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Inhibition of calcium oxalate crystal growth *in vitro* by uropontin: Another member of the aspartic acid-rich protein superfamily

(biomineralization/osteopontin)

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ABSTRACT **The majority of human urinary stones are primarily composed of calcium salts. Although normal urine is frequently supersaturated with respect to calcium oxalate, most humans do not form stones. Inhibitors are among the multiple factors that may influence the complex process of urinary stone formation. We have isolated an inhibitor of calcium oxalate crystal growth from human urine by monoclonal antibody immunoaffinity chromatography. The N-terminal amino acid sequence and acidic amino acid content of this aspartic acid-rich protein, uropontin, are similar to those of other pontin proteins from bone, plasma, breast milk, and cells. The inhibitory effect of uropontin on calcium oxalate crystal growth *in vitro* supports the concept that pontins may have a regulatory role. This function would be analogous to that of other members of the aspartic acid-rich protein superfamily, which stereospecifically regulate the mineralization fronts of calcium-containing crystals.**

Urinary tract stone disease is a common human malady, and the vast majority of stones formed in the urinary space are mineralized with calcium salts (1, 2). The elements contained in urine also provide a potential model system for evaluating the biologic control of mineralization in other body fluids. Although normal urine is frequently supersaturated with respect to calcium oxalate, most humans do not form stones. Urinary stone formation is a complex process involving multiple factors, and the precise role of the inhibitors that are present within urine is uncertain. The majority of the inhibition of crystal growth observed in normal urine is due to the presence of protein macromolecules rather than to the presence of lower molecular weight molecules (3). We approached the problem of identifying other crystal inhibitor proteins by preparing monoclonal antibodies from rats immunized with the main inhibitory peak of human urine protein (3). One of these monoclonal antibodies was used to purify an inhibitor of calcium oxalate crystal growth from human urine by immunoaffinity chromatography.

METHODS

Protein Purification. Human urine samples were carried through all procedures in the presence of 0.02% sodium azide and two protease inhibitors, 0.5 mM phenylmethanesulfonyl fluoride and 1.0 mM *N*-ethylmaleimide, and were partially depleted of the most abundant protein in normal urine, Tamm–Horsfall protein (TH), by salt precipitation followed by centrifugation at 5000 × *g* for 30 min (4). TH-depleted urine was adsorbed to DEAE-cellulose, batch eluted, and fractionated by DEAE-cellulose column chromatography, using a 0.1–0.4 M NaCl linear gradient in Tris buffer (3). The TH depletion step was performed since we found that TH is present within the main inhibitory peak from DEAE-cellulose and does not inhibit crystal growth in the assay used in the present study (5). Inhibitory activity of fractions was assayed by measuring the inhibition of incorporation of [^{14}C]oxalate (Amersham) into calcium oxalate monohydrate seed crystals from a metastable calcium oxalate solution (6), using a final incubation volume of 2.2 ml and an incubation period of 180 min. The percent residual radioactivity in 200 μl of the centrifuged supernatant was used to calculate the inhibitory activity (IA) of samples by the formula IA [units/ml] = 5 × (percent residual radioactivity of sample − percent residual radioactivity of standard)/(100 − percent residual radioactivity of sample).

Monoclonal Antibody. Hybridoma cells were derived from fusions of Sp2/0-Ag myeloma cells with cells from inbred Lewis rats immunized with the main inhibitory peak from DEAE-cellulose chromatography (3). Hybridomas were selected for subcloning by limiting dilution on the basis of differential reactivity in ELISA and used to produce monoclonal antibodies to an inhibitory protein and to TH in *nude* mice (7). ELISAs were performed as previously described (8), using microtiter plates coated with antigens and a 0.01 M Tris·HCl/0.154 M NaCl/0.5% casein, pH 7.6, blocking buffer. SDS/PAGE was performed by the slab technique (9). Proteins were transferred to 0.2-μm nitrocellulose membranes for Western blotting (10). Solid-phase immunoabsorbents were prepared by coupling IgG fractions of antibodies to cyanogen bromide-activated Sepharose 4B (5 mg of protein per ml of beads). After exposure to portions of inhibitory fractions of human urine, the monoclonal antibody beads were extensively washed with phosphate-buffered saline, pH 7.4, and proteins were eluted with a 0.2 M glycine pH 2.8 buffer. Eluates were neutralized and dialyzed against a 0.05 M Tris·HCl/0.05 M NaCl, pH 7.3, buffer prior to characterization of IA and immunologic reactivity. The protein isolated by immunoaffinity chromatography was further purified by reverse-phase HPLC prior to analysis of amino acid composition and N-terminal sequence (11).

