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MINI REVIEW

N-Glycans carried by Tamm-Horsfall glycoprotein have a crucial role in the defense against urinary tract diseases*

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Tamm-Horsfall glycoprotein (THGP), produced exclusively by renal cells from the thick ascending limb of Henle's loop, is attached by a glycosyl-phosphatidylinositol (GPI)-anchor to the luminal face of the cells. Urinary excretion of THGP (50–100 mg/day) occurs upon proteolytic cleavage of the large ectodomain of the GPI-anchored form. *N*-Glycans, consisting of a large repertoire of sialylated polyantennary chains and high-mannose structures, account for approximately 30% of the weight of human urinary THGP. We describe: (i) the involvement of urinary THGP high-mannose glycans in defense against infections of the urinary tract, caused by type-1 fimbriated *Escherichia coli,* which recognize high-mannose structures, (ii) the role of GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcNAcβ1-3Gal (Sd^a determinant) carried by human THGP in protecting the distal nephron from colonization of type-S fimbriated *E. coli* which recognise NeuAcα2-3Gal, (iii) the inhibitory effect of sialylated THGP on crystal aggregation of calcium oxalate and calcium phosphate, thus preventing nephrolithiasis. Finally, we outline the importance of *N*-glycans in promoting the polymerization of THGP, a process resulting in the formation of homopolymers with an M_r of several million in urine. Since THGP defense against diseases of the urinary tract mainly consists in binding damaging agents, its ability to behave as a multivalent ligand significantly enhances this protective role.
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Abbreviations: **COD, calcium oxalate dihydrate; COM, calcium oxalate monohydrate; *E. coli*, *Escherichia coli*; GPI, glycosyl-phosphatidylinositol; MCKD2, autosomal dominant medullary cystic kidney disease 2, PMN, polymorphonuclear leukocytes; TAL, thick ascending limb of Henle's loop; THGP, Tamm-Horsfall glycoprotein; UPI, uroplakins; UTI, urinary tract infections.**

Introduction

In 1950 Igor Tamm and Frank Horsfall at the Rockefeller Institute, New York, used a salt precipitation procedure on urine from healthy individuals to isolate a protein, which subsequently A. Gottschalk in Australia and L. Odin in Sweden demonstrated to have a very high carbohydrate content, including sialic acid [1–3]. The glycoprotein has since become known as Tamm-Horsfall glycoprotein (THGP). THGP is the most abundant protein in normal urine, about 50 mg of it being excreted daily [4]. Immunofluorescent microscopic analysis and THGP mRNA identification have demonstrated that biosynthesis of the glycoprotein occurs exclusively in cells of the thick ascending Henle loop (TAL) [5–9]. Consistently, THGP has been predominantly found by Western blot analysis in the outer medulla of the human kidney, where the TAL is located [10]. The cloning of human THGP-gene indicated that the predicted mature protein was of 616 amino acid residues in length [11]; after transfection of human-THGP cDNA into HeLa carcinoma cells, Rindler *et al.* [12] demonstrated that THGP is a glycosyl phosphatidylinositol (GPI)-anchored glycoprotein. After

*Dedicated to Winifred M. Watkins, who died on 3rd October 2003, and who contributed so much to identifying the Sd^a determinant structure expressed by Tamm-Horsfall glycoprotein.

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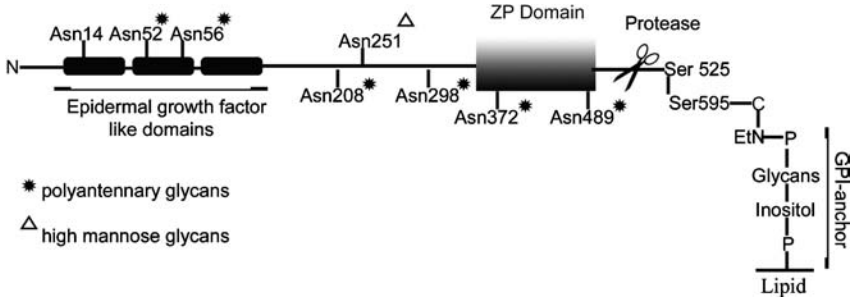

Figure 1. Schematic drawing of human THGP structure

the cleavage of the *C*-terminal hydrophobic peptide, which acts as a signal for the ER-transpeptidase responsible for the GPI-anchor addition, the length of the THGP protein-backbone has been estimated to be 595 amino acids (Figure 1). The GPI anchor ensures exposure of THGP at the cell plasma membrane [13] and, more importantly, vectorial delivery to the apical face in polarized cells [14]. These observations are consistent with evidence that THGP is almost exclusively excreted into the urinary fluid. Studies from our laboratory have shown that a proteolytic cleavage at the juxtamembrane ectodomain of GPI-anchored THGP is responsible for the release of THGP from both human kidney cells and THGP cDNA transfected cells [13,15]. According to Fukuoka and Kobayashi [16] the proteolysis occurs between amino acids 524 and 525 of the mature THGP-peptide.

