Properties of a Glycopeptide Isolated from Human Tamm–Horsfall Glycoprotein

INTERACTION WITH LEUCOAGGLUTININ AND ANTI-(HUMAN TAMM–HORSFALL GLYCOPROTEIN) ANTIBODIES

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A sialylated glycopeptide isolated after Pronase digestion of human Tamm–Horsfall glycoprotein behaves as a powerful monovalent hapten in the precipitin reaction between human Tamm–Horsfall glycoprotein and leucoagglutinin, but fails to inhibit the interaction of the glycoprotein with rabbit anti-(human Tamm–Horsfall glycoprotein) antibodies. The glycopeptide is much less active than the intact glycoprotein as an inhibitor of lymphocyte transformation induced by leucoagglutinin.

Human Tamm–Horsfall glycoprotein is excreted in the urine at the rate of approx. 50 mg/day by adult humans (Grant & Neuberger, 1973). It has been suggested that Tamm–Horsfall glycoprotein is associated with the plasma membrane of the cells of the ascending limb of the loop of Henle and the distal convoluted tubule, and that its biological role might be related to electrolyte and water transport in the kidney (Sikri *et al.*, 1979). The glycoprotein has a subunit mol.wt. of 80 000 and contains 25–30% of carbohydrate (Fletcher *et al.*, 1970*a,b*). By Pronase digestion of human Tamm–Horsfall glycoprotein, Afonso & Marshall (1979) purified a glycopeptide that contains more than 70% of the carbohydrate of the original protein and only 50–60 out of the 420 amino-acid residues of the peptide backbone; gel filtration indicated a mol.wt. of about 18 000. These results suggested that human Tamm–Horsfall glycoprotein, like glycophorin A (Marchesi *et al.*, 1976) and potato (*Solanum tuberosum*) lectin (Allen *et al.*, 1978), has all the carbohydrate chains confined to restricted regions of the polypeptide chain.

Recently, Serafini-Cessi *et al.* (1979) found that human Tamm–Horsfall glycoprotein interacts specifically with leucoagglutinin [a purified isolectin of the red kidney bean (*Phaseolus vulgaris*)], and that the precipitin reaction is similar to a typical antigen–antibody reaction in which leucoagglutinin recognizes the carbohydrate chains of human Tamm–Horsfall glycoprotein.

Present experiments were designed to investigate whether the glycopeptide purified from human Tamm–Horsfall glycoprotein maintains the ability to

interact with leucoagglutinin and with rabbit anti-(human Tamm–Horsfall glycoprotein) antibodies. Since human Tamm–Horsfall glycoprotein is a powerful inhibitor of lymphocyte transformation induced by leucoagglutinin, the effect of the glycopeptide on blastogenesis was investigated.

Experimental

Materials

Human Tamm–Horsfall glycoprotein was isolated from pooled urine (Tamm & Horsfall, 1952), and digested with purified Pronase as suggested by Afonso & Marshall (1979). The glycopeptide (identified by the orcinol method) was eluted with 0.1 M-acetic acid in the exclusion volume of a column (1 cm × 45 cm) of Sephadex G-25. The final purification was achieved by ion-exchange chromatography on DEAE-Sephacel (from Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.1 M-acetic acid. The column (1 cm × 20 cm) was washed with 60 ml of starting solution and eluted with a linear gradient of NaCl (100 ml of 0.1 M-acetic acid and 100 ml of 0.5 M-NaCl). Leucoagglutinin was purified from Bacto-Phytohaemagglutinin P (Difco Laboratories, Detroit, MI, U.S.A.) as previously described (Serafini-Cessi *et al.*, 1979). ¹⁴C-labelled human Tamm–Horsfall glycoprotein and ¹⁴C-labelled leucoagglutinin were prepared by the method of Means & Feeney (1968) with [¹⁴C]formaldehyde (sp. radioactivity 9 mCi/mmol) supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Antiserum to human Tamm–Horsfall

glycoprotein was raised in rabbits as described by Bloomfield *et al.* (1977).

Purification of anti-(human Tamm-Horsfall glycoprotein) antibodies by affinity chromatography

(Human Tamm-Horsfall glycoprotein)-Sephacel was prepared as previously described (Serafini-Cessi *et al.*, 1979). The conjugated gel was equilibrated with 0.02 M-phosphate buffer, pH 7.4, containing 0.15 M-NaCl. Rabbit anti-(human Tamm-Horsfall glycoprotein) antiserum (10 ml) diluted with an equal volume of equilibrating buffer was loaded on the column (1 cm $\times$ 15 cm). After extensive washing with the same buffer in order to remove the non-adsorbed serum proteins, the column was eluted with 0.05 M-glycine/HCl, pH 3.0, containing 0.5 M-NaCl. Fractions (2 ml) were collected, immediately neutralized with NaOH and the A_{280} was measured. A single protein peak was obtained. The fractions under the peak were pooled and dialysed for 72 h against 500 vol. of 0.02 M-phosphate buffer, pH 7.0, with three changes. The pooled material gave a precipitin reaction with human Tamm-Horsfall glycoprotein and was assumed to comprise anti-(human Tamm-Horsfall glycoprotein) antibodies. When normal rabbit serum was put on the column of (human Tamm-Horsfall glycoprotein)-Sephacel, all proteins were eluted in the washing buffer.

