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Urinary secretion and extracellular aggregation of mutant uromodulin isoforms: new insight for the understanding of uromodulin-associated kidney diseases pathogenesis

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ABSTRACT

Uromodulin is the most abundant protein secreted in urine where it is found as high molecular weight polymers. It is exclusively expressed in the thick ascending limb of Henle's loop and its biological function is still elusive. Mutations in uromodulin are responsible for autosomal dominant kidney diseases characterised by defective urine concentrating ability, hyperuricaemia and gout, tubulo-interstitial fibrosis, renal cysts and chronic kidney disease. Previous *in vitro* studies have pointed at retention in the endoplasmic reticulum as a common feature of all uromodulin mutant isoforms. We here report *in vitro* and *in vivo* evidence that mutant uromodulin isoforms can partially escape retention in the endoplasmic reticulum and reach the plasma membrane. Here mutant protein forms large extracellular aggregates that play a dominant negative effect on co-expressed wild-type protein. Notably, we demonstrate for the first time that mutant uromodulin excretion can be detected in patients carrying uromodulin mutations. Overall, these results open the interesting possibility that the proteotoxic effect of mutant uromodulin expression could be exerted both by intra- and extracellular gain of function mechanisms and have implications for therapeutic strategies.

Keywords: uromodulin, Tamm-Horsfall protein, urinary protein secretion, aggregation

INTRODUCTION

Uromodulin, also known as Tamm-Horsfall protein, was discovered in 1950 by Tamm and Horsfall (1). It is a large glycoprotein of about 105 kDa that is exclusively expressed in the kidney in the thick ascending limb (TAL) of Henle's loop. Uromodulin is a glycosylphosphatidylinositol (GPI)-anchored protein mainly localised at the apical plasma membrane of epithelial tubular cells (2), from which it is released into the tubular lumen through a proteolytic cleavage by a yet to be identified protease (3). In the urine, it constitutes the most abundant protein and it is mainly found as a high-molecular-weight polymer (M_r 1–10 x 10^6) (4).

Mutations in the *UMOD* gene encoding uromodulin have been shown to cause medullary cystic kidney disease type 2 (MCKD2; MIM 603860), familial juvenile hyperuricemic nephropathy (FJHN; MIM 162000) (5) and glomerulocystic kidney disease with hyperuricemia (GCKD; MIM 609886) (6, 7), collectively referred to as uromodulin-associated kidney diseases (UAKD). UAKD are autosomal-dominant disorders characterised by alteration of urinary concentrating ability and by tubulo-interstitial fibrosis, hyperuricaemia and gout, and in few cases by renal cysts at the cortico-medullary junction (8). Although UAKD are heterogeneous in terms of age at onset, clinical features and presence of cysts, they invariably lead to chronic kidney disease (CKD) during the third to fifth decade of life.

To date 51 mutations have been described. All but three (in-frame deletions) are missense changes that are clustered in the N-terminal half of the protein. *In vitro* studies performed in diverse cell lines have shown that mutant uromodulin isoforms are defective in trafficking to the plasma membrane and are retained in the ER (9-12). Enrichment and aggregation of mutant uromodulin in the ER have been confirmed in a transgenic mouse model that we recently reported and in patient biopsies (10, 13). All together these results suggest ER retention of mutant uromodulin as a key primary event in UAKD pathogenesis. Interestingly, both *in vitro* and *in vivo* models show that mutant uromodulin can in part reach the plasma membrane carrying mature, post-ER type of glycans (9, 10, 13).

In this study, we investigate the effect of mutant uromodulin escaping ER quality control. We show that both in *in vitro* and *in vivo* models it forms aggregates at the plasma membrane and in the tubule lumen. Moreover, we provide novel evidence supporting that these changes in the extracellular compartment might be relevant in the human disease, as we identified mutant uromodulin secretion in urine samples from UAKD patients. All together, our data suggest the possibility of an extracellular gain of function and dominant negative effect of mutant uromodulin isoforms.

RESULTS

Mutant uromodulin is trafficked to and aggregates at the plasma membrane

We investigated if mutant uromodulin can partially escape quality control in the ER and is trafficked to the plasma membrane. To do so, wild-type and mutant (R212C, C256Y and C317Y) human uromodulin isoforms were stably expressed in MDCK cells. The C317Y mutation was previously reported and partially characterised *in vitro* (6, 14). Uromodulin mutations R212C and C256Y are novel and were identified after genetic screening in Italian UAKD families (Figure 1a, b). Similarly to previous studies on uromodulin mutants, all analysed mutations lead to partial ER retention of mutant protein (Figure 2a, b). Interestingly, wild-type protein reaching the plasma membrane assembles into polymeric filaments as described for urinary protein (15) (Figure 3). Although less efficiently, all mutant isoforms were also trafficked to the plasma membrane and secreted in the culturing medium. Notably, mutant protein is unable to assemble into ordered filaments but rather forms large aggregates on the plasma membrane. Mutant uromodulin reaching the plasma membrane and released by phospholipase C (PLC) digestion, carries mature post-ER glycosylation, demonstrating that it escaped the ER quality control and entered the secretory pathway (Figure S1).

