Identification of human urinary trefoil factor 1 as a novel calcium oxalate crystal growth inhibitor

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Previous research on proteins that inhibit kidney stone formation has identified a relatively small number of well-characterized inhibitors. Identification of additional stone inhibitors would increase understanding of the pathogenesis and pathophysiology of nephrolithiasis. We have combined conventional biochemical methods with recent advances in mass spectrometry (MS) to identify a novel calcium oxalate (CaOx) crystal growth inhibitor in normal human urine. Anionic proteins were isolated by DEAE adsorption and separated by HiLoad 16/60 Superdex 75 gel filtration. A fraction with potent inhibitory activity against CaOx crystal growth was isolated and purified by anion exchange chromatography. The protein in 2 subfractions that retained inhibitory activity was identified by matrix-assisted laser desorption/ionization–time-of-flight MS and electrospray ionization–quadrupole–time-of-flight tandem MS as human trefoil factor 1 (TFF1). Western blot analysis confirmed the mass spectrometric protein identification. Functional studies of urinary TFF1 demonstrated that its inhibitory potency was similar to that of nephrocalcin. The inhibitory activity of urinary TFF1 was dose dependent and was inhibited by TFF1 antisera. Anti-C-terminal antibody was particularly effective, consistent with our proposed model in which the 4 C-terminal glutamic residues of TFF1 interact with calcium ions to prevent CaOx crystal growth. Concentrations and relative amounts of TFF1 in the urine of patients with idiopathic CaOx kidney stone were significantly less (2.5-fold for the concentrations and 5- to 22-fold for the relative amounts) than those found in controls. These data indicate that TFF1 is a novel potent CaOx crystal growth inhibitor with a potential pathophysiological role in nephrolithiasis.

Introduction

Nephrolithiasis remains a public health problem around the world, affecting 1–20% of the adult population (1). Of all types of renal stones, calcium oxalate (CaOx) is the most common composition found by chemical analysis (2). To date, the pathogenic mechanisms of stone formation remain unclear. One long-standing hypothesis is that stone formation is related to intratubular crystal nucleation, growth, and aggregation (3). The urine from patients with nephrolithiasis is commonly supersaturated with calcium and oxalate ions (4), favoring CaOx crystal nucleation and growth (5). Nucleated crystals can be retained in the kidneys of these patients by adhering to renal tubular epithelial surfaces (6, 7). Within the environment of supersaturated calcium and oxalate ions, the stone can then be formed. In contrast, nucleated crystals are not retained in the normal kidney (8). Calculation of the flow rate of renal tubular fluid and the rate of crystal growth in normal subjects suggests that nucleated crystals are eliminated from the normal kidney before they attach to tubular epithelial surfaces (9, 10). Additionally, there are urinary substances known as “stone inhibitors” in the normal renal tubular fluid that inhibit intratubular crystal growth, aggregation, and/or adhesion to renal epithelial surfaces (11). These substances include proteins, lipids, glycosaminoglycans, and inorganic compounds. Abnormality in function and/or expression levels of these molecules, especially proteins, in the urine and renal tubular fluid has been proposed to be associated with stone formation (12–14).

Another hypothesis, first described by Alexander Randall (15), is that the locale of crystal deposition is at a renal interstitium near or at the tip of renal papillae. Randall’s plaques, which contain apatite crystals, are usually found in CaOx stone formers (16). Examination of biopsies obtained during percutaneous nephrolithotomy has shown that apatite crystallization initially occurs in the basement membranes of the thin loop of Henle, then subsequently extends to vasa recta, spreads to the interstitial tissue surrounding inner medullary collecting ducts, and finally extends to the renal papillae (17, 18). Erosion of Randall’s plaques into the urinary space, which is supersaturated with calcium and oxalate ions, can occur and may promote heterogeneous nucleation and formation of CaOx kidney stones (17, 18). Although the CaOx stone formers produce interstitial apatite crystals that form the well-known Randall’s plaques, they do not develop epithelial damage, interstitial inflammation, or fibrosis (17).

