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Mechanism of Release of Urinary Tamm-Horsfall Glycoprotein from the Kidney GPI-Anchored Counterpart

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Human Tamm-Horsfall glycoprotein (THP) is synthesised in the thick ascending limb of Henle and convoluted distal tubules, inserted into luminal cell-surface by the glycosyl-phosphatidylinositol (GPI)-anchor and excreted in urine at a rate of 50–100 mg per day. Up to date there is no indication on the way in which THP is excreted into the urinary fluid. In this study, we examined by Western blotting THP from human kidney in comparison to urinary THP. As expected for a GPI-anchored protein, THP was recovered from the kidney lysate in a Triton X-100 insoluble form, which moved in a sucrose gradient to a zone of low density. The apparent molecular weight of kidney THP appeared greater than that of urinary THP, but no difference in the electrophoretic mobility was observed when the former was subjected to GPI-specific phospholipase-C treatment, strongly suggesting that a proteolytic cleavage at the juxtamembrane-ectodomain of kidney THP is responsible for the urinary excretion. © 2001 Academic Press

Key Words: **urinary protein; Tamm-Horsfall glycoprotein; urinary excretion; GPI-anchor; GPI-specific phospholipase.**

In 1950 Igor Tamm and Frank Horsfall (1) isolated from urine of healthy individuals a glycoprotein which has been subsequently referred to as Tamm-Horsfall glycoprotein (THP). THP is synthesised in kidney cells of the thick ascending loop of Henle (TAL) and of early distal convoluted tubules and in normal urine is excreted at the rate of 50–100 mg per day (2). The carbohydrate moiety represents about 30% of THP weight. *N*-Glycans have a poly-antennary sialylated structure, but one of the eight potential *N*-glycosylation sites predicted by the DNA-sequence encoding THP carries a high-mannose glycan (3–5). Recently, it has been reported that THP is also *O*-glycosylated (6). In 1990, Rindler *et al.* (7) after transfection of human-THP cDNA into carcinoma cells demonstrated that THP is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein.

The physiological role of THP on the cell surface of TAL is unknown. The addition of the GPI anchor and the high occurrence of *N*-glycans act as sorting signals to deliver glycoproteins to the apical membrane of epithelial cells (8–9). It is thus reasonable to postulate that THP plays a role at the cell-surface facing the filtrate of the TAL and distal nephron.

Up to now there is no indication about the mechanism by which THP is excreted in urine. Using a HeLa cell line with a stable expression of human THP (10), we observed that the release in the cell medium of recombinant THP exposed at the cell surface is largely dependent on the action of HeLa cell-associated protease(s) (11). The present study extends this observation to establish the way in which THP is excreted into the urine. Specifically we wanted to known if the release of urinary THP (uTHP) from the form bound to human kidney cells (kTHP) is due to the action of protease(s) or a GPI-specific phospholipase. The importance in clarifying this point is also related to the observation that GPI-anchor degradation products, inosytol-phosphate glycan and diacylglycerol derived from the phospholipase hydrolysis, mediate the signal transduction, and hormone activities (12).

MATERIALS AND METHODS

Materials. Urinary THP, from pooled urine or single individuals, was prepared by the diatomaceous earth filter method as previously described (13). Antiserum to human THP was raised in rabbits as described by Bloomfield *et al.* (14). Human immunoglobulins G (IgG) and anti-human IgG antibodies from rabbit were from Sigma as well as, octylglucoside, Triton X-100, Triton X-114, Earle's Balance Salts (EBSS) buffer, and anti-rabbit IgG conjugated with horseradish peroxidase (HRP). GPI-specific phospholipase C from *Tripanosoma brucei* (GPI-PLC) was from Oxford Glycosciences, UK. ECL immunodetection system was from Amersham International.

Abbreviations used: THP, human Tamm-Horsfall glycoprotein; GPI, glycosylphosphatidylinositol; TAL, thick ascending limb of Henle; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; GPI-PLC, GPI-phospholipase C; OcG, octylglucoside; TLCK, *N*-α-p-Tosyl-L-lysine-Chloromethyl-ketone.

