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Variation of High Mannose Chains of Tamm-Horsfall Glycoprotein Confers Differential Binding to Type 1-fimbriated *Escherichia coli**

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Daniela Cavallone‡, Nadia Malagolini‡, Angela Monti‡, Xue-Ru Wu§, and Franca Serafini-Cessi‡¶

From the ‡Department of Experimental Pathology, University of Bologna, Bologna 40126, Italy and the §Departments of Urology and Microbiology, New York University School of Medicine, New York, New York 10016

Tamm-Horsfall glycoprotein (THP), the most abundant protein in mammalian urine, has been implicated in defending the urinary tract against infections by type 1-fimbriated E*scherichia coli*. Recent experimental evidence indicates that the defensive capability of THP relies on its single high mannose chain, which binds to *E. coli* FimH lectin and competes with mannosylated uroplakin receptors on the bladder surface. Here we describe several major differences, on both structural and functional levels, between human THP (hTHP) and pig THP (pTHP). pTHP contains a much higher proportion (47%) of $Man_5GlcNAc_2$ than does hTHP (8%). FimH-expressing *E. coli* adhere to monomeric pTHP at an approximately 3-fold higher level than to monomeric hTHP. This suggests that the shorter high mannose chain ($Man_5GlcNAc_2$) is a much better binder for FimH than the longer chains ($Man_{6-7}GlcNAc_2$) and that pTHP is a more potent urinary defense factor than hTHP. In addition, unlike hTHP whose polyantennary glycans are exclusively capped by sialic acid and sulfate groups, those of pTHP are also terminated by Galα1,3Gal epitope. This is consistent with the fact that the outer medulla of pig kidney expresses the α1,3-galactosyltransferase, which is completely absent in human kidney. Finally, pTHP is more resistant to leukocyte elastase hydrolysis than hTHP, thus explaining why pTHP is much less prone to urinary degradation than hTHP. These results demonstrate for the first time that the species variations of the glycomoiety of THP can lead to the differential binding of THP to type 1-fimbriated *E. coli* and that the differences in high mannose processing may reflect species-specific adaptation of urinary defenses against *E. coli* infections.

Escherichia coli is the major causative agent of urinary tract infection, the most common nonepidemic bacterial infection in humans and domestic animals (1). This pathogen enters the urinary tract by an ascending route from the intestinal flora, and the critical first step in colonization relies on bacterial binding to carbohydrate sequences carried by glycoproteins and glycolipids exposed at the luminal surface of the urinary tract (2). This binding is mediated by lectin-like adhesins on the tip of *E. coli* fimbriae, which are classified according to their sugar specificity. Thus, type 1, P and S fimbriae recognize high mannose glycans, Galα1,4Galβ terminal disaccharide of glycolipids, and NeuAcα2,3Gal sequence-capping sialylated glycans, respectively (3). Within the type 1 fimbriae, phenotypic variants of FimH adhesin have been identified, based on their binding affinity to high mannose glycans (4). Interestingly, FimH variants exhibiting low affinity binding to high mannose glycans (M_1L) predominate in *E. coli* isolates from the large intestine, whereas those exhibiting high affinity to high mannose glycans (M_1H) predominant in *E. coli* isolates from the urinary tract (5). These results suggest that there is a selective advantage in the urinary tract for the *E. coli* strains expressing a particular type of FimH adhesin.

During the advanced stage of cellular differentiation, mammalian urothelium elaborates a group of integral membrane proteins called uroplakins (6–9). Together, these glycoproteins constitute the major protein building blocks of the asymmetric unit membrane, a rigid-looking structure that covers over 90% of the luminal surfaces of the proximal urethra, bladder, ureter, and renal pelvis (10, 11). Although uroplakin III carries complex-type *N*-glycans whose NeuAcα2,3Gal sequences may serve as receptors for S-fimbriated *E. coli*, uroplakins Ia and Ib carry a single high mannose glycan (7, 12). *In vitro* experiments have demonstrated that type 1-fimbriated *E. coli* binds to uroplakins Ia and Ib in a mannose-specific fashion (9). Infection of mouse bladders with type 1-fimbriated *E. coli* showed that the FimH-containing tip regions of type 1 fimbriae adhere to the central depression of asymmetric unit membrane (AUM) plaques, where uroplakin Ia and Ib reside (13). These findings indicate that uroplakins serve as the major urothelial receptors for type 1-fimbriated *E. coli*.

If the expression of adhesins recognizing various carbohydrates comes about as a natural selection for *E. coli* to adapt to specific habitats, the host itself, under selective pressure, has developed defense mechanisms against bacterial adhesion and colonization. In the respiratory and intestinal epithelium, for instance, the abundant mucus covering the epithelium prevents the adhesion of pathogens to glycoproteins and glycolipids exposed at the luminal membrane. This type of mucus defense is largely absent in the urothelial surface; however, it has been recently suggested that urinary defense against bacterial adhesion may depend on soluble glycoprotein receptors in the urine (1, 14, 15). In fact, the kidney cells of the thick ascending Henle's limb release into the urine from the GPI-anchored counterpart a protein at a rate of approximately 50 mg daily in humans, which is particularly rich in carbohydrates (30% of total weight) (16–19). This glycoprotein was first purified by Igor Tamm and Frank Horsfall (20) from healthy individuals, hence the name Tamm-Horsfall glycoprotein

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¶ To whom correspondence should be addressed: Dept. Experimental Pathology, Via S. Giacomo 14, 40126 Bologna, Italy. Tel.: 39-051-2094723; Fax: 39-051-2094746; E-mail: serafini@alma.unibo.it.