Carbohydrate composition and structural analysis, first performed in Albert Neuberger's laboratory at St. Mary's Hospital in London, demonstrated that *N*-glycans represent about 30% of THGP weight and the apparent molecular weight of the monomer is close to 80 kDa [17–19]. The primary structure of several polyantennary chains carried by human THGP was elucidated in the laboratory of JFG Vliegenthart in Utrecht and a large repertoire of sialylated, fucosylated and sulfated polyantennary chains was described [20–25]. Interestingly, a tetrantennary species with one antenna linked by β1-6GlcNAc to the trimannosyl core, is largely present in human THGP, a result consistent with its high affinity-interaction with leukoagglutinin, the major lectin from *Phaseolus vulgaris*, frequently used as a leukocyte mitogen [26–28]. Our laboratory was the first to identify and characterize high-mannose chains in human urinary THGP [29,30]. Van Roojien *et al.* [24] have shown that 7 of the 8 potential *N*-glycosylation sites are actually glycosylated in human THGP and that high-mannose chains are carried by a single *N*-glycosylation site (Figure 1).

Various hypotheses have been formulated as to the physiological role(s) of THGP. For instance, on the basis of results in the late seventies indicating that only epithelial cells of the TAL (but not of the macula densa) were "covered" by THGP, the hypothesis was formulated that THGP functions as a water barrier and electrolyte transporter [6]. The rationale for this idea related to the fact that the TAL forms the nephron compartment in which water permeability is remarkably low whereas salts are efficiently absorbed. Since GPI-anchored proteins are only present in the outer leaflet of plasma membrane, it is unlikely that THGP functions as an ionic channel, and recent evidence indicates that water reabsorption is regulated by transmembrane proteins (aquoporins), which have a different efficiency in water transport along nephron segments [31]. More plausibly, GPI-anchored proteins are often involved in cell surface protection and recognition functions [32].

On the basis of *in vitro* studies indicating that THGP, particularly that from pregnant women, which it is also known as uromodulin, binds to recombinant interleukins-1 and -2 and recombinant tumor necrosis factor (TNF-α), it has been suggested that THGP may exert an immunoregulatory activity [33]. In fact, binding of THGP to native cytokines has not been observed [34], neither has any *in* vivo interaction been demonstrated. As mentioned above, it is worth noting that, under normal conditions, GPI-anchored THGP is exclusively exposed at the luminal face of TAL cells, from which it is released into urinary fluid by a proteolytic cleavage [35]. Because of this localization, it is unlikely that its physiological function is related to immunoregulation; it is more probable that the biosynthesis and urinary excretion of THGP are associated with the functionality of the distal nephron.

This review was prompted by recent results demonstrating that urinary THGP is indeed involved in defense against the most frequent diseases of the urinary tract, such as *Escherichia coli* infections [36–39] and nephrolithiasis [40,41]. These results are relevant in the context of this journal because *N*-linked glycans carried by THGP are actually responsible for protection against the above mentioned diseases. That is, high-mannose chains and the Sd^a carbohydrate antigen are involved in defense against *E. coli* infections, whereas THGP sialylation appears to play a role in the inhibition of urinary stone formation. The last section of this review reports observations suggesting that *N*-glycans contribute to the tendency of urinary THGP to form gels or to polymerise [42,43], a fact relating to its capacity to interact efficiently with causative agents of urinary tract diseases.