Nonstandard abbreviations used: CaOx, calcium oxalate; Cr, creatinine; dI, deionized; ESI-Q-TOF, electrospray ionization–quadrupole–TOF; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MS/MS, tandem MS; TFF1, trefoil factor 1; TOF, time-of-flight.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J. Clin. Invest. 115:3613–3622 (2005). doi:10.1172/JCI25342.

http://www.jci.org

Volume 115 Number 12 December 2005 3613
CaOx stone formation is also associated with intestinal bypass that promotes hyperoxaluria. Histopathological examination reveals no plaque at the interstitium, but some apatite crystals plugged inside the terminal collecting duct lumens that are associated with epithelial cell damage, interstitial inflammation, and fibrosis (17). Another group of stone formers produce predominantly (>50%) calcium phosphate stones; of these, one-quarter contain brushite (CaHPO$_4$·2H$_2$O), which represents an early phase of calcium phosphate stone formation (19, 20). The degree of brushite supersaturation depends directly on urinary calcium (21), and patients with brushite stones have associated absorptive hypercalciuria type I and distal renal tubular acidosis (20). Brushite stone formers undergo histopathological changes that combine the interstitial plaques of CaOx stone formers with the intratubular apatite plugs found in bypass stone formers; in other words, their histopathology is an amalgam of CaOx and bypass stone disease (19). A nidus of brushite can elicit heterogeneous nucleation or epitaxial growth of CaOx; thus, brushite has been implicated in the formation of both hydroxyapatite [Ca$_{10}$(PO$_4$)$_6$(OH)$_2$] and CaOx stones (20). On the other hand, many patients with brushite stones initially had CaOx stones (19).

Whether intratubular or interstitial deposition of crystals is the main pathogenic mechanism of renal stone disease remains to be elucidated. It is unlikely that a single mechanism or pathway can explain all forms of kidney stones. Perhaps several mechanisms of stone formation occur in 1 patient. In this study, we have focused our attention on purification, identification and functional analysis of a novel CaOx crystal growth inhibitor and on its potential role in the pathogenic mechanisms of nephrolithiasis. Currently known stone inhibitory proteins are nephrocalcin (22), Tamm-Horsfall protein (23), uropontin (24), inter-α-trypsin inhibitor (bikunin) (25), and urinary prothrombin fragment 1 (crystal matrix protein) (26). Interestingly, these stone inhibitors have similar physical and chemical properties; they are small anionic proteins that bind to calcium and inhibit either growth or aggregation of CaOx crystals. Only a few additional proteins have been identified as inhibitors of stone formation (27–29). In the present study, we have purified and identified a novel CaOx crystal growth inhibitor, namely urinary trefoil factor 1 (TFF1), from normal human urine using conventional biochemical methods and recent advances in mass spectrometry (MS). The presence of TFF1 in the urine was confirmed by Western blot analysis, and its inhibitory activity against CaOx crystal growth was characterized. Finally, differences in concentrations and relative amounts of urinary TFF1 between patients with idiopathic CaOx stone and normal healthy individuals were evaluated.

**Results**

Isolation of anionic proteins and screening for potential inhibitors of CaOx crystal growth. The present study focused on anionic urinary proteins because all proteins known to inhibit CaOx crystal growth are anionic and because urinary proteins may interfere directly with crystal growth and stone formation. The purification

![Figure 1](http://www.jci.org) The schematic summary of analytical procedures in the present study. Normal human urinary proteins were purified using DEAE adsorption (isolation of anionic proteins), HiLoad 16/60 Superdex 75 gel filtration (size separation), and Resource Q anion exchange chromatography with 0–0.5 M NaCl gradient elution (charge separation). The protein in the pooled fraction with potent inhibitory activity against CaOx crystal growth was identified by MALDI-TOF MS and ESI-Q-TOF MS/MS. Expression and function of the identified proteins were then validated.

