Evidence that calgranulin is produced by kidney cells and is an inhibitor of calcium oxalate crystallization

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Pillay, Sokalingum N., John R. Asplin, and Fredric L. Coe. Evidence that calgranulin is produced by kidney cells and is an inhibitor of calcium oxalate crystallization. Am. J. Physiol. 275 (Renal Physiol. 44): F255–F261, 1998.—Urine produced by normal human kidneys is almost always supersaturated with respect to calcium oxalate (CaOx), the most common constituent of human kidney stones. Crystallization, with risk of renal damage and kidney stones, appears to be affected by molecules in urine that retard nucleation, growth, aggregation, and renal cell adherence of CaOx. The repertoire of such molecules is incompletely known. We have purified a 28-kDa protein from urine using salt precipitation, preparative isoelectric focusing, and sizing chromatography. Amino acid composition and NH₂-terminal amino acid sequence analysis showed complete homology to calgranulin. Calgranulin was found to be a potent inhibitor of CaOx crystal growth (44% of control) and aggregation (50% of control) in the nanomolar range. Calgranulin cDNA was cloned from a human kidney expression library. Western analysis of human and rat kidney homogenates and mRNA temporal expression from two independent renal epithelial cell lines showed that calgranulin is produced in the kidney. Given its urinary abundance and potency, calgranulin may contribute importantly to the normal urinary inhibition of crystal growth and aggregation and therefore to the renal defense against clinical stone disease.

Human kidneys have evolved to conserve water and eliminate essentially all solutes in as little as 350 ml of urine a day. Water conservation concentrates insoluble salts in urine and tubule fluid, creating supersaturations (SS) with respect to calcium oxalate monohydrate (COM), calcium phosphate solid phases such as brushite, urate phases such as dihydrogen urate, and the sodium, potassium, and ammonium salts of monohydrogen urate (1). Patchy calcifications can be found at autopsy in ~30% of kidneys from normal people and 60% of kidneys from stone formers (1, 9), presumably reflecting a lifetime of high urinary, and perhaps intratubular, SS values. Approximately 3% of adult humans in the Western hemisphere form overt kidney stones (9), ~80% of which contain calcium oxalate (CaOx) solid phases, variably admixed with calcium phosphates in the form of apatite, or brushite, or urates (9). In general, stone-forming people produce higher urine SS (11), because of a mixture of inherited and acquired disorders that increase urine calcium or oxalate excretion, alter urine pH, or reduce urine levels of citrate. Presumably, their higher SS values magnify the common patchy renal calcium deposits seen at autopsy into larger calcifications that can obstruct urine flow and cause pain and bleeding (11).

Opposing the high SS values in urine is a group of partly characterized molecules that delay or reduce the rates of nucleation, growth, and aggregation of crystal nuclei. These molecules appear to form a significant defense that may reduce crystallizations in normal people and could, perhaps, account for stone formation when defective (24, 45). Among the better characterized urine crystallization modifiers are uropontin, Tamm-Horsfall protein (25), and glycosaminoglycans (25, 45). Nephrocalcin, an acidic glycoprotein, has been extensively studied (33) but has not as yet been sequenced. Recently, the prothrombin F1 peptide (39) and bikunin (2–4) have been shown to reduce CaOx crystallization. Here, we present evidence that calgranulin, an S100 protein, is made in human kidney, is present in human urine, and is inhibitory of COM crystal growth and aggregation, at concentrations below those found in human urine.

The S100 family of calcium binding proteins contains ~16 members, each of which exhibits a unique pattern of tissue- and cellular-specific expression (14, 46). They are small, ubiquitous, acidic proteins that range in molecular mass from 9 to 20 kDa with a few exceptions (30). S100 proteins affect cell development, growth, function, and structure and have been implicated in some human diseases (23, 29, 47). All S100 molecules have two hydrophobic pockets at both the NH2 and COOH terminus, two EF hands, and one basic and one acidic motif. The linker region, a stretch of 12–13 amino acids in between the EF hands, has been reported to be responsible for binding to target molecules (46). The linker region has also been found to contribute 6–8 atoms of oxygen for coordination to calcium (46). Since S100 proteins share some characteristics of recognized urine inhibitors, e.g., acidic amino acid composition, low molecular weights, and calcium binding domains, we chose to look for S100-type proteins in urine.