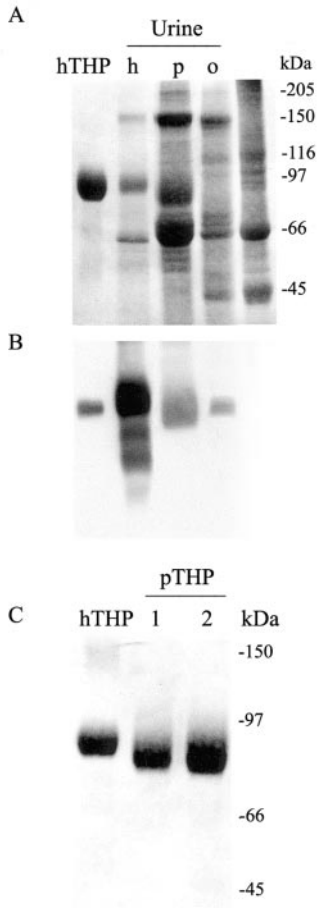


FIG. 1. **Electrophoretic analyses of total urinary proteins and purified THPs from different species.** *A*, Thirty microliters of 10-fold concentrated urine samples from human (*h*), pig (*p*), and oxen (*o*) were applied onto an 8.5% SDS-PAGE under nonreducing conditions. The proteins were visualized by Coomassie Blue staining. *B*, urinary THPs were identified by Western blotting using an anti-hTHP antibody. *C*, electrophoretic mobility of hTHP purified by the DEF method and of pTHP purified by the DEF or the salt precipitation method (*lanes 1* and *2*, respectively). Note that pTHP migrated faster than hTHP.

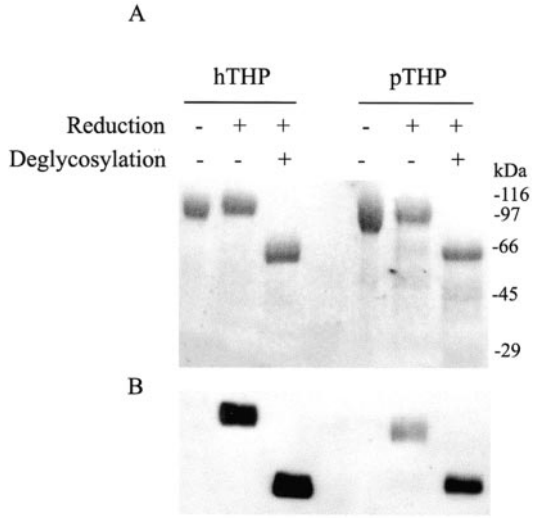


FIG. 2. **Effect of deglycosylation on electrophoretic mobility of hTHP and pTHP.** Reduced and alkylated THPs were incubated with PNGase F, electrophoresed in an 8.5% SDS-PAGE, and visualized either by Coomassie Blue staining (*A*) or by Western blotting using an anti-hTHP antibody (*B*). Note that deglycosylation abolished the difference of electrophoretic mobility between hTHP and pTHP.

(THP).[1] It was later detected in the urine of all mammals studied, and the amino acid sequences predicted by cDNA show a high degree of homology among THPs from various species (21–24). The glycomoiety of human THP (hTHP) consists mainly of polyantennary *N*-glycans, but a single *N*-glycosylation bears high mannose sequences in both native THP and recombinant THP expressed in transfected cells (25–30). One of our laboratories (15) demonstrated that (i) hTHP is the main urinary protein binding specifically to type 1-fimbriated *E. coli*; (ii) removal of high mannose glycans from hTHP annuls this binding; and (iii) binding of the *E. coli* to uroplakin receptors is blocked by purified THP. These results suggest that by competing with urothelial receptors for type 1-fimbriated *E. coli*, urinary THP can prevent the *E. coli* from binding to the urothelial surface and that THP can serve as a major urinary defense factor against bacterial infections.

In the present study, we reported several major species-specific differences in the chemical properties of THPs. We provided evidence that pTHP and hTHP differ significantly in their high mannose composition and that this difference results in their differential binding to the type 1-fimbriated *E. coli*. In addition, we demonstrated that pTHP and hTHP have different susceptibility to leukocyte elastase, thus providing an explanation for the different degrees of urinary degradation of the THPs. Finally, we showed that the species-specific expression of glycosyltransferases is responsible for different terminal sugar modifications of THPs in different species. These results document the species variations of the THPs and have functional implications on host adaptation to bacterial colonization and infections.

EXPERIMENTAL PROCEDURES

Materials—Human urine was collected over 24 h from four healthy individuals. Bovine and pig urine was removed immediately postmortem from the bladders of two and three animals, respectively. Antiserum to human THP was raised in rabbits as previously described (31). Reduction/alkylation of THPs was performed as described by van Rooijen *et al.* (30). Biotin-labeled concanavalin A (ConA), biotin-labeled *Griffonia simplicifolia* isolectin B4 (GS-IB4), anti-rabbit IgG conjugated with horseradish peroxidase, and pancreatic and leukocyte elastase were purchased from Sigma. Endoglycosidase H was from the Seikagaku Corporation. Glycopeptidase F (PNGase F) was from Roche Applied Science. Horseradish peroxidase-labeled streptavidin blocking reagent and ECL™ Western blotting reagent were from Amersham Biosciences, UK. [^{3}H]KBH_4 (67 mCi/mmol) and UDP[^{14}C]Gal (325 Ci/mol) were from Amersham Biosciences. Bio-gel P-10 (fine) was from Bio-Rad. All other chemicals were of reagent grades. Modified glucose-free Eagle's medium was from Invitrogen.