![Figure 2](http://www.jci.org) Expression and functional profiles of anionic urinary proteins. (A) After completion of DEAE adsorption, anionic proteins were separated using HiLoad 16/60 Superdex 75 gel filtration, and peptides were quantitatively analyzed by spectrophotometry at λ214 nm. (B) The inhibitory activity against CaOx crystal growth of each fraction was measured (see Methods). Fraction X was a low abundant compartment but had a potent inhibitory activity. This pooled fraction was then separated by Resource Q anion exchange chromatography with a 0–0.5 M NaCl gradient elution (charge separation).
form of human TFF1 is at the fifty-eighth residue (serine in 1pS2 versus cysteine in native TFF1) (30). We did not identify the C-terminal tryptic peptide of native TFF1 in any of the protein subfractions of human urine (Figure 4D). This is probably because the C-terminal tryptic peptide of TFF1 can only be identified in negative ion mode. We have therefore identified the native mature form of human TFF1 in the protein subfractions of normal human urine that contain the greatest CaOx crystal growth–inhibitory activity. TFF1 is a small secreted protein expressed predominantly in the gastric mucosa and thought to have an important role in mucosal protection. It has not been implicated previously in normal renal function.

Validation of mass spectrometric data. To confirm the identity of human TFF1 in subfractions X1, X2, X1’, and X2’ isolated from 2 normal donors, we performed Western blot analysis using a monoclonal anti-TFF1 antibody. Figure 5A illustrates that Western blotting identified a TFF1 immunoreactive protein band in all 4 subfractions, in the original fraction X, and in the crude anionic sample that migrated with the same apparent molecular mass as recombinant TFF1. These results confirmed the protein identification by MALDI-TOF MS and ESI-Q-TOF MS/MS and indicated that the CaOx crystal growth–inhibitory protein in fraction X and its subfractions is TFF1. The presence of TFF1 in urine was also confirmed in 4 other normal healthy individuals (Figure 5B).

Absence of known stone inhibitors in the urinary TFF1 fraction. Because the purification of urinary TFF1 in the present study used methodology similar to that employed previously for the purification of other stone inhibitors, particularly nephrocalcin and uropontin (24, 31), we performed Western blot analyses of fraction X to examine whether nephrocalcin, uropontin, bikunin, and Tamm-Horsfall protein were present or contaminated in the purified urinary TFF1 fraction. Figure 6 clearly illustrates that these stone inhibitors were detected only in the total anionic protein fraction, not in the purified fraction X, whereas TFF1 was present in both fraction X and the total anionic protein fraction.

Characterization of the inhibitory activity of urinary TFF1 against CaOx crystal growth. CaOx crystal growth–inhibitory activity of TFF1 purified from human urine was compared with that of known protein inhibitors of crystal growth. Nephrocalcin, a potent CaOx crystal growth inhibitor, was used as a positive control, whereas lysozyme, a protein without Ca2+−binding activity, was used as a negative control. Figure 7A shows the relative inhibitory activities of lysozyme, Tamm-Horsfall protein, nephrocalcin, and urinary TFF1. As expected, nephrocalcin had the most potent inhibitory activity against CaOx crystal growth, which was consistent with data reported previously (32, 33). Interestingly, the inhibitory activity of urinary TFF1 was very similar to that of nephrocalcin (36.1% ± 2.0% versus 39.8% ± 1.9% relative inhibitory activity) when an equal amount of protein was used (3 µg). Tamm-Horsfall protein had much weaker inhibitory activity compared with nephrocalcin and urinary TFF1, even though the total amount of Tamm-Horsfall protein used in this experiment was much greater (50 µg, approximately 16-fold more than nephrocalcin and urinary TFF1). Figure 7B demonstrates that the inhibitory activity of urinary TFF1 was dose dependent.

To confirm that the CaOx crystal growth–inhibitory activity of fraction X was specific to TFF1, the CaOx crystal growth–inhibitory activities of recombinant TFF1 and TFF3, another member in the trefoil factor family, were examined. Figure 7C shows that the CaOx crystal growth–inhibitory activity of recombinant TFF1 was significantly greater than that of recombinant TFF3 (36.1% ± 2.0% versus 11.1% ± 1.4% relative inhibitory activity), indicating that the inhibitory activity in fraction X was specific to TFF1.
dose dependent and similar to that of purified urinary TFF1 when the same amount of protein was used. The inhibitory activity of recombinant TFF3 was approximately half that of recombinant TFF1 and urinary TFF1.