Analytical methods

Neutral sugars were determined by orcinol/H₂SO₄ reagent (Francois *et al.*, 1962) with galactose as standard, and hexosamines by the Cessi & Philiego (1960) method after hydrolysis with 4 M-HCl at 100°C for 4 h. The galactosamine was determined by the procedure of Cessi & Serafini-Cessi (1963) and *N*-acetylneuraminic acid by the method of Warren (1959). Amino acids were determined by ninhydrin reagent as described by Moore & Stein (1954). Proteins were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Interaction experiments

For the quantitative determination of the precipitin reaction, ¹⁴C-labelled leucoagglutinin was used. ¹⁴C-labelled leucoagglutinin (25 μ g; 150 d.p.m./ μ g) in 0.02 M-sodium phosphate buffer, pH 7.0, was mixed in plastic tubes with increasing amounts of human Tamm-Horsfall glycoprotein or glycopeptide dissolved in the same buffer; the final volume was 0.5 ml. The tubes, including the control sample with ¹⁴C-labelled leucoagglutinin only, were incubated for 1 h at room temperature. The tubes were centrifuged at 19200 g for 1 h. The supernatants were discarded immediately and 0.5 ml of 0.1 M-HCl was added to the precipitates. After the tube contents were mixed, the tubes were incubated

for 2 h at room temperature. Then 0.4 ml portions were transferred to scintillation vials and the radioactivity was measured as previously described (Serafini-Cessi *et al.*, 1979).

The inhibition of precipitation by the hapten was studied by using ¹⁴C-labelled human Tamm-Horsfall glycoprotein. The precipitin reaction with anti-(human Tamm-Horsfall glycoprotein) antibodies or leucoagglutinin was performed as detailed elsewhere (Serafini-Cessi *et al.*, 1979). The glycopeptide was added to the reaction mixtures just before the labelled glycoprotein.

Culture of lymphocytes

Isolation of peripheral-blood human lymphocytes, their stimulation *in vitro* in microculture, [³H]thymidine pulsing, cell harvesting and radioactivity measurement were performed as previously described (Serafini-Cessi *et al.*, 1979). Human Tamm-Horsfall glycoprotein or glycopeptide dissolved in the culture medium were added at the start of the culture.

Results and Discussion

The chromatographic separation on DEAE-Sephacel of the glycopeptide material eluted in the exclusion volume of the Sephadex G-25 columns gave two components. The first peak (eluted by the washing eluent) contained only 10% of the neutral sugars of the starting material and was devoid of *N*-acetylneuraminic acid. Approximately two-thirds of the sugars bound to human Tamm-Horsfall glycoprotein appeared in a very narrow second peak eluted by the linear gradient of NaCl. Similar results were obtained by Afonso & Marshall (1979). The chemical characterization of this major glycopeptide gave about 70% carbohydrate (neutral sugars 24.6%; *N*-acetylhexosamine 26.4%; *N*-acetylglucosamine 2.9%; *N*-acetylneuraminic acid 18.4%) and 30% peptide. The ratio between neutral sugars and *N*-acetylhexosamines was the same (1:1) in the glycopeptide and in the original glycoprotein, whereas the ratio between *N*-acetylneuraminic acid and neutral sugars was higher in glycopeptide (1:1.5) than in human Tamm-Horsfall glycoprotein (1:2). This result is consistent with the finding that a asialylated glyco-compound was separated with the first peak.

Fig. 1 shows the precipitation of ¹⁴C-labelled leucoagglutinin with human Tamm-Horsfall glycoprotein or glycopeptide. The precipitin curve between lectin and glycoprotein shows that the precipitation of lectin is a linear function of the concentration of glycoprotein over the range explored. Other glycoproteins, such as fetuin and α_1 -acid glycoprotein, did not precipitate the lectin at all (results not shown), confirming the specificity of

Fig. 1. Precipitation of ^{14}C -labelled leucoagglutinin. In each tube, $25\mu\text{g}$ of ^{14}C -labelled leucoagglutinin was present in a final volume of 0.5ml . The experimental conditions are described in the Experimental section. ●, Human Tamm-Horsfall glycoprotein; ○, glycopeptide.

Fig. 2. Effect of glycopeptide on the precipitation of ^{14}C -labelled human Tamm-Horsfall glycoprotein by leucoagglutinin and anti-(human Tamm-Horsfall glycoprotein) antibodies.