Dominant negative effect of extracellular mutant uromodulin on the polymerisation of the wild-type isoform

To verify if aggregation of mutant uromodulin isoforms on the plasma membrane could be detrimental for the polymerisation of the wild-type one, wild-type and mutant proteins were stably co-expressed in MDCK cells, mimicking the patient heterozygous state. Equal expression level of the two isoforms was ensured by using a bicistronic expression vector. On western-blot, co-expression of both isoforms did not alter the band pattern observed when wild-type and mutants were expressed alone, nor secretion of the wild type protein (Figure S2). This shows that despite ER retention of mutant protein, trafficking of the co-expressed wild-type one is not altered or at least not to an extent that can be detected in our experiments. Interestingly, immunofluorescence analysis on non-permeabilised cells shows that mutant protein reaching the plasma membrane co-localises with the wild-type one in shortened and less structured polymers, when compared to cells expressing wild-type protein only (Figure 4a). This was confirmed both by the quantification of the average area of filament clusters (Figure 4b) and by differential migration on sucrose gradient (Figure 5). Indeed, sedimentation of wild type protein polymers into higher density fractions suggests that they are larger that the ones containing also mutant isoforms (16). This effect is mediated by co-assembly of the two isoforms as demonstrated by co-immunoprecipitation of secreted wild-type and mutant uromodulin (Figure 6). Taken together, these results demonstrate that mutant uromodulin isoforms that reach the plasma membrane exert an extracellular dominant negative effect on wild-type protein polymerisation.

Mutant uromodulin is secreted in the urine of a transgenic mouse model of UAKD and forms intraluminal casts

We verified whether mutant uromodulin could indeed escape ER retention and form extracellular aggregates *in vivo* in a transgenic mouse model of UAKD ($\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$) that expresses HA-tagged mutant uromodulin (C147W) (13), corresponding to the patient mutation C148W that we already characterised *in vitro* (6, 9). As a first evidence of mutant uromodulin trafficking to the plasma membrane, we detected its presence in the urine of 12 weeks-old $\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$ mice that still have preserved renal function (unpublished data) and no tubular damage. At this age secretion of transgenic uromodulin is similar to the one in expression-matched mice transgenic for HA-tagged wild-type uromodulin ($\mathrm{Tg}^{\mathit{Umod}\mathrm{wt}}$) (Figure 7a). Interestingly, urinary mutant uromodulin migrates slightly slower than the wild-type one on SDS-PAGE. This size difference is eliminated by PNGase F digestion, suggesting that the two isoforms carry different N-glycosylation. Immunofluorescence performed on mouse kidney sections confirms the presence of extracellular mutant uromodulin in the lumen of TAL tubules. Similarly to what observed *in vitro*, mutant protein tends to form aggregates that are never observed in $\mathrm{Tg}^{\mathit{Umod}\mathrm{wt}}$ mice (Figure 7b, c).

Mutant uromodulin is secreted in the urine of UAKD patients

We assessed the relevance of our *in vitro* and *in vivo* observations by investigating whether secretion of mutant uromodulin could also be observed in UAKD patients. We analysed the secretion of mutant uromodulin in 3 patients bearing different uromodulin mutations (R212C, C256Y or C317Y) (Figure 1a) and with preserved renal function (Table 1). Mass spectrometry analyses show that in all cases, mutant isoforms are secreted in urine (Figure S3-S5). Because summed peptide intensity can be used as a good proxy for absolute protein abundance (17), the intensity signals of peptides specifically assigned to the wild-type protein were compared to the ones of peptides specifically assigned to the mutant proteins, whereas signal intensities of common peptides of both isoforms were not considered. This method was used to have an estimate of the abundance of the urinary wild-type and mutant uromodulin isoforms in each patient. The ratio of the summed intensities of the specific peptides belonging to each uromodulin form is reported in Table 2. Mutant isoforms R212C, C256Y and C317Y secretion was estimated to represent about 1.3%, 7.6% and 35% respectively of the wild-type one.

DISCUSSION

To date 51 mutations in the *UMOD* gene coding for uromodulin have been described, all leading to UAKD. Previous *in vitro* studies have pointed at ER retention as a common feature of all investigated mutants (9-12). This was confirmed in a recently generated UAKD mouse model and on patient renal biopsy analysis (10, 13). All together these data led to propose that mutant uromodulin intracellular aggregation could play a key role in the pathogenesis of the disease, likely by exerting a gain-of-function effect through activation of cell stress responses.

In this study through *in vitro* and *in vivo* analyses we demonstrate that part of mutant protein can escape the ER quality control, is trafficked to the plasma membrane and secreted. Interestingly, mutant protein tends to form large extracellular aggregates that interfere with wild-type protein polymerisation, suggesting extracellular gain of toxic function as an additional consequence of uromodulin mutations.