Further characterization of the inhibitory activity of urinary TFF1 against CaOx crystal growth was performed using polyclonal anti-N-terminal and anti-C-terminal TFF1 antibodies to neutralize the inhibitory activity of TFF1. Both antibodies significantly reduced the CaOx crystal growth–inhibitory activity of urinary TFF1. Blocking the inhibitory activity of urinary TFF1 at its C terminus was more effective than blocking at the N terminus (Figure 7D). These data indicate that the C terminus of TFF1 is particularly important for its inhibitory activity against CaOx crystal growth.

Inhibitory activity of urinary TFF1 at physiological levels. Normal concentrations of urinary TFF1 in 30 normal healthy individuals were measured by ELISA (6.67 ± 0.93 ng/ml). Our results were similar to the data reported recently (7.04 ± 6.43 ng/ml) in 100 normal individuals using radioimmunoassay (34). The protein recovery yield of purification of urinary TFF1 using our protocol was approximately 73.50% ± 17.27%. Because the characterization of the CaOx crystal growth–inhibitory activity of urinary TFF1 shown in Figure 7 was performed using much...
had 2 stones, and the other 14 patients had 5 or more stones. Size control (without TFF1). These data demonstrate significant inhibition of the stones varied from 1.0 to 5.0 cm. Equal amounts of the total episode) renal stone disease. Five patients had 1 stone, 4 patients Additionally, there were significant differences among the 3 concentrations of crystal growth by a physiological concentration of TFF1 (7 ng/ml) of urinary TFF1 at of 7, 70, and 700 ng/ml concentrations as well as a different subfractions, the main fraction X, and crude anionic sample obtained from 2 normal healthy donors (DEAE). (B) Validation was performed for fraction X isolated from 4 normal donors (N1–N4), indicating consistent expression of urinary TFF1 in normal human urine. In each lane, 30 µg total protein was loaded.

higher concentrations of TFF1 than normal, the inhibitory activity of urinary TFF1 at physiological concentrations was also examined. Figure 8 shows the kinetics of CaOx crystal growth–inhibitory activity of urinary TFF1 at of 7, 70, and 700 ng/ml concentrations as well as a control (without TFF1). These data demonstrate significant inhibition of crystal growth by a physiological concentration of TFF1 (7 ng/ml) from 10 minutes of incubation through the end of the assay (1 hour). Additionally, there were significant differences among the 3 concentrations of urinary TFF1 tested.

Differential levels of urinary TFF1 in patients with idiopathic CaOx kidney stone compared with normal healthy controls. Urinary concentrations of TFF1 in 23 patients (12 males and 11 females) with idiopathic CaOx kidney stones and in 30 normal healthy individuals (14 males and 16 females) were measured using ELISA. Clinical characteristics of patients with idiopathic CaOx kidney stones are summarized in Table 1. Eighteen patients had new onset (first episode) of renal stone disease, whereas the remaining 5 had recurrent (second episode) renal stone disease. Five patients had 1 stone, 4 patients had 2 stones, and the other 14 patients had 5 or more stones. Size of the stones varied from 1.0 to 5.0 cm. Equal amounts of the total anionic proteins isolated from 10 ml urine of each donor were analyzed in this experiment. Figure 9A clearly illustrates that urinary concentrations of TFF1 in patients with idiopathic CaOx kidney stone were significantly lower than those in the normal controls (2.66 ± 0.46 versus 6.67 ± 0.93 ng/ml; P < 0.0001; approximately 2.5-fold difference). After normalization with urine creatinine (Cr; which would represent the total amount of TFF1 excreted within a day), the relative amounts of TFF1 in the urine of CaOx stone formers remained significantly less (3.24 ± 0.62 versus 17.19 ± 3.49 ng/mg Cr; P < 0.0001; approximately 5-fold difference; Figure 9B). This difference remained highly significant after normalization with total protein (11.08 ± 3.21 vs. 247.27 ± 53.68 ng/mg total protein; P < 0.0001; approximately 22-fold difference; Figure 9C).

