Annexin II Is Present on Renal Epithelial Cells and Binds Calcium Oxalate Monohydrate Crystals

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Abstract. Attachment of newly formed crystals to renal epithelial cells appears to be a critical step in the development of kidney stones. The current study was undertaken to identify potential calcium oxalate monohydrate (COM) crystal-binding proteins on the surface of renal tubular cells. Apical membranes were prepared from confluent monolayers of renal epithelial cells (MDCKI line), and COM crystal affinity was used to isolate crystal-binding proteins that were then subjected to electrophoresis and electroblotting. Microsequencing of the most prominent COM crystal-binding protein (M_r of 37 kD) identified it as annexin II (Ax-II). When exposed proteins on the surface of intact monolayers were biotinylated and then isolated using streptavidin agarose beads, Ax-II was detected, suggesting that at least a portion is exposed on the apical cell surface. Ax-II was not completely extracted by 0.1 M Na_2CO_3, suggesting that at least a portion of cellular Ax-II is an intrinsic membrane-bound protein. Using confocal immunofluorescence microscopy, Ax-II was visualized together with Caveolin-1 (Cav-1) on the apical membrane of intact MDCKI cells. Cells pretreated with a monoclonal anti-Ax-II antibody bound significantly fewer COM crystals, whereas anti-LDL receptor antibody did not decrease COM binding, further suggesting a functional role for Ax-II during adhesion of crystals to intact cells. These results suggest that Ax-II avidly binds COM crystals and is present on the apical surface of MDCKI cells. Therefore, in the intact nephron, Ax-II could mediate adhesion of COM crystals to cells, and altered exposure of Ax-II on the surface of renal tubular cells could promote crystal retention and possibly kidney stone formation.

Nephrolithiasis is common, affecting up to 10% of the population at some point during their life (1,2). Although many patients have metabolic abnormalities, such as over-excretion of calcium in the urine, that seem to explain their tendency toward kidney stone formation, many do not (3). In addition, it appears mathematically unlikely that nucleation and growth of an individual crystal could produce a particle large enough to occlude a nephron lumen (4). Therefore, our laboratory (5–11) and others (12–17) have investigated the mechanism(s) whereby urinary crystals can interact with renal epithelial cells; once adherent to a cell, smaller particles could be retained and serve as a nidus for renal stone formation.

Indeed, studies using diverse cultured renal epithelial cells demonstrate that calcium oxalate monohydrate (COM) crystals, the most common crystal in human stones, rapidly adhere to molecules on the apical cell surface (8,9,12,13,16). Internalization (5,7) and potential dissolution within lysosomal bodies (10) follow adhesion of COM crystals. Earlier evidence suggested that anionic glycoproteins could mediate COM crystal adhesion to cultured tubular cells (9). More recently, renal cell surface nucleolin-related protein (NRP) (18) and hyaluronan (17) have been identified as potential crystal-binding molecules. Hyaluronan appears to be present on the surface of cultured cells that are proliferating in response to injury (17), and anionic phospholipids may also mediate COM crystal adhesion to injured and possibly apoptotic cells (19,20).

Evidence to date suggests that more than one apical membrane molecule could mediate adhesion and internalization of crystals; the current study was therefore undertaken to identify additional potential candidates, because cell surface crystal-binding molecules could be a critical factor that mediates the earliest events in kidney stone formation.

Materials and Methods

Monoclonal antibodies against annexin II (Ax-II) were obtained from BD Transduction Labs (Franklin Lakes, NJ), against nucleolin from MBL Co. Ltd., Japan, and against LDL receptor from Oncogene Research Products, La Jolla, CA, and a polyclonal antibody against caveolin-1 was obtained from Santa Cruz Biotechnology, CA. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated.

Cell Culture

Renal epithelial cells of the Madin-Darby Canine Kidney (MDCK) line, type I, were a gift from Carl Verkoelen (Erasmus University, Rotterdam, The Netherlands). Cells were grown in Dulbecco-Vogt modified Eagle's medium (DMEM) containing 25 mM glucose at

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38°C in a CO_2 incubator as described previously (5). To prepare high-density, quiescent cultures, 1×10^6 cells/35-mm plastic plate (9.62 cm^2; Nunc, Naperville, IL) were plated in DMEM containing 10% calf serum and 1.6 μM biotin. Two days later, when confluent, cells were harvested to prepare apical membrane vesicles.