THGP high-mannose glycans in the defense against urinary tract infections

Urinary tract infections (UTI) are currently among the most common bacterial infections occurring in humans and *E. coli* is

the major causative agent [44]. Usually this pathogen originates in intestinal flora and enters the urinary tract by an ascending route via the urethra, a fact that explains the prevalence of UTI in women because of the anatomical difference between the sexes in the terminal portion of the urinary tract [45]. The vast majority of *E. coli* isolated from patients with UTI expresses type-1 fimbriae (also known as type 1 pili) which contain lectin-like adhesins, called FimH, that recognize high-mannose structures exposed by specific glycoproteins at the luminal face of urothelial cells [46–49]. This interaction is crucial for bacterial colonization [44]. All natural FimH variants recognize trimannosyl structures but mutated FimH adhesins also bind monomannosyl structures [50]. On the basis of their affinity binding to monomannosyl structures, FimH variants have been classified as high- and low-mannosyl binding type (M1H and M1L) and glycoproteins carrying high-mannose glycans, such as THGP or RNAase, bind to *E. coli* strains expressing M1H-FimH more efficiently than to strains expressing M1L-FimH [51]. Interestingly, the vast majority of uropathogenic type-1 *E. coli* express M1H fimbriae, whereas M1L variants are predominantly expressed by type-1 *E. coli* isolated from the large intestine [52].

High-mannose glycans have been found to be present in uroplakin 1 [53,54], which is one of a new class of integral membrane glycoproteins, called uroplakins (UPI), exposed at the luminal face of urothelial cells [55,56]. It has been shown that UPI 1 serves as a cell receptor for type-1 fimbrited *E. coli* both *in vivo* and *in vitro* and the binding is mannose specific and crucial for the colonization and invasion of pathogens into deeper tissues [36,52,57]. The last event is consistent with the frequent recurrence of UTI and the observation that more than 30% of *E. coli* isolates from patients with recurrent UTI are due to this pathogen [58].

Studies in the last century suggested that urinary THGP might be involved in the defense against type-1 *E. coli* infection of the urinary tract [59–61]. Both the location of biosynthesis and the structural characteristics of THGP appear suited to facilitating its protective role against UTI. For instance: (i) the synthesis of THGP occurs in the first tract of the distal nephron in which there is no protein reabsorption, a condition favoring the persistence of THGP in the urinary tract and (ii) the addition of a GPI anchor and the high level of *N*-glycosylation are supposed to mediate the sorting of GPI-anchored proteins to the luminal cell surface, a condition resulting in abundant excretion into the urinary fluid [32,62]. In respect to glycan structure, it is also relevant that high-mannose glycans are carried by THGP in all mammals analysed to date [63]. Moreover, recombinant THGP expressed by HeLa cells bears high-mannose chains very similar to that found in urinary form, indicating that the interruption of high-mannose processing at a single glycosylation site (Asp 252 in human THGP) is host cell independent, i.e., dictated by its peptide primary structure [64]. Finally, since THGP occurs in urine as large polymers [65], it behaves as a multivalent ligand which binds with high affinity type-1 fimbriated *E. coli.*

Table 1 shows the results from various studies on the binding of type-1 fimbriated *E. coli* to mannosyl structures with differing numbers of mannose residues [37,66–68]. The data clearly indicate that $Man_5GlcNAc_2$-Asn-R and Man6GlcNAc2-Asn-R bind to FimH adhesins and that the former structure (the final glycan structure formed by the action of Golgi α1,2-mannosidase) behaves as the best ligand for type-1 fimbriated *E. coli*. A possible explanation for these observations has been proposed by Nathan Sharon [68]: the high binding efficiency of $Man_5GlcNAc_2$-Asn- might depend on the fact that each monosaccharide of the Manα1,3Manβ1,4GlcNAc- branch is bound to one of three adjacent subsites of the FimH lectin-pocket. This explanation is consistent with the observation that Manα1-3Manβ1-4GlcNAc trisaccharide serves as an efficient inhibitor of hemagglutination induced by type-1 *E. coli* (Table 1).

Two recent studies by Bates *et al.* [38] and Mo *et al.* [39] have demonstrated that ablation of the THGP gene (THGP-knockout) results in an increased susceptibility of mice to bladder infection induced by type-1 fimbriated *E. coli*, but not by P-fimbriated *E.coli*. While this last result is consistent with evidence that P-fimbriated bacteria recognize the terminal Galα1-4Gal moiety of globosides, the two studies also provide convincing evidence that, due to its mannosyl chains, THGP strongly contributes to the defense against the most common urinary diseases in wild- type mice. It is worth noting that these results have been obtained for the first time by using an *in vivo* system. Previously, the effect of the interaction between urinary THGP and type-1 fimbriated *E-coli* was almost exclusively examined by means of *in vitro* systems with contrasting results. For instance, Kuriyama and Silverblatt [69] found that the ability of polymorphonuclear leukocytes (PMN) to ingest type-1 fimbriated *E. coli* was significantly reduced if the bacteria have been coated by THGP isolated from human urine. This observation may be interpreted as a condition which increases the bacterium virulence in urine. On the other hand, the authors in the Discussion of their article have pointed out that PMN are practically not active in the urinary tract because of the hyperosmolarity and acidic pH of urine and thus the reduced phagocytosis by PMN of bacteria coated with THGP is an event that very likely does not occur *in vivo*.