This effect was clearly evident in *in vitro* studies that were carried out in MDCK cells that to our knowledge are the only cell model able to induce proper uromodulin polymerisation into highly-ordered filaments. This allowed us to show that mutant isoforms are unable to polymerise but rather form aggregates at the plasma membrane. Moreover, when wild-type and mutant uromodulin were co-expressed at equal levels, thereby mimicking the patient condition, we observed that mutant protein co-assembles with the wild-type one and exerts a dominant negative effect over polymer formation and/or stability. Indeed, the presence of the mutant protein significantly shortens the polymers, as demonstrated by their quantification in immunofluorescence and their differential sedimentation on sucrose gradient. The dominant negative effect on protein polymerisation suggests that even if mutant protein has escaped the cellular quality control, it is still adopting a non-native conformation. ER quality control escape has already been observed for other disease-causing mutant proteins, e.g. alpha-1-antitrypsin (18) and CFTR (19). Interestingly, CFTR folding mutant ΔF508 on escape from the ER quality control is altering cellular lipid trafficking (20), showing that misfolded proteins that gain access to the distal secretory pathway, in addition of being non functional, can have a detrimental effect.

In the case of mutant uromodulin, this effect is not only evident in *in vitro* models but also *in vivo*. Indeed, immunofluorescence analyses performed on kidney sections of a mouse model of UAKD ($\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$ mice) show intraluminal casts containing mutant uromodulin. These structures are an early finding in the mouse disease that can be found in virtually all TAL segments at time points (1 and 4 weeks of age) when renal function and nephron integrity are still preserved (Figure S6 and data not shown).

We believe that secretion of mutant uromodulin as seen in MDCK cells and in $\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$ mice is not merely due to overexpression. The relevance of these findings is indeed demonstrated by the detection of mutant uromodulin in the urine of three UAKD patients carrying three different mutations. Urinary mutant protein is likely derived from active protein secretion from the plasma membrane and not due to the presence of cell debris in the urine sample. Indeed, if that was the case, one would expect mutant uromodulin to be mainly present as the shorter ER-glycosylated isoform, which was never detected in western-blots of patient urine. Mutant uromodulin was not found in the urine of UAKD patients in two earlier studies (21, 22). This discrepancy could be ascribed to the different *UMOD* mutations analysed. However, it should be noticed that in both previous studies uromodulin secretion was assessed at a time point when renal function of UAKD patients was already severely compromised. In this condition, total uromodulin secretion was highly reduced, possibly to such an extent to hamper the specific detection of mutant protein. In our experience, this was indeed the case in affected relatives of the patients analysed in the current study that had reduced renal function (data not shown). This suggests that a mutation-specific effect is unlikely. Rather mutant uromodulin secretion (ranging from about 1 to 30 percent of the wild-type protein level in our work) is evident in UAKD patients when renal function is still preserved.

What could be the consequences of extracellular mutant uromodulin aggregation? It is well known that uromodulin constitutes the matrix for the formation of hyaline casts (23). Mutant uromodulin with its high propensity to aggregation could increase the number of hyaline casts formed in the TAL leading to tubule obstruction, an event that could be upstream of inflammation and interstitial fibrosis. On a more speculative ground, we hypothesise that aggregation of mutant uromodulin could affect wild-type protein function by interfering with its polymerisation or by sequestering it in large extracellular aggregates. This could affect the very recently described role of uromodulin in the regulation of water/salt balance in the TAL (24), contributing to urinary concentrating defect and hyperuricemia in UAKD phenotype.

We believe that our findings have relevance when considering potential therapeutic strategies for UAKD, in particular the use of chemical chaperones to rescue mutant protein retention in the ER. The chemical chaperone sodium-phenylbutyrate (PBA) was shown to restore mutant uromodulin trafficking *in vitro* (25) and the use of this drug has already been extensively tested for the treatment of other ER-storage disorders such as alpha-1 antitrypsin deficiency (26) or cystic fibrosis (27). In the case of UAKD, chemical chaperones rescuing intracellular retention would likely increase mutant protein delivery to the plasma membrane thereby enhancing extracellular aggregation. We think that additional work would be needed to understand the potential negative consequences of such an effect that would be predicted to affect the TAL segment and kidney function.

In conclusion, our work shows that mutant uromodulin isoforms are secreted in the urine of patients with preserved renal function. Mutant uromodulin seems to have a higher propensity to aggregation than the wild-type protein due to either misfolding or to different post-translational modification, e.g. glycosylation. The formation of extracellular casts could induce tubule obstruction, cast formation and interfere with the function of wild-type uromodulin and possibly other membrane proteins. Thus, these data suggest that the proteotoxic effect of mutant uromodulin isoforms could be exerted through both intra-and extracellular gain of function mechanisms. This should be taken into account when envisaging therapeutic strategies for uromodulin-associated renal disorders.

METHODS

Uromodulin expression constructs

Wild-type uromodulin tagged with HA (pcDNA_HA_UMOD) was expressed from pcDNA 3.1(+) cloning vector (Invitrogen, Carlsbad, CA). Missense mutations were introduced in pcDNA_HA_UMOD by using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Primers were designed using the software QuikChange® Primer Design Program. For co-expression experiments, myc- or Flag-tagged and HA-tagged uromodulin isoforms were stably expressed from bicistronic vector pVITRO-hygro-mcs under the control of the elongation factor 1 alpha promoter of mouse and rat respectively (Invivogen, San Diego, CA). The HA, myc or Flag tag was inserted after the leader peptide, in between T26 and S27 in the protein sequence as previously described (28).