Preparation of MDCKI Cell Apical Membrane

MDCKI cell apical membrane vesicles were prepared according to a differential centrifugation procedure (21). Confluent monolayers were rinsed with ice-cold phosphate-buffered saline (PBS; pH 7.4) and then scraped into ice-cold PBS and maintained at 4°C for all subsequent steps. These detached cells were washed twice with ice-cold PBS via alternating centrifugation (500 $\times$ *g* for 5 min) and resuspension. The cell pellet was resuspended in 10 volumes of buffer A (50 mM D-mannitol, 15 mM Tris-HEPES, pH 7.2, containing 1 mM PMSF and 0.1 μg/ml pepstatin A) and treated with a Potter-Elvehjem homogenizer (20 strokes for 1 min $\times$ 3) followed by a Polytron homogenizer for 1 min (Brinkmann Instruments, Westbury, NY). Magnesium chloride was added to the resulting homogenate (final concentration, 12 mM), which was stirred continuously for 10 min on ice. The suspension was then centrifuged (3000 $\times$ *g* for 20 min), and the resulting supernatant further centrifuged (28,000 $\times$ *g* for 20 min). The pellet obtained was homogenized in buffer B (280 mM D-mannitol, 20 mM Tris-HEPES, pH 7.4, containing 1 mM PMSF and 0.1 μg/ml pepstatin A) and centrifuged (28,000 $\times$ *g* for 20 min), resuspended in buffer B, and centrifuged once again (28,000 $\times$ *g* for 20 min). This final pellet was reconstituted in buffer C (50 mM NaCl, 10 mM Tris, pH 7.4) and stored at −80°C until used.

Measurement of Enzyme Markers

Purity of the resulting apical membranes was assessed via activities of apical and basolateral membrane marker enzymes. Alkaline phosphatase (22), Na^+/K^+-ATPase (23), γ–glutamyl transpeptidase (24), succinate dehydrogenase (25), and glucose-6-phosphatase (26) activities were all measured as described previously. The protein content of the homogenates and apical membrane preparation was determined using the BCA-Protein estimation kit (Pierce, Rockford, IL).

Solubilization of Apical Membrane Proteins and COM Crystal Binding

The non-ionic detergent n-octyl-β-D-glucopyranoside (NOG) was used to solubilize apical membrane proteins because of its high critical micellar concentration. Apical membrane proteins were solubilized in 20 mM NOG by end-over-end rotation for 4 h at 4°C. This suspension was centrifuged (28,000 $\times$ *g* for 60 min), and the supernatant containing the solubilized proteins was collected. COM crystals (5 mg/mg of protein) were added to the solubilized protein and mixed end-over-end at 4°C overnight. COM crystals were sedimented by centrifugation (5000 $\times$ *g* for 10 min at 4°C) and washed twice with ice-cold PBS. These crystals were dissolved by end-over-end rotation in EDTA (0.3 M, pH 8.0) containing 10 mM NOG and Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The resulting solution that contained solubilized proteins was concentrated using a Centricon microconcentrator with 10 kD cutoff (Millipore, Bedford, MA). The detergent was removed by washing three times with 10 mM Tris, pH 7.4, containing 0.1 mM PMSF and 0.1 μg/ml pepstatin A in a Centricon microconcentrator.

SDS-PAGE and Western blot of Solubilized COM-Binding Proteins

Concentrated fractions containing COM crystal-binding proteins were electrophoresed on a 1.5-mm thick, 10% polyacrylamide gel at 100 V constant current using a Mini-Protean II apparatus (Bio-Rad, Hercules, CA). After electrophoresis, the gel was either stained using 0.1% Coomassie blue or transferred onto a Sequi-Blot membrane (Bio-Rad) in CAPS buffer (10 mM CAPS, 40% methanol, pH 11.0) at 250 mA constant current for 60 min using a Trans-Blot apparatus (Bio-Rad). Blots were stained using Coomassie blue, or probed with a monoclonal anti-Ax-II antibody (BD Transduction Labs) and detected using an HRP-conjugated secondary antibody and an Enhanced Chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Microsequencing of COM-Binding Protein

In the Mayo Clinic Protein Core, the most prominent COM crystal-binding protein (M_r, 37 kD) was subjected to *in situ* enzymatic digestion with trypsin at 37°C overnight. The resulting peptide fragments were separated by HPLC, collected directly onto a strip of polyvinylidinedifluoride (PVDF) membrane that was subsequently treated with Biobrene (Applied Biosystems, Foster City, CA) in methanol and applied to Applied Biosystems Procise 492 HT (Applied Biosystems). The data were analyzed with the ABI Model 610A data analysis software (Applied Biosystems). Mass spectrometry analysis of the digest mixtures was also performed at the Mayo Clinic Protein Core.

