, 0.05% Tween 20, and 0.1% NaN_3, in PBS) and incubated with anti-FAT or antimegalin antibody overnight. The membranes were then washed in several changes of washing buffer (0.05% Tween 20 in PBS), incubated for three hours with a 1:50 diluted ENVISION of anti-rabbit IgG (Dako Co., Kyoto, Japan), and colored with diaminobenzidine.

Immunofluorescence microscopy

The indirect immunofluorescence technique was applied to frozen kidney sections and cultured NRK52E cells as described previously [15, 21]. In brief, the rat kidneys were snap frozen at −70°C, sectioned at a thickness of 3 μm in a cryostat, fixed in 2% paraformaldehyde in PBS for five minutes, and processed for double-label immunostaining. NRK52E cells cultured on eight-well glass chamber slides (Lab-Tek, Miles Scientific, Naperville, IL, USA) were fixed in 2% paraformaldehyde in PBS for five minutes, permeabilized with 0.3% Triton X-100 in PBS for three minutes, and stained with antibodies. For double-label immunofluorescence microscopy, rabbit antisera and murine monoclonal antibodies or TRITC-conjugated phalloidin were mixed and applied as primary antibodies simultaneously. After washing with PBS, the sections were stained with fluorescence isothiocyanate (FITC)-conjugated anti-rabbit IgG, rewashed with PBS, and subsequently reacted with TRITC-conjugated anti-mouse IgG. PBS, preimmune rabbit serum or murine IgG_1 mAb (against rotavirus), shown not to react to rat glomeruli, were used as negative controls for the primary antibodies. Immunofluorescence of the sections and cultured cells were observed by a laser scanning confocal microscope (MRC-1024; Bio-Rad Laboratories, Hercules, CA, USA).

Immunogold labeling

Rat kidneys were perfused with 0.5% paraformaldehyde fixative buffered with 0.1 mol/L sodium phosphate buffer (PB; pH 7.4) and immersed in the same fixative for 30 minutes at 4°C. The samples were rinsed with 5% sucrose for 30 minutes at 4°C. Tissue samples were then infiltrated with 40% polyvinylpyrrolidone (Sigma)/2.3 mol/L sucrose buffered with 0.1 mol/L PB, embedded on nails, and frozen quickly in liquid nitrogen. Ultrathin cryosections were cut with a LEICA Ultracut UCT equipped with the LEICA EM FCS cryoattachment (Wien, Austria) at −110°C. Sections were transferred to nickel grids (150 mesh), which had been coated with Formvar. Subsequent incubation steps were carried out by floating the grids on droplets of the filtered solution. After quenching free aldehyde groups with PBS-0.01 mol/L glycine, sections were incubated overnight with PBS containing 10% FBS, mouse monoclonal anti-ZO-1 antibody (1:100 dilution with PBS containing 10% FBS; Zymed Laboratories, South San Francisco, CA, USA), or mAb 5-1-6 (1:50 dilution of culture supernatant). Next, the grids were incubated with affinity purified anti-FAT antibody (12 μg/mL) for two hours at room temperature. They were then incubated with anti-rabbit IgG coupled to 10 nm gold (diluted 1:100; British BioCell, Cardiff, UK) and anti-mouse IgG coupled to 5 nm-gold (diluted 1:100; British BioCell) for one hour. After immunostaining, they were fixed with 2.5% glutaraldehyde buffered with 0.1 mol/L PB (pH 7.4). The sections were then contrasted with 2% uranyl acetate solution for 20 minutes and absorption stained with 3% polyvinyl alcohol containing 0.2% uranyl acetate for 10 minutes. All sections were observed with a JEOL 1200-EX electron microscope.

RESULTS

Expression of FAT mRNA in the rat kidney

To clone cadherins expressed in rat glomeruli, PCR was performed on glomerular cDNA. PCR products of predicted size of approximately 450 and 130 bp were purified and cloned. Sequencing the clones and homology research revealed that some of the clones were 100% identical to rat FAT.