Bates *et al.* [38] have also reported that mice with an ablated THGP gene grow and breed normally, do not have changes in serum electrolytes and have a normal urinary composition. Moreover, no cysts or fibrosis in the kidney have been observed in THGP knockout mice up to 24 months of age. Recently, however, it has been demonstrated that autosomal dominant medullary cystic kidney disease 2 (MCKD2) and familial juvenile hyperuricemic nephropathy are associated with mutations of the THGP gene, which preferentially alter the number of cysteine residues of THGP [70–72]. Rampoldi *et al.* [73] have reported that in patients with MCKD2 there is an accumulation

Table 1. Oligomannoside-binding activity of type 1 fimbriated *E. coli* strain

Bacterium strain	Chemical nature	Oligomannoside structure	Relative activity
E. coli 346(025)	oligomannoside[a]	Manα1,2Manα1,6 Manα1,6 ManαOMe Manα1,3 Man	4.7
		Manα1,6 Manα1,3 Manα1,6 ManαOMe Manα1,3	30
		Manα1,3Manβ1,4GlcNAc	21
E. coli L74-30	glycopeptide[b]	$Man_6GlcNAc$-R Manα1,2Manα1,3 Manα1,3 Manβ1,4GlcNAcβ1,4GlcNAc—Asn Manα1,6 Manα1,6	7.5
		$Man_5GlcNAc$-R Manα1,6 Manα1,3 Manα1,6 Manβ1-4GlcNAcβ1–4GlcNAc—Asn Manα1,3	40
E. coli J96	human THP[c]	$Man_6GlcNAc$-R= 75% $Man_5GlcNAc$-R= 8%	1.04
	pig THP[c]	$Man_6GlcNAc$-R= 53% $Man_5GlcNAc$-R= 47%	6.22

[a] Compileted from data of Firon *et al.*,(66). The activity of each oligomannoside corresponds to inhibition of yeast cells agglutination relative to that of methyl-α-mannoside

[b] Compilated from data of Nesser *et al.*, (67). The activity of each ovoalbumin glycopeptide corresponds to inhibition of guinea-pig erythrocyte agglutination relative to that of methyl-α-mannoside

[c] Compilated from data of Cavallone *et al.*, (37). The relative activity corresponds to the capability of type-1 fimbriated *E. coli* to bind human- and pig-THP. The molar percentage of each oligomannoside assigned to human- and pig-THP was calculated assuming as 100% the total amount of oligomannosides carried by corrisponding THP

of misfolded THGP in the ER of TAL cells. This observation is consistent with our previous data indicating that when recombinant THGP is expressed by HeLa cells in the presence of exogenous reducing agents, which decrease the formation of intrachain disulfide bonds in THGP, the result is an intracellular accumulation of THGP bearing partially processed *N*-glycans and a delayed exit of THGP from ER to the Golgi apparatus [13]. Altogether these results support the notion that, just as bacteria, under evolutionary adaptation, have selected virulence factors, particularly adhesins with specific carbohydrate specificity, so the host has also developed appropriate defenses to withstand colonization.

Sd[a] carbohydrate antigen

In the late sixties Macvie *et al.* [74] and Renton *et al.* [75] first described a new isoantibody capable of causing agglutination of red cells among most of the adult Caucasian population. This antibody gave a weak hemagglutination and only a proportion of red cells of the same donor appeared to be agglutinated (mixed field agglutination). The antibody and antigen have been termed anti- Sd[a] antibody and Sd[a] antigen, respectively. Sd[a] antigen is not expressed in about 10% of the Caucasian population [Sda(−) individuals], but Sd[a] antibodies are detectable only in 2% of individuals. A few years after

its discovery, Sd^a antigen was found to be abundantly present in tissues and fluids, particularly in the urine of humans and other mammals [76]. This observation persuaded Walter Morgan and Winifred Watkins to identify the chemical structure of the Sd^a antigen present in urine, and they focused the attention on the THGP glycomoiety. In their laboratory, the carbohydrate analysis of THGP from Sda(+)- and Sda(−)-individuals first demonstrated that presence of GalNAc is associated with the Sda(+) phenotype, i.e. that GalNAc is the immunodominant sugar of the Sd^a antigen [77]. The same laboratory [78–80] established that the pentasaccharide GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcNAcβ1-3Gal has the highest activity in inhibiting the agglutination of Sda(+) erythrocytes by anti Sd^a antiserum (Table 2).