Biotinylation and Streptavidin Precipitation of Cell Surface Proteins

Exposed surface proteins on intact confluent monolayers were biotinylated using EZ-link Sulfo-NHS-Biotin and isolated using Streptavidin agarose beads (Pierce) essentially as described (27). Confluent MDCKI cell monolayers were placed on ice and washed three times with 0.1 M phosphate-buffered saline (PBS-C/M, pH 7.4: 0.1 mM $CaCl_2$; 1.0 mM $MgCl_2$). Cells were then incubated with EZ-link Sulfo-NHS-Biotin (Pierce) at a final concentration of 1.5 mg/ml into PBS-C/M, pH 8.0, for 60 min at 4°C, followed by glycine (100 mM) in PBS-C/M, pH 8.0, to quench unbound labeling reagent, and then washed two times with PBS-C/M to completely remove any remaining quenching buffer. Biotinylated cells were scraped off the plates in lysis buffer-RIPA (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, and a EDTA-free protease inhibitor mix), passed ten times through a 23-G needle, and agitated on a shaker for 30 min at 4°C. The cell lysate was centrifuged for 10 min at 10,000 $\times$ *g*, and the resulting supernatant was incubated with prewashed streptavidin-agarose beads, suspended in 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, and a protease inhibitor mix, pH 7.4, and mixed end-over-end at 4°C overnight. The beads were recovered by centrifugation (5000 $\times$ *g* for 15 s) and then washed three times in wash solution A (50 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4), two times in wash solution B (500 mM NaCl, 20 mM Tris-HCl, pH 7.4), and once with wash solution C (10 mM Tris-HCl, pH 7.4); all the wash solutions contained the protease inhibitor mix. Precipitated proteins were dissociated from the streptavidin-agarose beads by heating to 95°C for 5 min in an equal volume of 2X sample loading buffer (Bio-Rad). The resulting biotinylated cell surface proteins were resolved by SDS-PAGE on a 12.5% gel, transferred onto an Immobilon-P membrane (Millipore), and probed with anti-Ax-II antibody to detect the presence of Ax-II.

Removal of Extrinsic Apical Membrane Proteins

Extrinsic apical membrane proteins were extracted using 100 mM sodium carbonate as described by Fujiki *et al.* (28). The apical

membrane pellet obtained after differential centrifugation was suspended in a small volume of sucrose-Tris buffer containing 10 mM Tris-Cl, 300 mM sucrose, pH 7.5, diluted 100-fold in ice-cold 100 mM Na_2CO_3 and mixed by end-over-end rotation at 4°C for 30 min. The suspension was then layered on a cushion of sucrose-Tris buffer and centrifuged (150,000× *g* for 60 min at 4°C). The pellet devoid of extrinsic proteins was suspended in small volume of sucrose-Tris buffer and stored at −80°C until used.

Confocal Microscopy

MDCKI cells grown to confluence on coverslips were rinsed twice with PBS and fixed using 3.7% formaldehyde in PBS for 10 min. The fixed, nonpermeabilized cells were then rinsed twice with PBS (10 min × 2) and incubated with 1% goat serum for 1 h to block nonspecific binding sites. The goat serum was aspirated, and the cells were incubated overnight with anti-Ax-II and anti-Cav-1 antibodies (Santa Cruz Biotechnology Inc.) at 4°C in a humidified chamber. Cells were then washed with PBS (10 min × 3) and incubated for 1 h with an anti-mouse IgG conjugated with Alexa-488 and anti-rabbit IgG conjugated with Texas-Red (BD Transduction Labs). The cells were then washed with PBS (10 min × 3), and the coverslips were mounted onto glass slides using Slow-Fade (Molecular Probes, Eugene, OR). The *XY* and *XZ* sections were scanned using an LSM 510 confocal microscope (Carl Zeiss, Inc. Oberkochen, Germany) equipped with an Axiovert 100M Microscope and a c-Apochromat 63 37 /1.2na water immersion lens. Alexa488 and Texas-Red were excited with the 488 nm and 568 nm lines, respectively, from an Argon/Krypton laser. Emissions for Alexa488 and Texas-Red were collected at 505 to 550 nm and >585 nm, respectively.

Effect of Antibody Pretreatment on COM Crystal Binding

Cells grown on coverslips were rinsed twice with PBS and incubated for 15 min with 1% goat serum, anti-Ax-II, anti-LDL receptor, or anti-Nucleolin antibody (0, 0.2, 5, and 20 μg/ml in 1% goat serum). Cells were then rinsed four times with PBS, and ^{14}C-COM binding was measured after 2-min exposure as described previously (5).

Effect of Calcium on COM Crystal Binding

^{14}[C]-COM binding to MDCKI cells was quantitated in the presence of various concentrations of calcium (0 to 500 mM) as described previously (9).

Results

Isolation and Identification of a COM Crystal-Binding Apical Membrane Protein

Apical membrane vesicles were prepared as described in the Materials and Methods section. There was a 4- and 11-fold enrichment in the apical membrane markers alkaline phosphatase and γ-glutamyl transpeptidase, respectively, compared with the total cell homogenate. Conversely, the activity of basolateral membrane marker (Na^+/K^+ATPase) was de-enriched (approximately 0.4-fold). In addition, the apical membrane preparation had very low activities of succinate-dehydrogenase and glucose-6-phosphatase, indicating negligible contamination with mitochondrial or endoplasmic reticulum membranes. Therefore, the apical membrane preparation was sufficiently pure and contained acceptable basolateral membrane contamination (21). The non-ionic detergent NOG was used at a 20 mM final concentration that resulted in solubilization of approximately 85% of total membrane proteins.