The expression of the mRNAs for FAT was examined in the adult rat kidney by ribonuclease protection assay (Fig. 1). Significant signals for FAT were found in the every fraction of the kidney, that is, glomerulus, cortex, medulla, and whole kidney. mRNAs from glomeruli showed the most intense signals and those from cortices showed the weakest. The levels of FAT mRNA in glomeruli of Wistar rats were higher than twice those of WKY rats. We examined FAT expression in glomeruli of male and female rats and of two- and seven-month-old rats (data not shown). The difference among these samples was less than one and a half times.

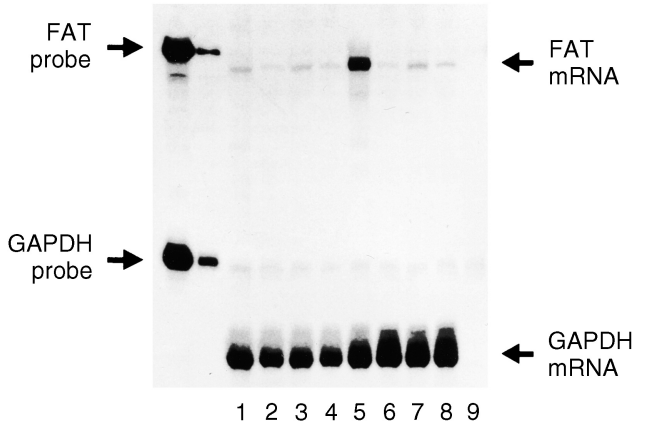


Fig. 1. Expression of FAT mRNA detected by ribonuclease protection assay. RNA samples (10 μg each) were obtained from fractions of WKY and Wistar rat kidneys. Abbreviation is GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Lane 1, WKY glomerulus; lane 2, WKY cortex; lane 3, WKY medulla; lane 4, WKY whole kidney; lane 5, Wistar glomerulus; lane 6, Wistar cortex; lane 7, Wistar medulla; lane 8, Wistar whole kidney; lane 9, tRNA as negative control. FAT mRNA is distinctly expressed in glomeruli and remarkably intense in Wistar glomeruli. The level of FAT expression is different between the rat strains. mRNA expression can be seen also in cortex and medulla of both rat strains.

To elucidate which cells in the glomerulus expressed FAT, the distribution of FAT mRNA was accessed by in situ hybridization (Fig. 2). FAT mRNA was observed predominantly in podocytes of the both rat strains, although Wistar rats showed more distinct signals. FAT expression was not detected in mesangial cells, but rarely in endothelial cells. In addition, parietal cells of Bowman's capsule and some of tubular epithelial cells were also positive for FAT mRNA.

Immunolocalization of FAT

To localize FAT protein in the glomerulus, we prepared anti-FAT antibody by immunizing rabbits against an oligopeptide corresponding to near the C-terminus of rat FAT. On immunoblot analysis, the antibody detected a single band in samples of glomerulus of Wistar rats and cultured rat kidney epithelial cells (NRK52E), but not in samples of cortices, probably due to a relatively low amount of FAT protein in the cortex (Fig. 3). Its molecular size was comparable with that of megalin whose predicted molecular weight is 516,715 excluding glycosylation [22]. This size is slightly larger than the molecular weights obtained from the deduced amino acid sequence of rat FAT (503,576) [14]. This discrepancy between predicted and actual molecular weights is common among various cadherins and is attributable to the glycosylation.

