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Guinea-pig kidney β-*N*-acetylgalactosaminyltransferase towards Tamm–Horsfall glycoprotein

Requirement of sialic acid in the acceptor for transferase activity

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A β-*N*-acetylgalactosaminyltransferase that preferentially transferred *N*-acetylgalactosamine to Sd(a–) Tamm–Horsfall glycoprotein was found in guinea-pig kidney microsomal preparations. This enzyme was kidney-specific and was able to transfer the sugar to other glycoproteins, such as fetuin and α_1-acidic glycoprotein. The presence of sialic acid in the acceptors was essential for the transferase activity when either glycoproteins or their Pronase glycopeptides were used as acceptors. Two glycopeptides (Tamm–Horsfall glycopeptides I and II) with a different carbohydrate composition were separated by DEAE-Sephacel chromatography from Pronase-digested Tamm–Horsfall glycoprotein. The amount of *N*-acetylgalactosamine transferred to glycopeptides by the enzyme correlated with their degree of sialylation. Enzymic digestion of *N*-[^{14}C]acetylgalactosamine-labelled Tamm–Horsfall glycopeptide II showed that the transferred sugar was susceptible to β-*N*-hexosaminidase. The amount of sugar cleaved by β-hexosaminidase was strongly increased when the labelled Tamm–Horsfall glycopeptide II was pretreated with mild acid hydrolysis, a procedure that removed the sialic acid residues. Alkaline borohydride treatment of the labelled Tamm–Horsfall glycopeptide II did not release radioactivity, thus indicating that enzymic glycosylation took place at the *N*-asparagine-linked oligosaccharide units of Tamm–Horsfall glycoprotein.

T-H glycoprotein, the most abundant glycoprotein in normal human urine, contains 30% of carbohydrates (Fletcher *et al.*, 1970). Analytical studies of the glycoprotein and its glycopeptides (T-H glycopeptides) suggest that the carbohydrate moiety consists mainly of *N*-asparagine-linked oligosaccharide units formed by a di-*N*-acetylchitobiose-mannosyl core that carries poly-branched sialylated chains (Afonso *et al.*, 1981; Serafini-Cessi & Dall'Olio, 1982). *N*-Acetylgalactosamine is present but its content is variable and depends on the individual source of glycoprotein; the content is almost 2% in T-H glycoprotein preparations from Sd(a+) donors, whereas it is negligible in the preparations from Sd(a–) individuals (Soh *et al.*, 1980). The Sd^a character is an inherited human blood group (Race & Sanger, 1975) and the Sd^a antigen found in the urine appears to be closely associated with T-H glycoprotein (Morgan *et al.*, 1979). Very low concentrations of Sd(a+) T-H glycoprotein inhibit haemagglutination of Sd(a+) red cells induced by naturally occurring anti-Sd^a antibodies, whereas Sd(a–) T-H glycoprotein lacks this inhibitory activity. Moreover Sd(a+) glycoprotein gives a precipitin reaction with anti-Sd^a sera (Serafini-Cessi & Conte, 1982). Donald *et al.* (1982) found that *N*-acetylgalactosamine in Sd(a+) T-H glycoprotein is mostly present as terminal non-reducing sugar in (β1-4)-linkage to galactose. Cartron & Blanchard (1982) suggested that Cad specificity, which appears closely similar to Sd^a specificity, is defined by *N*-acetylgalactosamine residues carried by glycans, probably *O*-linked to glycoproteins of membrane erythrocytes.

No information is available on the biosynthesis of the carbohydrate structure related to Sd^a specificity, namely on the *N*-acetylgalactosaminyltransferase, which is involved in the addition of the terminal *N*-acetylgalactosamine residues to T-H glycoprotein. To

Abbreviation used: T-H glycoprotein, Tamm–Horsfall glycoprotein.

approach this point two observations have been considered; (i) the great amount of Sd^a activity detected in guinea-pig urine and kidney (Morton *et al.*, 1970); and (ii) the presence of β-*N*-acetylgalactosaminyltransferase activity specific for trihexosylceramide in guinea-pig kidney (Ishibashi *et al.*, 1974).