COM crystal affinity was used to identify apical membrane protein(s) that may mediate COM crystal binding to intact cells. As shown in Figure 1, the protein most closely associated with COM crystals had a M_r of 37 kD. After trypsin digestion, microsequencing revealed that it was Ax-II (Table 1). In addition, mass spectrometry analysis of the tryptic digest mixture identified 17 peptides matching the expected profile of Ax-II. Furthermore, Western blotting demonstrated that this protein was recognized by a monoclonal antibody against Ax-II (Figure 2). Therefore, multiple lines of evidence suggest that the 37 kD protein isolated from apical membrane proteins that bound avidly to COM crystals was Ax-II.

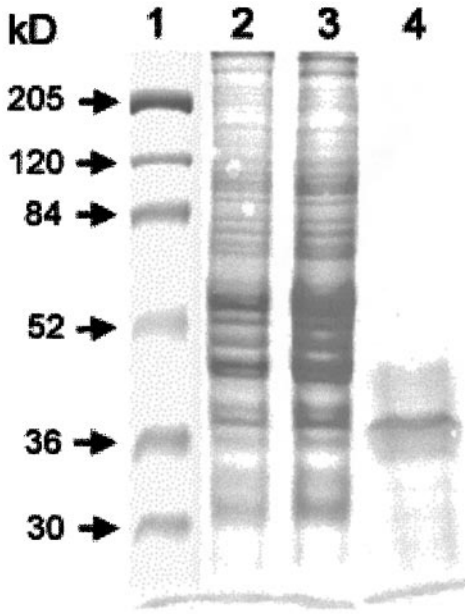

Figure 1. Calcium oxalate monohydrate (COM) crystal-binding proteins present in MDCKI cell apical membranes. Homogenate, apical membrane and COM crystal-binding proteins were prepared from MDCKI cells and resolved by SDS-PAGE, and the gel was stained with Coomassie blue as described in Materials and Methods. Lane 1, molecular weight standards (kD); lange 2, homogenate; lane 3, apical membrane proteins; lane 4, affinity-purified COM crystal-binding proteins.

Table 1. Amino acid sequence of peptide fragments obtained upon digestion of 37-kD COM crystal-binding protein

Protein/Peptide Fragment	Amino Acid Sequence (N to C Terminal)
37-kD fragment # 1	S V C H L Q K
Mouse Ax-II (amino acids 221 to 227)	S V C H L Q K
37-kD fragment # 2	L Y D S M K
Mouse Ax-II (amino acids 274 to 279)	L Y D S M K

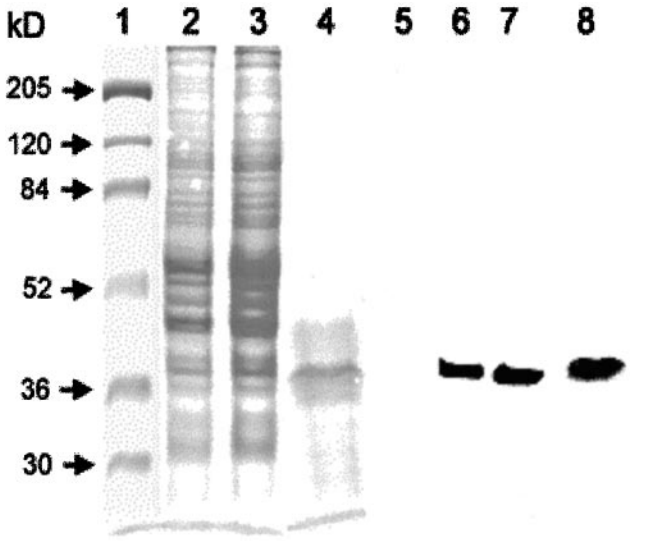

Figure 2. Western blot of MDCKI cell homogenate, apical membrane proteins, and COM crystal-binding proteins. Homogenate, apical membrane, and COM crystal-binding proteins were prepared as described, subjected to SDS-PAGE, electroblotted, and stained with Coomassie blue (lanes 1 to 4) or probed with a monoclonal antibody against annexin II (Ax-II) (lanes 5 to 8). Lanes 1 and 5, molecular weight standards (kD); 2 and 6, homogenate; 3 and 7, apical membrane; 4 and 8, affinity-purified COM crystal-binding protein.

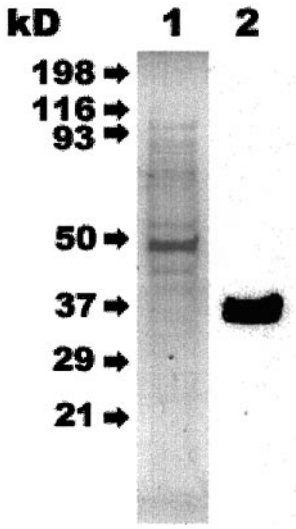

Figure 3. Biotinylation of cell surface proteins. Exposed apical surface proteins were biotinylated using EZ-link Sulfo-NHS-Biotin and precipitated using streptavidin-agarose beads. The precipitated proteins were electrophoresed, transferred onto Immobilon-P membrane, and probed with anti-Ax-II antibody. Lane 1, Coomassie-stained blot of biotinylated apical surface proteins; lane 2, blot probed using anti-Ax-II antibody.