Immunofluorescence microscopy showed significant

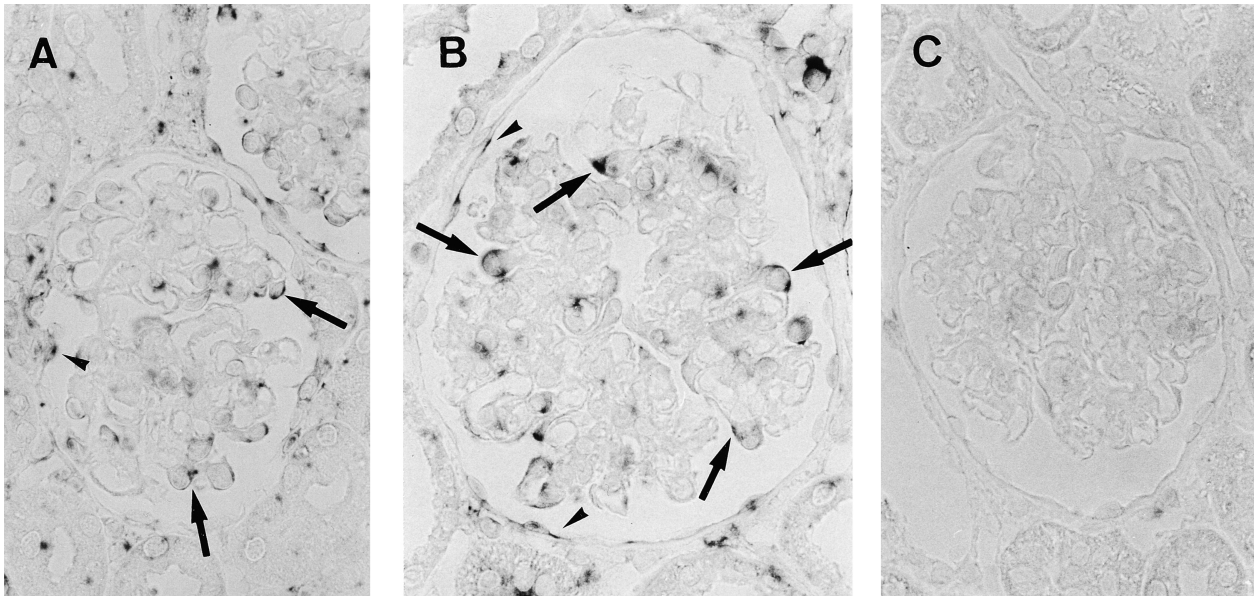


Fig. 2. Localization of FAT mRNA in glomeruli of WKY (*A*) and Wistar (*B* and *C*) rats detected by in situ hybridization using digoxigenin-labeled cRNA probe. Podocytes (arrows) show distinct expression of FAT mRNA in addition to parietal epithelial cells of Bowman's capsule (arrowheads) and some of the tubular epithelial cells when hybridized with antisense probe (A and B), but not with sense probe (C). Magnification ×550.

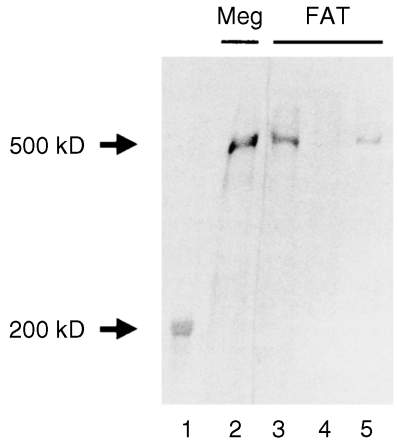
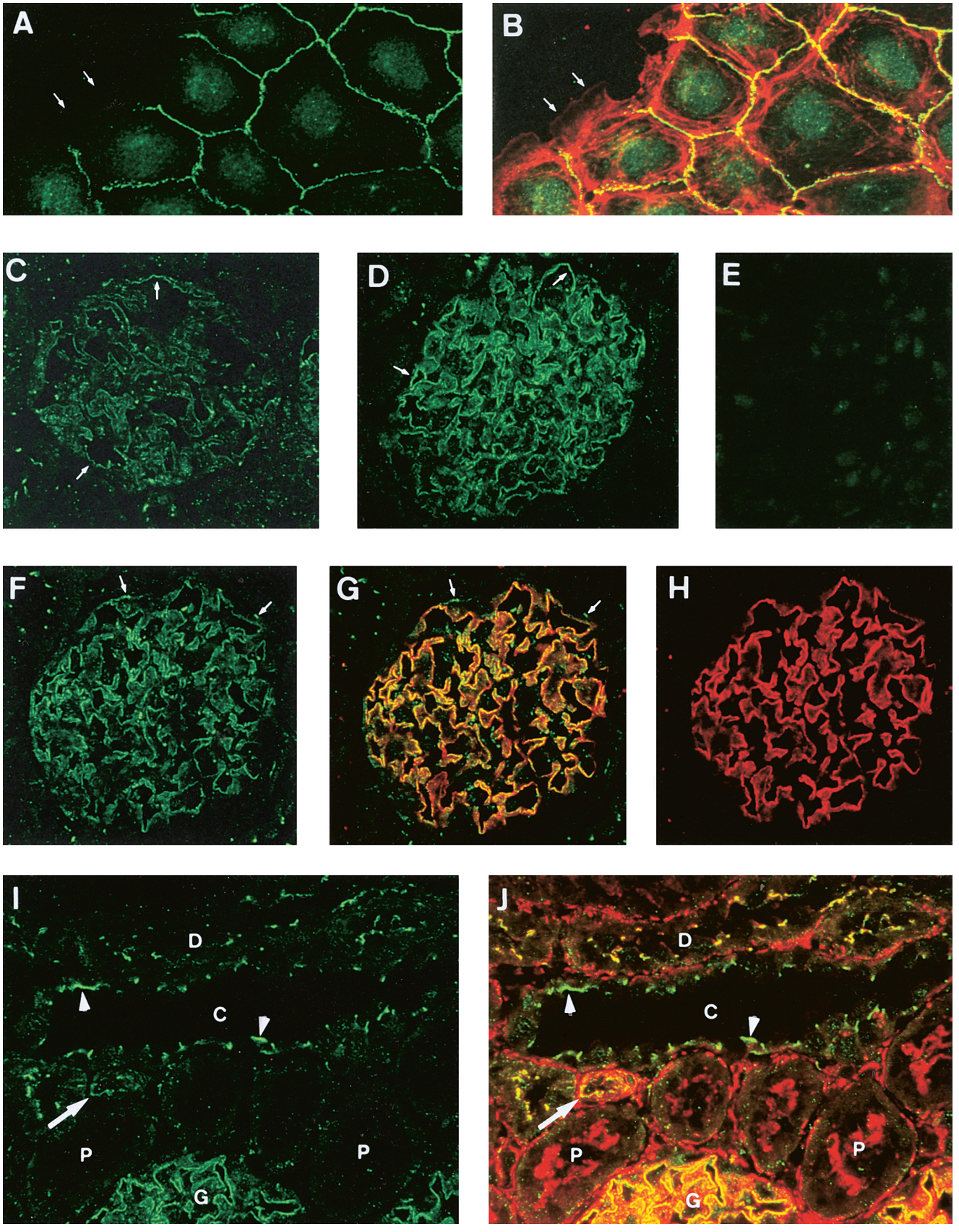