In subsequent studies Winifred Watkins and coworkers [81–83] found that urinary THGP from Sda(+) individuals, but not that from Sda(−) individuals, behaves as efficient receptor for lectins which recognize terminal GalNAc β-linked to Gal or GlcNAc. Interestingly, the interaction between ricin and Sda(+) THGP increased significantly when sialic acid was removed from the glycoprotein.

In our laboratory, it has been shown that β1,4-*N*-GalNAc-transferase requires the presence of NeuAc (not NeuGc) α2-3-linked to Gal for its task of adding the immunodominant sugar [84–86]. The tissue distribution of this β1,4-*N*-GalNAc-transferase (also termed Sd^a-transferase and homologous to the murine CT transferase [87]) correlates with the tissue localization of the Sd^a antigen, namely, it is particularly abundant in the human kidney and colon, and a soluble form is present in urine from Sd(a+) individuals [88–90]. In the human kidney, Sd^a-transferase has been exclusively detected in the outer medulla, the region comprising the thick ascending Henle limb, which expresses THGP [10]. In contrast to the modification of the *N*-glycans of THGP by this enzyme, the Sd^a antigen in the descending colon is present on core 3 O-glycans [91]; the distribution of the transferase decreases from the proximal to the distal portion [89]. The region-specific distribution of the Sd^a antigen in the human colon is thought by some to be an ecological system modulating bacterial colonization [92].

Like other glycosyltransferases responsible for the assembly of blood group antigens, Sd^a-transferase is onto/onco-logically regulated: thus for example the activity is very low in the kidney from neonatal guinea-pig, and in sucking rat colon, and is dramatically reduced in human colon carcinomas [89,93,94]. Recently it has been shown that the molar ratio between NeuAcα2-3Galβ1-4GlcNAcβ1-3Gal tetrasaccharide (THGP substrate for Sd^a-transferase) and GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcNAcβ1-3Gal pentasaccharide is very similar in two pairs of homozygous twins [95], whereas the same ratio varies significantly in genetically unrelated individuals [22]. These observations support the notion that Sd^a -transferase activity is genetically strictly regulated.

In human THGP, Sd^a epitopes predominate at the non-reducing terminal end of tetrantennary chains elongated by repeating *N*-acetyllactosamine units, suggesting that exposure of the NeuAcα2-3Galβ1-4GlcNAc-substrate on a longer carbohydrate chain facilitates the action of Sd^a-transferase towards THGP during its progress through the secretory pathway [23]. On this basis, one may speculate that the NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-pentasaccharide from GPI-anchored THGP is exposed at the luminal cell membrane in a way that facilitates its recognition by specific ligands and that the addition of GalNAc is an event evolutionarily selected by the host in order to hinder the binding of damaging agents which might recognize α2-3-sialylated glycans [82]. The dominance of the most important carbohydrate blood group-characters, i.e.,

Table 2. Structure and Sd^a activity of various oligosaccharides in the hemagglutination test[a]

Structure	Inhibition of Sd^a+ erythrocyte agglutination by Sd^a antiserum (Sd^a antiserum µg/µl)
GalNAcβ1,4 \ Galβ1,4GlcNAcβ1,3Gal / NeuAcα2,3	1.5
GalNAcβ1,4 \ Galβ1,4GlcNAc / NeuAcα2,3	3
GalNAcβ1,4 \ Galβ1,4Glc / NeuAcα2,3	50

[a]Compilated from data of W. M. Watkins [79]

the A, B, O system, has actually been correlated with resistance to specific bacterium and virus infections [96]. Considering that the large intestine and distal kidney are the main locations for *E. coli* infections, one may postulate that this type of infection has served as a selective agent for the high expression of Sd^a-transferase in these tissues in order to interfere with adhesion by type-S *E. coli* expressing adhesins recognizing the NeuAcα2-3Galβ sequence [68]. The predominant expression of Sd^a antigen and Sd^a- transferase in the colon of humans and other mammals is consistent with this idea. In fact, type-S fimbriated *E. coli* with a high affinity for sialylated chains have been found to adhere to intestinal cells only in neonatal animals, remaining until they are a few weeks old, a time period in which the expression of Sd^a-transferase is either low or non-existent [97].