Ax-II Is Present on the Apical Surface of MDCKI Cells

To determine if Ax-II was present and exposed on the apical surface of MDCKI cells, proteins exposed on the apical surface of intact MDCKI cell monolayers were biotinylated before precipitation by streptavidin-agarose beads. The streptavidin-precipitated proteins were transferred onto Immobilon-P membrane and probed for Ax-II. The monoclonal anti-Ax-II antibody detected a streptavidin-precipitated protein band at 37 kD (Figure 3). These results indicate that Ax-II is present and exposed on the surface of MDCKI cells.

Ax-II belongs to a family of structurally related proteins that are mainly cytosolic and bind to the inner face of the membrane. To determine if extracellular Ax-II is an intrinsic or extrinsic protein, apical membrane was treated with 0.1 M Na_2CO_3 to extract extrinsic and loosely associated membrane proteins. Na_2CO_3-treated apical membrane still contained Ax-II (Figure 4), suggesting that at least a portion of MDCKI cell Ax-II is an integral apical membrane protein, perhaps explaining its exposure on the apical surface. Ax-II is known to be present in the cytosol; nonpermeabilized cells were therefore probed with anti-Ax-II antibody under conditions where the cytosol would not be accessible to the antibodies. Cells were visualized using confocal microscopy, and *XZ* scans were performed that confirmed the presence of Ax-II on the apical surface of the MDCKI cells (Figure 5). In addition, Ax-II co-localized with Cav-1, a key protein involved in endocytosis and exocytosis that is present on the apical membrane of MDCK cells (29–31).

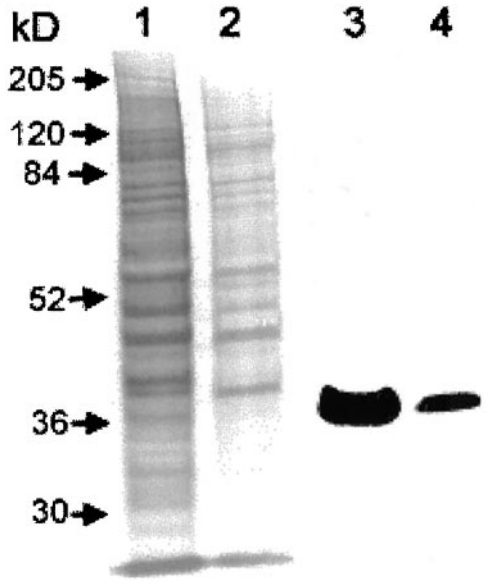

Figure 4. Extraction of extrinsic proteins of MDCKI cell apical membranes. MDCKI apical membranes were extracted using 0.1 M Na_2CO_3, electrophoresed, transferred to Immobilon-P membrane, and stained with Coomassie blue (lanes 1 and 2) or probed for Ax-II (lanes 3 and 4). Lanes 1 and 3, apical membrane proteins; lanes 2 and 4, Na_2CO_3-extracted apical membrane.

Effect of Antibody Pretreatment on COM Crystal Binding

As a final measure to assess whether or not Ax-II exposed on the apical surface of intact MDCKI cells can bind COM crystals, MDCKI cells were treated with antibodies against

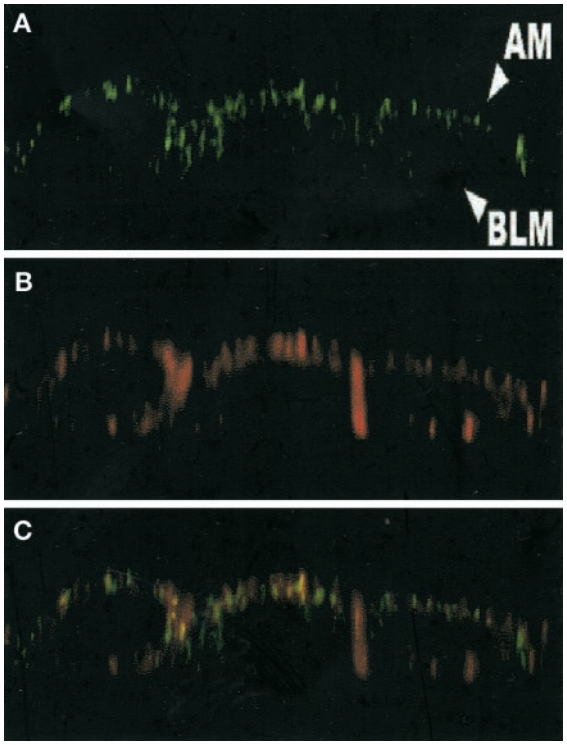

Figure 5. Visualization of MDCKI cell surface Ax-II by confocal microscopy. Non-permeabilized confluent MDCKI cell monolayers on glass coverslips were probed for Ax-II and Caveolin-1 (Cav-1). Cross-sectional XZ scans (perpendicular to cell layer) were obtained using an LSM 510 Confocal Microscope (Magnification, 630(). Ax-II and Cav-1 were visualized using secondary antibodies conjugated to Alexa-488 and Texas-Red, respectively. (A) Ax-II visualized as green fluorescence; (B) Cav-1 visualized as red fluorescence; (C) overlay of green and red fluorescence visualized as yellow fluorescence, depicting colocalization of Ax-II and Cav-1. AM, apical membrane; BLM, basolateral membrane.