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Kidney dissection and tissue preparations for immunoblotting. The bottom poles of two human kidneys with a carcinoma confined to the upper pole were removed at surgery and dissected to obtain the cortex and the outer stripe of the medulla as detailed by Eveloff *et al.* (15). Kidney portions were cut into small pieces and homogenised in EBSS (0.2 g/ml). The homogenate was digested with collagenase (0.2% final concentration) in EBSS for 90 min at 37°C, then centrifuged at 50*g* for 3 min and the pellet washed with EBSS to remove the collagenase. The pellet was then extracted with 60 mM octylglucoside in 20 mM Na phosphate buffer, pH 7.5, containing 150 mM NaCl (PBS) for 16 h at 4°C. After centrifugation the insoluble material was again extracted for 3 h at room temperature in PBS containing 1% SDS.

Electrophoresis and Western blotting. SDS–PAGE was performed at 8.5% acrylamide or on a gradient of acrylamide (see the legends of figures) as previously described (16). β-Mercaptoethanol, when present, was at 1.5% (v/v). Proteins separated by SDS–PAGE were transferred electrophoretically onto nitro-cellulose membrane and the blot incubated with anti-THP antibodies or anti-human IgG antibodies followed by anti-rabbit IgG conjugated with horseradish peroxidase as previously described (17). The blot was developed with the ECL detection system as recommended by supplier. The protein concentration of the samples was determined by Lowry method (18) using bovine serum albumin as a standard.

Sucrose gradient of kidney homogenate. Kidney homogenate (11 mg of proteins) in 0.5 ml of 25 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 (TNE/TX-100) was lysed for 20 min on ice. After that, the kidney suspension was homogenised again with a Dounce homogenizer, brought to 40% sucrose and placed in an ultracentrifuge tube. A linear gradient of sucrose (5–30%) was layered over the lysate and centrifuged overnight as described by Brown and Rose (19). Fractions of 0.5 ml were collected by bottom puncture and the percentage of sucrose was detected by phenol assay in each fraction (20). Each fraction was separately dialysed against water and an aliquot subjected to SDS–PAGE and Western blotting. The blots were incubated with anti-THP antibodies or with anti human IgG antibodies and developed as described above.

GPI-Phospholipase C treatment. Fractions 10 and 11 of the sucrose gradient containing kTHP were pooled and an aliquot (100 μl) subjected to enzymatic digestion with 0.1 U of GPI-PLC (*Tripanosoma brucei*) in 20 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 1 mM TLCK, and 0.1% Triton X-114 for 1 h to 30°C. An aliquot of treated sample and of the control sample, similarly incubated without the enzyme, were dialysed against water and subjected to the sucrose gradient as described above.

RESULTS

Western blotting of uTHP and kTHP. Figure 1 shows that while uTHP migrated as a single band, kTHP migrated as a doublet (Fig. 1). The slower major band, probably corresponds to the fully glycosylated THP (mature form) whereas the minor one is the partially-glycosylated precursor which resides only inside the cell. We have demonstrated that during the transport along the Golgi complex the majority of *N*-glycans carried by recombinant THP are converted from the high-mannose to polyantennary-sialylated type, an event resulting in an increase of the glycoprotein molecular weight (10). Figure 1 shows also that the mobility in SDS–PAGE of both uTHP and kTHP in nonreducing conditions was faster than that observed in mercaptoethanol-reducing conditions. Moreover, in

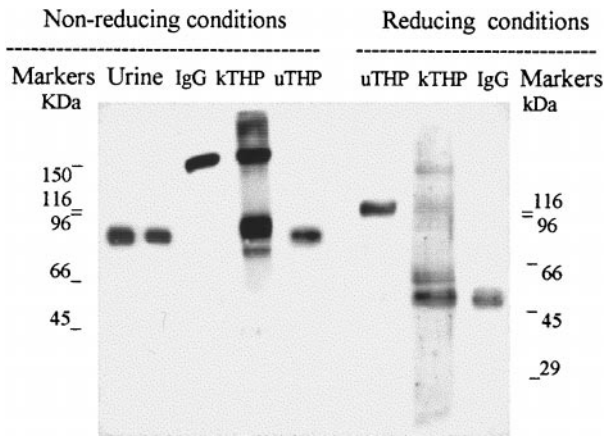

FIG. 1. Western blot of uTHP and kTHP. Twenty-four hours urine of healthy individuals (5 μl), human IgG, uTHP purified from pooled urine, and kidney homogenate were subjected to SDS–PAGE (8.5% polyacrylamide), blotting, incubated with anti-THP antibodies, and developed as described under Materials and Methods. The amount of IgG, protein-kidney homogenate, and uTHP applied to the gel were 0.3, 9, and 0.24 μg in nonreducing conditions and in reducing conditions were 0.6, 90, and 2.4 μg, respectively.

nonreducing conditions uTHP and kTHP exhibited a several fold higher reactivity with polyclonal anti-THP antibodies than the reduced forms. Because of the higher immunoreactivity of nonreduced THP, the SDS–PAGE was performed in nonreducing conditions throughout the study. The Western blot of kidney homogenate visualised also a band with the mobility of IgG. This band was also detected when the blot was exclusively incubated with the secondary antibodies, indicating an interspecies immunoreactivity of rabbit anti-IgG antibodies towards the human IgG (results not shown).