Cell line and culture condition

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% CO_2. Stable clones were generated by transfecting MDCK cells with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For pcDNA and pVITRO constructs, selection was started 24 h after transfection by adding 1 mg/ml G418 (Invitrogen) or 200 µg/ml hygromycin (Invitrogen) respectively, and was pursued for 2 weeks to obtain a population of resistant cells.

Tg^{Umod} mice and murine urine collection

Generation and characterisation of Tg^{Umod} mice is described elsewhere (13). Murine urine was obtained on age- and gender-matched transgenic and control mice (females, aged 10-12 weeks). They were housed in light- and temperature-controlled room with *ad libitum* access to water and standard chow. Twenty-four hours urine collections were obtained at baseline in individual metabolic cages. All animal procedures were carried out at San Raffaele Scientific Institute, Milan, Italy, according to and approved by the San Raffaele Institutional Animal Care and Use Committee.

Patient and urine collection

Diagnostic criteria for patient collection and *UMOD* mutational screening procedures have been previously described (6). Uromodulin was purified from 20 ml of urine as already reported (3), and resuspended in distilled water. Purified protein was deglycosylated with peptide-N-glycosidase F (PNGase F) (New England Biolabs, Ipswich, MA) in denaturing/reducing conditions according to the manufacturer’s instructions, alkylated in 55 mM iodoacetamide (IAA) or N-ethylmaleimide (NEM) for 20 min at room temperature, separated on 8% acrylamide SDS–PAGE and stained with Coomassie Brilliant Blue (Sigma–Aldrich Corporation, St Louis, MO).

Western-blot

MDCK cells were grown to 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 were added, and precipitated proteins were resuspended in 100 µl of phosphate-buffered saline (PBS). When indicated, cells were incubated with Pi-PLC digestion medium (DMEM; 20mM Tris-HCl pH 7.4; Pi-PLC 0.3U/ml (Invitrogen)) for 3 h in a 5% $C0_2$

atmosphere at 37°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. Unsoluble fractions were resuspended in 50 µl of Laemmli buffer while soluble fractions were quantified by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). When indicated, soluble fractions or Pi-PLC digestion medium were deglycosylated with peptide-N-glycosidase F (PNGase F) or endoglycosidase H (EndoH) (New England Biolabs). Thirty µg of each protein lysate, 10 µl (1/5) of each unsoluble fraction, and 20 µl (1/5) of medium-precipitated proteins 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 Reseach Products, Princeton, NJ) or myc (1:1000 dilution; Covance) followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:7500 dilution; GE Healthcare). Anti alpha-tubulin mouse mAb (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal anti-transferrin goat antibody (1:1000 dilution; Bethyl laboratories, inc, Montgomery, TX) were used as a loading control for lysate and precipitated medium respectively. Protein bands were visualized with the Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate kit (Millipore, Billerica, MA).

Sucrose gradient

Pi-PLC digestion medium was overlaid on a discontinuous gradient consisting of 1, 1.5 and 2 M sucrose in DMEM and centrifuged at 100 000 g for 2 h. After centrifugation, the gradient was separated in 6 fractions that were precipitated with acetone and analysed by western-blot using a sheep anti-uromodulin antibody (1:1000 dilution, Abcam, Cambridge, UK).

Immunoprecipitation

MDCK cells grown to confluence (3 100 mm dishes/line) were kept for 30 h in Opti-MEM (Invitrogen). The conditioned medium was collected, pooled and concentrated on Amicon-Ultra-15 3K (Millipore) to obtain a total volume of 700 µl. 500 µl of the concentrated medium was added to 40 µl of anti-Flag M2 affinity gel (Sigma). After an overnight incubation at 4°C under rotation, the resin was washed 3 times with 1 mL PBS, resuspended in 60 µl Laemmli buffer and boiled. 50 µl of the concentrated medium and 30 µl of the immunoprecipitate were loaded on SDS PAGE and analysed by western-blot as described above using a rabbit anti-Flag antibody (1:2000 dilution, Sigma) or a mouse anti-HA antibody (1:1000 dilution, Covance).

Immunofluorescence

Cells grown on coverslip in 12-wells plate were fixed in 4% paraformaldehyde (PFA) for 30 min. When needed, cells were permeabilised 10 min at room temperature with 0.5% Triton X-100. Cells were labelled for 1 h 30 min at room temperature with anti-HA (1:500; Covance) and anti-myc (1:500; Novus Biologicals, Littleton, CO) and anti-calnexin (dilution 1:500; Sigma-Aldrich) antibodies. After incubation with the appropriate secondary antibody (1:500; Invitrogen) cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and mounted using FluorSave Reagent (Calbiochem, San Diego, CA).

To collect renal tissue, animals were anaesthetised with avertin and perfused with a 4% PFA solution. Kidneys were taken and fixed in 4% PFA solution for additional 16 h and embedded in optimum cutting temperature embedding medium (Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA), snap-frozen in a mixture of isopentane and dry ice, and stored at –80°C. Immunofluorescence was carried out on tissue sections (10 µm thick) stained with rat anti-HA antibody (Roche, Indianapolis, IN) and anti-NKCC2 antibody (kind gift from Prof. Sebastian Bachmann, Charité University, Berlin).