Bacterial Strains, Culture, and Metabolic Labeling—The P678-54 strain is a minicell-producing *E. coli* K12 derivative that expresses no fimbriae; J96 strain is a human pyelonephritis *E. coli* isolate expressing both type 1 and P fimbriae, whereas SH48 and HU849 are two recombinant strains derived by transfecting the nonfimbriated P678-54 strain with J96 genomic DNA fragments encoding the type 1 and P fimbriae (PapG1), respectively (32–34). KB96 and KB91 are two recombinant strains obtained by transfecting a *fimH*-null *E. coli* AAEC191A strain with *fimH* genes isolated from urinary tract infection or from the intestine, respectively; of these, the former strain expresses M_1H fimbriae, and the latter M_1L fimbrial variants (5). The fimbrial expression of clinical and recombinant *E. coli* strains was determined by yeast aggregation and hemagglutination as previously described (5, 9). All strains were cultured in Lennox-Broth medium (Sigma) at 37 °C for 16 h, in methionine and cysteine-free Eagle's medium for 2 h, and then in Eagle's medium containing [^{35}S]methionine and [^{35}S]cysteine (PerkinElmer Life Sciences) for 2 h at room temperature. The labeled *E. coli* were washed four times in 0.02 M sodium phosphate buffer, pH 7.5, containing 0.14 M NaCl (PBS) and stored in PBS containing 30% glycerol at −80 °C until use.

[1] The abbreviations used are: THP, Tamm-Horsfall glycoprotein; hTHP, human THP; pTHP, pig THP; ConA, concanavalin A; GS-IB4, *G. simplicifolia* isolectin B4; PNGase F, glycopeptidase F; PBS, phosphate-buffered saline; DEF, diatomaceous earth filter; HPLC, high pressure liquid chromatography; BSA, bovine serum albumin.

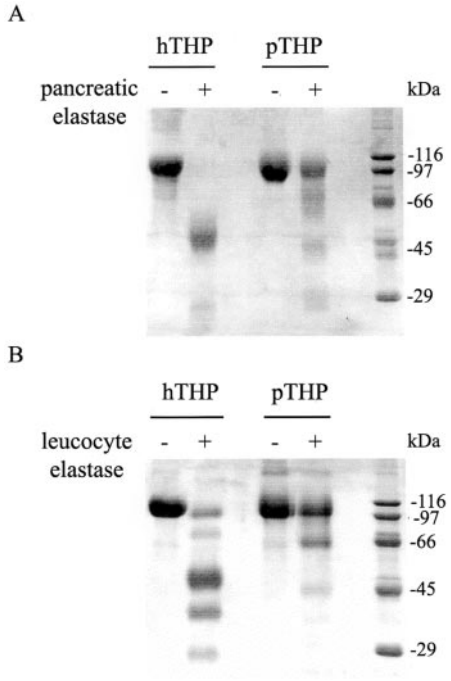
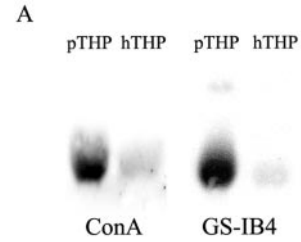


FIG. 3. **Resistance of hTHP and pTHP to pancreatic or leukocyte elastase.** Purified THPs in native forms were treated with pancreatic elastase (*A*) or with leukocyte elastase (*B*), followed by electrophoresis in a 10% SDS-PAGE in reducing conditions and Coomassie Blue staining. Note that pTHP is more resistant to the digestion by both enzymes than hTHP.



B

Source of enzyme	α1,3Gal-T activity (nmol/mg/h)
h-kidney (OM)	< 0.01
p-kidney (OM)	2.20

FIG. 4. **Reactivity of THPs to plant lectins and detection of oligosaccharide transferase in the kidneys.** *A*, equal amounts (15 μg) of pTHP and hTHP were resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and probed with ConA or GS-IB4 as described in the text. Note the much stronger reaction of ConA and GS-IB4 to pTHP. *B*, the activity of α1,3Gal-transferase in the outer medulla (*OM*) of human and pig kidney was assayed. Note the strong activity of the enzyme in pig kidney but not in human kidney.

Purification of Urinary THPs and Preparation of THP Monomers—THPs were purified from pooled urine by either a diatomaceous earth filter (DEF) method (35) or by the Tamm and Horsfall method (20) with minor modifications. The urine was diluted with an equal volume of distilled water and brought to 0.58 M NaCl. After incubation at 4 °C for 16 h, the insoluble material was collected by centrifugation, resuspension in deionized water, and reprecipitation in the presence of 0.58 M NaCl. This step was repeated twice, and the final suspension was dialyzed exhaustively against deionized water. The monomeric forms of hTHP and pTHP were prepared by dissolving DEF-purified THPs (20 mg each) in phosphate buffer containing 8 M urea (pH 6.8) (36). The solutions were then extensively dialyzed against deionized water and lyophilized.

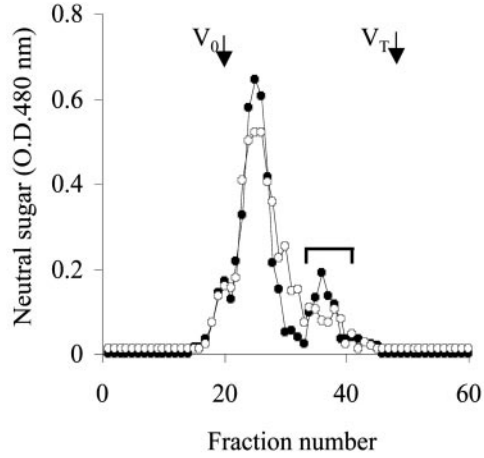


FIG. 5. **Detection of neutral sugars in Pronase-glycopeptides from hTHP and pTHP.** Glycopeptides of hTHP (●) and pTHP (○) were resolved by Bio-Gel P10 fractionation, and neutral sugars were detected in 50 μl of each fraction. The fractions under the *bracket* corresponding to the elution volume of high mannose glycopeptides from ovalbumin were pooled and lyophilized for further analysis.