Fig. 3. Immunoblot analysis of lysates from glomeruli of Wistar rats (lane 3), cortex of WKY rats (lane 4), and NRK52E cells (lane 5) stained with anti-FAT antibody. Lane 1 is a prestained molecular marker, myosin. Lane 2 is immunoblot of cortex stained with anti-megalin antibody.

staining with the antibody against FAT cytoplasmic domain in NRK52E cells. FAT protein was concentrated only at cell–cell contact site and was absent from the free edge of cells (Fig. 4 A, B). In the renal cortex, a distinct immunofluorescent signal for FAT was associated with glomeruli of both WKY and Wistar rats (Fig. 4 C–J). Intensity of FAT staining in glomeruli was higher in Wistar rats than WKY rats, although the pattern of the antibody labeling was essentially identical in the both strains. Within glomeruli, most of fine granular dotted lines of FAT staining appeared to outline the capillary loops (Fig. 4 C, D). The staining was removed when the antibody was preincubated with the synthetic FAT peptide (Fig. 4E). To determine whether the immunoreactivity located in podocytes, especially in the slit diaphragm, sections were double labeled with a murine mAb directed against slit diaphragms (mAb 5-1-6; Fig. 4 F–H). As shown by immunofluorescence microscopy, FAT staining colocalized with mAb 5-1-6 labeling. In addition, TRITC-conjugated phalloidin was used to identify podocytes and proximal tubules by its actin staining of foot processes and brush border, respectively (Fig. 4 I, J) [23]. FAT and actin along the glomerular capillary walls were expressed in a similar distribution. Bright fluorescent dots and strings of FAT staining were seen in parietal layers of Bowman's capsule and distal and collecting tubules, but proximal tubule cells were not labeled by anti-FAT antibody. A significant signal was also found in endothelial cells of arterioles and of glomerular capillary walls.