Discussion

CaOx crystal growth inhibitors (proteins, lipids, glycosaminoglycans, and inorganic compounds) have been proposed to play an important role in renal stone disease for several decades (35, 36). While some progress has been made in characterization of these inhibitors, knowledge of proteins that can inhibit stone formation is limited to a relatively small number of proteins. Identification of additional stone-inhibitory proteins was hampered in the past by limitations in protein identification methods, which meant that low abundance components or novel proteins could not be identified without some prior knowledge of their involvement. Exploratory studies using modern technologies to define novel CaOx crystal growth inhibitors are therefore necessary and may lead to better understanding of the pathogenesis and pathophysiology of nephrolithiasis.

In the present study, we have identified human urinary TFF1 as a novel CaOx crystal growth inhibitor by using a combination of conventional biochemical purification methods and recent advances in mass spectrometric protein identification. We initially screened for candidate proteins from a large volume of normal human urine in order to examine all anionic proteins with inhibitory activity against CaOx crystal growth. This approach allowed us to purify low-abundance proteins that could not be identified in previous studies, in which a smaller volume of urine was utilized (37, 38). The purification was performed systematically, including anionic adsorption, size separation, and finally, charge-density separation. We have focused on fraction X in the present study. This fraction was one among several fractions that had inhibitory activity against CaOx crystal growth. We have initiated extensive profiling of the fractions with inhibitory activity against CaOx crystal growth in a larger number of samples. Characterization and functional analyses of other fractions will have additional significant impact on current knowledge of modulators of stone formation.

TFF1 is generally known as an estrogen-inducible gene product with 60 amino residues (39–41). It belongs to the trefoil factor family of proteins, is expressed predominately in gastric mucosa, and is synthesized by mucosal epithelial cells (42, 43). It has antiapoptotic and motogenic activities, and its main functions...
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Characterization of the inhibitory activity of urinary TFF1 against CaOx crystal growth. (A) The CaOx crystal growth–inhibitory activity of urinary TFF1 was similar to that of nephrocalcin when an equal amount of protein was used and more potent than that of Tamm-Horsfall protein, even though a smaller amount of TFF1 was used. Lysozyme was used as a negative control. (B) The inhibitory activity of urinary TFF1 was dose dependent. (C) The inhibitory activity of recombinant TFF1 was also dose dependent and comparable with that of urinary TFF1. With an equal amount of protein used, the inhibitory activity of recombinant TFF3 was significantly less (approximately 2-fold) than that of recombinant or urinary TFF1. (D) Incubation of urinary TFF1 with an anti-C-terminal TFF1 antibody significantly reduced the CaOx crystal growth–inhibitory activity of TFF1. The neutralizing effect of anti-C-terminal TFF1 antibody was more potent than that of anti-N-terminal TFF1 antibody. These data indicate that the C terminus of TFF1, which contains multiple glutamic acid residues, is particularly important in mediating the CaOx crystal growth–inhibitory function of TFF1. *P < 0.001. n = 4 per sample.

Methods

The present study was approved by the Institutional Ethical Committee of Faculty of Medicine Siriraj Hospital, Mahidol University, and by the Ethical Review Committee at Ministry of Public Health, Thailand. Methodologies employed in the present study included DEAE anionic adsorption, gel filtration using HiLoad 16/60 Superdex 75 (Amersham Biosciences), Resource Q anion exchange chromatography with linear-gradient salt elution (Amersham Biosciences), CaOx crystal growth–inhibitory assay, MALDI-TOF MS, ESI-Q-TOF MS/MS, Western blot analysis, and ELISA. The schema of these analytical procedures is shown in Figure 1.