Electrophoresis and Western Blotting—SDS-PAGE, at 8.5% or 10% acrylamide (see the figure legends), was performed as described (16). When electrophoresis was performed in reducing conditions, β-mercaptoethanol was added to a final concentration of 1.5% (v/v). The proteins were either stained with Coomassie Brilliant Blue or transferred electrophoretically onto nitrocellulose membrane and probed with anti-THP antibodies followed by anti-rabbit IgG conjugated with horseradish peroxidase (16). When the reactivity to lectins was analyzed, the blots were treated with 2.5% blocking reagent (Amersham Biosciences) and then incubated with biotinylated ConA lectin or with biotinylated GS-IB4 followed by streptavidin conjugated with horseradish peroxidase as previously described (37). All of the blots were developed with ECL™ Western blotting reagent as recommended by the supplier.

PNGase F and Elastase Digestion—Reduced/alkylated THPs were treated with PNGase F in 200 mM sodium phosphate buffer (pH 7.5) containing 50 mM EDTA, 0.1% Triton X-100 at 37 °C for 24 h. Alternatively, THPs were digested with pancreatic elastase or leukocyte elastase at an enzyme:protein ratio of 1:25 for 16 h as described by Jovine *et al.* (38).

Preparation and Analysis of High Mannose Glycans—Approximately 35 mg of hTHP or pTHP were digested with Pronase (2 mg) at 60 °C for 48 h as previously described (39). The Pronase glycopeptides were fractionated on a column (1 × 80 cm) of Bio-Gel P10 (fine) equilibrated with 0.1 M NH_4HCO_3. The distribution of glycopeptides was identified by the phenol-sulfuric acid test (40). The fractions corresponding to the elution position of high mannose glycopeptides were pooled, desalted on a Bio-Gel P2 column, and lyophilized. The high mannose glycopeptides were solubilized in 0.2 ml of 0.2 M sodium citrate buffer at pH 5 and subjected to endoglycosidase H treatment (27). After reduction by $[^3H]KBH_4$, the oligosaccharides released were separated by HPLC with a water apparatus equipped with a LiChrosorb diol column eluted with 70:30 (v/v) acetonitrile:water. The 0.5-ml fractions were collected and counted for radioactivity.

Assay of α1,3Gal-transferase from Human and Pig Kidney—Homogenates of outer medulla from human and pig kidney were obtained as previously described (16). The assay mixture contained 0.1 M sodium cacodylate buffer, pH 6.0, 5 mM $MnCl_2$, 0.5 mM UDP-[^{14}C]Gal (5.6 dpm/pmol), 0.5% Triton X-100, 6.4 mM ATP, and 0.65 μmol of *N*-acetyllactosamine as an acceptor in a total volume of 50 μl and 70–80 μg of protein homogenate. Incubation was performed at 37 °C for 2 h and then stopped with 1 ml of cold water, and the mixture was passed through a column (1 × 3 cm) of Dowex 1 × 8 (Cl^-) equilibrated with water. The column was eluted with 3 ml of water. The eluted sample was lyophilized and analyzed by HPLC as previously described (41). The fractions with the retention time of the trisaccharide (Galα1,3Galβ1,4GlcNAc) were counted for radioactivity.

Solubility of THPs in Both Polymeric and Monomeric Forms—Purified THPs in polymeric and monomeric forms were dissolved in deionized water at a concentration of 0.4 mg/ml to obtain an optical density

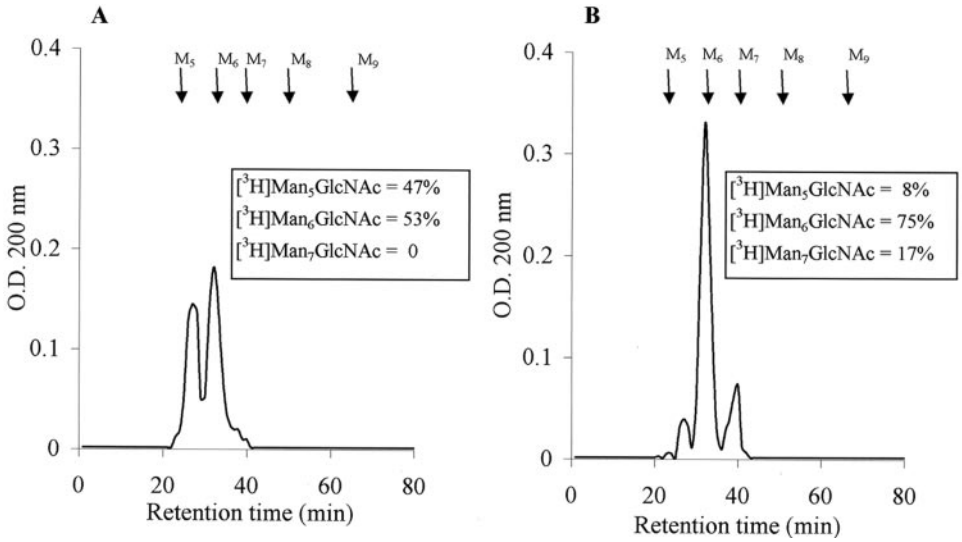


FIG. 6. **Fractionation of high mannose-derivatives from pTHP (*A*) and hTHP (*B*).** ^{3}H-Labeled high mannose-derivatives released by endoglycosidase H were fractionated by HPLC and detected by UV absorbance at 200 nm. An aliquot of all fractions was counted for radioactivity, and the molar percentage of each high mannose sequence (*inset*) was calculated assuming the sum of the radioactivity of all fractions as 100%. M_{5-9} are abbreviations for Man$_{5-9}$GlcNAc, respectively. The oligomannoside standards were isolated from unit A of thyroglobulin as previously described (47). Note the much higher proportion of Man$_5$GlcNAc in pTHP than in hTHP.