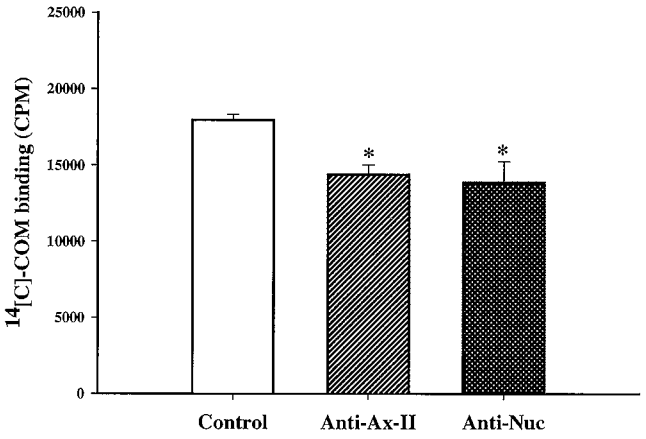

Figure 6. MDCKI cells were rinsed twice with PBS and incubated with either 1% goat serum (control), anti-Ax-II, anti-nucleolin, or anti-LDL receptor antibodies (5 μg/ml in 1% goat serum) for 15 min, and rinsed four times with PBS, and ^{14}C-COM binding during a 2-min exposure was measured. Data are mean ± SEM of three independent observations in the presence of 5 μg/ml of various antibodies. Both anti-Ax-ii (20%) and anti-nucleolin antibodies (23%) decreased COM binding significantly, whereas anti-LDL receptor antibody had no effect (not shown). * $P < 0.01$ *versus* control.

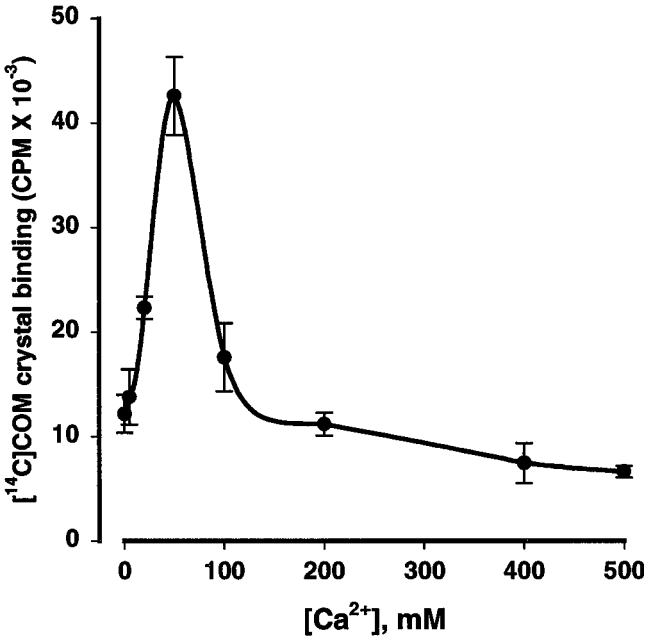

Figure 7. Effect of the ambient calcium concentration on COM crystal binding to MDCKI cells. ^{14}[C]-COM binding to MDCKI cells in PBS was measured in the presence of increasing concentrations of calcium (0 to 500 mM). Crystal binding increased as the calcium concentration was initially raised from 0 to 50 mM; it then fell below baseline as the calcium concentration further increased. Data are mean ± SEM.

Ax-II, nucleolin (which recognizes NRP, another proposed cell surface crystal-binding protein), or the LDL receptor (which recognizes a control cell surface molecule not thought to bind crystals). Pretreatment of cells with anti-Ax-II or anti-nucleolin antibody significantly decreased COM crystal binding compared with control, whereas anti-LDL receptor antibody had no effect (Figure 6). The effects of anti-Ax-II and anti-nucleolin were not additive (not shown); however, both antibodies were equally effective, and neither completely abolished crystal adhesion, suggesting multiple cell surface molecules mediate crystal adhesion. These results provide functional evidence that Ax-II is exposed on the apical surface of intact MDCKI cells and plays a role during COM crystal adhesion to intact cells.

Effect of Calcium on COM Crystal Binding

Ax-II is known to bind calcium, and calcium molecules in the crystal structure could mediate COM crystal adhesion (33); therefore, we next evaluated the effect of free calcium concentration on COM crystal binding to MDCKI cells. Calcium had a biphasic effect on COM binding to MDCKI cells, whereby COM binding increased as the calcium concentration increased from zero, reaching a peak at 50 mM (Figure 7). Crystal binding then decreased as the calcium concentration was further raised. Therefore, at low concentrations, calcium enhances cell crystal interactions; at higher, supraphysiologic concentra-

tions calcium inhibits crystal binding to cells, perhaps in part by occupying cell surface Ax-II calcium-binding sites.

