Analysis of Uromodulin Polymerization Provides New Insights into the Mechanisms Regulating ZP Domain-mediated Protein Assembly

Céline Schaeffer,* Sara Santambrogio,*[†] Simone Perucca,* Giorgio Casari,[‡] and Luca Rampoldi*

*Dulbecco Telethon Institute, Molecular Genetics of Renal Disorders, San Raffaele Scientific Institute, 20132 Milan, Italy; and [‡]Human Molecular Genetics, San Raffaele Scientific Institute, 20132 Milan, Italy

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Uromodulin is the most abundant protein secreted in urine, in which it is found as a high-molecular-weight polymer. Polymerization occurs via its zona pellucida (ZP) domain, a conserved module shared by many extracellular eukaryotic proteins that are able to assemble into matrices. In this work, we identified two motifs in uromodulin, mapping in the linker region of the ZP domain and in between protein cleavage and glycosylphosphatidylinositol (GPI)-anchoring sites, which regulate its polymerization. Indeed, mutations in either module led to premature intracellular polymerization of a soluble uromodulin isoform, demonstrating the inhibitory role of these motifs for ZP domain-mediated protein assembly. Proteolytic cleavage separating the external motif from the mature monomer is necessary to release the inhibitory function and allow protein polymerization. Moreover, we report absent or abnormal assembly into filaments of GPI-anchored uromodulin mutated in either the internal or the external motif. This effect is due to altered processing on the plasma membrane, demonstrating that the presence of the two modules has not only an inhibitory function but also can positively regulate protein polymerization. Our data expand previous knowledge on the control of ZP domain function and suggest a common mechanism regulating polymerization of ZP domain proteins.

INTRODUCTION

Uromodulin, also known as Tamm-Horsfall protein, is a large glycoprotein of ~105 kDa that is exclusively expressed in the thick ascending limb (TAL) of Henle's loop and the early distal convoluted tubule (DCT) of the kidney. It is a glycosylphosphatidylinositol (GPI)-anchored protein mainly localized at the apical plasma membrane of epithelial tubular cells (Bachmann *et al.*, 1985), from which it is released into the tubular lumen through a proteolytic cleavage by a yet to be identified protease (Santambrogio *et al.*, 2008). Uromodulin constitutes the most abundant protein in urine, in which it is found as a high-molecular-weight polymer (M_r 1–10 $\times$ 10⁶) (Serafini-Cessi *et al.*, 2003).

Although uromodulin was discovered in 1950 (Tamm and Horsfall, 1950), its biological function is still not well defined. Based on the phenotype in *umod*^{-/-} mice, it has been proposed to have a protective role against urinary tract infections and calcium oxalate crystals damage (Bates *et al.*, 2004; Mo *et al.*, 2004). Due to its ability to assemble into a meshwork of fibers forming a gel-like structure, uromodulin

has been hypothesized to have also a role in water/salt balance in the TAL and DCT (Wiggins, 1987; Matthey and Naftalin, 1992).

Electron microscopy (EM) studies on purified urinary uromodulin showed that it forms a three-dimensional matrix with pores. This matrix is formed by long filaments having a width of ~100 Å and measuring 2,500 to 40,000 Å in length, with an average of 25,000 Å (Porter and Tamm, 1955). High-resolution EM imaging shows fibrils with frequent occurrence of "zig-zag" configuration suggestive of a three-dimensional double helical structure. The same analysis of a uromodulin C-terminal fragment, essentially consisting of the ZP domain, confirmed these observations and strongly suggests that the ZP domain is responsible for protein polymerization (Jovine *et al.*, 2002).

The ZP domain is shared by many extracellular eukaryotic proteins, including zona pellucida sperm receptors ZP1, ZP2, and ZP3, tectorial membrane components α - and β -tectorin or transforming growth factor- β receptor type III (TGF- β R3) (Jovine *et al.*, 2005; Wassarman, 2008). ZP domain proteins play diverse functions and are found in various organisms ranging from jellyfish to mammals. The ZP domain consists of ~260 amino acids with poor sequence conservation, mainly restricted to either eight or 10 invariant cysteines, and predicted to have a high β -strand content. It has a bipartite structure with ZP-N and ZP-C subdomains separated by a linker region (Llorca *et al.*, 2007). The ZP-N subdomain was shown to be sufficient for polymerization (Jovine *et al.*, 2006). The ZP domain is located at the C terminus of the polypeptide, generally near protein membrane-anchoring (Bork and Sander, 1992; Wassarman, 2008). As uromodulin, the majority of ZP domain proteins

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[†] Present address: Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, 20132 Milan, Italy.

Address correspondence to: Luca Rampoldi (rampoldi.luca@hsr.it).

Abbreviations used: DMBT1, Deleted in Malignant Brain Tumors 1; GPI, glycosylphosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; PFA, paraformaldehyde.

are targeted to the plasma membrane and secreted via a proteolytic cleavage that allows their extracellular assembly into filaments or matrices. Previous studies on ZP3 protein polymerization identified two conserved short hydrophobic motifs in its sequence, one motif internal to the ZP domain (internal hydrophobic patch, IHP) (Jovine *et al.*, 2004) and the other motif localized between the cleavage site and the transmembrane domain (external hydrophobic patch, EHP) (Zhao *et al.*, 2003; Jovine *et al.*, 2004). The presence of intact IHP and EHP motifs in full-length ZP3 is necessary for protein assembly into zona pellucida, whereas it is required for protein secretion in a ZP3 isoform lacking a transmembrane domain. Based on these findings, it was hypothesized that the two motifs might function by keeping the domain in an "inactive" conformation, possibly by direct interaction, and that the ZP domain would reach an "active" state allowing polymerization after release of the EHP through proteolytic cleavage (Jovine *et al.*, 2004).

So far, uromodulin assembly into filaments has been studied through electron microscopy only (Porter and Tamm, 1955; Delain *et al.*, 1980; Wiggins, 1987; Jovine *et al.*, 2002). In this work, we identified molecular mechanisms that regulate uromodulin assembly into filaments. Our data expand previous knowledge on the control of ZP domain function and suggest a common mechanism regulating polymerization of ZP domain proteins.