All slides were visualized under a DM 5000B fluorescence upright microscope (Leica DFC480 camera, Leica DFC Twain Software, 40X/0.75 lens; Leica Microsystems, Deerfield, IL) or with an UltraVIEW ERS spinning disk confocal microscope (UltraVIEW ERS-Imaging Suite Software, Zeiss 63X/1.4; PerkinElmer Life and Analytical Sciences Boston, MA). All images were imported in Photoshop CS (Adobe Systems, Mountain View, CA) and adjusted for brightness and contrast.

The area of polymers covering the plasma membrane in cells co-expressing myc-tagged wild-type uromodulin together with either wild-type or mutant HA-tagged protein was quantified by using Image J software (http://imagej.nih.gov/ij/) (Figure S7). The analysis was performed on pictures taken at 10X magnification that were adjusted for brightness and contrast and after setting a threshold to exclude the membrane signal background. Only polymers positive for both tags were quantified by setting a selection mask on the HA channel and restoring it on the myc one. The selected area was quantified by setting an object size limit of 300 pixels. For each experiment, about 1000 objects were quantified per line. Data are expressed as average ± SEM and compared by unpaired two-tailed *t* test analysis (Prism software; GraphPad, San Diego, CA).

Mass-spectrometry analysis

LC-MS/MS analysis

Bands of interest were excised from gels of patients, subjected to reduction by 10 mM dithiothreitol (DTT) and alkylation by 55 mM IAA or NEM, and finally digested overnight with trypsin (Roche) (29). After acidification, up to 10% formic acid, peptide mixtures were concentrated and desalted on StageTips µC18 (Proxeon Biosystem, Odense, Denmark) (30) and injected in a capillary chromatographic system (EasyLC, Proxeon Biosystem, Odense, Denmark). Peptide separations occurred on a RP homemade 15 cm reverse-phase spraying fused silica capillary column (75 µm i.d. x 15 cm), packed with 3 μm ReproSil-Pur C18-AQ (Dr. Maisch GmbH, Germany). A gradient of eluents A (H_2O with 2% v/v ACN, 0.5% v/v acetic acid) and B (80%ACN with 0.5% v/v acetic acid) was used to achieve separation, from 7% B (at 0 min 0.2 µL/min flow rate) to 35% B (in 70 min, 0.2 µL/min flow rate). The LC system was connected to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a nanoelectrospray ion source (Proxeon Biosystems). MS and MS/MS spectra were acquired selecting the ten most intense ions per survey spectrum acquired in the orbitrap from *m*/*z* 300-1750 with 60 000 resolution. Target ions selected for the MS/MS were fragmented in the ion trap and dynamically excluded for 45s. Target value were 1 000 000 for survey scan and 100 000 for MS/MS scan. For accurate mass measurements, the lock-mass option was employed (31).

Data Processing and Analysis

For peptide identifications Mascot (Matrix Science; version 2.2.07) search engine was used. Raw MS files were converted into peaklist (.msm files), and searched against the database IPI_human_20100628 (86 397 sequences; 35 097 130 residues) supplemented with the on purpose created sequences of human uromodulin variants with mutations R212C (IPI99999999), C256Y (IPI99999996) and C317Y (IPI99999995).

Enzyme specificity was set to trypsin, allowing a maximum of two missed cleavages. Cysteine alkylation by IAA or NEM or acrylamide, oxidation of methionine, deamidation of asparagine and protein N-acetylation were all considered as variable modifications. Mass tolerance was set to 5 ppm and 0.6 Da for precursor and fragment ions respectively. The criteria used to evaluate the quality of MS/MS data was a Mascot ion score cut-off of at least 20.

Data Analysis by MaxQuant

To measure the intensity of peptides, especially the intensity of the mutated peptides and the correspondent wild-type peptides in each sample from urine of patients, MS data were analyzed using the MaxQuant software, (32, 33) version 1.1.1.25 applying the Label-free algorithms.

For MS/MS peak list file construction, up to top 6 peaks per 100 Da window were included for database searching. The generated peak lists were searched against the concatenated forward and reverse database IPI_human_20100628 (86 397 sequences; 35 097 130 residues) supplemented with the on purpose created sequences of human uromodulin variants with mutations R212C (IPI99999999), C256Y (IPI99999996) and C317Y (IPI99999995). Mass tolerance was set to 7 ppm and 0.6 Th for precursor and fragment ions respectively. Cysteine alkylation by IAA or NEM and acrylamide, protein N-terminal acetylation, asparagine deamidation and methionine oxidation were set as variable modifications for the database search. The cut-off false discovery rate for proteins and peptides was set to 0.01, and peptides with a minimum of six amino acids were considered for identification. The derived peptides intensities were used for a rough estimation of relative peptide abundances.