Solubilization of kTHP by detergents. The GPI-anchor proteins are preferentially solubilized by octylglucoside (19). Therefore, homogenate of kidney cortex and outer medulla were first extracted with octylglucoside and then with SDS. Electrophoresis performed on a gradient of polyacrylamide (7–10%) showed that in both kidney regions mature kTHP solubilized by octylglucoside migrated slower than uTHP (Fig. 2). The result was constantly obtained using various preparations of uTHP from pooled urine or uTHP isolated from different single individuals. A relatively greater amount of kTHP was present in outer medulla samples, a result consistent with the prevalent localisation of THP in cell of TAL. Interestingly, both bands of kTHP solubilized by SDS from outer medulla migrated with a mobility faster than those solubilized by octylglucoside. In this case, the slower band (mature kTHP) exhibited the same apparent molecular weight of uTHP. Since the solubility of a protein in octylglucoside is largely dependent on the addition of the GPI-anchor, one may suggest that a minor amount of kTHP

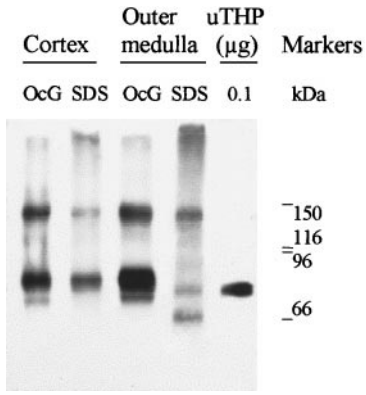

FIG. 2. Solubility in octylglucoside (OcG) and SDS of kTHP from the cortex and outer medulla. SDS–PAGE (acrylamide gradient 7–10%) was in nonreducing conditions. Twenty micrograms of protein homogenate were applied in each lane.

without the GPI-moiety and with a truncated peptide-moiety is present in kidney cells.

Fractionation of kidney THP by sucrose gradient and GPI phospholipase-C treatment. The addition of a GPI-anchor to proteins results in the loss of solubility in Triton X-100 and in the property of moving in a sucrose gradient to a region of low density (19). Figure 3 shows Western blotting of sucrose-gradient fractions from kidney lysed in the presence of Triton X-100. IgG and cross-reacting proteins were visualised at the bottom of the gradient (Fig. 3B), whereas kTHP was recovered in the region of low density (fractions 9–11) in which a 10–20% sucrose concentration was determined (Fig. 3A). Interestingly, in this case kTHP migrated as a single-narrow band with a very clear slower mobility than that of uTHP.

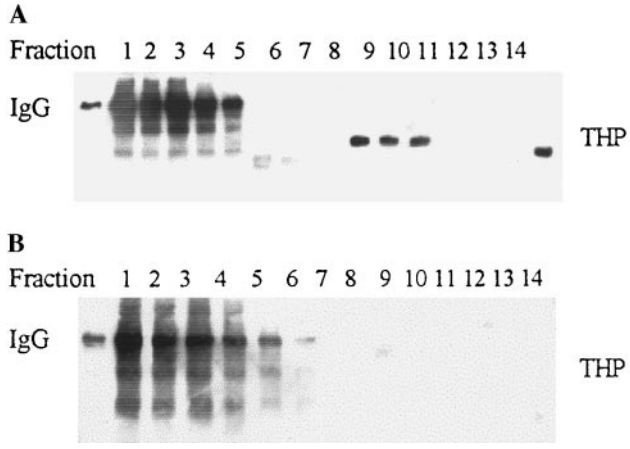

FIG. 3. Fractionation of kTHP by sucrose gradient. Aliquots of 30 μl of fractions from sucrose gradient were subjected to SDS–polyacrylamide gradient gel (6–15%) in nonreducing conditions. The blot was incubated with anti-THP antibodies (A) or with anti-human IgG antibodies (B) and developed as in Fig. 1.