RESULTS AND DISCUSSION

Isolation of an Inhibitory Protein by Immunoaffinity Purification. Hybridomas derived from the fusion of cells from

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Abbreviations: TH, Tamm–Horsfall protein; AARP, aspartic acid-rich protein; IA, inhibitory activity.

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rats immunized with the main inhibitory peak of human urine (3) were screened by ELISA. Two of 42 initial supernatants of hybridomas had greater reactivity with the main inhibitory peak than with TH by ELISA, and these hybridomas were selected for subcloning. The monoclonal antibodies produced by one subclone, ZH2, were studied in greatest detail because the supernatant of this clone reacted strongly with the inhibitory protein peak but not with TH. Analysis of the fractions obtained by DEAE-cellulose column chromatography and by acid elution from monoclonal antibody immunoaffinity columns demonstrated that the greatest immunoreactivity with antibody ZH2 was present in the same tubes as those with the greatest inhibitory activity (Fig. 1). Absorption of aliquots of the inhibitory protein peak (tubes 70–90 in Fig. 1*A*) with 200 μl of monoclonal antibody beads decreased the ELISA reactivity detected by ZH2, but not by anti-TH antibody, in the supernatants of ZH2 beads. Similar absorptions using anti-TH beads and normal rat IgG beads did not decrease reactivity detected by ZH2, but they did eliminate anti-TH reactivity in the supernatant absorbed with anti-TH beads. Acid eluates from the antibody beads showed reactivity by ELISA with their respective antibodies (Fig. 2 *A* and *B*). IA was detected only in the eluate from ZH2 beads (Fig. 2*C*).

Affinity columns of ZH2 beads were used for inhibitory protein purification based on the above results. The elution patterns for IA and ELISA reactivity with ZH2 of these larger columns were essentially identical to those shown in Fig. 1*B*. Approximately 30% of the protein in 3- to 9-liter lots of

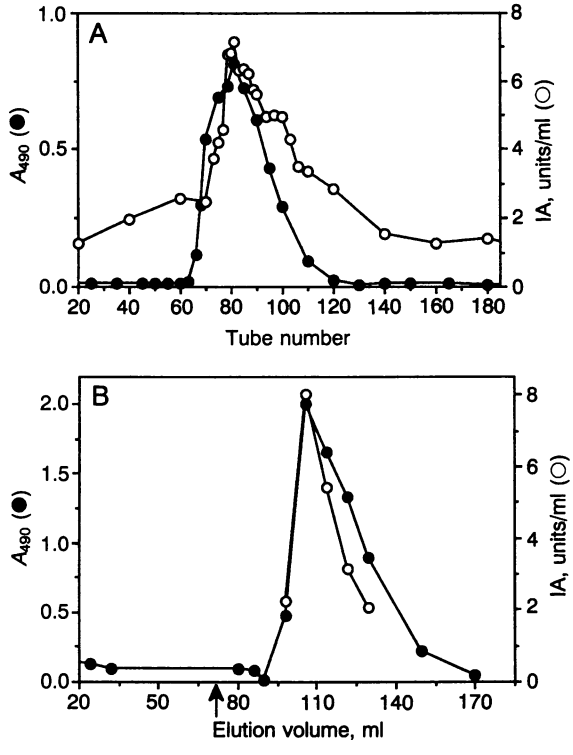

FIG. 1. (*A*) Typical DEAE-cellulose chromatogram of salt gradient elution (0.1–0.4 M NaCl) of the DEAE-cellulose batch eluate (3) obtained from normal human urine partially depleted of TH by salt precipitation (4). The greatest ELISA reactivity detected with monoclonal antibody ZH2 (●) using microtiter plates coated with column fractions coincided with the main inhibitory peak (○) identified by functional assay of [^{14}C]oxalate incorporation into seed crystals (6). (*B*) Elution of ELISA reactivity to ZH2 (●) and crystal growth IA (○) from an affinity column of monoclonal antibody ZH2 beads that had been exposed to an aliquot of the main inhibitory peak and then extensively washed with phosphate-buffered saline, pH 7.4. Elution with a 0.2 M glycine pH 2.8 buffer was started at the arrow.