close to 0.400 at 277 nm. Each solution was divided into samples of 0.9 ml, to which 0.1 ml of a NaCl solution was added at increasing concentrations ranging from 0.4 to 1.6 M. In the control samples, 0.1 ml of deionized water was added. The samples were left at room temperature for 30 min and then centrifuged in a microcentrifuge (ALC International) for 30 min at 15,000 rpm, and the optical density of supernatants read at 277 nm.

E. coli Binding Assay—The monomeric forms of hTHP and pTHP and BSA were dissolved in distilled water to different concentrations, and 100-μl aliquots were applied to a 96-well polystyrene microtiter plate. The solutions were left at room temperature for 30 min and at 4 °C for 16 h. After washing, the unoccupied sites were blocked with 3% BSA made in PBS and 0. 1% NaN_3 for 2 h and then incubated for 2 h at room temperature with [^{35}S]methionine/cysteine-labeled *E. coli* strains reconstituted in PBS containing 3% BSA and 0.1% NaN_3 (5×10^5 cpm). After washing four times with PBS, the bound bacteria were dissolved in 1% SDS and quantified using a scintillation counter. All of the bindings were performed in triplicate.

Analytical Methods—The protein concentration was determined by Lowry's method (42) using BSA as a standard. Sugar content was determined by the phenol methods as described by Dubois *et al.* (40).

RESULTS

Difference in N-linked Glycosylation Accounts for Different Electrophoretic Mobility of pTHP and hTHP—When total urinary proteins were resolved by SDS-PAGE under nonreducing conditions (Fig. 1*A*) and THPs were subsequently identified by Western blotting (Fig. 1*B*), pTHP clearly exhibited a faster electrophoretic mobility than that of hTHP and oxen THP. There were several minor, lower molecular weight bands in human urine that were also reactive with the THP antibody (Fig. 1*B*). These bands were, however, absent from pig urine, suggesting that hTHP is more prone to enzymatic degradation than pTHP (see below). Consistent with their urinary counterparts, pTHP purified either with the salt precipitation method of Tamm and Horsfall (20) or with the diatomaceous earth retention method migrated faster than hTHP (Fig. 1*C*).

Because one of the possibilities of different electrophoretic mobility of the glycoproteins may lie in the different degrees of glycosylation, we subjected both hTHP and pTHP to *N*-glycosidase (PNGase F) treatment. Under reducing conditions and prior to enzyme treatment, both pTHP and hTHP migrated more slowly than the nonreduced forms (Fig. 2), with pTHP migrating faster than hTHP. PNGase F treatment resulted in a dramatic decrease of the apparent molecular weights of both THPs and, more interestingly, abolished the difference of the electrophoretic mobility between the two THPs (Fig. 2). These data strongly suggest that pTHP and hTHP differ in *N*-linked glycosylation.

Different Susceptibility of hTHP and pTHP to Proteases—It has been recently shown that the *N*-terminal portion of hTHP (amino acids 1–291) is particularly susceptible to enzymatic degradation by pancreatic elastase (38). The fact that hTHP and pTHP exhibited different degrees of urinary degradation (Fig. 1*B*) prompted us to examine the relative susceptibility of purified THPs to enzymatic degradation. Fig. 3*A* shows that although hTHP was completely degraded by pancreatic elastase to a core peptide of approximately 48 kDa, pTHP was largely resistant. Because leukocytes are frequently present in human urine, we also subjected both THPs to leukocyte elastase treatment. Again, the bulk of hTHP was degraded, whereas the majority of pTHP was resistant to the enzyme digestion (Fig. 3*B*).

Reactivity of hTHP and pTHP to Lectins—The glycosylation type of hTHP and pTHP was examined by testing their reactivity with two different plant lectins: (i) ConA, which specifically recognizes the high mannose sequences; and (ii) GS-IB4, which recognizes the Galα1,3Gal sequence (43). When the same amounts of pTHP and hTHP were used, ConA reacted with pTHP much more strongly than with hTHP (Fig. 4*A*; see below). A strong reactivity with GS-IB4 was also observed with pTHP, suggesting that the Galα1,3Galβ1,4GlcNAc epitope is present at the terminal nonreducing ends of pTHP polyantennary glycans. Consistent with this result, α1,3-galactosyltransferase, which is responsible for the assembly of Galα1,3Galβ1,4, was found to be highly expressed in the outer medulla of the pig kidney but not at all in the human kidney.