Subjects Urine samples were collected from 30 normal healthy individuals who had not recently taken medication (14 males and 16 females, aged 26.1 ± 2.9 years) and 23 idiopathic CaOx kidney stone patients (12 males and 11 females, aged 45.3 ± 10.7 years). Eighteen patients had new-onset
TFF1 levels in all donors (50 ml each) collected from 4 normal healthy individuals. To measure urinary for 30 minutes. The slurry was filtered through a sintered glass filter, and with a HiLoad 16/60 Superdex 75 gel filtration column (Amersham Biosciences) was used. A small volume of urine samples identity with Western blot analysis, we used a larger volume of urine samples (5 l each) collected from 2 normal healthy anionic proteins with inhibitory activity against CaOx crystal growth, we used the cake was washed with 0.01 M Tris-HCl in 0.05 M NaCl (pH 7.3) until the running buffer. The flow rate was 1 ml/min, and absorbance of the eluate was monitored at λ214 nm. The resultant fractions were examined for activity in the CaOx crystal growth–inhibitory assay. Eight consecutive fractions with the lowest OD at λ214 nm — but with the highest inhibitory activity against CaOx crystal growth — were combined and termed fraction X. After dialysis with dH2O, fraction X was lyophilized to reduce its volume.

To validate the initial mass spectrometric data and to confirm the protein identity with Western blot analysis, we used a smaller volume of urine samples (50 ml each) collected from 4 normal healthy individuals. To measure urinary TFF1 levels in all donors (n = 53) by ELISA, only 10 ml urine from each donor was used. The larger volume was processed in the initial screening because we wished to examine all anionic proteins with inhibitory activity against CaOx crystal growth, including low abundance components. Cell debris and particulate matter were removed from the urine samples by 1,000 g centrifugation at 4 °C for 30 minutes, and the supernatant was recovered.

DEAE cellulose anionic adsorption (isolation of anionic proteins). Because all known urinary proteins that inhibit CaOx crystal growth are anionic (perhaps to bind with Ca2+), we isolated anionic proteins using DEAE adsorption. The urine was diluted 4 times with deionized (dH2O) water, and its pH was adjusted to 7.3. DEAE cellulose (DE-52; Whatman), which had been equilibrated with a buffer containing 0.01 M Tris-HCl and 0.05 M NaCl (pH 7.3), was added into the urine and stirred at room temperature for 30 minutes. The slurry was filtered through a sintered glass filter, and the cake was washed with 0.01 M Tris-HCl in 0.05 M NaCl (pH 7.3) until the filtrate became colorless. The adsorbed proteins were then eluted with 1 1 of 0.01 M Tris-HCl in 0.6 M NaCl (pH 7.3). Thereafter, the eluate was dialyzed against 12 l of dH2O water for 24 hours twice and then lyophilized to reduce the sample volume and to concentrate the proteins.

Gel filtration using HiLoad 16/60 Superdex 75 Column (size separation). After DEAE adsorption, size separation of the anionic proteins was performed with a HiLoad 16/60 Superdex 75 gel filtration column (Amersham Biosciences) using a buffer containing 0.15 M NaCl and 0.01 M Tris-HCl (pH 7.3) as a running buffer. The flow rate was 1 ml/min, and absorbance of the eluate was monitored at λ214 nm. The resultant fractions were examined for activity in the CaOx crystal growth–inhibitory assay. Eight consecutive fractions with the lowest OD at λ214 nm — but with the highest inhibitory activity against CaOx crystal growth — were combined and termed fraction X. After dialysis with dH2O, fraction X was lyophilized to reduce its volume.

Figure 8
Kinetics of the CaOx crystal growth–inhibitory activity of urinary TFF1 at physiological levels. The inhibitory activities of urinary TFF1 at concentrations of 7, 70, and 700 ng/ml were evaluated to address whether urinary TFF1 at physiological levels could inhibit CaOx crystal growth. The inhibitory assay was performed for a longer duration (up to 1 hour) to simulate the long-term effect of interaction between crystals and low protein concentration during the normal physiological state. Data are presented as the rate of free oxalate depletion. Significant inhibition of crystal growth by a physiological concentration of TFF1 (7 ng/ml) was observed from 10 minutes of incubation through the end of the assay (1 hour). Additionally, there were significant differences among the 3 concentrations of urinary TFF1 tested. n = 3 per group.