The present paper demonstrates that microsomal preparations of guinea-pig kidney transfer *N*-[^{14}C]-acetylgalactosamine from labelled UDP-*N*-acetylgalactosamine to the *N*-linked glyco-moiety of T-H glycoprotein and to other glycoproteins such as fetuin and α_1-acidic glycoprotein. This transferase, which attaches *N*-acetylgalactosamine in the β-configuration, strictly requires the presence of sialic acid in the acceptor for its activity.

Experimental

Microsomal preparation

Guinea-pigs were from a rural breeding; the animals were killed by decapitation and the kidneys and liver immediately removed and placed on ice. Microsomal preparations were obtained by the procedure of Keller & Zamecnik (1956). The pellet was suspended in 0.2M-Tris/HCl, pH 7.5. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of glycoprotein acceptors

Individual preparations of T-H glycoprotein from persons of different Sd^a phenotype were prepared by a procedure that involves three salt precipitation steps as described by Tamm & Horsfall (1952). Two preparations of Sd(a—) T-H glycoprotein (R.D. and A.S.) were kindly provided by Dr. W. M. Watkins, Clinical Research Centre, Harrow, Middx., U.K. The Sd^a activity of T-H glycoproteins was measured as detailed by Serafini-Cessi & Conte (1982) and was the minimum concentration (μg/ml) giving complete inhibition of Sd(a+) red cells induced by human anti-Sd^a serum. Other glycoproteins were purchased from Sigma.

Preparation of Pronase-labelled glycopeptides

One T-H glycoprotein preparation from pooled urine of Sd(a+) individuals and one preparation from the urine of a Sd(a—) donor (B.N.) were subjected to Pronase digestion as previously described (Abbondanza *et al.*, 1980). The digests were freeze-dried, dissolved in 1 ml of water and then chromatographed on a column (1 cm × 75 cm) of Bio-Gel P-10. Blue Dextran was used to define the void volume (V_0) and 0.1M-NH_4HCO_3 to elute the column. A large peak, containing about 90% of the neutral sugars of T-H glycoprotein, emerged after the fractions that contained Blue Dextran. All the fractions of this peak were pooled, dialysed against water and freeze-dried. The material solubilized in 2ml of water was chromatographed on a DEAE-Sephacel column (1 cm × 16 cm) equilibrated with 0.05M-acetic acid. The column was washed with 10ml of water and then eluted with a linear gradient of NaCl (50ml of 0.05M-acetic acid and 50ml of 0.05M-acetic acid containing 0.5M-NaCl).

The chromatography was performed at 4°C. Fractions (1 ml) were collected at a rate of 10ml/h and 0.1 ml was used to detect neutral sugars by the orcinol method. At the end of the chromatography the fractions corresponding to the peaks were pooled, immediately dialysed against water and freeze-dried.

Fetuin glycopeptide was prepared from the Pronase digest of fetuin with a procedure substantially similar to that described for T-H glycopeptides. Its elution position from the DEAE-Sephacel column was identical with that of T-H glycopeptide II.

N-Acetylgalactosaminyltransferase assay

Glycoprotein acceptors. The standard mixture contained 0.04mM-UDP-*N*-acetyl-D-[1-^{14}C]galactosamine (sp. radioactivity 61.5Ci/mol; The Radiochemical Centre, Amersham, Bucks, U.K.), 20mM-$MnCl_2$, 0.5% Triton X-100, 0.08M-Tris/HCl buffer, pH 7.5, microsomal preparation (0.1mg of protein) and the glycoprotein acceptor (0.1mg) in a final volume of 50μl. Incubation was usually at 37°C for 45min and the reaction was stopped by the addition of 1 ml of 1% phosphotungstic acid in 0.5M-HCl. The precipitates were collected on glass-fiber filters, washed and counted for radioactivity as previously described (Serafini-Cessi, 1977).