FIG. 4. Effect of GPI-specific phospholipase-C (GPI-PLC) treatment on the SDS–PAGE mobility of kTHP and uTHP. Aliquots of kTHP fractionated by sucrose gradient (fraction 10 and 11 of Fig. 3) and uTHP purified from pooled urine were treated with GPI-PLC as described in the text. The corresponding control samples were similarly treated but in the absence of the enzyme. The SDS–PAGE (polyacrylamide gradient 6–15%) was in nonreducing conditions and the blot developed as in Fig. 1.

To ascertain whether the faster migration of uTHP relative to that of kTHP was because of the action of a protease or GPI-specific phospholipase, kTHP isolated by sucrose gradient was subjected to GPI-PLC treatment and then analysed by SDS–PAGE. After the treatment, the kTHP sample could not be fractionated in the low density region of sucrose gradient, a result indicating that the hydrophobic portion of the GPI-anchor has been indeed cleaved (results not shown). In spite of this, no shift in the mobility of treated kTHP was observed (Fig. 4, cf. lane 2 and lane 3).

DISCUSSION

Current results show that the apparent molecular weight of both uTHP and kTHP shifts from 80 to 95 kDa when they migrate in SDS–PAGE under reducing conditions. Such a difference may be explained by two observations: (i) in native THP all the cysteine-S-H groups are present as disulphide bridges (21) and (ii) the DNA sequence predicts as many as 48 cysteine residues in the THP molecule (22). When such a large number of S-S bridges are reduced, the THP molecule acquires, in all likelihood, a more linear conformation, with a consequent increment in the SDS binding and a decrease in the SDS–PAGE mobility. A similar shift in the apparent molecular weight of uTHP and kTHP supports the notion that the former derives from kTHP after the acquisition of a correct peptide folding and full glycan-processing, two events occurring in the proximal- and distal-compartments of exocytic pathway, respectively (23, 24). Up to date, the demonstration that cell-bound THP is a GPI-protein has been based on the analysis of recombinant THP expressed by carcinoma cell lines after the transfection of human-THP cDNA (7, 10). Current results confirm that the vast majority of THP is bound to kidney cells by a GPI anchor. Indeed, kTHP, as expected for a GPI-protein, is insoluble in cold Triton X-100 and in the sucrose gradient moves to a zone of low density. This behaviour has been related to the property of GPI-proteins to associate with sphingolipids and cholesterol (25). Ac-

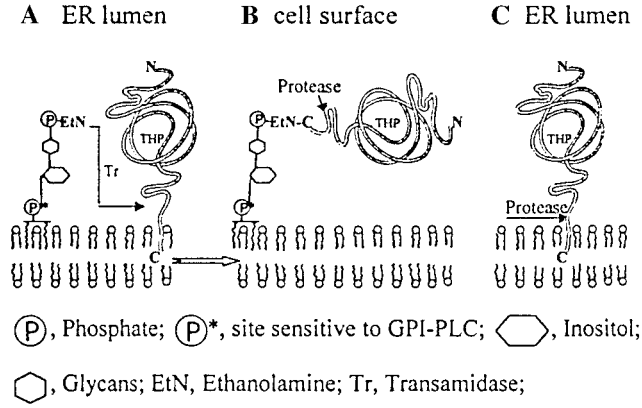

FIG. 5. Model depicting the formation of GPI-anchored THP (A) and two possible ways responsible for the excretion of urinary THP (B and C).

cording to Simons and van Meer (26), GPI-proteins associate with sphingolipid microdomains (sphingolipid rafts) in the distal compartment of Golgi apparatus. Our results are consistent with this model. Indeed, whereas in the kidney homogenate both precursor and mature kTHP are present, in the kTHP separated by sucrose gradient only the latter is visualised (cf. Fig. 2 and Fig. 3) indicating that the association between sphingolipids and kTHP occurs when the glycoprotein has been fully processed by the Golgi glycosyltransferases.