Table 1
Clinical data of patients with idiopathic CaOx kidney stone

<table>
<thead>
<tr>
<th>Patients’ characteristics</th>
<th>No. patients (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (average, 45.3 ± 10.7 yr; range, 25–67 yr)</td>
<td></td>
</tr>
<tr>
<td>&lt;30 yr</td>
<td>1 (4.35%)</td>
</tr>
<tr>
<td>31–40 yr</td>
<td>8 (34.78%)</td>
</tr>
<tr>
<td>41–50 yr</td>
<td>8 (34.78%)</td>
</tr>
<tr>
<td>51–60 yr</td>
<td>5 (21.74%)</td>
</tr>
<tr>
<td>&gt;60 yr</td>
<td>1 (4.35%)</td>
</tr>
<tr>
<td>Gender (male/female ratio, 1.09)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (52.17%)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (47.83%)</td>
</tr>
<tr>
<td>No. kidney stones per patient (average, 5.70 ± 3.97; range, 1–10)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5 (21.74%)</td>
</tr>
<tr>
<td>2</td>
<td>4 (17.39%)</td>
</tr>
<tr>
<td>3–5</td>
<td>2 (8.70%)</td>
</tr>
<tr>
<td>&gt;5</td>
<td>12 (52.17%)</td>
</tr>
<tr>
<td>Stone type*</td>
<td></td>
</tr>
<tr>
<td>CaOx monohydrate</td>
<td>18 (78.26%)</td>
</tr>
<tr>
<td>CaOx dihydrate</td>
<td>5 (21.74%)</td>
</tr>
<tr>
<td>No. stone events (frequency)</td>
<td></td>
</tr>
<tr>
<td>1 episode (new onset)</td>
<td>18 (78.26%)</td>
</tr>
<tr>
<td>2 episodes (recurrent)</td>
<td>5 (21.74%)</td>
</tr>
<tr>
<td>≥2 episodes (multiple recurrent)</td>
<td>0 (0.00%)</td>
</tr>
<tr>
<td>Stone size* (average diameter, 2.87 ± 1.11 cm; range, 1.0–5.0 cm)</td>
<td></td>
</tr>
<tr>
<td>&lt;2.0 cm</td>
<td>3 (13.04%)</td>
</tr>
<tr>
<td>2.0–4.0 cm</td>
<td>17 (73.92%)</td>
</tr>
<tr>
<td>&gt;4.0 cm</td>
<td>3 (13.04%)</td>
</tr>
</tbody>
</table>

*The chemical compositions of stones were analyzed by fourier transform infrared spectrometry. Only patients with CaOx stones (either monohydrate or dihydrate form) were included. Patients with secondary causes of CaOx kidney stone or systemic disorders (e.g., primary hyperparathyroidism, sarcoidosis, vitamin D excess, hyperthyroidism, and renal tubular acidosis) and those who had taken diuretics were excluded. Staghorn renal calculi and stones containing uric acid, struvite, cystine, or over 50% calcium phosphate were ruled out. No donor had a recent urinary tract infection.
Anion exchange chromatography using Resource Q column with linear-gradient salt elution (charge separation). Fraction X was purified by anion exchange chromatography using a Resource Q column (Amersham Biosciences). The column was run in 0.02 M Tris-HCl (pH 8.0) at a flow rate of 1 ml/min, and the bound proteins were eluted with a 0–0.5 M NaCl linear gradient. All of the resultant subfractions were tested for activity in the CaOx crystal growth–inhibitory assay, and the 2 subfractions with potent inhibitory activity against CaOx crystal growth (subfractions X1 and X2) were collected. The protein in subfractions X1 and X2 was separated by SDS-PAGE and identified by MS. Salts were removed from the purified protein prior to analysis; the sample was dialyzed with dI water and lyophilized to reduce the volume.