Materials and Methods

Chemical purification scheme. Twenty-four hour urine was collected from healthy individuals (who did not suffer from any ailments at the time of the study) using thymol as a preservative and 10 mM phenylmethylsulfonyl fluoride to prevent enzymatic protein degradation. The urine was dialyzed extensively against water containing 0.02% sodium azide, for 72 h, and then concentrated 50-fold by evaporation under vacuum. Dialysis tubing of molecular mass cutoff 3,500 kDa was obtained from Spectra/Por (Gardena, CA). All chemicals were purchased from Sigma Chemical (St. Louis, MO). The urinary samples were sequentially precipitated using increasing amount of ammonium sulfate, (NH₄)₂SO₄ at pH 7.4. Supernatant from the 85% (NH₄)₂SO₄ precipitation was dialyzed against water, concentrated 20-fold, and focused isoelectrically (Rotorfor Cell; Bio-Rad, Hercules, CA). Briefly,
and two electrode strips were placed on the ends of the Immobiline tray. The swelled Immobiline strip was aligned, the cooling plate was adjusted on the Multiphor system and rehydrated overnight in a solution of 8 M urea, 0.5% sodium chloride, 20 mM Tris, pH 7.6, containing 0.1% Tween-20. The two-dimensional electrophoresis of calgranulin was performed using the Multiphor system (Pharmacia Biotech, Uppsala, Sweden). The purified proteins were ultimately used for further studies and are referred to as our purified S100 protein.

SDS-PAGE analysis. One-dimensional discontinuous polyacrylamide gel electrophoresis (PAGE, 17.5% separating and 3.9% stacking gels) was performed using the method of Laemmli (28) with the Mini-Protean II electrophoresis cell (Bio-Rad). Gels were run under denaturing and reducing conditions. Protein samples (1–5 µg) were solubilized by boiling for 3 min in the presence of SDS and 2-mercaptoethanol and loaded in their respective wells. Silver stain was used to detect protein bands in the polyacrylamide gels as described by Blum et al. (7). Briefly, following gel electrophoresis, the gels were fixed overnight in 50% methanol containing 12% acetic acid and 0.02% formaldehyde. They were rinsed with 50% ethanol and subsequently equilibrated for 3 min in 0.02% sodium thiosulfate. Incubation was carried out in 0.02% silver nitrate solution containing 0.03% formaldehyde. The gels were developed using a solution of 6% sodium carbonate, 0.02% formaldehdye, and 0.004% sodium thiosulfate.

Western analysis. Proteins were separated by PAGE under constant current. After the run, gels were immersed for 5 min in a continuous buffer system composed of 39 mM glycine, 48 mM Tris, 0.0375% SDS, and 10% methanol. The proteins were ultimately transferred under semi-dry conditions onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) using the Multiphor II Novablot unit for electrophoretic transfer apparatus (Pharmacia). Western analysis was carried out using the ECL chemiluminescence kit of Amer- sham International (Arlington, MA). A 2-ml aliquot of the crystall slurry was transferred to a quartz cuvette, maintained at 37°C, and magnetically stirred. Oxalate consumption was initiated by the seed crystals and was monitored for 400 s with a continuous recording spectrophotometer at 214 nm. The rate of oxalate consumption follows second-order kinetics, and from the integrated rate equation we derive the velocity of the reaction. The assay was run with and without protein samples in an alternating fashion, such that each sample was sandwiched between two control measurements. Inhibitory activity was expressed as a dissociation constant calculated from a Langmuir-type isotherm.