Relative to the way in which THP is excreted in urine two important observations arise from current results: (i) the apparent molecular weight of uTHP is lower than that of kTHP and (ii) the treatment of kTHP with GPI-specific phopholipase-C does not result in any change of the electrophoretic mobility. On this basis we propose that a proteolytic cleavage occurring in the juxtamembrane ectodomain of kTHP is responsible for the release of uTHP (see Fig. 5B). This is consistent with the way in which recombinant THP is released from transfected HeLa cells. Indeed, THP accumulating in the cell medium, was found to be mainly devoid of ethanolamine, the residue responsible for the binding of the GPI-anchor to THP C-terminus (13). As shown in Fig. 5A the acquisition by a protein of the GPI-anchor comprises a proteolytic cleavage of the carboxyl terminal peptide, containing mainly lipophilic amino acids, and the addition to the "new" C-terminus of the preformed GPI-unit. Such a process occurs in the ER and is catalysed by a specific transamidase (27). The release in soluble form of GPI-proteins occurs usually at the cell-surface, but an alternative pathway has been proposed in that small amounts of truncated forms of a GPI-protein lacking the GPI-anchor have been found to accumulate inside the cells (28). The cleavage of the C-terminal peptide without the anchor addition has been indicated to generate the intracellularly truncated form which is eventually excreted through the exocytic pathway (28). In the SDS-solubilized sample of the outer medulla (Fig. 2) we have found a small proportion of kTHP with the same mobility of uTHP and a faster migrating band. It is possible that these two bands consist of the intracellularly-truncated mature THP and the partially glycosylated precursor. The relative high amount of the precursor vs the mature form suggests that the transit along the exocytic pathway of the intracellularly-truncated THP is much less efficient than that of the GPI-anchored THP (cf. lanes 3 and 4 of Fig. 2). Therefore, the intracellularly cleavage of the C-terminus peptide without the addition of the GPI-anchor should represent a minor alternative pathway for the excretion of THP in urine (Fig. 5C).

Since current results do not support the notion that a GPI-specific phospholipase is involved in the release of uTHP, degradative products of the GPI-anchor should not be formed and no signal transduction should be associated with the THP excretion. It has been proposed that uTHP, because of the large and heterogeneous glycosylation pattern, may represent a host protection against *E. coli* infections of the urinary tract (29). Indeed, the excreted uTHP, may compete with the glycans exposed at the cell-surface of urinary tract in binding type 1- and type S-fimbriated *E. coli* responsible for the colonisation of pathogens (30, 31). In this context, the urinary infections could have served as selective agents to promote excretion of THP in the host.

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REFERENCES

1. Tamm, I., and Horsfall, F. L., Jr. (1950) A mucoprotein derived from human urine which reacts with influenza, mumps, and Newcastle disease viruses. *Proc. Soc. Exp. Med.* **74,** 108–114.
2. Kumar, S., and Muchmore, A. (1990) Tamm-Horsfall protein-uromodulin (1950–1990). *Kidney Int.* **37,** 1395–1401.
3. Serafini-Cessi, F., Dall'Olio, F., and Malagolini, N. (1984) High-mannose oligosaccharides from human Tamm-Horsfall glycoprotein. *Biosci. Rep.* **4,** 269–274.
4. Hard, K., Van Zadelhoof, G., Moonen, P., Kamerling, J. P., and Vliegenthart, J. F. G. (1992) The Asn-Linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. *Eur. J. Biochem.* **209,** 895–915.
5. Rooijen, J. J., Voskamp, A. F., Kamerling, J. P., and Vliegenthart, J. F. G. (1999) Glycosylation sites and site-specific glycosylation in human Tamm-Horsfall glycoprotein. *Glycobiology* **9,** 21–30.
6. Easton, R. L., Patankar, M. S., Clark, G. F., Morris, H. R., and Dell, A. (2000) Pregnancy associated changes in the glycosylation of Tamm-Horsfall glycoprotein. Expression of sialyl Lewis^x sequences on core-2 type O-glycans derived from uromodulin. *J. Biol. Chem.* **275,** 21928–21938.