The precise localization of FAT molecules was detected by the immunogold technique with ultrathin cryosections. In tubular epithelial cells, FAT molecules localized at the junctional complex. Gold particles were mainly concentrated at the adherens junction. Some signals were also found at the tight junction above the adherens junction (Fig. 5A). In podocytes, the gold particles associating with FAT cytoplasmic domain were found at the position of slit diaphragms (Fig. 5 B, C). We used double-label immunoelectron microscopy to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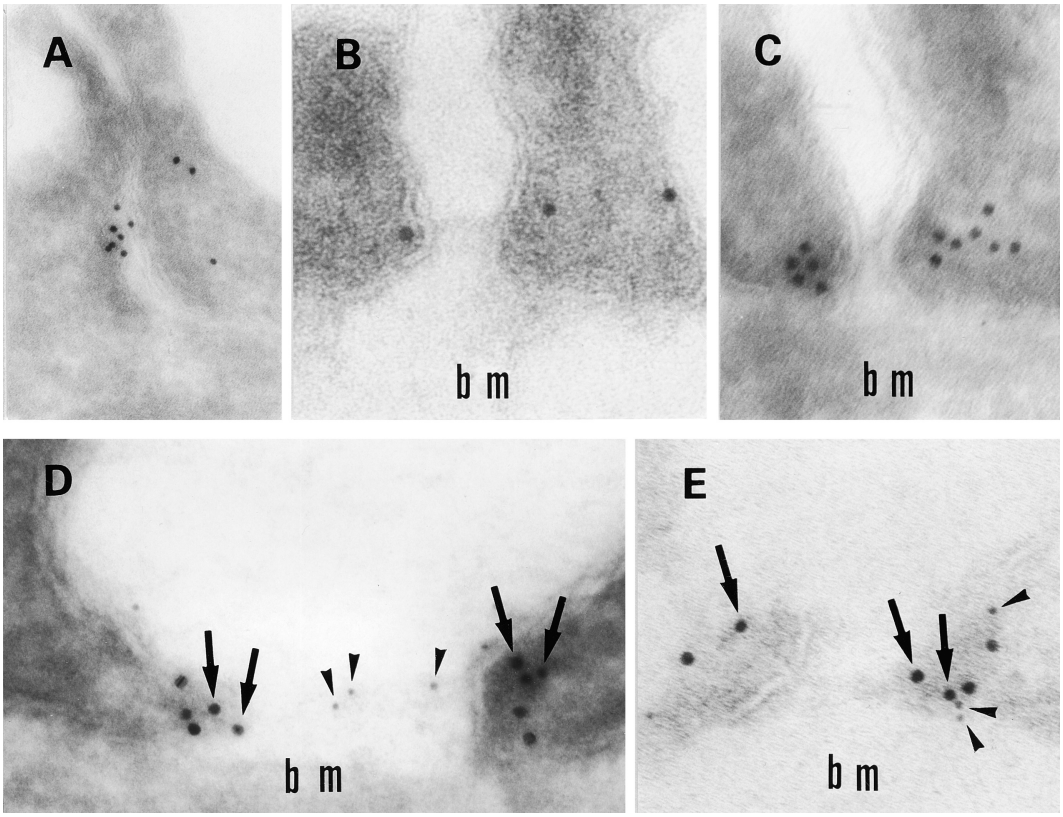

Fig. 5. Immunogold localization for the cytoplasmic domain of FAT in the renal cortex of Wistar rats. Ten nanometer gold particles for FAT were found at the junctional complex of tubular epithelial cells (*A*) and at the position of slit diaphragms of podocytes (*B* and *C*). Double-label immunogold staining of the cortex using anti-FAT antibody (10 nm gold, arrows in *D*) and mAb 5-1-6 (5 nm gold, arrowheads in D) reveals that 5-1-6 antigen and FAT cytoplasmic domain are located on slit diaphragms and at the base of slit diaphragms, respectively. Double immunogold labeling with anti-FAT antibody (10 nm gold, arrows in *E*) and anti–ZO-1 mAb (5 nm gold, arrowheads in E) shows colocalization of the both antibody in foot processes of podocytes. Abbreviation b m is glomerular basement membrane. Magnification A ×97,000; B ×200,000; C ×180,000; D ×190,000; and E ×180,000.