MATERIALS AND METHODS

Protein Sequence Analysis

Sequence alignments were obtained by using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) (Thompson *et al.*, 2000).

Secondary structure prediction was carried out by using PSIPred (<http://bioinf.cs.ucl.ac.uk/psipred/psiform.html>) (McGuffin *et al.*, 2000), APSSP2 (<http://www.imtech.res.in/raghava/apssp2/>) (Raghava, 2002), Jpred (<http://www.compbio.dundee.ac.uk/~www-jpred/>) (Cole *et al.*, 2008), Sspro (<http://scratch.proteomics.ics.uci.edu/>) (Pollastri *et al.*, 2002; Cheng *et al.*, 2005), and Porter (<http://distill.ucd.ie/~porter/>) (Pollastri and McLysaght, 2005). Consensus sequences were calculated by using CONSENSUS (<http://coot.embl.de/Alignment/consensus.html>).

Uromodulin Expression Constructs

Wild-type uromodulin cDNA was cloned in HindIII and EcoRI sites of pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) as described previously (Bernascone *et al.*, 2006). Hemagglutinin (HA)-tagged uromodulin construct (pcDNA_HA_UMOD) was generated by creating a new AgeI site at position 78 in the uromodulin cDNA. HA tag was produced by phosphorylating and annealing the two primers: HA_F 5'-CCGGTTACCCATACGATGTTCCAGATTACGCTA-3' and HA_R 5'-CCGGTAGCGTAATCTGGAACATCGTATGGGTAA-3' and inserted in the AgeI site. The tag was inserted after the leader peptide, in between T26 and S27 in the protein sequence.

Truncated mutants were produced by polymerase chain reaction (PCR) using pcDNA_HA_UMOD as template. PCR products were gel purified, digested with KpnI (unique internal site in uromodulin cDNA sequence) and EcoRI, and inserted in pcDNA_HA_UMOD in KpnI and EcoRI sites.

The forward primer used for all the truncated mutants was Mut-Forw 5'-CTGGTACCGCTTCGTGGGCCAGGGCGG-3'. Mutation-specific reverse primers were as follows: m1-589, 5'-CGGAATTCCTCAACTTCGGAATCTGTCCAG-3'; m1-597, 5'-CGGAATTCCTCAACGGGATTGATCTATGACACT-3'; m1-604, 5'-CGGAATTCCTCAGATGGGACCAAGTTCAGGAC-3'; m1-607, 5'-CGGAATTCCTATTTCCGTGTGATGGGACCCAA-3'; and Wt_s, 5'-CGGAATTCCTCAGACTGTGGCCTCGACACC-3'.

Missense mutations and internal in-frame deletions were introduced in pcDNA_HA_UMOD or Wt_s by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Primers were designed using the software QuikChange Primer Design Program. Sequences of all the mutagenesis primers are available upon request.

Insertion of the 6xHis tag in the C-terminal region (C-His1 and C-His2) was performed using the QuikChange mutagenesis kit (Stratagene). The 6xHis tag was inserted in between amino acids I593 and D594 (C-His1) and in between K607 and G608 (C-His2). The N-terminally His-tagged uromodulin construct (N-His) was obtained by replacing the HA tag in pcDNA_HA_UMOD construct by digesting with AgeI enzyme and inserting the 6xHis tag obtained by phosphorylating and annealing the two following primers: NhisF 5'-CCGGT-

CATCATCACCATCACCACA-3' and NhisR 5'-CCGGTGTGGTGATGGT-GATGATGA-3'.

Mutants mWt_s and mTRK/AAA_s were obtained by insertion of a myc tag downstream of residue V613 in Wt_s and TRK/AAA_s mutants by using the QuikChange mutagenesis kit (Stratagene). All the constructs were sequence-verified before transfection.

Cell Lines and Culture Conditions

Madin-Darby canine kidney (MDCK) cells were grown in DMEM supplemented with 10% fetal bovine serum, 200 U/ml penicillin, 200 µg/ml streptomycin, and 2 mM glutamine (complete medium) at 37°C, 5% CO₂. Stable clones were generated by transfecting MDCK cells by using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Selection was started 24 h after transfection by adding 1 mg/ml G418 (Invitrogen) and was pursued for 1–2 wk to obtain a population of G418-resistant cells.

Western Blot and Immunoprecipitation

Stably transfected MDCK cells were grown in six-wells plate in complete medium with 1 mg/ml G418. When cells reached confluence, the complete medium was replaced with 2 ml of Opti-MEM (Invitrogen). After 24 h, the conditioned medium was collected, 4 volumes of acetone was added, and precipitated proteins were resuspended in 100 µl of phosphate-buffered saline (PBS). For deglycosylation experiments, 10 µl of resuspended proteins were incubated 15 min at 55°C in denaturing buffer (New England Biolabs, Ipswich, MA), 3 h at 37°C in G7 buffer containing 1% NP-40 and 0.5 µl of peptide-N-(N-acetyl-β-glucosaminyl)asparagine amidase (PNGase F) (New England Biolabs) and 15 min at 55°C. Cells were lysed in 300 µl of octylglucoside lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 60 mM octylglucoside, and 1 mM phenylmethylsulfonyl fluoride) for 1 h at 4°C under rotation. The lysate was centrifuged for 10 min at 10,000 × g to separate soluble and insoluble fractions.

The insoluble fraction was resuspended in 50 µl of Laemmli buffer. Soluble fractions of protein lysate were quantified by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Then, 50 µg (~1/10) of each protein lysate, one half of each insoluble fraction, and 20 µl (~1/5) of the proteins precipitated from each medium were loaded onto reducing 8% SDS-polyacrylamide gel electrophoresis (PAGE). Transblotted nitrocellulose membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) were incubated with mouse monoclonal antibody (mAb) against HA (1:2000 dilution; Covance Research Products, Princeton, NJ) followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:7500 dilution; GE Healthcare). Anti-α-tubulin mouse mAb was used as a loading control and to exclude cellular contamination in the precipitated medium (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized with the Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate kit (Millipore, Billerica, MA).