Crystal aggregation inhibition. COM crystal slurries of 0.8 mg/ml were prepared in 200 mM NaCl, pH 5.7, and magnetically stirred at 1,100 rpm overnight at 37°C. The crystals and solution came to equilibrium so crystals no longer dissolved or grew. The slurry was then added to multiple tubes, each used for a single assay. Protein samples were added to the stirred slurry 1 h prior to assay. A 2-ml aliquot of the crystal slurry was transferred to a quartz
cuvette maintained at 37°C and was constantly monitored at 620 nm. In this system, the optical density at 620 nm (OD_{620}) was proportional to the mass of crystal per unit volume. The slurry was initially stirred at 1,100 rpm to obtain a stable baseline, then crystal aggregation was induced by slow stirring at 500 rpm for 180 s. Stirring was stopped, and particle sedimentation was monitored. The rate of fall in OD_{620}, measured as the slope of the straight portion of the plot of OD versus time, reflects the average particle size and was taken as a measure of aggregation. The assay was run with and without protein sample added to the slurry in an alternating fashion. Sample inhibition of aggregation was quantified by plotting log sample concentration vs. log aggregation inhibition and estimating the 50% inhibition concentration from the slope of the regression line.

Protein measurements. Total protein was measured using nihydrin, as prior studies have shown that many common reagents fail to recognize all the urine proteins (33). A 25- to 75-μl aliquot was hydrolyzed in 0.5 ml of 1 N NaOH for 8 h. Thereafter, pH was adjusted to 5.7 with 0.5 ml of 7.5% acetic acid, and 25 μl of nihydrin was added. The resulting color development during 15 min of incubation in a boiling water bath was measured at 570 nm. A standard curve was constructed for each run using bovine serum albumin.

Amino acid sequence analysis. After SDS-PAGE electrophoresis, proteins were transferred onto PVDF membranes. Amino acid sequence was determined by automated Edman degradation on an Applied Biosystem protein sequencer by the Protein Core Facility at the University of Chicago and the Department of Biochemistry at the Medical College of Wisconsin.

Quantitative Western analysis. Known amounts of urinary calgranulin (28 kDa) ranging from 2.5 to 25 ng were loaded on a 17.5% PAGE and run as described above (SDS-PAGE analysis). The gel was transferred to a PVDF membrane, and Western analysis was performed using an anti-S100 antibody, as described above. Only the band at 28 kDa was analyzed in the quantitation of urine calgranulin concentration.

Northern blot analysis. Total RNA from BSC-1 and MDCK renal epithelial cell lines was isolated using guanidine thiocyanate as described by Chomczynski and Sacchi (10). Samples of total RNA (15 μg) were electrophoresed on a 1.2% agarose-6% formaldehyde gel and transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH). The portion of total RNA (15 μg) was electrophoresed on a 1.2% agarose-6% formaldehyde gel and transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH). The 480-bp calgranulin fragment was labeled using [α-32P]dCTP by random hexamer priming and hybridized to Northern blots at 42°C in a solution containing 1 M NaCl, 1% SDS, 50% formamide, and 10% dextran sulfate. The blots were washed at 65°C in 2× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate) containing 0.1% SDS. An autoradiogram of the blot was obtained by exposing the blot to an X-ray film at −70°C for 24 h.

Human cDNA library screening. A human kidney cDNA expression library (Clontech Laboratories, Palo Alto, CA) was screened using the S100 antibody (1:2,000) per the manufacturer's instructions. Positive clones were rescreened three times. The insert was characterized using the Clontech LD-insert Screening amplimer set (Clontech Laboratories).