Glycopeptide acceptors. The incubation mixture contained 0.04mM labelled sugar nucleotide, 0.750mg of microsomal proteins and 0.05mg of glycopeptide acceptor in a total volume of 100μl; other components were as described above. After 45min incubation at 37°C, the mixture was diluted 10 times with water and immediately centrifuged at 100000 ***g*** at 4°C for 1h. The supernatant was loaded on a Bio-Gel P-10 column (1 cm × 75 cm) and eluted with 0.1M-NH_4HCO_3. Fractions (1ml) were collected and the radioactivity measured in each fraction. The fractions corresponding to the elution position of the glycopeptide were pooled for further analysis.

Enzymic and chemical treatment of N-[^{14}C]acetylgalactosamine-labelled T-H glycopeptides

The labelled T-H glycopeptide II separated by Bio-Gel filtration as described above was subjected to α-*N*-acetylgalactosaminidase digestion; 1ml of pooled fractions corresponding to the elution position of the glycopeptide was dialysed against 0.1M-citrate buffer, pH 4, and then incubated with 0.1 unit of α-glycosidase from *Charonia lampus* (Seikagaku

Kogio Co., Tokyo, Japan) for 24h at 37°C. At the end of this period the sample was chromatographed on the same Bio-Gel column with unlabelled *N*-acetylgalactosamine. The radioactivity was measured in each fraction and the elution position of monosaccharide was determined by the Morgan & Elson (1934) reaction.

Digestion of the same sample with β-*N*-acetylhexosaminidase from beef kidney (Boehringer–Mannheim G.m.b.H.) was carried out with 4 units of enzyme/ml in 0.01 M-citrate buffer, pH 4, for 24h at 37°C. In order not to increase the salt concentration in the incubation mixture, the enzyme solution, before the incubation, was dialysed against the same buffer for 8h at 4°C. At the end of the incubation time free *N*-acetylgalactosamine was separated from T-H glycopeptide by gel filtration as described above.

To remove sialic acid from T-H glycopeptide, the mild acid treatment was performed with 0.05 M-H_2SO_4 for 1h at 80°C. Digestion with neuraminidase from *Vibrio cholerae* was carried out at 0.1 unit/ml for 24h at 37°C.

The labelled T-H glycopeptide II was also subjected to mild alkaline degradation. The sample was treated with 0.05 M-NaOH containing 1 M-sodium borohydride at 45°C for 16h. The excess of borohydride was destroyed by addition of acetic acid to pH 4 and the sample, degassed, was rechromatographed on Bio-Gel P-10.

Carbohydrate analysis

This was performed by g.l.c. after methanolysis as described by Reinhold (1972). Neutral sugars were determined by the orcinol method of Francois *et al.* (1962). The removal of sialic acid from the glycoprotein acceptors was controlled by the Warren (1959) method.

Results

Glycoprotein acceptors

Microsomal preparations from guinea-pig kidney transferred *N*-[^{14}C]acetylgalactosamine from the sugar nucleotide into endogenous acceptor(s) precipitable by acid. A large increase of acid-precipitated radioactivity occurred if the T-H glycoprotein preparation from an Sd(a–) individual was added as an exogenous acceptor. The requirements for the transfer are given in Table 1. Both Mn^{2+} and Triton X-100 were necessary. A concentration of detergent higher than 0.5% reduced incorporation. When Triton X-100 was substituted by Emulphogene (0.5%) the activity was reduced by 20%. Maximal transferase activity towards the exogenous acceptor was observed at pH 7.5 with a steep decline in the acidic and basic ranges. The incorporation of sugar reached a maximum after 45min of incubation at 37°C.

Table 1. *Requirements for UDP-N-acetylgalactosamine: T-H glycoprotein N-acetylgalactosaminyltransferase activity of guinea-pig kidney*

Details of the incubation mixture are described in the text. Incubations were performed for 45min at 37°C in duplicate and the average values are shown. The Sd(a–) T-H glycoprotein was from R.D. donor. The 100% value was 12630d.p.m.