MDCK cells stably expressing His-tagged uromodulin were grown in T-75 flasks. When cells reached full confluence, the complete medium was replaced by 10 ml of Opti-MEM (Invitrogen). After 24 h, the conditioned medium was collected, 4 volumes of acetone was added, and precipitated proteins were resuspended in 400 µl of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, and 1% Igepal CA-630). Fifty microliters of protein G-Sepharose beads (Sigma-Aldrich, St. Louis, MO) were incubated for 4 h with 5 µl of anti-uromodulin antibody (MP Biomedicals, Irvine, CA) in 500 µl of RIPA buffer. Resuspended proteins were added onto the beads coupled to the antibody and incubated overnight at 4°C. After three washes in RIPA buffer, beads were resuspended in 80 µl of denaturing buffer (New England Biolabs), and deglycosylation was carried out as described above. The samples were then analyzed by Western blot using either the PentaHis antibody (dilution 1:1000; QIAGEN, Valencia, CA) following the manufacturer's instructions or the anti-uromodulin antibody (MP Biomedicals).

Immunofluorescence

Cells grown on coverslip in 12-wells plate were fixed in 4% paraformaldehyde (PFA) for 30 min. When needed, cells were permeabilized 10 min at room temperature with 0.5% Triton X-100. After washing in PBS, cells were incubated 30 min at room temperature in 10% preimmune donkey serum in PBS. Cells were then incubated for 1 h 30 min at room temperature with the goat anti-uromodulin antibody (dilution 1:500 in PBS with 1% donkey serum; MP Biomedicals) and with the PentaHis antibody (dilution 1:50; QIAGEN) (His-tagged constructs). Permeabilized cells were costained with either rabbit anti-calnexin antibody (dilution 1:500; Sigma-Aldrich) or rabbit anti-giantin antibody (dilution 1:500; Covance Research Products). Cells stably expressing HA- and myc-doubly tagged constructs were costained with mouse anti-HA antibody (dilution 1:500; Covance Research Products) and goat anti-myc antibody (dilution 1:500; Novus Biologicals, Littleton, CO). Cells were washed in PBS and incubated for 1 h at room temperature with the appropriate secondary antibody: Alexa Fluor 594-conjugated donkey secondary antibody against goat or mouse immunoglobulin G (IgG) (dilution 1:500; Invitrogen); or Alexa Fluor 488-conjugated donkey secondary antibody against rabbit, goat or mouse IgG (dilution 1:500; Invitrogen). Cells were then

Figure 1. Identification of putative EHP and IHP motifs in uromodulin sequence. (A) A schematic representation of uromodulin is shown. The leader peptide is shown as a black box, EGF-like domains are displayed as numbered white boxes, and the ZP domain is shown in blue and divided in ZP-N and ZP-C subdomains (Boja *et al.*, 2003). Urinary consensus cleavage site (CCS) is shown in pink and GPI-anchoring site in green. Glycosylation sites are represented as Y. The sequence of human uromodulin C terminus is shown below. Highlights show the C-terminal end of the ZP domain (blue), the consensus cleavage site (pink), the predicted EHP motif (red) and the GPI-anchoring site (green). The alignment (ClustalW) of C-terminal sequences of human (Hs, gi 56550049), murine (Mm, gi 22128623), rat (Rn, gi 8394509), canine (Cf, gi 50950249), and bovine (Bt, gi 27806359) uromodulin is shown. Each residue is highlighted in color, ranging from red for highly hydrophobic residues to blue for the most hydrophilic residues. The predicted secondary structure for human uromodulin obtained by using five different programs is shown. β -Strands are depicted as green arrows; α -helices as gray cylinders. (B) Uromodulin domain structure is represented as above. Alignment of sequences of the ZP domain linker region from human (Hs, gi 56550049), murine (Mm, gi 22128623), rat (Rn, gi 8394509), canine (Cf, gi 50950249), and bovine (Bt, gi 27806359) uromodulin is given. The two putative IHP motifs are boxed in yellow. Hydrophobicity of each residue is shown as described above. The prediction of secondary structure for human uromodulin obtained by using five different programs is shown: β -strands are represented by green arrows.

stained for 5 min with 4,6-diamidino-2-phenylindole and mounted using FluorSave Reagent (Calbiochem, San Diego, CA). All slides were visualized under a DM 5000B fluorescence upright microscope (Leica DFC480 camera, Leica DFC Twain Software, 40 $\times$ /0.75 lens; Leica Microsystems, Deerfield, IL) except for the cells in Supplemental Figure S1, visualized with an UltraVIEW ERS spinning disk confocal microscope (UltraVIEW ERS-Imaging Suite Software, Zeiss 63 $\times$ /1.4; PerkinElmer Life and Analytical Sciences Boston, MA). All images were imported in Photoshop CS (Adobe Systems, Mountain View, CA) and optimized for brightness and contrast.

RESULTS

Identification of Putative EHP and IHP in Uromodulin Sequence by *In Silico* Analysis

Studies on mouse ZP3 protein have shown that two short sequences in the C-terminal region of the protein as well as membrane-anchoring play a role in protein secretion and assembly into the zona pellucida (Zhao *et al.*, 2003; Jovine *et al.*, 2004).

In particular, the EHP is a short stretch of seven amino acids localized in between the protein cleavage site and the transmembrane domain. Based on conserved features among ZP proteins, it has been defined as a motif containing the sequence (X)GP followed by four amino acids, mainly

hydrophobic, predicted to have a β -strand secondary structure (e.g., 362VGPLIFL368 in ZP3 human sequence). We carried out sequence alignment and secondary structure prediction analysis on uromodulin of different mammalian origin (Figure 1A). By applying the same criteria used to define ZP3's EHP, we identified two putative, partially overlapping motifs, one motif rich in hydrophobic residues (598VLNLGP1604) and the other motif defined by (X)GP and the four following residues (601LGPITRK607), localized between the urinary protein cleavage site (F587) (Santambrogio *et al.*, 2008) and the GPI-anchoring site. Both sequence stretches flanking GP residues, likely representing a turn region, were predicted to have a β -strand structure. Taking these observations into account and considering that the role of the three amino acids N-terminal to ZP3's EHP (359DVT361) was not assessed by site-specific mutagenesis, we hypothesized that the EHP could be longer than reported and that the sequence 598VLNLGP1607 could be part of the motif. Therefore, we considered the whole sequence 598VLNLGPITRK607 as the putative EHP motif for further mutagenesis studies (see below).