Characterization of High Mannose Glycans from THPs—The greater reactivity of ConA to pTHP than to hTHP prompted us to ascertain whether the two THPs differed in the high mannose glycans. After exhaustive Pronase digestion, pTHP and hTHP glycopeptides were fractionated by gel filtration. Neutral sugar determination gave similar profiles: a major peak in the elution volume of polyantennary glycopeptides and a minor one in that of high mannose glycopeptides (Fig. 5). Even the ratio of neutral sugar content between the two peaks of each gel filtra-

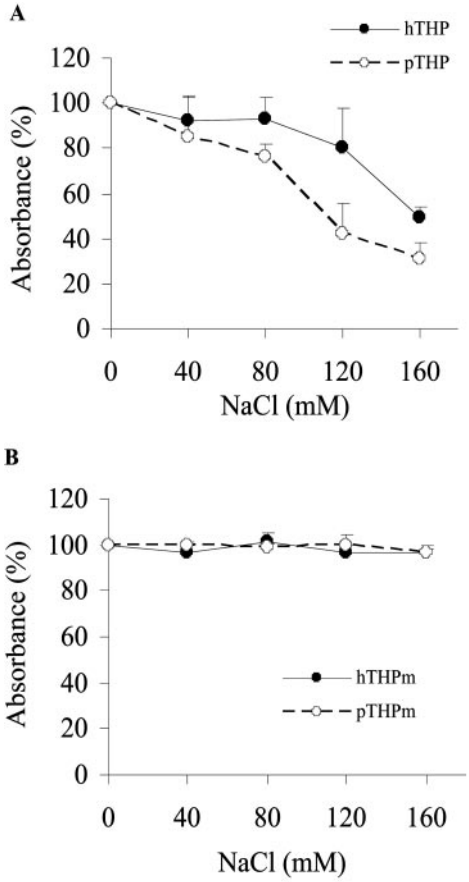


FIG. 7. **Solubility of hTHP and pTHP in polymeric and monomeric forms.** The water solutions of THP polymers (*A*) and monomers, obtained by urea treatment (*B*), were adjusted to an optical density close to 0.400 at 277 nm. After the addition of NaCl to concentrations as indicated, each sample was centrifuged, and the supernatant was read at 277 nm. Each *bar* represents the mean ± S.D. from three or four independent experiments. Note that urea-treated THP monomers were incapable of reforming polymers even at high concentrations of NaCl.

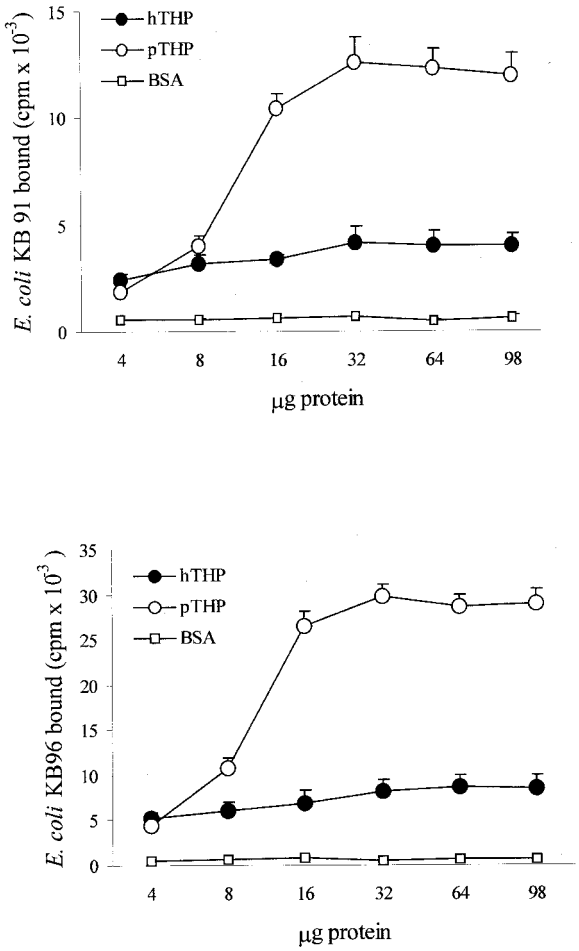


FIG. 8. ***In vitro* binding of type 1-fimbriated *E. coli* to purified THPs.** ^{35}S-Labeled *E. coli* strains that express a low affinity variant of FimH (KB91) and a high affinity variant of FimH (KB96) were incubated with increasing amounts of immobilized BSA or monomeric pTHP and hTHP. Note that although there was little binding to BSA, both strains bound to THPs with KB96 bound with greater numbers and that both strains bound to pTHP approximately 3-fold higher than to hTHP.

tion was very similar, suggesting that pTHP, like hTHP, carries a single high mannose glycan. To characterize the high mannose structure of pTHP, we treated the glycopeptides of the minor peaks with endoglycosidase H, radiolabeled the released high mannose moieties by [^{3}H]KBH$_4$, and fractionated them by HPLC. High mannose glycans from hTHP yielded $Man_6GlcNAc$ as the predominant form (75%), consistent with what was previously reported (27). In contrast, pTHP contained almost equal proportions of $Man_5GlcNAc$ (47%) and $Man_6GlcNAc$ (53%), with no $Man_7GlcNAc$ detected (Fig. 6). Therefore, the relative proportion of $Man_5GlcNAc$ in pTHP is significantly greater than that in hTHP (8%).

Binding of Type 1-fimbriated E. coli to Monomeric hTHP and pTHP—Type 1-fimbriated *E. coli* is known to be able to bind to purified THP in a mannose-specific manner (15). The different high mannose moieties contained in pTHP and hTHP raised the interesting possibility that the *E. coli* may bind differentially to the two THPs. We chose to use monomeric THPs for *E. coli* binding, because monomeric THPs yielded much more reproducible results than polymeric THPs in our preliminary experiments. We observed a different pattern of solubility of the two polymeric THPs in the presence of different concentrations of NaCl, particularly at values closed to iso-osmolarity (Fig. 7*A*). In contrast, the solubility of the monomeric forms of hTHP and pTHP obtained by urea treatment appeared to be entirely unaffected by the NaCl concentration (Fig. 7*B*).