Incubation mixture	Activity %
Complete system with Sd(a–) T-H glycoprotein	100
Without enzyme	0
Without exogenous acceptor	34
Stopped at zero time	2
Without Mn^{2+}	2
Without Triton X-100	7

Table 2. *N-Acetylgalactosaminyltransferase activity towards individual preparations of T-H glycoprotein with different Sd^a activity*

Incubation conditions were as described in the text. The values of sugar transferred were corrected for endogenous incorporation. The values are averages of three separate experiments with each acceptor.

Individual source of T-H glycoprotein	Sd^a red-cell phenotype	Sd^a activity of T-H glycoprotein (μg/ml)	Transferase activity (d.p.m./mg of microsomal protein)
R.D.	Sd(a–)	>1000	82540
A.S.	Sd(a–)	>1000	50180
B.N.	Sd(a–)	200	27870
R.L.	Sd(a+)	0.2	19470
E.C.	Sd(a+)	0.1	20250
M.S.	Sd(a+)	0.1	5700
F.D.	Sd(a+)	0.1	5280

In order to determine the *N*-acetylgalactosaminyltransferase activity towards T-H glycoproteins according to the Sd^a activity, individual preparations of glycoproteins from donors with different Sd^a phenotypes were tested. Whereas the content of *N*-acetylgalactosamine in three Sd(a–) T-H glycoproteins used was negligible, the content of this sugar was about 2% in all preparations with Sd^a activity. Table 2 shows that the highest incorporation of *N*-acetylgalactosamine was observed in two preparations completely devoid of Sd^a activity. Although a remarkable variability in the sugar incorporation was observed between different individual preparations, all the values for Sd(a+) T-H glycoproteins fell in a lower range than those for Sd(a–) T-H glycoproteins.

Even though *N*-acetylgalactosamine is not a

Table 3. *N-Acetylgalactosaminyltransferase activity towards various glycoproteins*

Incubation conditions were as described in the legend to Table 2. The enzyme activity was corrected for the endogenous values. The results are means of duplicate experiments.

Acceptor	Transferase activity (d.p.m./mg of microsomal protein)
Fetuin	81530
Bovine α_1-acidic glycoprotein	38850
Human transferrin	11420
Bovine thyroglobulin	6090
Sd(a+) T-H glycoprotein (human pooled urine)	5160
Rabbit T-H glycoprotein	840
Ovalbumin	0

Table 4. *Effect of cleavage of sialic acid from glycoprotein acceptors on the N-acetylgalactosaminyltransferase activity*

Sd(a−) T-H glycoprotein was from the urine of B.N. donor. Mild acid treatment was performed with 0.05 M-H_2SO_4 at 80°C for 1 h. Transferase activity was determined as described in the legend to Table 2.

Acceptor	Mild acid treatment	Transferase activity (d.p.m./mg of microsomal protein)
Sd(a−) T-H glycoprotein	−	34130
	+	2080
Fetuin	−	70840
	+	4220
α_1-Acidic glycoprotein	−	41960
	+	450

sugar frequently found in glycoproteins that carry *N*-linked glycans, we investigated to see if other glycoproteins could serve as acceptors. Fetuin and α_1-acidic glycoprotein incorporated a large amount of *N*-acetylgalactosamine, comparable with that found in Sd(a−) T-H glycoproteins (Table 3). When these three effective acceptors were tested in the *N*-acetylgalactosaminyltransferase activity, using guinea-pig liver microsomal preparations, no increase over endogenous incorporation was observed, indicating that this transferase is kidney-specific.

Unexpectedly, the cleavage of sialic acid by mild acid hydrolysis from all the three glycoproteins strongly affected their ability to incorporate *N*-acetylgalactosamine. Practically no *N*-acetylgalactosaminyltransferase activity could be detected towards desialylated glycoproteins (Table 4).