All samples contained $32\mu\text{g}$ of ^{14}C -labelled human Tamm-Horsfall glycoprotein in a final volume of 0.5ml of 0.02M -phosphate buffer, pH 7.0; the glycopeptide was added to the reaction mixtures just before the labelled glycoprotein. ○, Precipitin reaction obtained with leucoagglutinin ($50\mu\text{g}$); ●, precipitin reaction obtained with anti-(human Tamm-Horsfall glycoprotein) antibodies ($50\mu\text{g}$).

the interaction between leucoagglutinin and human Tamm-Horsfall glycoprotein. The glycopeptide also failed to precipitate labelled lectin when present at high concentration.

The ability of the glycopeptide to act as hapten in the test of precipitation of ^{14}C -labelled human Tamm-Horsfall glycoprotein with rabbit specific antibodies or leucoagglutinin is shown in Fig. 2. The glycopeptide did not inhibit the precipitation induced by rabbit anti-(Tamm-Horsfall glycoprotein) antibodies, but had a very high hapten inhibitory activity in the system leucoagglutinin-(^{14}C -labelled human Tamm-Horsfall glycoprotein). A 50% inhibition of precipitation was obtained when the weight ratio between glycopeptide and glycoprotein was approx. 1:2. Serafini-Cessi *et al.* (1979) demonstrated that *N*-acetylgalactosamine is the only monosaccharide that inhibits the precipitin reaction of human Tamm-Horsfall glycoprotein with leucoagglutinin; 50% inhibition was obtained at 30mm *N*-acetylgalactosamine. On a molar basis, the glycopeptide (mol.wt. 18 000; Afonso & Marshall, 1979) is 10 000 times more potent than the hapten *N*-acetylgalactosamine. This result confirms the complexity of the carbohydrate specificity of leucoagglutinin (Hellström *et al.*, 1976) and the high affinity of oligosaccharide chains of human Tamm-Horsfall glycoprotein for this lectin.

The blastogenesis of human peripheral lymphocytes was induced by optimal dose of leucoagglutinin ($1\mu\text{g}/\text{ml}$). Table 1 reports the dose-response inhibition by human Tamm-Horsfall glycoprotein

Table 1. Inhibition of lymphocyte transformation by human Tamm-Horsfall glycoprotein and its glycopeptide.

Lymphocyte transformation was induced by leucoagglutinin ($1\mu\text{g}/\text{ml}$). Results are expressed as radioactivity (d.p.m.) of [^3H]thymidine incorporated per culture and are means $\pm$ S.E.M. for four determinations.

Inhibitor ($\mu\text{g}/\text{ml}$)	[^3H]Thymidine incorporated			
	Human Tamm-Horsfall glycoprotein		Glycopeptide	
	d.p.m.	Inhibition (%)	d.p.m.	Inhibition (%)
None	77913 $\pm$ 3795	—	68551 $\pm$ 3540	—
1	27650 $\pm$ 2604	58	72708 $\pm$ 1487	0
5	20999 $\pm$ 3818	68	61540 $\pm$ 8344	10
10	6950 $\pm$ 1472	89	75497 $\pm$ 5472	0
50	401 $\pm$ 43	99	46568 $\pm$ 7375	32
100	138 $\pm$ 33	100	29339 $\pm$ 1914	57
200	—	—	19959 $\pm$ 2714	71

and its glycopeptide. The latter shows a lower inhibitory activity. A little more than 50% inhibition of thymidine incorporation was produced by the glycoprotein at $1\mu\text{g}/\text{ml}$ and by the glycopeptide at $100\mu\text{g}/\text{ml}$.

In conclusion, the results show that (i) a sialylated glycopeptide with a total carbohydrate

content of 70% can be isolated after Pronase digestion of human Tamm-Horsfall glycoprotein; (ii) the glycopeptide does not precipitate leucoagglutinin, but behaves as powerful univalent hapten in the precipitin reaction between human Tamm-Horsfall glycoprotein and lectin; (iii) the glycopeptide does not inhibit the interaction between glycoprotein and rabbit anti-glycoprotein antibodies; this lack of inhibition is consistent with the hypothesis that rabbit anti-(human Tamm-Horsfall glycoprotein) antibodies are essentially directed against the peptide backbone of glycoprotein; (iv) the glycopeptide is much less active than whole glycoprotein as an inhibitor of lymphocyte transformation induced by leucoagglutinin. Similar results have been obtained by Adair & Kornfeld (1974). They observed that Pronase digestion caused a dramatic fall in the inhibitory activity of glycoprotein receptors isolated from erythrocytes on the binding of wheat-germ (*Triticum*) agglutinin to erythrocytes. The high inhibitory activity of human Tamm-Horsfall glycoprotein on leucoagglutinin-induced blastogenesis has been interpreted as a competition between the glyco moiety of human Tamm-Horsfall protein and the carbohydrate receptors on lymphocytes for the mitogen (Serafini-Cessi *et al.*, 1979). The strong binding of human Tamm-Horsfall glycoprotein with mitogen depends on the multivalent interaction of the two macromolecules; therefore the much lower inhibitory activity of the glycopeptide on lymphocyte transformation may result from its behaviour as a monovalent ligand to the mitogen.

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