DISCLOSURE

None to declare

REFERENCES

1. Tamm I, Horsfall FL. Characterisation and separation of an inhibitor of viral hemagglutination present in urine. *Proc Soc Exp Biol Med* 1950; 108-114.
2. Bachmann S, Koeppen-Hagemann I, Kriz W. Ultrastructural localization of Tamm-Horsfall glycoprotein (THP) in rat kidney as revealed by protein A-gold immunocytochemistry. *Histochemistry* 1985; **83:** 531-538.
3. Santambrogio S, Cattaneo A, Bernascone I, *et al.* Urinary uromodulin carries an intact ZP domain generated by a conserved C-terminal proteolytic cleavage. *Biochem Biophys Res Commun* 2008; **370:** 410-413.
4. Serafini-Cessi F, Malagolini N, Cavallone D. Tamm-Horsfall glycoprotein: biology and clinical relevance. *Am J Kidney Dis* 2003; **42:** 658-676.
5. Hart TC, Gorry MC, Hart PS, *et al.* Mutations of the UMOD gene are responsible for medullary cystic kidney disease 2 and familial juvenile hyperuricaemic nephropathy. *J Med Genet* 2002; **39:** 882-892.
6. Rampoldi L, Caridi G, Santon D, *et al.* Allelism of MCKD, FJHN and GCKD caused by impairment of uromodulin export dynamics. *Hum Mol Genet* 2003; **12:** 3369-3384.
7. Lens XM, Banet JF, Outeda P, *et al.* A novel pattern of mutation in uromodulin disorders: autosomal dominant medullary cystic kidney disease type 2, familial juvenile hyperuricemic nephropathy, and autosomal dominant glomerulocystic kidney disease. *Am J Kidney Dis* 2005; **46:** 52-57.
8. Scolari F, Caridi G, Rampoldi L, *et al.* Uromodulin storage diseases: clinical aspects and mechanisms. *Am J Kidney Dis* 2004; **44:** 987-999.
9. Bernascone I, Vavassori S, Di Pentima A, *et al.* Defective intracellular trafficking of uromodulin mutant isoforms. *Traffic* 2006; **7:** 1567-1579.

10. Vylet'al P, Kublova M, Kalbacova M, *et al.* Alterations of uromodulin biology: a common denominator of the genetically heterogeneous FJHN/MCKD syndrome. *Kidney Int* 2006; **70:** 1155-1169.

11. Jennings P, Aydin S, Kotanko P, *et al.* Membrane targeting and secretion of mutant uromodulin in familial juvenile hyperuricemic nephropathy. *J Am Soc Nephrol* 2007; **18:** 264-273.

12. Williams SE, Reed AA, Galvanovskis J, *et al.* Uromodulin mutations causing familial juvenile hyperuricaemic nephropathy lead to protein maturation defects and retention in the endoplasmic reticulum. *Hum Mol Genet* 2009; **18:** 2963-2974.

13. Bernascone I, Janas S, Ikehata M, *et al.* A transgenic mouse model for uromodulin-associated kidney diseases shows specific tubulo-interstitial damage, urinary concentrating defect and renal failure. *Hum Mol Genet* 2010; **19:** 2998-3010.

14. Scolari F, Puzzer D, Amoroso A, *et al.* Identification of a new locus for medullary cystic disease, on chromosome 16p12. *Am J Hum Genet* 1999; **64:** 1655-1660.

15. Jovine L, Qi H, Williams Z, *et al.* The ZP domain is a conserved module for polymerization of extracellular proteins. *Nat Cell Biol* 2002; **4:** 457-461.

16. Ksiezak-Reding H, Wall JS. Mass and physical dimensions of two distinct populations of paired helical filaments. *Neurobiol Aging* 1994; **15:** 11-19.

17. de Godoy LM, Olsen JV, Cox J, *et al.* Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature* 2008; **455:** 1251-1254.

18. Miranda E, Perez J, Ekeowa UI, *et al.* A novel monoclonal antibody to characterize pathogenic polymers in liver disease associated with alpha1-antitrypsin deficiency. *Hepatology* 2010; **52:** 1078-1088.

19. Hegedus T, Aleksandrov A, Cui L, *et al.* F508del CFTR with two altered RXR motifs escapes from ER quality control but its channel activity is thermally sensitive. *Biochim Biophys Acta* 2006; **1758:** 565-572.

20. Gentzsch M, Choudhury A, Chang XB, *et al.* Misassembled mutant DeltaF508 CFTR in the distal secretory pathway alters cellular lipid trafficking. *J Cell Sci* 2007; **120:** 447-455.

21. Dahan K, Devuyst O, Smaers M, *et al.* A cluster of mutations in the UMOD gene causes familial juvenile hyperuricemic nephropathy with abnormal expression of uromodulin. *J Am Soc Nephrol* 2003; **14:** 2883-2893.

22. Rezende-Lima W, Parreira KS, Garcia-Gonzalez M, *et al.* Homozygosity for uromodulin disorders: FJHN and MCKD-type 2. *Kidney Int* 2004; **66:** 558-563.

23. Wenk RE, Bhagavan BS, Rudert J. Tamm-Horsfall uromucoprotein and the pathogenesis of casts, reflux nephropathy, and nephritides. *Pathobiol Annu* 1981; **11:** 229-257.

24. Renigunta A, Renigunta V, Saritas T, *et al.* Tamm-Horsfall glycoprotein interacts with renal outer medullary potassium channel ROMK2 and regulates its function. *J Biol Chem* 2011; **286:** 2224-2235.