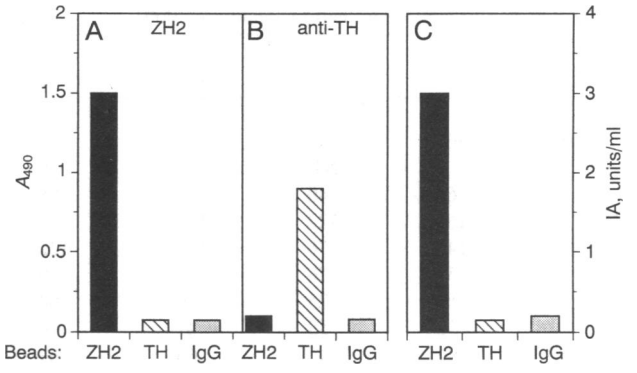

FIG. 2. (*A*) ELISA reactivity detected by monoclonal antibody ZH2 in acid eluates from monoclonal ZH2 beads, anti-TH beads, and normal IgG beads after equivalent exposure to aliquots of the main inhibitory peak. (*B*) ELISA reactivity detected by monoclonal anti-TH in the same eluates. (*C*) Crystal growth IA in the same eluates as in *A* and *B*.

TH-depleted urine (n = 4) was isolated by 0.4 M NaCl elution after batch adsorption to DEAE-cellulose. Approximately 4% of the protein in DEAE batch eluates applied to ZH2 affinity columns was recovered in acid eluates. The specific activity (IA units/mg) of ZH2 eluates was 3.3- to 11-fold greater than that of corresponding DEAE eluates (n = 4).

Characterization of the Inhibitory Protein. The comparison of the N-terminal sequence of our protein with protein sequences from the literature and from the Swissprot data base (Fig. 3) revealed identity with human osteopontin (12) and lactopontin (18) and extensive homology with rat (13) and porcine (17) osteopontins and with other pontin proteins from mouse cells (14–16). To denote its source, we refer to our urine protein as uropontin.

The amino acid composition determined for uropontin (Table 1) includes a very high percentage of aspartic residues and corresponds well to the distribution of amino acids in human osteopontin (12, 20). Uropontin is quite distinct from nephrocalcin, another protein inhibitor of urine crystal

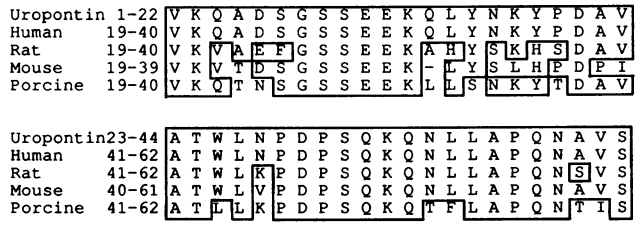

FIG. 3. Comparison of the aligned N-terminal amino acid sequences of human uropontin isolated by immunoaffinity chromatography in the present study, human osteopontin (12), rat osteopontin (13), mouse (osteo)pontins from skin, macrophages, and thymocytes (14–16), and porcine osteopontin (17) derived from cDNA sequences. Conserved amino acids in this sequence are indicated by enclosure. The amino acid sequences of the four osteopontins shown start with position 19 of the precursors and extend to residue 62 (residue 61 for mouse). With the exception of an indeterminate residue 25, the entire N-terminal sequence from residue 2 to residue 30 of uropontin isolated from a second individual was identical to that shown. The last five of the seven amino acids in the N terminus of human lactopontin (18) are identical to the first five amino acids of uropontin. The last four amino acids (Asn-Ala-Val-Ser) of the uropontin sequence are deleted in one of the isoforms encoded by mRNA from human bone, decidua, and kidney (ref. 19; J.R.H., unpublished observations).§§

§§The nucleotide sequence(s) referred to in this paper as unpublished observations (J.R.H.) has been deposited in the GenBank data base (accession no. M83248).