Glycopeptide acceptors

The transferase activity of guinea-pig kidney was assayed towards T-H glycopeptides prepared after exhaustive digestion of T-H glycoproteins. Since glycopeptides with a different carbohydrate composition have been isolated from T-H glycoprotein (Afonso *et al.*, 1981; Abbondanza *et al.*, 1980), we investigated the enzymic addition of *N*-acetylgalactosamine to separated glycopeptides and compared the transferase activity with their degree of sialylation.

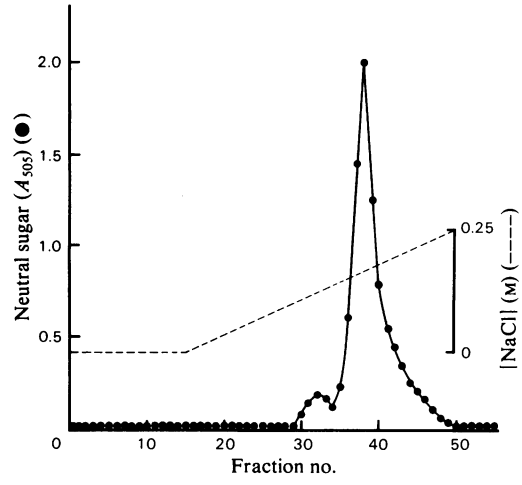

Fig. 1. *DEAE-Sephacel chromatography of T-H glycopeptides*

The acidic Pronase-digested glycopeptides, separated as a single peak by Bio-Gel P-10 column, were chromatographed on DEAE-Sephacel as described in the text. The elution position of glycopeptides was detected by the orcinol method. Fractions 30–33 and fractions 36–40 were pooled separately. The first pool was designed as T-H glycopeptide I, and the second one as T-H glycopeptides II; 1 ml fractions were collected.

Two different glycopeptides were separated by DEAE-Sephacel chromatography on the basis of their anionic charge. The major, T-H glycopeptide II, contained about 80% of the neutral sugars present in the glycoprotein, whereas the minor, T-H glycopeptide I, accounted for less than 10% (Fig. 1). Most of the Sd^a activity associated with T-H glycoproteins was recovered in the T-H glycopeptide II (F. Serafini-Cessi & F. Dall'Olio, unpublished work).

Table 5 shows the carbohydrate composition of the two glycopeptides. As previously reported (Afonso *et al.*, 1981; Serafini-Cessi & Dall'Olio, 1982) the presence of mannose and the ratio between this sugar and *N*-acetylglucosamine suggested for both glycopeptides an *N*-linked complex-type structure. The higher content of sialic acid in T-H glycopeptide II is consistent with a more branched structure of this species in comparison with T-H glycopeptide I. A peculiar feature of both glycopeptides was the presence of *N*-acetylgalactosamine,

which occurred in both species at a similar concentration. Apart from the negligible content of *N*-acetylgalactosamine, the carbohydrate composition of T-H glycopeptide II purified by the same procedure, from an Sd(a—) T-H glycoprotein, was similar to that of T-H glycopeptide II reported in Table 5.

In order to ascertain the extent of enzymic addition of *N*-acetylgalactosamine into various T-H glycopeptide preparations, their separation from the incubation mixture of transferase assay was carried out. Fig. 2 shows the profile of a typical gel filtration in which the labelled T-H glycopeptide was separated from the endogenous acceptors and the sugar nucleotide and its hydrolysis products. The large amount of radioactivity eluted as free *N*-acetylgalactosamine depended on the activity of pyrophosphatases that hydrolysed the sugar nucleotide and that are present at high concentration in the kidney microsomal preparations (Ishibashi *et al.*, 1976).

As expected the T-H glycopeptide II isolated from Sd(a—) T-H glycoprotein incorporated the highest amount of *N*-acetylgalactosamine. In contrast with the coresponding original T-H glycoprotein, prepared from the pooled urine of Sd(a+) individuals (see Table 3), a significant amount of sugar was transferred into its T-H glycopeptide II. T-H glycopeptide I, purified from the same Sd(a+) T-H glycoprotein preparation, incorporated less sugar than the more sialylated species, and a marked decrease in incorporation occurred when the desialylated glycopeptide was used as acceptor (Table 6). The mild acid hydrolysis of T-H glycopeptide II, which removed about 90% of sialic acid, did not change the concentration of other sugars, no *N*-acetylgalactosamine being lost during the treatment.