25. Choi SW, Ryu OH, Choi SJ, *et al.* Mutant tamm-horsfall glycoprotein accumulation in endoplasmic reticulum induces apoptosis reversed by colchicine and sodium 4-phenylbutyrate. *J Am Soc Nephrol* 2005; **16:** 3006-3014.

26. Burrows JA, Willis LK, Perlmutter DH. Chemical chaperones mediate increased secretion of mutant alpha 1-antitrypsin (alpha 1-AT) Z: A potential pharmacological strategy for prevention of liver injury and emphysema in alpha 1-AT deficiency. *Proc Natl Acad Sci U S A* 2000; **97:** 1796-1801.

27. Rubenstein RC, Zeitlin PL. A pilot clinical trial of oral sodium 4-phenylbutyrate (Buphenyl) in deltaF508-homozygous cystic fibrosis patients: partial restoration of nasal epithelial CFTR function. *Am J Respir Crit Care Med* 1998; **157:** 484-490.

28. Schaeffer C, Santambrogio S, Perucca S, *et al.* Analysis of uromodulin polymerization provides new insights into the mechanisms regulating ZP domain-mediated protein assembly. *Mol Biol Cell* 2009; **20:** 589-599.

29. Shevchenko A, Wilm M, Vorm O, *et al.* M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996; **68:** 850-858.

30. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 2007; **2:** 1896-1906.

31. Olsen JV, de Godoy LM, Li G, *et al.* Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics* 2005; **4:** 2010-2021.

32. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 2008; **26:** 1367-1372.

33. Cox J, Matic I, Hilger M, *et al.* A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat Protoc* 2009; **4:** 698-705.

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FIGURE LEGENDS

Figure 1. (**a**) Schematic representation of uromodulin. The three EGF domains are represented by orange boxes, the D8C domain by a pink box and the ZP domain (composed of ZP_N and ZP_C subdomains) is represented in blue. The GPI anchoring site as well as the glycosylation sites (Y) are shown. Studied mutations are indicated in red. (**b**) Pedigree charts of the three families D123, MCKD1 and CM1 bearing uromodulin mutations R212C, C256Y and C317Y respectively. *, urine mass-spectrometry analysis.

Figure 2. (**a**) Immunofluorescence analysis showing uromodulin in permeabilised MDCK cells stably expressing HA-tagged wild-type or mutant uromodulin isoforms. Calnexin is shown as an ER marker. Bar = 35 µm (**b**) Western-blot detection of HA-tagged wild-type uromodulin or indicated mutants from the medium (M) and the soluble (L) and unsoluble (P) fractions of protein extracts from stably transfected MDCK cells. Tubulin is shown as a loading control (left panel). Western-blot detection of uromodulin present in the cell lysate after deglycosylation with Endo H (E) or PNGase F (P); C, untreated sample (right panel).

Figure 3. Immunofluorescence showing uromodulin distribution on the plasma membrane in unpermeabilised MDCK cells stably expressing HA-tagged wild-type or mutant uromodulin isoforms. Wild-type isoform is mainly present as large polymeric filaments while mutant isoforms form extracellular bulky aggregates. Bar = 35 µm.

Figure 4. (**a**) Immunofluorescence analysis showing uromodulin on the plasma membrane of unpermeabilised MDCK cells stably expressing myc-tagged wild-type isoform and either wild-type or mutant HA-tagged isoforms. Staining with anti-HA and anti-myc antibodies are

shown. The presence of the mutant isoform at the plasma membrane interferes with the polymerisation of the wild-type one, as polymers look shortened and more aggregated. Bar = 55 µm. (**b**) Quantification of the average surface of uromodulin polymers on the membrane of MDCK cells co-expressing HA- and myc-tagged uromodulin isoforms. Only polymers containing co-transfected isoforms (positive for both tags) were quantified (about 1000 counts per cell line per experiment, 2 independent experiments). Filaments formed by wild-type and mutant protein are significantly shortened. **, $p < 0.001$.

Figure 5. Western-blot analysis after separation on sucrose gradient of uromodulin digested by Pi-PLC from MDCK cells expressing the myc-tagged wild-type isoform and either wild-type or mutant HA-tagged isoforms. Fractions numbers are indicated from the top (1M sucrose, fraction 1) to the bottom (2M sucrose, fraction 6) of the gradient. I, input (1/5 of the amount loaded on the gradient). Uromodulin sediments deeper in the gradient when wild type protein is expressed alone.

Figure 6. Top panel: Co-immunoprecipitation experiments on protein from the conditioned medium of MDCK cells co-expressing Flag-tagged wild-type and HA-tagged uromodulin isoforms. Immunoprecipitation was carried out with an anti-Flag antibody. The presence of co-immunoprecipitated HA-tagged uromodulin was assessed by western-blot analysis. Mutant uromodulin co-assembles with wild-type protein. Bottom panel: Same experiment performed on protein from the conditioned medium of MDCK cells expressing HA-tagged wild-type uromodulin isoform. The absence of co-immunoprecipitated HA-tagged uromodulin in the absence of Flag-tagged isoform demonstrates the specificity of the assay. *, unspecific signal, IP: immunoprecipitate.