All these observations support the notion that the expression of the Sd^a determinant in THGP may result in some form of protection against type-S *E. coli* infections. On the other hand, the mechanism of protection appears to differ from that utilized by urinary THGP in the defense against UTI caused by type-1 fimbriated *E. coli.* In this case, urinary THGP interacts strongly by way of its high-mannose glycans, with the latter pathogens and this binding facilitates their being washed away with urine. In contrast, the addition of β1-4GalNAc to the NeuAcα2-3Galβ moiety of THGP results in a reduced interaction between type-S fimbriated *E. coli* and THGP. We suggest that addition of the Sd^a determinant in GPI-anchored THGP, rather than in urinary THGP, is an event favourable to the host, in that the reduced adhesion of S-fimbriated *E. coli* to TAL cells inhibits their invasion in renal tissue. This hypothesis is consistent with experimental and clinical evidence that type-S fimbriated *E. coli* have been prevalently isolated from patients with pyelonephritis rather than bladder infections [44,98]. The second hypothesis, which is entirely speculative, is that the conformation of urinary THGP in large polymers results in exposure of more accessible high-mannose than that of α2-3-sialylated glycans, so that in urinary THGP the latter structure is not available for binding pathogens.

THGP sialylation and the defense against nephrolithiasis

Urine supersaturation with components forming crystals, such as calcium oxalate, calcium urate and calcium phosphate, is a consequence of one of the most important physiological functions of the kidney: water conservation. Supersaturation of the urinary fluid starts in Henle's loop and distal nephrons and when it is high enough crystal nucleation of the supersaturated salts takes place and crystals are discarded with the urine (crystalluria) or deposited in renal tissue without any apparent ill effect [40]. Neprolithiasis occurs when the microscopic crystals undergo aggregation and grow into macroscopic stones [99]. To maintain homeostasis along the urinary tract, urine contains several inhibitors of crystal aggregation and stone formation, but the inhibitory activity is predominately associated with high-molecular weight components having a polyanionic structure, such as heparin and chondroitin sulfate and proteins with acidic isoelectric points [100]. The proteins either belong to the family of aspartic acid-rich phosphorylated proteins or are glycoproteins carrying sialylated, sulfated glycans and uronic acid [100–105]. There is a consensus that these urinary components are very effective in inhibiting the aggregation of calcium oxalate and calcium phosphate crystals, but their involvement in the crystal nucleation step cannot be excluded [106].

Since THGP is, by far, the predominant glycoprotein occurring in the urine and carries several NeuAc residues and sulfate groups, close attention has been paid to understanding how exactly it contributes to preventing nephrolithiasis. For instance, several studies have investigated whether nephrolithiasis patients have reduced urinary excretion of THGP, but the results have been controversial [107–109]. In fact, reduced excretion of urinary THGP is very often associated with damage to the tubular epithelial cells expressing THGP, and this event may be an effect rather than a cause of stone formation. Other studies have aimed to ascertain whether deficient THGP sialylation occurs in former stone patients, but again divergent results have been observed [110–113]. THGP excretion and structure have also been investigated in a rat neprolithiasis model based on a diet supplemented with ethylene glycol (an oxalate precursor) and vitamin D3 which enhances calcium absorption [9,40,114]. These studies have shown that (i) urinary THGP reduces the aggregation of calcium oxalate crystals *in vitro*, (ii) THGP excretion is decreased only after deposition of aggregate crystals in rat kidneys, a finding suggesting that cell damage is an effect induced by crystal aggregates and (iii) in nephrolithic rats the content of sialic acid THGP is slightly less than in control rats.