Figure 2. Soluble uromodulin mutated in the EHP motif assembles into intracellular filaments. (A) Schematic representation of the C-terminus of truncated and soluble (indicated by *_s*) uromodulin EHP mutants. The putative EHP motif is highlighted in red. (B) Western blot detection of uromodulin in the medium (M) and in the soluble (S) and insoluble (P) fraction of cell lysates from stably transfected MDCK cells. Uromodulin is found as a 100- and an 80-kDa isoform, corresponding to the mature and ER precursor form, respectively. Mutations in the EHP motif with the exception of the mutations affecting TRK residues (TRK/AAA_s and Δ TRK_s) dramatically impair protein secretion. Tubulin is shown as a loading control. (C) Immunofluorescence analysis of permeabilized MDCK cells stably expressing WT_s, m1-589 or TRK/AAA_s isoforms and stained for uromodulin and calnexin (ER marker). Mutations in the EHP sequence lead to the formation of intracellular filaments of mutant uromodulin (magnified in the insets). In the merged picture, the uromodulin signal is shown in red and the calnexin signal in green. Colocalization with calnexin shows the presence of mutant protein in the ER. However, uromodulin intracellular filaments are calnexin negative. Bar, 40 μ m.

ZP3's IHP was defined previously as a short stretch of seven amino acids, containing hydrophobic residues, adjacent to the end of the ZP-N subdomain (167EEKLTFS173 in human ZP3 sequence) and partially arranged in a β -strand (Jovine *et al.*, 2004). Based on these criteria, we identified two putative IHP motifs in uromodulin sequence, 430DMKVSLK436 and 456FTVRMAL462, the latter motif being localized at a position corresponding to ZP3's IHP (Figure 1B).

A Motif External to the ZP Domain Is Needed for the Regulation of Uromodulin Polymerization

To assess the role of the putative EHP sequence 598VLNLGPITRK607 in regulating uromodulin polymerization, we carried out mutagenesis analysis in soluble (Figure 2A) and full-length (Figure 3A) uromodulin isoforms. All constructs were stably expressed in MDCK cells, a canine kidney epithelial cell line that is able to properly process transfected uromodulin to form extracellular filaments/polymers (Figures 3C, 4D, and 6B and Supplemental Figure S3).

We first generated progressive uromodulin C-terminal truncations. Mutant m1-589, truncated at the consensus cleavage site (586RFRS589), was retained in the endoplasmic reticulum (ER) and not secreted. Indeed, this mutant was mainly detected as an 80-kDa precursor (Figure 2B) that was endoglycosidase H sensitive (data not shown) and almost completely colocalized with calnexin in immunofluorescence analysis (Figure 2C). Very interestingly, part of the intracellular protein assembled into filaments suggestive of premature polymerization. No signal could be detected on the plasma membrane of unpermeabilized cells (data not shown). The same observations were made with mutant m1-597, truncated just before the EHP, and mutant m1-604, missing residues TRK of the putative EHP sequence (Figure 2B and Supplemental Figure S1). On the contrary, mutant m1-607, containing the entire putative EHP sequence and the isoform Wt_s, truncated immediately upstream of the GPI-anchoring site (S614), did not show intracellular filaments. Both isoforms entered the secretory pathway, as shown by colocalization with calnexin (ER marker) (Figure 2C and

Figure 3. Polymerization defect in GPI-anchored EHP mutants. (A) Domain structure of uromodulin protein. A schematic representation of the C terminus of all GPI-anchored EHP mutants is given. (B) Western blot detection of wild-type or mutant uromodulin in the medium (M) and in the soluble (S) and insoluble (P) fractions of cell lysates from stably transfected MDCK cells. Uromodulin is found as a 100-kDa mature form and an 80-kDa precursor. Wild-type as well as mutant isoforms are fully glycosylated and secreted in the culturing medium, although to a different extent. Tubulin is shown as a loading control. (C) Immunofluorescence analysis showing wild-type and mutant (VLN/AAA, GP/AA, DQ/AA) uromodulin on the cell surface of stably transfected MDCK cells fixed with PFA. All isoforms are trafficked to the plasma membrane, but only wild-type uromodulin and the mutant not affecting the EHP motif (DQ/AA) can properly polymerize. On the contrary, mutations in the EHP motif impair protein polymerization. Bar, 40 μm.

Supplemental Figure S1) and giantin (Golgi marker) (data not shown) and by the presence of a fully glycosylated mature form of ~100 kDa in the cellular lysate and were efficiently secreted in the medium (Figure 2B, lane M). These results map the C-terminal end of the EHP motif to residue K607 in uromodulin sequence. To better define the motif, we produced in-frame deletions and point mutations in soluble uromodulin (Wt_s) and assessed the presence of intracellular filaments as a read-out of dysregulated polymerization. Partial deletions (Δ598-604_s, Δ601-607_s) in the sequence 598VLNLGPITRK607 led to the same results observed for mutants m1-589, m1-597, and m1-604, i.e., secretion impairment, ER retention and intracellular polymerization (Figures 2B and Supplemental S1). Additional mutants on each portion of the EHP were produced by deleting or inserting alanine residues instead of VLN (ΔVLN_s and VLN/AAA_s), GP (GP/AA_s), and TRK (ΔTRK_s and TRK/AAA_s) (Figure 2A). All of them formed intracellular filaments (Supplemental Figure S1 except for mutant TRK/AAA_s, shown in Figure 2C) confirming the importance of the entire EHP sequence to regulate polymerization of the protein. Western blot experiments (Figure 2B) showed impaired secretion and a significant enrichment in the ER form of the protein for mutants ΔVLN_s, VLN/AAA_s, and GP/AA_s. Mutants affecting 604TRK607 residues (ΔTRK_s and TRK/AAA_s) did not seem to impair protein secretion (Figure 2B). Mutations in the sequence 594DQSR597 (SR/AA_s and DQ/AA_s), immediately upstream of the EHP motif (Figure 2A), did not lead to intracellular filaments (Supplemental Figure S1) nor to secretion impairment (Figure 2B). These results map the EHP N-terminal boundary, demon-

strating that residues upstream of V598 are not involved in regulating uromodulin polymerization.