To confirm that the presence of sialic acid was the essential requirement for the acceptors to incorporate *N*-acetylgalactosamine, fetuin *N*-linked glycopeptide was added in the transferase assay before and after desialylation. No enzyme activity was observed towards the desialylated form, whereas the untreated fetuin glycopeptide behaved as an efficient acceptor (Table 6).

Table 5. *Carbohydrate composition of T-H glycopeptides from Sd(a+) T-H glycoprotein*

T-H glycoprotein was prepared from pooled urine of Sd(a+) individuals. The acidic glycopeptides from Pronase-treated glycoprotein were isolated by gel filtration and subsequently by DEAE-Sephacel chromatography (see Fig. 1). Carbohydrate analysis was performed by g.l.c. and the monosaccharide content is given as residues/3.0 residues of mannose. The values for total carbohydrate are given as g/100g of glycopeptide and were obtained by summing the values for the individual carbohydrate.

Glycopeptide	... T-H glycopeptide I	T-H glycopeptide II
Fucose	1.0	1.3
Mannose	3.0	3.0
Galactose	3.8	4.9
N-Acetylglucosamine	4.7	5.7
N-Acetylgalactosamine	1.5	1.6
N-Acetylneuraminic acid	2.1	3.6
Total carbohydrate	89.5	94.9

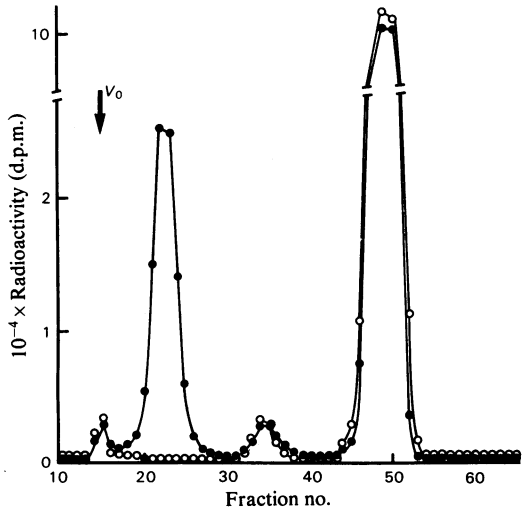

Fig. 2. *Separation of N-[^{14}C]acetylgalactosamine-labelled T-H glycopeptide*

Bio-Gel P-10 profiles of supernatants from incubation mixtures of microsomal preparations with UDP-*N*-[^{14}C]acetylgalactosamine in the presence (●) and in the absence (○) of Sd(a—) T-H glycopeptide II. The incubation conditions were as given in the text. Blue Dextran was used for determination of V_0. The elution position of unlabelled T-H glycopeptide II was between fractions 20 and 25 (1ml fractions were collected). The radioactivity between fractions 33 and 36 corresponded to the elution position of the sugar nucleotide and that between 46 and 52 to that of free *N*-acetylgalactosamine.

Analysis of N-[^{14}C]acetylgalactosamine-labelled T-H glycopeptide

Enzymic digestion of the labelled Sd(a+) and Sd(a—) T-H glycopeptide II with α-*N*-acetylgalactosaminidase indicated that the sugar added by the guinea-pig kidney transferase was resistant to this enzyme. After digestion all the radioactivity loaded on the Bio-Gel column was recovered in the elution position of the glycopeptide. In contrast, β-*N*-acetylhexosaminidase digestion released labelled material

Table 6. *N-Acetylgalactosaminyltransferase activity towards various Pronase-digested glycopeptides*
The glycopeptides were isolated as described in the text. Sd(a−) T-H glycopeptide II was from the T-H glycoprotein preparation of Sd(a−) B.N. donor; the Sd(a+) T-H glycopeptides were all from the T-H glycoprotein preparation from human pooled urine. Sialic acid and *N*-acetylgalactosamine were determined by g.l.c. Transferase activity was determined by measuring the *N*-[^{14}C]acetylgalactosamine transferred to the glycopeptide, which was then separated from the incubation mixture by gel filtration. Acid treatment was performed with 0.05 M-H_2SO_4 at 80°C for 1 h.