Table 1. Amino acid composition, residues/1000

Amino acid	Uropontin*	Osteopontin†	Osteopontin (cDNA)‡	Nephrocalcin§
Cys	0	ND	0	17
Asn + Asp	207	223	201	106
Met	7	ND	13	6
Thr	66	40	47	88
Ser	162	122	141	97
Glu + Gln	140	176	138	122
Pro	55	76	50	59
Gly	69	25	20	108
Ala	52	55	47	76
Val	46	42	60	64
Ile	15	23	23	24
Leu	47	60	54	63
Tyr	26	9	27	10
Phe	16	8	23	31
His	36	46	54	20
Lys	39	64	64	36
Arg	17	28	30	41
Trp	ND	ND	7	9
Gla¶	0	ND	ND	20

ND, not determined.
*Uropontin isolated from human urine.
†Osteopontin isolated from human bone (20).
‡Human osteopontin predicted from cDNA for a mature protein sequence of 298 amino acids. This sequence predicts that 164 of the 201 (Asp + Asn) residues/1000 are aspartic acid and that 103 of the 138 (Glu + Gln) residues/1000 are glutamic acid (12).
§Nephrocalcin isolated from human urine (3).
¶γ-Carboxyglutamic acid. In contrast with uropontin, other proteins also present in the main inhibitory peak contained both γ-carboxyglutamic acid and β-hydroxyasparagine (data not shown).

growth. Their amino acid compositions and molecular weights differ substantially (3), and none of the glutamic residues in uropontin are γ-carboxylated as they are in nephrocalcin, osteocalcin, and other vitamin K-dependent proteins (11). The amino acid and nucleotide sequences of nephrocalcin are not yet known. Furthermore, in contrast to TH and coagulation proteins with calcium-binding epidermal growth factor-like domains (11), none of the aspartic and asparagine residues in uropontin were β-hydroxylated, a finding consistent with the lack of epidermal growth factor-like domains in pontin sequences. The relative abundance and role of individual proteins in urinary stones is not yet known. However, the overall amino acid compositions of proteins extracted from calcium oxalate stones demonstrate a striking preponderance of acidic amino acids and more closely resemble uropontin than nephrocalcin (21–23), and preliminary studies indicate that uropontin is a major component of the matrix of calcium oxalate monohydrate stones (J.R.H., unpublished observations).

The migration of uropontin on SDS/PAGE varied according to the gel composition. The major band detected by silver staining and in Western blots migrated at M_r ≈50,000 on 16% gels (Fig. 4) and at M_r ≈72,000 in 5–18% gradient gels. This unusual pattern of migration is very similar to the behavior reported previously for rat osteopontin (24, 25). Human osteopontin migrates at M_r ≈80,000 on 4–20% gels (20), while lactopontin migrates at M_r ≈75,000 in 10% gels (18). The peptide cores of mature rat osteopontin and two isoforms of human pontin proteins predicted from cDNA sequences have M_r values of ≈35,000 (13) and ≈33,500 and ≈32,000 (refs. 12 and 19; J.R.H., unpublished observations), respectively. Although the differences in migration of these human proteins isolated from different sources may reflect differences in the extent of phosphorylation and/or glycosylation of isoforms (26, 27), they may also be due to alternative RNA splicing. This latter possibility is supported by heterogeneity

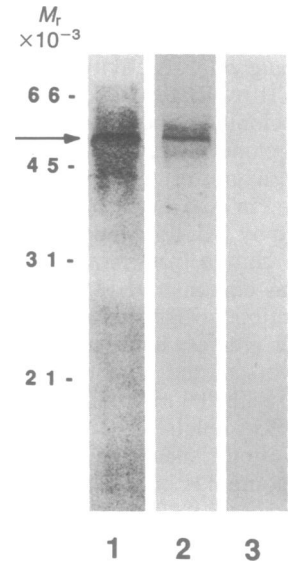

FIG. 4. SDS/16% polyacrylamide gel electrophoresis of uropontin purified by immunoaffinity chromatography using ZH2 beads. The position of migration of molecular weight markers is shown on the left. Lanes 1 and 2 contain 6-μg samples of uropontin per lane and lane 3 contains 6 μg of DEAE-cellulose batch eluate. Lane 1 was stained with silver. Lanes 2 and 3 are Western blots (10) that used monoclonal antibody ZH2 for detection.

of mRNA transcripts detected in kidney, bone, and decidua (ref. 19; J.R.H., unpublished observations). Exon 4 of the mouse osteopontin gene encodes 14 amino acids, (28) a sequence that is highly conserved across species and deleted in one of the isoforms encoded by cDNAs from human kidney (J.R.H., unpublished observations), bone, and decidua (19). While all tissue sources of uropontin are not known, renal synthesis of uropontin is suggested by the demonstration of transcripts on Northern hybridization, by *in situ* hybridization of the kidneys of rats and mice using labeled probes encoding osteopontin (29, 30), and by immunochemical detection of osteopontin secreted from renal cells in culture (26, 27).