The last observation is relevant considering that partial removal of sialic acid by sialidase treatment from human urinary THGP results in a loss of inhibition of crystal calcium oxalate aggregation *in vitro* [115,116]. Very recently, an important study [41] has provided the first evidence that absence of THGP in mice (i.e., in THGP knockout animals) significantly increases the formation of calcium oxalate stones in the kidney. This study has shown that calcium oxalate stones are formed at a high level when knockout mice are fed with ethylene glycol and vitamin D3, but a significant presence of crystal aggregates is found also in knockout mice on a normal diet. Osteopontin (OPN)-knockout mice too develop renal calcium oxalate deposits under a diet supplemented with ethylene glycol and vitamin D3, but not under a normal diet [117]. Comparison of the two studies supports the notion that, under normal conditions, THGP is the main actor in the defense against nephrolithiasis. Recently, it has been reported that the removal of sialic acid by sialidase from glycoproteins and glycolipids exposed at the luminal face of renal cells disturbs the stereospecific interaction between cells and calcium oxalate dihydrate (COD) crystals, the most common crystals in human urine [118]. COD crystals employ a different face in nucleating onto sialidase-treated renal cells than towards untreated cells, a result indicating that sialic acid residues exposed by surface glycoproteins are crucial

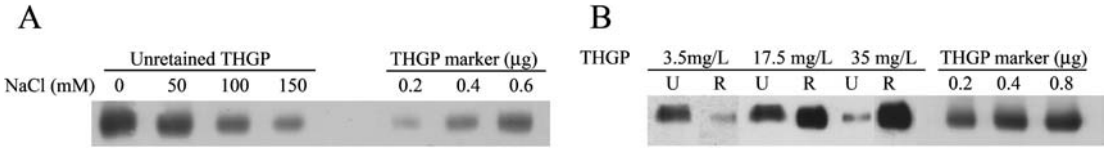

Figure 2. Effect of NaCl addition and THGP concentration on the ability of a diatomaceous earth filter to retain the glycoprotein isolated from human urine. (A) Aliquots of 0.1 ml of NaCl solutions containing ten times the salt concentration indicated in the figure were added to 0.9 ml of a stock solution of THGP (35 mg/L) in deionized water. Samples were filtered through diatomaceous earth layer and filtrated fluids containing unretained THGP collected. Aliquots of 30 μl were applied to SDS-PAGE (8.5% acrylamide) performed in non reducing conditions. Western blotting was developed with anti-human IgG antibodies and anti-THGP antibodies. (B) Aliquots of 1 ml of 150 mM NaCl solution containing increasing concentrations of THGP (from 3.5 mg/L to 35 mg/L) were filtered through diatomaceous earth layer. The retained THGP (R) was removed by diatomaceous earth layer by means of deionised water as described in [21]. The unretained THGP (U) was collected as in A. Aliquots of 0.5 ml of solutions containing either unretained- or retained-THGP were dialyzed against water, lyophilized and solubilized in 100 μl of distilled water. Aliquots of 30 μl of each sample were applied to of SDS-PAGE and western blotting was carried out as in (A). Note that the proportion of retained THGP versus the unretained form drammatically increased as a function of THGP concentration.

in determining a specific chemical binding to COD crystals. In another study [119] the interaction of sialic acid exposed at the surface of MDCK cells with calcium oxalate monohydrate (COM) crystal was analyzed at different times of cell maturation. This study demonstrates that (i) the expression of NeuAc at the MDCK cell surface increases with cell differentiation, (ii) COM crystal-binding to undifferentiated MDCK cells is reduced by desialylation and (iii) the higher sialylation observed in well differentiated cells is accompanied by a reduced extent of COM crystal binding. It is possible that a relatively low presence of sialic acid or a peculiar exposure at the cell surface may be crucial in modulating the binding of COM crystals to the renal cell surface. Taking into account these observations and the fact that GPI-anchored THGP is abundantly exposed at the luminal face of the first tract of distal nephrons, its involvement in calcium oxalate nephrolithiasis, via sialic acid residues, might be relevant even before its release into the urinary fluid.