Figure 7. (**a**) Transgenic HA-tagged uromodulin secreted in urine of $\mathrm{Tg}^{\mathit{Umod}\mathrm{wt}}$ and $\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$ mice was analysed by western-blot. C, control non-transgenic animal. The amount of urine loaded is representative of a 24 h urine collection. Urinary uromodulin was also analysed after deglycosylation with PNGase F. (**b**) Immunofluorescence performed on kidney sections of $\mathrm{Tg}^{\mathit{Umod}\mathrm{wt}}$ and $\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$ mice showing transgenic uromodulin and NKCC2 (apical marker of TAL epithelial cells). Uromodulin aggregates are present exclusively in TAL lumen of $\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$ mice. Bar = 50µm (**c**) Immunofluorescence showing at higher magnification uromodulin aggregates present in TAL lumen of $\mathrm{Tg}^{\mathit{Umod}\mathrm{C147W}}$ mice and absent in $\mathrm{Tg}^{\mathit{Umod}\mathrm{wt}}$ mice. Bar = 50µm.

Table 1. Summary of clinical features in the UAKD patients analysed.

Uromodulin mutation	**Patient**	**Sex**	**Age at urine collection**	**Urine specific gravity**	**Urinary creatinine (mg/dl)**	**Serum creatinine (mg/dl)**	**Serum uric acid (mg/dl)**	**Gout**	**Renal ultrasound**
R212C	II 1	M	41	1010	65,5	1,08	6,2	No	NK
C256Y	II 4	M	35	1018	73	1,4	8,9	No	NK
C317Y	IV 5	M	18	1020	80	0,8	7,5	No	NK

NK, normal kidneys.

Table 2. Intensities of specific peptides belonging to wild-type and mutant uromodulin isoforms as detected in urine samples from UAKD patients and calculation of their relative abundance.

Sequence	Mass	Accession Number	PEP	Intensity sample	wt/mutant peptide intensity	%mutant *vs* wt
Patient II-1 (R212C)						
FVGQGGA**C**MAETCVPVLR	1836	IPI99999999	2,843E-3	703400	76,3	1,3%
FVGQGGAR	790	IPI00013945	9,630E-3	231460		
MAETCVPVLR	1117	IPI00013945	5,089 E-3	53459000		
Patient II-4 (C256Y)						
ACAHWSGHCCLWDASVQVK	2099	IPI00013945	7,90E-11	293380000	13,12	7,6%
ACAHWSGHC**Y**LWDASVQVK	2159	IPI99999996	3,85E-08	29253000		
KACAHWSGHCCLWDASVQVK	2228	IPI00013945	1,03E-14	90289000		
Patient IV-5 (C317Y)						
WHCQCK	803	IPI00013945	0,39716	2342100	2,80	35,7%
WHCQCKQDFNITDISLLEHR	2485	IPI00013945	6,92E-08	3591500		
WHCQ**Y**KQDFNITDISLLEHR	2545	IPI99999995	0,036102	2434900		
SNNGRWHCQCK	1331	IPI00013945	0,011313	281370		
SNNGRWHCQCKQDFNITDISLLEHR	3013	IPI00013945	0,025424	598510		

Mass: mass of the fragmented peptide. PEP: Posterior Error Probability (probability of a false hit given the peptide identification score and length of peptides. The mutated amino acid is highlighted in bold.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Western-blot analysis of HA-tagged uromodulin released after PLC cleavage from MDCK cell plasma membrane and deglycosylated with PNGase F or EndoH; control, untreated sample.

Supplementary Figure 2. Western-blot analysis of uromodulin from MDCK cells expressing myc-tagged wild-type and either wild-type or mutant HA-tagged isoforms. Both uromodulin present in the cell lysate (top row) and secreted in the medium (bottom row) are shown. Tubulin and transferrin are shown as loading controls.

Supplementary Figure 3. MS/MS spectra of the wild-type peptide (**a**) and the correspondent mutated peptide (**b**) obtained by analysis of uromodulin purified from the urine of patient with R212C mutation.

Supplementary Figure 4. MS/MS spectra of the wild-type peptide (**a**) and the correspondent mutated peptide (**b**) obtained by analysis of uromodulin purified from the urine of patient with C256Y mutation.

Supplementary Figure 5. MS/MS spectra of the wild-type peptide (**a**) and the correspondent mutated peptide (**b**) obtained by analysis of uromodulin purified from the urine of patient with C317Y mutation.

Supplementary Figure 6. Immunofluorescence performed on kidney sections of $\text{Tg}^{\textit{Umod}\text{wt}}$ and $\text{Tg}^{\textit{Umod}\text{C147W}}$ mice at 1 week of age showing transgenic HA-tagged uromodulin.

Uromodulin aggregates are present exclusively in TAL lumen of $Tg^{\mathit{Umod}\mathrm{C147W}}$ mice. Bar = 25 µm.

Supplementary Figure 7. Representative pictures for the quantification of polymer average area on unpermeabilised MDCK cells expressing myc-tagged wild-type and either wild-type or mutant (C317Y) HA-tagged uromodulin isoforms. Top row: merged myc (green) and HA (red) signal. Bottom row: selection outline defining quantified areas. Bar = 100 µm.