As shown in Fig. 8, both *E. coli* strains (KB91 and KB96) bound to the THPs in much greater numbers than the BSA, suggesting that the binding is mediated by the high mannose moieties of the THPs. Between the two strains, KB96 bound approximately 3-fold higher to both THPs than KB91, consistent with the fact that KB96, but not KB91, expresses high affinity FimH variant (M_1H) to high mannose moieties (Fig. 8). This result was confirmed by a high level of binding of another M_1H FimH variant (SH48) to the two THPs (Table I). Interestingly, all tested *E. coli* strains expressing type 1 adhesins bound in greater numbers to pTHP than to hTHP (Fig. 8 and Table I). These results provide evidence on a functional level that the $Man_5GlcNAc$ sequence, which exists in much higher percentage in pTHP than in hTHP, is a better binder for *E. coli* FimH adhesins than the $Man_{6-7}GlcNAc$ sequences.

Although in humans type 1-fimbriated *E. coli* are by far the most common strain responsible for urinary tract infections (1), a recent epidemiological study indicated P-fimbriated *E. coli* as one of more prevalent strains in pigs with bacteriuria (44). We have therefore determined the capability of two THPs in binding a *E. coli* strain that expresses exclusively the P fimbriae, which recognize the terminal Galα1,4Galβ sequence. We found that this P-fimbriated strain did not bind at all to the two THPs (Table I), thus ruling out the possibility that pTHP contains the P fimbria-interacting Galα1,4Galβ sequence.

TABLE I

Adhesive properties of various E. coli strains to hTHPm and pTHPm

The adhesion of BSA or THPs (32 μg) was performed as described in the text. The values were calculated by subtracting the radioactivity found in the immobilized BSA to that found in THP samples. The values are the means ± S.D. of two experiments, each performed in triplicate.

E. coli strain	Fimbriae type	hTHPm binding	pTHPm binding	pTHPm binding/hTHPm binding
		cmp × 10^{-3}	*cmp* × 10^{-3}	
P678	None	0.41 ± 0.18	0.20 ± 0.18	0.48
J96	Type 1 and type P	1.04 ± 0.33	6.47 ± 0.14	6.22
SH48	Type 1 (variant M_1H)	21.30 ± 6.17	66.64 ± 5.98	3.13
KB96	Type 1 (variant M_1H)	8.76 ± 1.44	27.96 ± 1.22	3.19
KB91	Type 1 (variant M_1L)	3.26 ± 0.78	11.65 ± 0.99	3.57
HU849	Type P	0.77 ± 0.35	0.48 ± 0.29	0.62

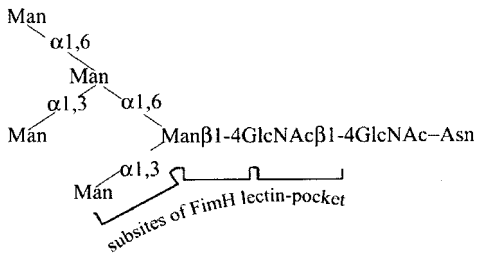


FIG. 9. **Schematic illustration of high affinity binding of FimH adhesins to the trisaccharide sequence of $Man_5GlcNAc_2$ accumulating in pTHP.**

DISCUSSION

In addition to its extraordinary abundance in mammalian urine, THP possesses another intriguing property of bearing one unprocessed high mannose chain. Structural and functional analyses indicated that such a high mannose chain exists in the THP of all animal species heretofore studied, including human, cattle, pig, and mouse (45). Additionally, forced expression of THP cDNA in cultured cells originally not expressing THP results in the synthesis of recombinant THP also bearing one high mannose chain (28). It is well known that the presence of one partially processed *N*-glycan in cell surface glycoproteins such as THPs is caused by steric hindrances that interfere with the glycan processing at a specific *N*-glycosylation site. More specifically, the arrest of glycan processing occurs because, along the routing in the secretory pathway, the *N*-glycan(s) is not accessible to enzymes of the Golgi complex, such as α1,2-mannosidase I or GlcNAc-transferase I (46). Previous investigation in one of our laboratories (27, 47) demonstrated that the major mannosyl isomer present in hTHP is the $Man_6GlcNAc_2$-glycan that retains an α1,2-mannose residue linked to α1,3-mannose of the trimannosyl core, very likely because the peptide hindrance close to Asn^{251} interferes with the removal of the last α1,2-mannose residue by the Golgi mannosidase-I. Previous (27) and current results show that $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ account for 75 and 8%, respectively, of all high mannose sequences occurring in hTHP. In striking contrast, the present study shows that pTHP contains a much higher proportion (47%) of $Man_5GlcNAc_2$ and a much lower proportion (53%) of $Man_6GlcNAc_2$. Experimental evidence suggests that the difference in the lengths of high mannose glycans confers functional consequences in the binding to type 1-fimbriated *E. coli*; Firon *et al.* (48) compared the inhibitory activity of mannose derivatives on yeast cell agglutination induced by *E. coli* strains and found that the Manα1,3-Manβ1,4GlcNAc trisaccharide and the pentamannoside derivative with the branched structure as shown in Fig. 9 are much stronger inhibitors than the six mannose homologous derivative. By digesting $Man_8GlcNAc_2$ glycopeptides from ovalbumin with α1,2-mannosidase, Neeser *et al.* (49) demonstrated that the $Man_5GlcNAc_2$Asn-R glycopeptide with the structure shown in Fig. 9 exhibits seven times more inhibitory activity on hemagglutination induced by type 1-fimbriated *E. coli* than $Man_6GlcNAc_2$Asn-R, a structure identical to that found in hTHP. These authors proposed that exposure of the α1,3Man residue from the trimannosyl core within the $Man_5GlcNAc_2$Asn chain is crucial for the high affinity binding between the high mannose chain and *E. coli* FimH adhesins. Based on these observations, Nathan Sharon (3) postulated that the binding pocket of FimH adhesins corresponds to the size of a trisaccharide and that the high affinity binding is due to the interaction of each monosaccharide of Manα1,3Manβ1,4GlcNAc with three adjacent subsites in the FimH lectin pocket (Fig. 9). Although the cDNA sequence of pTHP has yet to be elucidated, we propose that specific amino acid sequences surrounding the *N*-glycosylation site bearing high mannose glycans in pTHP results in a conformational structure that enables a more efficient removal of all α1,2-mannnose residues by Golgi α1,2-mannosidase I, so that the shortest oligomannoside is present in a higher proportion. Consistently, although $Man_7GlcNAc_2$ accounts for 17% of all oligomannosides in hTHP, it is virtually undetectable in pTHP. Importantly, the current study (i) extends the previous observation by showing that naturally occurring $Man_5GlcNAc_2$ within the THP is a more avid binder for *E. coli* FimH adhesins than the $Man_{6-7}GlcNAc_2$ counterparts and (ii) suggests that pTHP is a very effective defense factor in the pig urinary tract against infection by type 1-fimbriated *E. coli*.