To investigate the function of the motif 598VLNLGPITRK607 in full-length uromodulin and to assess the role of membrane-anchoring, shown previously to be sufficient to rescue intracellular retention of EHP mutants in ZP3 (Jovine *et al.*, 2004), we generated a similar set of mutations in the GPI-anchored protein (Figure 3A). EHP mutants (Δ598-604, Δ601-607, VLN/AAA, GP/AA, and TRK/AAA) did not show the presence of intracellular filaments that were detected for their soluble counterparts (Supplemental Figure S2A). All of them, with the exception of TRK/AAA, showed increased ER retention, as could be deduced from the ratio of mature versus immature bands in the soluble fractions of cell lysates (Figure 3B). All isoforms were nevertheless secreted in the medium to an extent that is proportional to the amount of mature protein (Figure 3B). Very interestingly, mutations inside the region 598VLNLGP604 led to the absence of uromodulin polymers (Figures 3C and Supplemental S2B). These results demonstrated that GPI anchoring is sufficient to prevent intracellular polymerization of EHP mutants and that the integrity of the region 598VLNLGP604 is necessary for the formation of uromodulin extracellular filaments.

Together, experiments on soluble and GPI-anchored mutant isoforms define the sequence 598VLNLGPITRK607 as a motif in uromodulin sequence that regulates protein polymerization. Despite the fact that mutations in the sequence TRK did not seem to affect protein polymerization in GPI-anchored uromodulin, they led to the formation of intracel-

Figure 4. Mutations in the IHP lead to polymerization dysfunction. (A) Domain structure of uromodulin protein. Amino acid sequence in the linker region 425–466 is shown. Putative IHP motifs 430DMKVSLK436 and 456FTVRMAL462 are highlighted in red and gray, respectively. Point mutations in both motifs are shown in red. (B) Immunofluorescence analysis of MDCK cells stably expressing the soluble isoforms of uromodulin IHP mutants (indicated by *_s*). Permeabilized cells were incubated with anti-uromodulin and anti-calnexin antibodies. In the merged picture, uromodulin and calnexin signals are shown in red and green, respectively. In cells expressing uromodulin that carries mutations in the first putative IHP (430DMKVSLK436), intracellular filaments of mutant protein similar to the ones observed for EHP mutants are visible (magnified in the insets). Intracellular assembly cannot be observed for mutants in the other putative IHP motif (456FTVRMAL462). As for EHP mutants, intracellular filaments formed by IHP mutants do not colocalize with calnexin. Bar, 40 μ m. (C) Western-blot detection of soluble and GPI-anchored uromodulin mutants in the putative IHP motifs. Culturing medium (M), soluble (S), and insoluble (P) fractions of cell lysates from stably transfected MDCK cells are loaded. All mutants are secreted in the culturing medium with the exception of isoforms carrying V458R mutation that is fully retained in the ER and shows only the precursor form of the protein. Tubulin is shown as a loading control. (D) Immunofluorescence analysis of unpermeabilized MDCK cells stably expressing wild-type or GPI-anchored IHP mutants. Wild-type uromodulin and L462S mutant assemble into polymers. Mutant V458R is barely trafficked to the membrane and does not show any polymerized protein. Mutants D430L and L435S form abnormally short and less organized polymers on the plasma membrane. Bar, 40 μ m.

lular filaments of the soluble counterparts suggesting that they still alter the conformational state of the ZP domain.

Mutations in a Motif in the Linker Region of Uromodulin ZP Domain Lead to Polymerization Abnormalities

Computational analysis identified two motifs in uromodulin, 430DMKVSLK436 and 456FTVRMAL462, that could be functionally related to the IHP motif of ZP proteins (Figure 1B). We generated mutant isoforms for both putative IHP

motifs (D430L; L435S; V458R; L462S) (Figure 4A) that were stably expressed in MDCK cells.

Interestingly, mutations in the 430DMKVSLK436 sequence (D430L_s and L435S_s) in the soluble uromodulin isoform (Wt_s) led to the formation of intracellular filaments (Figure 4B), suggesting that the sequence 430DMKVSLK436 is also implicated in the regulation of uromodulin polymerization and is necessary to prevent premature polymerization of soluble uromodulin. Secretion was not impaired in these

Figure 5. The EHP motif is lost upon uromodulin assembly into filaments. Immunofluorescence analysis on MDCK cells stably expressing N- and C-terminally tagged uromodulin. A schematic representation of each tagged isoform is shown above the respective immunofluorescence panel. Uromodulin domain structure is depicted as before. EHP motif is indicated as a red box, the position of 6xHis and HA tags as green and red triangles, respectively. Immunofluorescence analysis was carried out on unpermeabilized PFA-fixed cells that were stained for the 6xHis tag and uromodulin. In the merged picture, uromodulin signal is in red whereas the 6xHis tag one is in green. Uromodulin polymers are positive for both antibodies when the 6xHis tag is present at the N terminus (N-His). However, they are negative for the anti-His antibody when the tag is localized in the C terminus, either before (C-His1) or after (C-His2) the EHP motif. These data suggest that the EHP sequence is lost in polymeric uromodulin. Bar, 10 μ m.

mutants (Figure 4C, top). Mutations in the 456FTVRMAL462 sequence did not lead to any kind of polymerization abnormality, excluding its role in regulating uromodulin polymerization. In particular, mutation V458R led to full ER retention of the mutant protein with no evidence of intracellular filaments (Figure 4, B and C, top). Due to the low steady-state level and the absence of secretion of the mutant protein, it seems likely that this mutation causes protein misfolding. In contrast, mutant L462S behaved like the wild-type soluble protein Wt_s (Figure 4, B and C, top).