SDS-PAGE and visualization of protein bands. Aliquots of the concentrated subfractions X1 and X2 were mixed with Laemmli sample buffer and heated at 95°C for 10 minutes. The samples (30 µg per lane) were then resolved in 0.75-mm-thick, 12% SDS-polyacrylamide gel using a vertical electrophoresis apparatus (ATTO Corp.). Proteins were then visualized by staining with Coomassie Brilliant Blue R-250 (Fluka).

Protein identification by MALDI-TOF MS and ESI-Q-TOF MS/MS. Visualized protein bands were excised and subjected to in-gel tryptic digestion and identification by both MALDI-TOF MS and ESI-Q-TOF MS/MS (Imperial College London). Peptide matching was performed using the MASCOT search engine (http://www.matrixscience.com) assuming that peptides were monoisotopic, carbamidomethylated at cysteine residues, and oxidized at methionine residues. A mass tolerance was 120 parts per million, and only 1 maximal cleavage was allowed for peptide matching. Probability-based MOWSE (MOlecular Weight SEarch) score was calculated using the formula $[-10 \log(P)]$, where $P$ was the probability that the observed match was a random event. Peptide matching scores ≥75 (for the MALDI data) and individual ion scores ≥50 (for the Q-TOF or MS/MS data) were considered significant hits.

Anti-TFF1 antibodies. Murine monoclonal anti-TFF1 antibody was raised in the laboratory of F.E.B. May and used for validation of expression of TFF1 by Western blotting. For characterization of the inhibitory activity of urinary TFF1 against CaOx crystal growth, anti-N-terminal TFF1-specific (catalog no. sc-22501; Santa Cruz Biotechnology Inc.) and anti-C-terminal TFF1-specific (catalog no. sc-22502; Santa Cruz Biotechnology Inc.) goat polyclonal antibodies were used.

Western blotting. After SDS-PAGE, separated proteins were transferred onto nitrocellulose membrane using a semi-dry transfer apparatus (Amersham Biosciences) at 20 V for 30 minutes. Nonspecific binding was blocked with 5% milk in PBS at room temperature for 1 hour. The membranes were then incubated either with mouse monoclonal anti-TFF1, rabbit polyclonal anti-nephrocalcin (University of Chicago Pritzker School of Medicine), sheep polyclonal anti-Tamm-Horsfall (catalog no. AB733; Chemicon International), rat monoclonal anti-uropontin (catalog number 31831; BD Biosciences), or mouse monoclonal anti-lgA (catalog number 70105; BD Biosciences) antibodies.

Figure 9
Differential urinary levels of TFF1 in CaOx stone formers compared with normal healthy controls. (A) Urinary TFF1 concentrations were measured with ELISA using equal amounts (40 µg) of the total anionic proteins isolated from 10 ml urine of each donor. Urinary concentrations of TFF1 in patients with idiopathic CaOx kidney stone (2.66 ± 0.46 ng/ml; n = 23, 12 males and 11 females) were significantly lower than those in normal healthy individuals (6.67 ± 0.93 ng/ml; n = 30, 14 males and 16 females). (B) After normalization with urine Cr, the relative amounts of TFF1 in the urine of CaOx stone patients remained significantly less (3.24 ± 0.62 versus 17.19 ± 3.49 ng/mg Cr). (C) This difference remained highly significant after normalization with total protein (11.08 ± 3.21 versus 247.27 ± 53.68 ng/mg total protein). *P < 0.0001.

Figure 10
The proposed model of Ca2+-binding site in the TFF1 molecules. Based on the functional data in Figure 7D, and because the C terminus contains multiple glutamic residues that are negatively charged, we therefore hypothesize that this area is particularly important in mediating the CaOx crystal growth–inhibitory function of TFF1. Because dimerization usually occurs in the native form of TFF1 (Cys58–Cys58) (30), we also propose that dimerization of TFF1 may facilitate entrapment of Ca2+ ions in this area.