**RESULTS**

Commercially available bovine brain S100 protein, which we used as an initial test material, inhibited COM growth at 55% of control at 1 × 10−6 M and COM crystal aggregation at 75% of control at 1 × 10−7 M (Table 1). Western analysis of dialyzed urine from seven non-stone-forming subjects, using two different commercial anti-S100 antibodies, showed positive signals at 6, 28, and 42 kDa (Fig. 1A). The signal at 28 kDa (arrow on Fig. 1A) was found to be consistent, prominent, and abundant in all urine samples analyzed; thus we provisionally termed it “SLP28” (i.e., 28-kDa S100-like protein). Using quantitative Western analysis, we found that daily excretion of SLP28 varied from 1 to 3 µg/mg urinary protein (Fig. 1B). Immunoaffinity columns were prepared using the two different polygonal antibodies and urine from one individual. Immunoeluates from the two columns were strikingly similar and highly reactive to anti-S100 antibody (Fig. 1C). Both immunoeluates were strongly inhibitory to COM crystal growth at 35% and 31% of control at 1−2.5 × 10−9 M, respectively (Table 1). However, by using immunoaffinity, we could not purify SLP28 to complete homogeneity, as three closely associated spots were seen on a two-dimensional gel electrophoresis (results not shown).

Given these encouraging results for immunoreactive S100 proteins of urine, SLP28 was purified from urine using (NH4)2SO4 precipitation (a classic method of purifying S100 proteins, see MATERIALS AND METHODS) (31). The >85% supernatant material was dialyzed and subjected to preparative isoelectric focusing (Fig. 1D) followed by sizing chromatography on a P-60 column (Bio-Rad). The 28-kDa fraction eluted from the P-60 column was found to migrate as a single band on SDS-PAGE (Fig. 1E) and a single spot on two-dimensional gel (Fig. 1F), indicating that it had been purified to homogeneity. The purified protein was immunoreactive to S100 antibody (not shown), indicating that it was the same protein obtained by immunopurification. It inhibited COM growth and aggregation at concentrations as low as 1 × 10−9 M (Table 1). Langmuir-type plots of COM growth inhibition vs. protein concentration (Fig. 2A) gave a Kd value of 1.5 nM. A log plot of aggregation inhibition vs. protein concentration (Fig. 2B) gave a 50% inhibition concentration of 4 nM. Given concentrations in the range of 1−3 μg calgranulin per milligram of urine protein, and urine protein concentrations of 100 to 300 mg/dl (MATeRIALS AND METHODS), urine concentrations of SLP28 were approximately 3.5–10 nM, above the levels required for marked inhibition in both assays.

<table>
<thead>
<tr>
<th>Source</th>
<th>Assay</th>
<th>Protein, nM</th>
<th>Inhibition, % control</th>
</tr>
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<tbody>
<tr>
<td>Bovine brain S100</td>
<td>Growth</td>
<td>1,000</td>
<td>55</td>
</tr>
<tr>
<td>Purified SLP28</td>
<td>Aggregation</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>SLP product A</td>
<td>Growth</td>
<td>5</td>
<td>44</td>
</tr>
<tr>
<td>SLP product B</td>
<td>Growth</td>
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<td>30</td>
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Inhibition is expressed as % control. SLP28 (S100-like protein, 28 kDa) was purified using isoelectric focusing and size exclusion chromatography and was identified as calgranulin by amino acid sequencing. Products A and B refer to immunopurified urinary S100 proteins using antibodies from 2 separate commercial sources (see MATERIALS AND METHODS).
Ser-Ile. A search of available protein databases revealed complete homology to calgranulin. On sequencing, the lack of any significant minor sequences (not shown) supported two-dimensional gel evidence for purity of the preparation. Given the identification of SLP28 as calgranulin, we asked whether it could have its source in kidney cells themselves. Western analysis of kidney tissue homogenate from both rat and human sources revealed a prominent band at ~28 kDa on probing with an anti-S100 antibody (Fig. 3A). Its renal presence is further confirmed by Western and Northern analysis of two renal epithelial cell lines, BSC-1 and MDCK (Fig. 3, B and C). Its abundance in the kidney led us to screen a human kidney λgt11 expression library for possible S100 immunoreactive clones (Fig. 3, D–F). Secondary and tertiary screening (Fig. 3, E and F) of one positive clone (Fig. 3D) gave rise to a 480-bp fragment (Fig. 3G). This fragment was sequenced and found to be calgranulin.