As observed for mutants in the EHP sequence, GPI anchoring of D430L and L435S mutants prevented the formation of intracellular filaments (data not shown). These isoforms were correctly trafficked to the plasma membrane and secreted but showed abnormalities in the formation of extracellular polymers that looked shorter and less organized than wild-type polymers (Figure 4C, bottom; and D). As for their soluble counterparts, mutants in the 456FTVRMAL462 sequence did not show specific polymerization defects. Mutant L462S was correctly trafficked and processed, forming extracellular filaments that seemed identical to the wild-type filaments, whereas mutant V458R trafficking to the plasma membrane was barely rescued by membrane anchoring and the majority of mutant protein was ER retained and likely degraded (Figure 4C, bottom; and D).

In summary, mutations in the sequence 430DMKVSLK436 lead to polymerization abnormalities similar to the abnormalities observed for EHP mutants and pinpoint this region as uromodulin IHP motif.

Release of the EHP Is Needed to Allow Extracellular Polymerization of Uromodulin

The similar phenotype observed for EHP and IHP mutants suggests that these two motifs are functionally related and necessary to keep the ZP domain in an inactive conformation that prevents intracellular polymerization of soluble uromodulin isoforms. We assessed whether this function is lost in the polymerization process through cleavage of uromodulin monomers releasing the EHP motif to reach an active conformation. To study protein extracellular cleavage relative to the EHP motif position, we generated differently tagged wild-type uromodulin carrying 1) N-terminal 6xHis tag (N-His), 2) C-terminal 6xHis tag upstream of the EHP motif (between I593 and D594) (C-His1), and 3) C-terminal 6xHis tag immediately downstream of the EHP motif (between K607 and G608) (C-His2) (Figure 5). These isoforms were stably expressed in MDCK cells.

Immunofluorescence experiments on unpermeabilized cells showed the presence of polymers on the plasma membrane with all constructs when using a polyclonal anti-uromodulin antibody (Figure 5). These polymers looked identical to the ones observed in stable clones expressing untagged wild-type uromodulin (Supplemental Figure S3), suggesting that the presence of N- and C-terminal tags is not interfering with protein processing and assembly into filaments. Only polymers formed by the N-His isoform were positive for an anti-His antibody, suggesting that sequence downstream of I593, i.e., including the EHP motif, is lost in polymeric uromodulin.

Figure 6. Release of the EHP motif in polymeric uromodulin. (A) Western blot detection of wild-type and RFRS/AAAA uromodulin isoforms that are secreted in the medium of stably transfected MDCK cells. Where indicated (+), samples were deglycosylated with PNGase F before loading. The short uromodulin band is lost in RFRS/AAAA mutant. (B) Immunofluorescence analysis of unpermeabilized MDCK cells stably expressing either wild-type uromodulin or uromodulin mutated in the CCS (RFRS/AAAA). Mutation of the CCS abolishes the formation of uromodulin polymers on the plasma membrane. Bar, 20 μ m. (C) Western blot detection of N- and C-terminally tagged wild-type uromodulin isoforms that are immunoprecipitated from the medium of stably transfected MDCK cells. Samples were deglycosylated with PNGase F before loading. Although N-His uromodulin shows the presence of two bands with an anti-His and an anti-uromodulin antibody, for C-His1 and -2 only the upper band is positive for both antibodies. The lower band is negative for anti-His antibody, suggesting that in both cases the 6xHis tag is lost in the short uromodulin isoform that is incorporated into polymers.

To verify that the absence of anti-His reactivity of the filaments formed by isoforms C-His1 and 2 is indeed due to the absence of the tag and not to its inaccessibility, we performed a biochemical characterization of the secreted protein. In Western blot experiments, wild-type uromodulin that was secreted in the medium of MDCK stable clones showed one band of ~100 kDa corresponding to the fully glycosylated mature form. However, two isoforms were detected when the protein was deglycosylated, suggesting the existence of two different cleavages releasing uromodulin in the culturing medium (Figure 6A). Interestingly, the same experiment carried out on mutant RFRS/AAAA, where the cleavage site 586RFRS589 of urinary uromodulin was replaced by four alanine residues, showed the presence of the high-molecular weight isoform only. This mutant was trafficked to the plasma membrane as efficiently as the wild-type but was not able to form extracellular polymers, as assessed in immunofluorescence experiments (Figure 6B). These results suggest that only the short isoform, likely produced at the 586RFRS589 site, is the one assembled into polymers.

The Western blot analysis of secreted N-His uromodulin after deglycosylation displayed two bands with both anti-uromodulin and anti-his antibodies (Figure 6C). Secreted C-His1 and -2 showed two bands with the anti-uromodulin antibody, but only the long isoform was reactive with the anti-his antibody. These results demonstrated that the EHP is absent from the short isoform as it is produced by a proteolytic cleavage upstream of the tag (C-His1). Moreover, they showed that the EHP motif is still present in the long isoform that retains the His tag (C-His2). As the short isoform is the only one assembled into polymers, these experiments demonstrated that loss of the EHP motif is needed for uromodulin to polymerize into extracellular filaments.