Glycopeptide	Content of sugars (%) NeuAc	GalNAc	Transferase activity (d.p.m./mg of microsomal preparation)
Sd(a−) T-H glycopeptide II	25.2	<1	70040
Sd(a+) T-H glycopeptide II	25.0	7.8	46650
Sd(a+) T-H glycopeptide I	17.7	8.5	19890
Acid-treated Sd(a+) T-H glycopeptide II	3.1	8.4	2260
Fetuin glycopeptide	28.9	—	48650
Acid-treated fetuin glycopeptide	<1	—	1920

that emerged with unlabelled *N*-acetylgalactosamine. No more than 30% of the sugar was freed by simple exhaustive digestion with β-hexosaminidase, but when this treatment was performed after mild acid hydrolysis, which removed sialic acid, the amount of sugar released accounted for 85% of the label bound to T-H glycopeptide. No comparable result was obtained if the desialylation pretreatment was performed with neuraminidase, in that the amount of label released by β-hexosaminidase did not exceed 30% of that bound to the glycopeptide.

When the radiolabelled T-H glycopeptides II were subjected to mild alkaline borohydride treatment and then rechromatographed on Bio-Gel, more than 90% of the radioactivity was recovered in the original position of T-H glycopeptide. This result indicated that the group containing *N*-[^{14}C]acetylgalactosamine was not removed from serine and threonine by base-catalysed β-elimination.

Discussion

Several *N*-acetylgalactosaminyltransferases specific for glycoproteins, glycolipids and proteoglycans have been described (Beyer *et al.*, 1981). The most extensively studied has been the (α1-3) *N*-acetylgalactosaminyltransferase, which is involved in the synthesis of the A blood group antigen and is present in human serum of A type and in tissues of several mammalian species (Watkins, 1978). The results presented here indicate that guinea-pig kidney contains an *N*-acetylgalactosaminyltransferase, probably involved in the biosynthesis of Sd^a antigen in T-H glycoprotein, that strictly requires the presence of sialic acid in the acceptor for its activity. Indeed the cleavage of sialic acid from all the acceptors tested, either glycoproteins or glycopeptides, resulted in their inability to accept *N*-acetylgalactosamine. In this way this enzyme resembles that involved in the biosynthesis of A antigen, which requires the presence of fucose to non-reducing terminal galactose of the H substance for the transfer of α-*N*-acetylgalactosamine.

The relationship between the presence of *N*-acetylgalactosamine in the acceptors and the enzymic activity appears to be a more intriguing phenomenon. Whereas the presence of *N*-acetylgalactosamine at the concentration recovered in the Sd(a+) T-H glycoproteins seemed to hinder the enzymic transfer of the sugar into T-H glycoproteins, when the T-H glycopeptides were tested no correlation could be observed between the content of *N*-acetylgalactosamine and the ability to serve as an acceptor (Table 6). The difference in the behaviour of T-H glycoprotein and T-H glycopeptide indicates that the peptide backbone may affect the accessibility of the oligosaccharide chains to the transferase. The removal of protein may unmask other sites on the oligosaccharides, which, *in vitro*, become *N*-acetylgalactosamine acceptors.