N-Glycans are involved in the gelation/polymerization tendency of urinary THGP

Analyses performed by electron microscopy have shown that urinary THGP molecules are filamentous, having lengths of about 25000 Å, with extensive intertwining of fibrils that are organized prevalently in large networks [65,120]. This conformation depends on the property of THGP to aggregate and polymerise when the NaCl- and $CaCl_2$-concentration in the solvent is close to 100 mm and 2 mm, respectively [121,122]. Since both conditions occur in the urinary fluid circulating inside the distal nephron, the urinary THGP actually takes the form of large polymers with a *Mr* of several million [123] while the monomer has, according to SDS-PAGE under either non-reducing or reducing conditions, apparent molecular weights of 80 and 90 kDa, respectively [15]. Recently, Jovine *et al.* [65] have shown that the THGP *C*-terminal ZP domain of about 48 kDa, which has homology with both the ZP2- and ZP3-glycoproteins from zona pellucida of mammalian eggs, is responsible for the polymerisation of urinary THGP and other glycoproteins containing such domains. The polymerisation of THGP monomers released into the urine from the GPI− anchored counterpart is responsible for "THGP gelation", a condition which, in proteinuric patients, particularly those with multiple myeloma-containing immunoglobulins (IgG) and IgG light chains, results in the formation of hyaline casts [124]. A purification method based on the gelation tendency of THGP from urine of healthy individuals has been devised in our laboratory [125,126]. Figure 2 shows that, only at a NaCl concentration close to iso-osmolarity, the vast majority of urinary THGP is retained by diatomaceous earth, i.e., is aggregated in large polymers. The polymerization of THGP is also dependent on its concentration in solution. Figure 2B shows that only at a concentration close to that occurring in normal urine (35 mg/L) THGP is efficiently retained by the diatomaceous earth-layer (Monti, unpublished results). This result suggests that a poor urinary excretion of THGP, which has been found in various kidney diseases [127,128] may cause deficient THGP polymerization.

Under non-denaturing conditions, *N*-glycanase digestion of polymeric THGP from normal urine abolishes both the property of forming a gel and the capacity of interacting with pig renal tubular cells [42]. Moreover the reduced extent of sialylation in urinary THGP induced in humans by colchicine treatment results in a decreased ability of THGP to form gel as well as to aggregate with Bence-Jones protein [43]. Interestingly, colchicine treatment of two unrelated volunteers reduced the capping of THGP *N*-glycans by NeuAc in α2-3- and 2-6-linkages to the subterminal Gal residue [43]. The mechanism by which colchicine interferes with the activity of both sialyltransferases has not been clarified, but it may be of interest to note that, in healthy individuals, α2-3- and 2-6-sialyltransferases are preferentially expressed in the renal outer medulla where THGP is synthesized, rather than in the renal cortex [10].

Concluding remarks

Invading microorganisms take advantage of the carbohydrate moieties of glycoproteins exposed at the cell surface of epithelial cells to interact with the host's tissues, this binding being the first step of their invasive process. Uroplakins cover about 90% of the luminal face in urothelial cells and expose both high-mannose and α2-3- and α2-6-sialylated glycans [54,55]. Thus these glycoproteins are responsible for the adhesion and colonization of uropathogenic *E. coli* strains, particularly in the bladder [36,44]. In this review we have attempted to show that also urinary THGP represents a very effective defense against the most frequent infections of the urinary tract caused by type-1 fimbriated *E. coli*. This pathogen, because of its high-affinity binding to high-mannose chains, is trapped by urinary THGP and rapidly eliminated from the body with the urine. Thus, the excretion of THGP in urine at a rate of 30–50 mg /l appears to be an event with a high advantage for the host. Consistent with this idea, there are observations indicating that the affinity binding of urinary THGP to type-1 fimbriated *E.coli* is modulated in various species according to the respective risk of infection [37]. For example in the pig, a mammal with a high incidence of urinary tract infections caused by type-1 fimbriated *E.coli*, urinary THGP contains a very high proportion of the $Man_5GlcNAc_2$-Asn-R structure, which exhibits the highest affinity binding to this pathogen (Table 1).

Another remarkable characteristic of urinary THGP is its conformation. The high salt concentration in urine, usually over iso-osmolarity, causes aggregation of urinary THGP in large homopolymers, behaving as multivalent ligands. In this way the affinity binding between THGP and pathogens increases several fold and consequently so does its protective effect against infection.

To date, the majority of studies on THGP, regarding both its physiological function(s) and pathological implications, have only considered the urinary form. In this review, we have pointed out that the GPI-anchored counterpart may also play a direct role in the defense against infection caused by type-S *E. coli*. We have proposed that the addition of β1-4-N-GalNAc to NeuAcα2-3Galβ1-4-R by Sd^a- transferase interferes with the adhesion of type-S fimbriated *E. coli* to GPI-anchored THGP exposed at the luminal face of TAL cells. The high incidence of renal infections caused by this bacterium is consistent with its tendency to ascend to the upper tract of distal nephron [129], where THGP biosynthesis and assembly of the Sd^a determinant take place. In this case, the expression of Sd^a-transferase should be an evolutionary advantage selected by the host in order to prevent infection caused by type-S *E. coli*, which recognize the NeuAcα2-3Galβ1-4-sequence.

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