Mutations in EHP/IHP Motifs Affect Uromodulin Cleavage on the Plasma Membrane

Because the presence or absence of the EHP in the secreted protein seems to be tightly linked to the ability of the protein

to be incorporated into polymers, we studied cleavage of GPI-anchored EHP or IHP mutants. Protein secreted in the medium for all mutants was deglycosylated and analyzed by Western blot. As shown in Figure 7A, all EHP mutants that were not forming polymers (Δ 601-607, Δ 598-604, VLN/AAA, and GP/AA; see Figure 3C and Supplemental Figure S2) showed one isoform only with an intermediate molecular weight compared with the two isoforms present in the wild-type protein. In contrast, mutants forming polymers (TRK/AAA, SR/AA, and DQ/AA) showed a profile identical to the wild-type protein. Interestingly, IHP mutants

Figure 7. EHP/IHP mutations affect uromodulin cleavage. Western-blot detection of wild-type and EHP or IHP mutant uromodulin isoforms that are secreted in the medium of stably transfected MDCK cells. Samples were deglycosylated with PNGase F before loading. (A) EHP mutants that do not form polymers (*) (see Figure 3C) are abnormally processed as compared with wild-type and mutants that are able to polymerize. (B) IHP mutants (D430L and L435S) showing altered polymerization (*) (see Figure 4C) are abnormally processed.

that formed abnormal polymers (Figure 4D) were also processed differently than the wild-type protein (Figure 7B). These data suggest that mutations affecting IHP/EHP motifs interfere with protein assembly into filaments by altering its cleavage on the plasma membrane.

DISCUSSION

Uromodulin, the most abundant protein found in urine, belongs to the family of the ZP domain proteins. These proteins perform highly diverse functions but share the ability to extracellularly polymerize into filaments and matrices via their ZP domain (Jovine *et al.*, 2005). In this work, we aimed at gaining new insights into the molecular mechanisms that regulate uromodulin polymerization and identified two motifs that are required for protein assembly into filaments. Previous studies performed on mouse ZP3 protein have shown the presence of two hydrophobic patches involved in the regulation of ZP domain-mediated polymerization: an IHP that maps inside the ZP domain and an EHP that is localized in the C-terminal part between the cleavage site and the membrane-anchoring site (Zhao *et al.*, 2003; Jovine *et al.*, 2004). Through sequence alignment and secondary structure prediction analysis, we identified putative EHP and IHP motifs in human uromodulin sequence that could be related to the two patches found in ZP3. We assessed the role of predicted EHP/IHP motifs by extensive mutagenesis and characterization of the ability of mutant isoforms to polymerize in MDCK cells. Although urinary uromodulin polymers have been described previously (Porter and Tamm, 1955; Wiggins, 1987), to our knowledge this is the first time that uromodulin polymers can be reproduced and studied in a cellular model.

We first analyzed the phenotype induced by mutations in the putative EHP and IHP in nonmembrane-anchored uromodulin. The wild-type soluble isoform (Wt_s) was efficiently sorted in the secretory pathway and released in the culturing medium of stably transfected MDCK cells showing that membrane-anchoring is not required for uromodulin secretion. Mutations in the motifs 598VLNLGPITRK607 and 430DMKVSLK436 led to the formation of mutant protein intracellular filaments suggestive of premature intracellular polymerization. This phenotype did not fully correlate with protein secretion impairment. Although the majority of EHP mutations dramatically reduced protein secretion, mutants in the 605TRK607 region as well as IHP mutants were normally secreted. In contrast, mutant V458R_s that was not secreted did not assemble intracellularly. We believe that the presence of intracellular filaments rather than protein secretion should be considered as a more specific sign of polymerization dysfunction. The specificity of this effect was confirmed by the absence of intracellular filaments when inserting mutations on the flanking residues of the EHP sequence and on the second putative IHP (456FTVRMAL462). Membrane-anchoring of the protein was sufficient to prevent intracellular polymerization of EHP/IHP mutant monomers, likely by confining them to the membrane. Interestingly, mutations either in the EHP, with the exception of the one affecting residues 605TRK607, or in the IHP abolished or significantly altered extracellular protein polymerization, even though they did not block secretion. This is consistent with previous findings on ZP3's EHP/IHP membrane-anchored mutants that were secreted but not assembled in the zona pellucida of microinjected oocytes (Zhao *et al.*, 2003; Jovine *et al.*, 2004). These data define the motifs 598VLNLGPITRK607 and 430DMKVSLK436 as uromodulin EHP and IHP.

It has been proposed previously that the functional relationship between EHP and IHP motifs could underlie a direct interaction of the two modules during the trafficking of the protein to the plasma membrane to keep the ZP domain in an inactive conformation that does not allow polymerization. Once the protein has reached the plasma membrane where it is cleaved, the interaction would be lost, leading to an active conformation allowing protein assembly (Jovine *et al.*, 2004). Our results are consistent with this model and show for the first time evidence of premature polymerization of a ZP domain protein mutated in either IHP or EHP motif and lacking membrane anchoring. This effect was hypothesized but not demonstrated for ZP3 protein (Jovine *et al.*, 2004). Notably, uromodulin intracellular filaments were not colocalizing with calnexin and giantin, markers of ER and Golgi compartment, respectively. We hypothesize that they could be formed by mutant protein either in an intermediate compartment or in the cytoplasm after retrotranslocation from the ER. Additional experiments will be needed in order to further characterize the mechanism of intracellular polymerization. Preliminary results on C-terminally myc-tagged protein showed that filaments are formed without any further C-terminal processing of the mutant protein (Supplemental Figure S4). Because EHP/IHP combined action is likely keeping the ZP domain in an inactive conformational state, it needs to be lost to allow extracellular polymerization. Data on C-terminal mapping on mouse ZP1, ZP2, and ZP3 (Boja *et al.*, 2003) and on mouse and human urinary uromodulin (Santambrogio *et al.*, 2008) are consistent with this. Cleavage of these proteins occurs upstream of the EHP motif at a position adjacent to the end of the ZP domain. The relevance of release of the EHP/IHP inhibitory effect on polymerization is demonstrated by our findings in MDCK cells. Interestingly, in this cellular model uromodulin was found in the medium as two isoforms of different molecular weight, likely resulting from two distinct cleavages. By insertion of a histidine tag either upstream or downstream of the EHP motif, we demonstrated that the short isoform lacks the EHP and is the only one assembled into polymers. In contrast, the long isoform still retaining the EHP is not able to polymerize. Characterization of a mutant at the urinary cleavage site 586RFRS589 (RFRS/AAAA), showing absence of the short isoform in the culturing medium and of extracellular polymers, strongly suggests that this isoform is generated by cleavage at F587 as for urinary protein. Together, these results provided evidence that EHP/IHP inhibitory role has to be lost through a specific proteolytic cleavage in the region between the ZP domain and the EHP motif to allow protein polymerization. This could be a general mechanism to control protein polymerization, as suggested by the fact that C-terminal cleavage that separates the EHP motif from the mature protein occurs also in soluble ZP domain proteins. This is the case of some fish vitelline envelope proteins that are released by the liver into the bloodstream as precursors that undergo proteolytic cleavage upon their arrival at the egg. Mature protein lacking the EHP can then be assembled into the vitelline envelope (Darie *et al.*, 2004, 2005).