Our results also documented the species differences in the terminal glycosylation of polyantennary glycans of THP. Although glycans of hTHP are mostly capped by sialic acids (29), the current study shows that pTHP exposes also the Galα1,3Galβ1,4GlcNAc sequence at the terminal nonreducing end of polyantennary glycans. This difference may explain the slower electrophoretic mobility of hTHP, caused by the abundance of sialic acids, which are known to cause *N*-glycosylated proteins to migrate more slowly during SDS-PAGE. The fact that hTHP contains more sialic acid residues than THPs from other mammals, *e.g.* rabbits and hamsters (51), may be related to the status of α1,3-galactosyltransferase (52). This enzyme, which competes with α2,3-sialyltransferases, is not detectable in human kidney (Fig. 4), a result consistent with the fact that the human gene encoding α1,3-galactosyltransferase has been inactivated by nonsense mutations (53). The absence of the Galα1,3Galβ1,4 epitope in human species is accompanied with a relative large presence (approximately 1%) of circulating IgG directed to this epitope (52). However, our results show that this epitope, which is present in pTHP, is not recognized by P-fimbriated *E. coli*, the prevalent strain causing urinary tract infections in pigs (44).

Another peculiar property of urinary THPs is the tendency to aggregate, forming large polymers. The aggregation occurs when the concentration of NaCl is close to 0.15 M or that of Ca^{2+} is close to ~2 mM (54), conditions that normally occur in urine. The ability to form large polymers has been recently

attributed to the ZP domain, the large C-terminal peptide that hTHP shares with ZP2 and ZP3 glycoproteins, two glycoproteins forming the transparent coat surrounding the eggs of all placental mammals, called the zona pellucida (38). The ZP domain is highly species-conserved in all hitherto cloned THPs (21–24).

The DEF method used in this study to purify both hTHP and pTHP is based on the fact that when urine is filtered through a layer of diatomaceous earth, the THP polymers are selectively entrapped (35). THP polymers are solubilized in deionized water, so that by means of a two-step filtration at a concentration of NaCl of $\geq$0.15 M and by washing the diatomaceous earth with deionized water, THP is purified to homogeneity even from urine of proteinuric patients (50). In the present study, we observed that under increasing concentrations of NaCl, the solubility of polymeric pTHP is lower than that of polymeric hTHP, whereas the two monomers are similarly soluble even at a relatively high concentration of NaCl (Fig. 7*B*). Together, these observations indicate that although the depolymerization of urinary THPs induced by the lowering of salt concentration is a reversible phenomenon, the monomers produced by urea treatment are incapable of reforming the polymers.

Finally, our results show that pTHP is more resistant to proteolysis than hTHP. Upon PMN elastase treatment, the main nondegraded peptide from pTHP migrates as a 66-kDa peptide, whereas that from hTHP migrates as a 48-kDa peptide. Jovine *et al.* (38) have found that the amino acid 292 is the N terminus of the elastase-resistant 48-kDa peptide from hTHP. Because the high mannose chain is linked to the Asn^{251} residue, the nondegraded peptide does not carry any high mannose chain. One may therefore expect that the ability of native hTHP to behave as an efficient ligand for FimH adhesins dramatically decreases when leukocytes release their proteolytic enzymes in urine. The cDNA of pTHP has not been cloned; thus we do not yet know whether the 66-kDa peptide found in a small quantity after leukocyte elastase treatment carries any high mannose glycan. This and other aspects of the pTHP structure require further study. Nevertheless, the lesser susceptibility of pTHP to proteolysis (Fig. 3) may itself contribute to increased binding to type1-fimbriated *E. coli*.

Given the fact that different high mannose chains of THP confer differential binding affinities to type 1-fimbriated *E. coli*, other differences in the chemical composition observed here may also have biological and functional significance. The difference between pTHP and hTHP may reflect the species-specific adjustment in urinary defenses against bacterial infections. The molecular mechanisms underlying these types of species variation are currently unclear. This aspect and the structure of the pTHP glycomoiety are subjects for future studies.

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Variation of High Mannose Chains of Tamm-Horsfall Glycoprotein Confers Differential Binding to Type 1-fimbriated *Escherichia coli*

Daniela Cavallone, Nadia Malagolini, Angela Monti, Xue-Ru Wu and Franca Serafini-Cessi

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