Aspartic acid-rich proteins (AARP) constitute a superfamily of proteins that are closely associated with mineralization events in a broad range of organisms and tissues (29–44). Several studies have shown that osteopontin is present during events surrounding bone mineralization (29–33) and the production of calcified matrix within the inner ear (34, 35), and potential roles as a regulator of mineralization have been previously discussed. However, to our knowledge, uropontin is the first pontin protein shown to be an inhibitor of crystal growth by *in vitro* studies. It is inhibitory at the concentration (≈0.1 μM) present in normal human urine (J.R.H., unpublished observations). This concentration is too low for inhibition to be on the basis of chelation of calcium. Thus, it seems likely that uropontin inhibits crystal growth by interacting with crystals, although the precise nature of the interaction has not been defined. Very potent inhibition of crystal growth by AARPs appears to require specific structural conformations that produce a novel pattern of charge density. The affinity of calcium binding *per se* does not seem to predict inhibitory activity (45, 46).

An insight into the potential function of uropontin as well as other pontins is provided by a series of *in vitro* studies demonstrating the modulation of crystal growth by AARPs from other mineralizing tissues. Solutions of an aspartic acid-rich matrix protein from mollusk shells, for example, interact specifically with selected faces on calcium dicarboxylic acid crystals to promote the growth of these faces, while simultaneously slowing the overall rate of crystal growth (40).

Synthetic poly(aspartic acid) has an effect very similar to that of the aspartic acid-rich protein, while poly(glutamic acid) has only a weak nonspecific effect on crystallization (40).

The growth of calcium carbonate (calcite) crystals is also inhibited nonspecifically by solutions of this mollusk protein at concentrations above 0.5 μg/ml (40). When the protein is adsorbed onto a solid substrate, however, this AARP provides a template that regularly induces calcite crystal formation in a specific orientation characteristic of biological mineralization. This orientation is rarely demonstrable in the absence of the protein (40). Blocking the carboxylate groups of this adsorbed protein suppresses this orientation of calcite nucleation (41). Substitution of a less acidic AARP from the sea urchin for the mollusk protein causes biologically relevant differences in the internal texture of the calcite, and their presence alters the fracture properties of the crystals (42). The AARPs of dentin similarly exert an overall inhibition of crystallization in the fluid phase (43). Conversely, when immobilized on agarose beads, these dentin proteins provide a template for ordered crystal formation in solutions with mineral concentrations below those that support spontaneous crystal growth (44). Other studies using synthetic poly(aspartic acids) further support this concept. Solutions containing this synthetic peptide inhibit crystal growth, while the immobilized peptide promotes new crystallization (47).

It seems likely that the vitality of multicellular organisms is critically dependent on their ability to regulate mineralization within their fluid spaces that are supersaturated with respect to their mineral constituents. On the basis of our *in vitro* studies of uropontin, we suggest that the ubiquitous family of pontin proteins may contribute to this essential process. The responsiveness of pontins to vitamin D and parathyroid hormone (29, 48–50), as well as the multiplicity of forms resulting from post-translational modifications (26, 27, 32), favor a substantive regulatory role for this family of proteins. The presence of aspartic acid-rich sequences and of the functional -Arg-Gly-Asp- cell-binding sequence in osteopontin may facilitate interactions between cells and mineralized matrix (13, 51). We suggest that AARPs, such as the pontins, acting in concert with other proteins, such as nephrocalcin and osteocalcin, may exert either restrictive or permissive effects on the formation or remodeling of crystal surfaces in a variety of body fluids. Such protein signals for mineralization are based on the conformation and processivity of these proteins, and they may determine the characteristics of the rigid structures that are formed.

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