Absent/abnormal extracellular polymerization of uromodulin (this study) and ZP3 (Jovine *et al.*, 2004) mutated in the EHP/IHP motifs could not be explained by the inhibitory role of the interaction of these two modules on polymerization. Indeed, the interaction would already be lost in these mutants and these isoforms should therefore be able to polymerize once trafficked to the plasma membrane and secreted. Our work provides a possible explanation to these observations. We demonstrated that absent/abnormal extra-

cellular polymerization of uromodulin EHP/IHP mutants is accompanied by altered cleavage of GPI-anchored mutant protein likely releasing protein monomers that are not able to assemble into filaments or do so in a less stable manner. This suggests a novel function for EHP/IHP motifs related to proper protein cleavage. The conformation reached through interaction between internal and external motifs could create a recognition site for the protease or an environment that would allow activation of the cleavage in a way similar to what has been described for the cleavage of ephrins by ADAM10 (Janes *et al.*, 2005). More work will be needed to identify the protease responsible for uromodulin extracellular polymerization and to understand the molecular bases of protease-substrate recognition and cleavage.

Mapping of EHP/IHP elements in uromodulin sequence showed some discrepancies with previous results on mouse ZP proteins. The EHP motif we identified (598VLNLGPITRK607) is longer than the one mapped by mutational analysis in ZP3 (362VGPLIFL368 in the human sequence) (Jovine *et al.*, 2004). Because implication of ZP3 sequence 359DVT361, corresponding to 598VLN600 in uromodulin, was not assessed by mutational analysis, it seems possible that ZP3's EHP is longer than reported. The position of uromodulin IHP motif (430DMKVSLK436) is different relative to the one proposed

for ZP3. Indeed, ZP3's IHP spans a flanking beta-strand in the linker region, corresponding to 456FTVRMAL462 in uromodulin. We excluded the involvement of this region because no specific sign of polymerization dysregulation was observed for V458R and L462S mutants. This inconsistency in IHP localization could imply that the conservation of functionally related IHP motifs in different ZP domain proteins is limited to a β -strand, not necessarily the same one, in the ZP domain linker. Alternatively, the internal motif regulating protein polymerization might make up different residues on flanking β -strands.

As our results suggest that the molecular mechanism regulating protein polymerization is conserved between distantly related proteins as uromodulin and ZP3, we looked for the presence of EHP and IHP motifs in other ZP domain proteins. Interestingly, the analysis of secondary structure prediction of the C-terminal region showed the presence of two conserved β -strands in the otherwise unstructured region between the cleavage site and the membrane anchoring domain (Figure 8). This region corresponds to the EHP of uromodulin and ZP3, suggesting that the position relative to cleavage and membrane anchoring site as well as the structural organization of the EHP motif is conserved in ZP domain proteins. Conservation of β -strand organization was

Figure 8. Conservation of IHP and EHP motifs in different ZP domain proteins. Schematic representation of the secondary structure prediction (obtained by PSI-Pred analysis) of the ZP domain and the C terminus of different human ZP domain proteins. Predicted β -strands and α -helices are indicated as dark and light gray arrows and striped cylinders, respectively. For each protein the position of known or putative cleavage site is indicated with a striped box. The position of predicted EHP and IHP motifs is marked by dashed lines and their sequence is shown below. Consensus sequences are also indicated. Uppercase letters within consensus sequence specify the corresponding amino acid in one-letter code. Lowercase letter indicate the following grouping sets: l, aliphatic (I, L, V); s, small (A, C, D, G, N, P, S, T, V); h, hydrophobic (F, H, I, L, M, V, W, Y); p, polar (C, D, E, H, K, N, Q, R, S, T); and t, turnlike (A, C, D, E, G, H, K, N, Q, R, S, T). Despite the apparent lack of significant sequence conservation among different EHP and IHP motifs, their secondary structure is remarkably conserved in ZP domain proteins.

also observed for the linker region of ZP domain. According to our data, we propose that the IHP is localized at the first β -strand, adjacent to the end of ZP-N subdomain. The position of IHP and EHP motifs relative to the ZP domain and the cleavage site as well as their predicted secondary structure seem to be their only conserved features.

Based on our data, we would predict that mutations in EHP/IHP motifs would lead to premature intracellular polymerization of soluble ZP domain proteins and in defective processing in the membrane-anchored proteins. Because uromodulin (Hart *et al.*, 2002) and other ZP domain proteins, i.e., endoglin (McAllister *et al.*, 1994), α -tectorin (Verhoeven *et al.*, 1998), DMBT1 (Mollenhauer *et al.*, 1997), and TGF- β R3 (Dong *et al.*, 2007) are involved in human diseases, we analyzed the spectrum of reported mutations and searched for the mutations affecting predicted EHP and IHP motifs, to evaluate potential consequences of EHP/IHP dysfunction. Unfortunately, none of the reported mutations was localized to one of the two motifs.

In summary, our work investigated for the first time polymerization mechanisms in uromodulin and showed data that can be of relevance for other ZP domain proteins. We identified two short motifs in uromodulin sequence that are involved in the regulation of protein polymerization. We demonstrate the inhibitory role of these motifs on protein polymerization and their relevance for protein cleavage and assembly into filaments on the plasma membrane. Our study sheds new light on the functional significance of IHP and EHP motifs and suggests that the molecular mechanisms that regulate polymerization of ZP domain proteins is highly conserved despite apparent lack of functional and sequence conservation in this heterogeneous protein family.

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