DISCUSSION

Nephrolithiasis is a common disorder, and 75% of all kidney stones are mainly composed of CaOx (12) admixed with variable amounts of calcium phosphate, mainly in the form of apatite. The nucleation and growth of CaOx and calcium phosphates occur because urine, and presumably tubular fluid at some nephron sites (1, 26), are supersaturated with respect to these salts. The near universal SS of human urine with CaOx, combined with the fact that most people do not form stones, suggests that urine must contain inhibi-
tory molecules. Moreover, a long list of publications has offered abundant evidence that urine can indeed slow the growth, nucleation, and aggregation of COM (11). The nature of the molecules that confer inhibitory properties on urine may be of considerable importance in understanding the pathogenesis of renal stones, because SS alone never has been sufficient to separate normal from stone-forming subjects in a satisfactory manner (12, 35, 36).

We report evidence here that calgranulin is made by human kidney, is present in human urine, and is a potent inhibitor of COM growth and aggregation. Calgranulin belongs to the S100 family of calcium binding proteins. There are three monomers (A, B, and C) of calgranulin: A, growth inhibition; x-axis is the reciprocal of the concentration of calgranulin in nmol/l; y-axis is a measure of the inhibitory activity of the protein, where V₀ is the velocity of crystal growth in the absence of protein, and V is the velocity of crystal growth in the presence of SLP₂₈. This is a Langmuir-type plot in which the slope of the linear regression gives the dissociation constant, K_d = 1.5 nM. B: aggregation inhibition; x-axis is the SLP₂₈ concentration plotted on a log scale; y-axis (log scale) is the degree of aggregation in the presence of SLP₂₈ as a percent of the control without an inhibitor present. From the linear regression, we calculated the concentration at which SLP₂₈ reduced aggregation by 50% to be 4 nM.

Fig. 2. A: growth inhibition; x-axis is the reciprocal of the concentration of calgranulin in nmol/l; y-axis is a measure of the inhibitory activity of the protein, where V₀ is the velocity of crystal growth in the absence of protein, and V is the velocity of crystal growth in the presence of SLP₂₈. This is a Langmuir-type plot in which the slope of the linear regression gives the dissociation constant, K_d = 1.5 nM. B: aggregation inhibition; x-axis is the SLP₂₈ concentration plotted on a log scale; y-axis (log scale) is the degree of aggregation in the presence of SLP₂₈ as a percent of the control without an inhibitor present. From the linear regression, we calculated the concentration at which SLP₂₈ reduced aggregation by 50% to be 4 nM.

Fig. 3. A: presence of calgranulin in kidney extracts of rat and human tissue homogenates; lanes 1 and 2 are rat and human kidney homogenates. B: SLP₂₈ expression in two renal epithelial cell lines; lanes 1 and 2 represent cellular homogenates from BSC-1 and MDCK renal epithelial cell lines probed with a monoclonal antibody to calgranulin. C: Northern analysis of two renal epithelial cell lines to SLP₂₈ clone (Calgranulin); lanes 1 and 2 and lanes 3 and 4 contained 15 µg of total RNA from BSC-1 and MDCK cell lines, respectively, and were probed with the 480-bp fragment obtained through expression cloning. D–F: expression cloning of calgranulin cDNA, as 3 rounds (D, E, and F) of screening of a human kidney expression library. Clones were identified by incubating first with an anti-S100 antibody followed by horseradish peroxidase-linked anti-rabbit IgG and addition of chemiluminescence reagents. G: PCR of the isolated clone. PCR was carried out using the LD-insert screening amplimer sets (Clontech Laboratories). A clone of ~480 bp was thus isolated and subsequently cloned in PKS vector.
varying molecular weights. These proteins have been isolated, cloned, and mapped to chromosome 1 and are also referred to as S100A 8–12 (44). The calgranulins are also known under a puzzling array of names such as leukocyte antigen L1 (16), migratory-inhibitory factor (MRP) (17, 18), and cystic fibrosis-associated antigen (CFA) (20, 22). Additionally, the complexes formed by these monomers are also termed differently, such as protein complex (PC) (32), calprotectin (41), Mac 387 antigen (8), and 60B8 antigen (34) (the latter two refer to monoclonal identification). Given that the NH2-terminal sequence of SLP28 has 100% homology to calgranulin, we prefer to call the protein as calgranulin.