see whether FAT colocalizes with 5-1-6 antigen. The double labeling with the antibodies against FAT cytoplasmic domain and mAb 5-1-6 showed that 5-1-6 antigens localized on the slit diaphragm and the cytoplasmic tails of FAT at the base of slit diaphragm (Fig. 5D). Furthermore, we examined whether FAT cytoplasmic domain colocalizes with a protein that is located at the cytoplasmic side of slit diaphragm in the podocyte by using the double immunogold technique. It is known that one isoform of ZO-1 is located along the cytoplasmic face of the slit diaphragm [7]. Therefore, the cellular localization of ZO-1 and FAT in podocytes was tested. Double immunogold labeling with anti-FAT antibody and anti–ZO-1 mAb revealed that the cytoplasmic domain of FAT colocalized with ZO-1 at the cytoplasmic faces of slit diaphragms (Fig. 5E).

Fig. 4. Confocal immunofluorescence microscopy for FAT in NRK52E cells (*A* and *B*) and the renal cortex of WKY (*C*) or Wistar (*D–J*) rats. Cultured NRK52E cells (A and B) are double labeled by using anti-FAT antibody (green; A and B) and TRITC-conjugated phalloidin (red; B). FAT staining is seen at the cell–cell contact sites but is not found in the free edge of cells, which are demarcated by phalloidin staining (arrows). In the renal cortex, the intensity of FAT signal in glomeruli is different between WKY (C) and Wistar (D) rats in spite of their identical staining pattern of fine granular dotted lines (arrows). No staining is observed with antibody preincubated with synthetic FAT oligopeptide (E). Double-label immunostaining by using anti-FAT antibody (green; F and G) and mAb 5-1-6 (red; G, H) shows colocalization of the both antibodies along the glomerular capillary walls (yellow; G). Dotted fluorescence is observed between parietal epithelial cells of Bowman's capsule (arrows in F and G). Double-label immunostaining of the cortex by using anti-FAT antibody (green; I and J) and TRITC-conjugated phalloidin (red; J) reveals dots or strings (arrowheads) of FAT staining in endothelial cells of arteriole (arrow) and distal (D) and collecting (C) tubules, but not in proximal tubules (P) with brush border. Magnification ×500.

DISCUSSION

In the present study, we have demonstrated the expression of FAT, a novel member of the cadherin superfamily, in the adult rat kidney, especially in the glomerulus with the strain difference. In situ hybridization and immunofluorescence microscopy revealed that significant FAT signals in the glomerulus were located mainly in podocytes. Thus far, the studies of FAT in mammals have been undertaken by in situ hybridization but not by immunohistochemistry [13, 14]. Our study revealed, to our knowledge for the first time, that FAT protein is concentrated mainly at cell–cell contact sites as a huge transmembrane protein comparable in size with megalin (Mr more than 500 kD) [22].

The biological role of FAT in mammals is elusive, although recessive lethal mutations in the *Drosophila fat* cause hyperplastic, tumor-like overgrowth of larval imaginal discs [12]. A particular striking feature of the FAT sequence is the presence of 34 tandem cadherin repeats. This is the same number of cadherin repeats found in *Drosophila fat*. The majority of the cadherin repeats are conserved with respect to length and sequences, which most resemble the protocadherin consensus [13, 14]. It has been demonstrated that protocadherins are localized at cell–cell contact sites and show weak but significant cell aggregation activity [16, 24, 25]. A chimeric protocadherin in which the original cytoplasmic domain was replaced with cytoplasmic domain of E-cadherin was expressed in mouse L cells. The transfection experiments have clearly indicated that the extracellular domain of the protocadherin is capable of Ca^{2+}-dependent and specific homophilic interaction, as has been shown for classic cadherins [24]. In addition, it is hypothesized from a structural study that cadherin repeats mediate homophilic interaction [26]. The extracellular domain motif of FAT is essentially the same as those of protocadherins [13, 14]. Deduced amino acid sequencing has revealed that the majority of the FAT cadherin repeats have the ability to form a Ca^{2+}-binding pocket and that many of the strand–dimer interface residues are conserved. Thus, it is highly likely that FAT is capable of Ca^{2+}-dependent homophilic interaction.