7. Rindler, M. J., Naik, S. S., Li, N., Hopps, T. C, and Peraldi, M-N. (1990) Uromodulin (TH glycoprotein/Uromucoid) is a phosphatidylinositol-linked membrane-protein. *J. Biol. Chem.* **265,** 20784–20789.
8. Hannan, L. A., and Edidin, M. (1996) Traffic, polarity and detergent solubility of a anchored protein after LDL-deprivation of MDCK cells. *J. Cell. Biol.* **133,** 1265–1276.
9. Benting, J. H., Rietveld, A. G., and Simons, K. (1999) *N*-glycans mediate the apical sorting of a GPI-anchored, raft associated protein in Madin-Darby canine kidney cells. *J. Cell Biol.* **146,** 313–320.
10. Serafini-Cessi, F., Malagolini, N., Hoops, T. C., and Rindler, M. J. (1993) Biosynthesis and oligosaccharide processing of human Tamm-Horsfall glycoprotein permanently expressed in HeLa cells. *Biochem. Bioph. Res. Commun.* **194,** 784–790.
11. Malagolini, N., Cavallone, D., and Serafini-Cessi, F. (1997) Intracellular transport, cell-surface exposure and release of recombinant Tamm-Horsfall glycoprotein. *Kidney Int.* **52,** 1340–1350.
12. Sargiacomo, M., Sudol, M., Tang, Z. L., and Lisanti, M. P. (1993) Signal transducing molecules and glycosylphosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell. Biol.* **122,** 789–807.
13. Serafini-Cessi, F., Bellabarba, G., Malagolini, N., and Dall'Olio, F. (1989) Rapid isolation of Tamm-Horsfall glycoprotein (uromodulin) from human urine. *J. Immun. Methods* **120,** 185–189.
14. Bloomfield, F. J., Dunstan, D. R., Foster, C. L., Serafini-Cessi, F., and Marshall, R. D. (1977) Some factors affecting the production by cultured baby-hamster kidney cell of BHK glycoprotein 1 which cross-reacts immunologically with THP. *Biochem. J.* **164,** 41–51.
15. Eveloff, J., Haase, W., and Kinne, R. (1980) Separation of renal medullary cells: Isolation of cells from the thick ascending limb of Henle's loop. *J. Cell Biol.* **87,** 672–681.
16. Smilovich, D., Malagolini, N., Fagioli, C., de Lalla, C., Sitia, R., and Serafini-Cessi, F. (1998) Differential expression of Galα1,3Gal epitope in polymeric and monomeric IgM secreted by mouse myeloma cells deficient in α2,6-sialyltransferase. *Glycobiology* **8,** 841–848.
17. Cavallone, D., Malagolini, N., and Serafini-Cessi, F. (1999) Binding of human neutrophils to cell-surface anchored Tamm-Horsfall glycoprotein in tubulointerstitial nephritis. *Kidney Int.* **55,** 1787–1799.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
19. Brown, D. A., and Rose, J. K. (1992) Sorting of GPI-anchored protein to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68,** 533–544.
20. Dubois, M., Gilles, K. A., Hamilton, J. K., Roberts, P. A., and Smith, F. (1956) Colorimetric method for determination of sugar and related substances. *Anal. Chem.* **28,** 350–356.
21. Hamlin, L. M., and Fish, W. W. (1977) Physical properties of Tamm-Horsfall glycoprotein and its glycopeptide. *Int. J. Peptide. Protein Res.* **10,** 270–276.
22. Hession, C., Decker. J. M., Sherblom, A. P., Kumar, S., Yue, C., Mattaliano, R., Tizard, R., Kawashima, E., Schmeissner, V., Heletky, S., Chow, P., Burne, C., Shaw, A., and Muchmore, A. V. (1987) Uromodulin (Tamm-Horsfall glycoprotein): A renal ligand for limphokines. *Science* **237,** 1479–1484.
23. Braakman, I., Hoover-Litty, H., Wagnar, K. R., and Helenius, A. (1991) Folding of influenza hemagglutinin in the endoplasmic reticulum. *J. Cell Biol.* **114,** 401–411.
24. Kornfeld, R., and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* **84,** 631–664.
25. Muniz, M., and Riezman, H. (2000) Intracellular transport of GPI-anchored proteins. *EMBO J.* **19,** 10–15.
26. Simons, K., and van Meer, G. (1988) Lipid sorting in epithelia cells. *Biochemistry* **27,** 6197–6202.
27. Maxwell, E. S., Ramalingam, S., Gerber, L. D., Brink, L., and Udenfriend, S. (1995) An active carbonyl formed during glycosylphosphatidylinositol addition to a protein is evidence of catalysis by a transamidase. *J. Biol. Chem.* **270,** 19576–19582.
28. Wang, J., Shen, F., Yan, W., Wu, M., and Ratnam, M. (1997) Proteolysis of the carboxyl-terminal GPI signal independent of GPI modification as a mechanism for selective protein secretion. *Biochemistry* **36,** 14583–14592.
29. Parkkinen, J., Virkola, R., and Korhonen, T. K. (1988) Identification of factors in human urine that inhibit the binding of *Escherichia coli* adhesins. *Infect. Immun.* **56,** 2623–2630.
30. Sharon, N. (1987) Bacterial lectins, cell-cell recognition and infectious diseases. *FEBS Lett.* **217,** 145–157.
31. Malagolini, N., Cavallone, D., Wu, X-R., and Serafini-Cessi, F. (2000) Terminal glycosylation of bovine uroplakine III, one of the major integral-membrane glycoproteins of mammalian bladder. *Biochim. Biophys. Acta* **1475,** 231–237.