The ability of calgranulin to inhibit crystal growth is possibly related to its ability to bind to the crystal surface. What other role it may play in renal cells is as yet unknown, as is its exact quantitative importance in overall urine inhibition of CaOx growth and aggregation. In particular, calgranulin must eventually be considered alongside uropontin, Tamm-Horsfall protein, the prothrombin F1 fragment, bikunin, and nephrocalcin (27, 40, 42, 45) as one of potentially many urine COM inhibitors.

The inhibitory properties of calgranulin may be shared by other urinary S100 proteins. Bovine brain S100 protein inhibits COM growth and aggregation. It is this initial observation that led us to screen urine for SLPs. Indeed, 5–7 separate bands in urine have S100-like epitopes as determined by Western blotting. We have proven that the prominent band at 28 kDa (SLP28), which appears to be present in all urine analyzed so far, is calgranulin. However, the identity of the others, as well as whether any of them have inhibition effects separate from those of calgranulin, also remains to be determined. Although the cDNA has an open-reading frame of 279 nucleotides predicting a protein of 93 amino acids and M, 10,835, we have observed urine calgranulin associate as a multimer migrating at 28 kDa. This is consistent with the known molecular biology of calgranulin, which exists as three monomers of 10, 12, and 14 kDa molecular mass (17, 18, 37) that combine readily into hetero- and homodimers of 25–48 kDa molecular mass (6, 13, 15, 37, 38). Whether polymerization patterns affect COM inhibition properties and whether these differ among normal people and patients is unknown at this time.

Calgranulin is abundant in both rat and human kidney homogenates, and we were able to isolate the cDNA of calgranulin by screening a human kidney expression library. Its renal presence is further evidenced by Western and Northern analysis of two renal epithelial cell lines. In addition, calgranulin has also been identified in circulating neutrophils and monocytes of normal individuals (21, 22). Therefore, at the present time, we cannot be sure whether urinary calgranulin arises in part or entirely from renal cell production or whether some arises from glomerular filtration or even leukocytes in the renal interstitium. However, expression of calgranulin by renal cells indicates that urine calgranulin could be produced by kidney cells and perhaps be regulated by them in ways that are important in defense against crystallization. Umekawa and Kurita (43) found calprotectin (30 kDa) in the matrix of CaOx stones and reported that calprotectin isolated from granulocytes was a potent inhibitor of CaOx crystal growth in vitro by using the standard 14C CaOx assay. Recently, it has been found to be present in the matrix of struvite (5) and CaOx stones (21, 43), which may reflect either adsorption as part of inhibition or a possible role in nucleation or formation of a protein matrix in stones.

Although the exact role of calgranulin in stone formation has yet to be elucidated, we feel that its presence in the kidney and urine and its extremely high levels of inhibition of growth and aggregation of COM suggest calgranulin as such may prove to be an important defense against crystal formation and, when abnormal, an etiologic factor in calcium stones.

We are grateful to Dr. G. Toback, Dr. Y. Nakagawa, Dr. J. Lieske, and J. Parks for helpful suggestions and criticisms and to Amanda Rydel and Michael Martin for the excellent technical assistance.

This work was supported by O’Brien Kidney Research Center National Institute of Diabetes and Digestive and Kidney Diseases Grant P50-DK-47631.

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Received 21 November 1997; accepted in final form 30 April 1998.

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