Discussion

In this study, COM affinity was used to identify apical membrane proteins that might mediate crystal adhesion to renal epithelial cells. The major COM crystal-binding protein that was identified had an apparent molecular weight of about 37 kD. Amino acid microsequencing revealed that it was Ax-II, and it was identified on the apical surface of intact MDCKI cells (Figures 3 and 5). Functional evidence that Ax-II has a role as a COM crystal-binding molecule on the surface of intact cells was obtained because COM crystal adhesion decreased significantly after MDCKI cells were pretreated with a monoclonal antibody against Ax-II (Figure 6). However, crystal binding to antibody-treated cells was not abolished completely, suggesting that Ax-II may be one of several cell surface crystal-binding molecules.

Attachment of newly formed crystals to renal epithelial cells appears to be a critical step in the development of kidney stones, but only preliminary information is available about the molecules mediating this interaction (13,17,32). Pre-coating COM crystals with polyanions and pre-coating cells with polycations each inhibited crystal attachment to cultured renal epithelial cells (8,9), implying that anionic cell surface molecules play a prominent role in attachment of crystals to the cells. Crystal adhesion decreased markedly after neuraminidase and trypsin treatment of BSC-1 cells, suggesting a role for anionic sialic acid-containing proteins in the binding process. In support of this hypothesis, a concentration-dependent inhibition of crystal attachment by sialic acid-binding lectin from *T. vulgaris* was observed (9). However, the role of sialic acid during COM crystal binding to the cell surface appears to be more complex, because these groups may not directly bind to the crystals (34) and membrane glycoproteins could contribute a large number of anionic sites with the potential to bind crystals, including acidic amino acid residues or sulfated or phosphorylated residues, in addition to glycosidic side chain sialic acids. Sorokina and Kleinman (18) reported that the glycoprotein nucleolin-related protein (NRP) is present on the surface of cIMCD cells and appears to bind COM crystals avidly. Verkoelen *et al.* (17) identified hyaluronan associated with the surface of injured and migrating cells, and it appeared to mediate crystal binding to these cells. Finally, molecules other than proteins on the cell surface may at times mediate crystal adhesion to cells. Phosphatidylserine (PS), a negatively charged phospholipid, appears to mediate COM crystal binding to certain injured renal cells (14,19,20).

As a group, annexins are characterized by a conserved COOH-terminal protein "core" that mediates their membrane- and calcium-binding properties (35). Annexins form an α-helical and tightly packed disk with a slight curvature. The convex side faces the membrane and contains the calcium-binding sites. The NH_2-terminal tail, which varies between annexins, lies adjacent to the concave surface, where it can interact with binding partners, including cytoskeletal elements. The conserved COOH-terminal core contains four repeats of approximately 70 amino acids in length, each of which contains a calcium-binding motif G-X-G-T- (38 amino acids)-(D/E) (35). Members of the annexin family differ in binding specificity of the core for phospholipid headgroups (*e.g.*, PS, phosphatidic acid, or phosphatidylinositol), as well as the binding specificity of the NH_2-terminal tail.

Annexin V (Ax-V) is routinely used as a probe to demonstrate PS exposed on the surface of apoptotic cells. Several groups have demonstrated increased crystal binding to apoptotic renal epithelial cells that can be blocked by Ax-V (36). These observations suggest that PS can bind calcium oxalate crystals. When Ax-V is adherent to a plasma membrane, the four calcium-binding motifs are most likely occupied via interactions with cell-surface PS, in the process blocking a population of potential calcium oxalate crystal-binding sites, perhaps both on the cell surface as well as on Ax-V. Although Ax-V and Ax-II share similar calcium-binding motifs, as do all members of the annexin family (35), they differ in other important aspects, most notably in the composition of the NH_2-terminus. Ax-II has been demonstrated on the surface of many cell types, where it can serve receptor-like functions for molecules, including Tissue Plasminogen Activator (37). In addition, the NH_2-terminus of Ax-II can bind a p11 dimer and become linked to another Ax-II molecule via its NH_2 tail, a process that can mediate aggregation of membrane vesicles (35). Ax-V differs because, although it can bind to membrane vesicles, it does not aggregate them. Our experiments suggest that Ax-II also differs from Ax-V because it can bind calcium oxalate crystals while anchored on the cell surface. Under certain conditions, Ax-II is known to interact with membranes via cholesterol molecules in a calcium-*independent* mechanism (35), possibly leaving the calcium-binding motifs available to bind calcium oxalate crystals. Alternatively, an Ax-II-p11 tetramer might link a crystal to a membrane instead of linking two membranes. Future experiments will be necessary to further investigate these and other possibilities.