Immunolocalization study showed that FAT was localized at cell–cell contact sites of cultured epithelial NRK52E cells. Fluorescent dots or sometimes short strings of FAT labeling in tubular and endothelial cells in the kidney are essentially identical to those shown by ZO-1 staining [6, 7, 27]. In addition, immunogold particles for FAT were detected at the junctional complex in tubular epithelial cells. These findings support the idea that FAT is involved in the intercellular junctional complexes. In the glomerulus, FAT was expressed in podocytes, as shown by in situ hybridization. Podocytes have slit diaphragms as a unique intercellular junction. Distinct immunofluorescent signals for FAT were observed along the glomerular capillary wall, although the staining intensity for FAT was different among the strains. The FAT staining colocalized with labeling with mAb 5-1-6 that reacts with slit diaphragms. The precise localization study using immunoelectron microscopy revealed that the FAT cytoplasmic domain was located at the base of slit diaphragms and colocalized with ZO-1, a cytoplasmic component of slit diaphragms. Considering these findings and the molecular structure of FAT, we have concluded that FAT is a component of slit diaphragms.

The cytoplasmic domain of FAT contains sequences homologous to β-catenin binding region of the classic cadherins [13, 14]. We tried immunostaining for α- and β-catenins in podocytes (data not shown). However, no significant staining was detected in podocytes in the adult rat kidney, although immature podocytes in the developing kidney and glomerular endothelial cells expressed both α- and β-catenins, which are identical in the mouse kidney as described previously [28]. A putative PDZ-domain binding sequence (TEV, conforming to the X-Thr/Ser-X-Val-COOH motif [29]) has been found at the C-terminal end of FAT [13, 14]. PDZ domains are a conserved motif found in a diverse array of proteins and have been shown to mediate protein–protein interactions at the plasma membrane [30, 31]. The majority of the PDZ domains examined to date bind to consensus motifs located at the C terminus of transmembrane proteins [32], although several PDZ domains also interact directly with other PDZ domains [33, 34]. ZO-1, which is concentrated in slit diaphragms, contains three copies of the PDZ domain and has been demonstrated to interact with the components of cell–cell junctions (occludin, α-catenin, and ZO-2) and cytoskeletal networks (spectrin, F-actin) [35, 36]. It is fascinating to speculate that ZO-1 enhances FAT clustering and anchors FAT at sites of slit diaphragms.

Slit diaphragms adopt a unique morphology and are distinct from any intercellular junction of typical polarized epithelial cells. The presence of a wide intercellular space distinguishes slit diaphragms from tight junctions. There is no apparent accumulation of actin microfilaments or intermediate-sized filaments to slit diaphragms, which indicates the clear difference from adherens junctions and desmosomes. Disappearance of catenins and desmosomal proteins during maturation of podocytes corroborates the uniqueness of slit diaphragms as intercellular junctions [28, 37]. For the last three years, the molecular composition of slit diaphragms is coming to light. Nephrin and P-cadherin as transmembrane molecules and CD2-associated protein in addition to ZO-1 as intracellular molecules have been reported [4, 38, 39]. Nephrin is a member of the immunogloblin superfamily coded by the *NPHS1* gene, which is mutated in the congenital nephrotic syndrome of the Finnish type [3]. Re-

cently, mAb 5-1-6 has been demonstrated to recognize the extracellular domain of rat nephrin [40]. CD2-associated protein is an adapter protein that interacts with the cytoplasmic domain of CD2 [41]. It has been demonstrated that CD2-associated protein associates with nephrin and that mice lacking CD2-associated protein exhibit congenital nephrotic syndrome [39]. Expression of nephrin is extremely restricted to podocytes, whereas the other components, including FAT, are widely expressed in other tissues. Slit diaphragms are 30 to 45 nm wide, which is wider than an approximately 20 to 30 nm wide intercellular space of adherence junctions and desmosomes [2, 3]. We speculate that slit diaphragms contain longer intercellular adhesion molecules than adherence junctions and desmosomes. The huge extracellular domain of FAT seems to be consistent with the idea. However, to understand how FAT contributes to the unique configuration of slit diaphragms, the question of whether FAT associates with proteins or protein complexes specific to slit diaphragms like the interaction of CD2-associated protein to nephrin remains to be answered.

In summary, we have made the novel observation that FAT is located mainly at cell–cell contact sites of epithelial cells. FAT is expressed in podocytes in the adult rat, and its distribution indicates that FAT is a component of slit diaphragms.

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