Given its four calcium-binding sites, it is perhaps not surprising that Ax-II would bind to COM crystals. The biphasic effect of calcium on crystal adhesion to cells (Figure. 7) is of particular interest and is best considered in the context of the overall process of atomic adhesion. Calcium can assume an eightfold coordination, as is required by the structure of COM crystals; therefore, calcium can be adsorbed to the surface of COM crystals, yet it can also be available to form hydrated complexes with membrane proteins (33). It is expected that the overall process of adhesion of COM crystals will be optimized once the calcium-binding sites of proteins and those of the crystals (calcium equipoints, calcium voids, and surface calcium) are all optimally occupied. This is obviously a condition of high-calcium density per atomic volume; however, if calcium concentrations rise further, repulsion will result (38,39), perhaps explaining the decreased COM binding observed at $>$50 mM calcium concentration. It is also of potential physiologic and pathologic interest that the concentrations over which crystal binding to cells increases (0 to 50 mM) includes the range of urinary calcium concentrations observed in humans. Therefore, one factor by which hypercalcemia could

favor the formation of kidney stones (39) is by increased crystal adhesion to cells.

Although most annexins are abundant intracellular proteins and Ax-II lacks a signal peptide, Ax-II has been localized in extracellular sites within several tissues as both a soluble and membrane-bound protein (40). Ax-II has been visualized on the external membrane of a diversity of cell types, including keratinocytes (41), endothelial cells, glioma cells, and smooth muscles cells (42,43); however, its mechanism of secretion is unknown. When quantitated in the endothelial cells extracellular membrane-bound Ax-II constituted approximately 4% of the total cellular Ax-II (42). In this study, soluble Ax-II bound to endothelial cell surface in a saturable and equilibrium-based manner. Ax-II has also been localized on the external plasma membrane of some cells, where it can function as a receptor for lipid A (44), cytomegalovirus (45), $1,25(OH)_2D_3$ (46), and α_2-glycoprotein I (47). Therefore, it is not unreasonable to expect Ax-II to be present and exposed on the surface of renal cells. Furthermore, at least a part of MDCKI cell Ax-II is an integral apical membrane protein, perhaps explaining its exposure on the apical surface, as suggested by Siever and Erickson (40) because Na_2CO_3-treated apical membrane still contained Ax-II (Figure 4).

Conditions have been described in which Ax-II expression is increased on the surface of cells. Our results predict that cells would be more vulnerable to crystal adhesion under such circumstances. Ax-II expression is regulated during the mammalian cell cycle (48). In Chinese hamster ovary and HeLa cells, steady-state levels of both Ax-II mRNA and protein were high in mitotic cells. As the cells divided and entered G_1, there was a reduction in the levels of both Ax-II mRNA and protein. New synthesis of Ax-II occurred early in the G_1 phase, and the maximal level was attained as cells entered S-phase. A gradual reduction in steady-state levels of Ax-II mRNA and protein thereafter occurred as cells progressed through S-phase. MDCKI cells exhibit variable COM binding at different stages of growth. COM crystal binding is highest between days 2 and 4 after plating; by day 7, it decreases to about 20% of this value (49). Expression of Ax-II during various phases of MDCKI cell cycle has not been determined; however, it is tempting to speculate that this variable COM binding exhibited by MDCKI cells could be due, in part, to altered Ax-II expression during the MDCK cell cycle.

Various injuries increase COM crystal binding to renal cells. After mechanical injury, MDCKI cells that divide and migrate to fill up the wound exhibit greatly enhanced crystal binding (17); these cells demonstrate increased cell surface hyaluronan. Exposure of cIMCD cells to oxalate enhanced COM crystal binding and was associated with increased exposure of PS on the cell membrane (20,50). In addition, treatment of cIMCD cells with PS-containing liposomes enhanced COM crystal binding. Ax-II binds to anionic phospholipids, including PS, and our results demonstrate that Ax-II avidly binds to COM crystals; it is therefore possible that enhanced COM crystal binding to cells under conditions where PS is exposed could, in part, be related to increased Ax-II present on the cell surface. It is also possible that Ax-II is also exposed with PS after oxalate treatment, because it is known to bind to anionic phospholipids (51,52), especially PS.

In this study, Ax-II co-localized with Cav-1 on the surface of MDCKI cells. Cav-1 is a key protein involved during endocytosis and exocytosis, and it is also present on the apical membrane of MDCK cells (29,31). Ax-II has also been implicated during specific membrane fusion events involved in exocytosis (53). Therefore, not only might Ax-II mediate adhesion of COM crystals to renal cells, but it could also play a role in their subsequent internalization.

In conclusion, this study demonstrates that Ax-II is present on the apical surface of MDCKI cells and avidly binds COM crystals. We hypothesize that Ax-II may serve as a COM crystal-binding molecule on the surface of renal epithelial cells *in vivo*. Further studies regarding expression of Ax-II, especially in response to various stimuli, will be necessary to confirm its precise role in adhesion and/or internalization of COM crystals and subsequent development of kidney stones.

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