Our results indicate also that kidney microsomal transferase attaches *N*-acetylgalactosamine to T-H glycopeptides in the β-configuration, since only β-hexosaminidase is able to cleave the sugar incorporated via *N*-acetylgalactosaminyltransferase. Moreover, it was apparent from the selective susceptibility of the desialylated T-H glycopeptide to the β-glycosidase that the presence of sialic acid strongly affects the cleavage of *N*-acetylgalactosamine. This result suggests that at least some of the sialic acid residues present in T-H glycopeptide II are close to the transferred sugar and hinder glycosidase activity. In the ganglioside G_{M_2}, in which both β-*N*-acetylgalactosamine and sialic acid are linked to the same subterminal galactose residue, β-hexosaminidase treatment cleaves the sugar only if sialic acid has been removed (Li & Li, 1982). Furthermore we observed that after neuraminidase

treatment the enzymic cleavage of *N*-acetylgalactosamine from labelled T-H glycopeptide was not increased. It has been reported that the action of neuraminidase is inhibited if the 4-hydroxy group of galactose, which carries sialic acid, is substituted (Leeden & Salsman, 1965). Altogether these results suggest that this *N*-acetylgalactosaminyltransferase is responsible for the attachment of the sugar in the structure β-GalNAc(1→4)Gal, which was found in Sd(a+) T-H glycoprotein by Donald *et al.* (1982), and that sialic acid is probably bound to the subterminal galactose residue. The requirement of sialic acid in the acceptors suggests also that during the biosynthesis the transfer of *N*-acetylgalactosamine occurs after the addition of sialic acid. In various biosynthetic steps the addition of sialic acid prohibits action of other glycosyltransferases and becomes the termination signal of the oligosaccharide chains (Beyer *et al.*, 1979). The essential requirement of sialic acid in the acceptor appears a very peculiar feature of this β-*N*-acetylgalactosaminyltransferase.

The effect of mild alkaline degradation of T-H glycopeptides labelled via *N*-acetylgalactosaminyltransferase seems to indicate that non-reducing terminal β-*N*-acetylgalactosamine is attached to the *N*-linked oligosaccharide units. So far, *N*-acetylgalactosamine has been reported in *N*-linked oligosaccharides only in the α-anomeric linkage (Krusius *et al.*, 1978). Our results strongly support the occurrence in T-H glycoprotein of *N*-acetylgalactosamine in the β-configuration as part of *N*-linked oligosaccharide chains.

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References

Abbondanza, A., Franceschi, C., Licastro, F. & Serafini-Cessi, F. (1980) *Biochem. J.* **187**, 525–528

Afonso, A.-M. M., Charlwood, P. A. & Marshall, R. D. (1981) *Carbohydr. Res.* **89**, 303–319

Beyer, T. A., Rearick, J. I., Paulson, J. C., Prieels, J.-P., Sadler, J. E. & Hill, R. L. (1979) *J. Biol. Chem.* **254**, 12531–12541

Beyer, T. A., Sadler, J. E., Rearick, J. I., Paulson, J. C. & Hill, R. L. (1981) *Adv. Enzymol. Relat. Areas Mol. Biol.* **48**, 23–175

Cartron, J. P. & Blanchard, D. (1982) *Biochem. J.* **207**, 497–504

Donald, A. S. R., Soh, C. P. C., Watkins, W. M. & Morgan, W. T. J. (1982) *Biochem. Biophys. Res. Commun.* **114**, 56–65

Fletcher, A. P., Neuberger, A. & Ratcliffe, W. A. (1970) *Biochem. J.* **120**, 417–424

Francois, C., Marshall, R. D. & Neuberger, A. (1962) *Biochem. J.* **83**, 335–341

Keller, E. B. & Zamecnik, P. C. (1956) *J. Biol. Chem.* **221**, 45–59

Krusius, T., Finne, J. & Rauvala, H. (1978) *Eur. J. Biochem.* **92**, 289–300

Ishibashi, T., Kijimoto, S. & Makita, A. (1974) *Biochim. Biophys Acta* **337**, 92–106

Ishibashi, T., Atsuta, T. & Makita, A. (1976) *Biochim. Biophys Acta* **429**, 759–767

Leeden, R. & Salsman, K. (1965) *Biochemistry* **4**, 2225–2233

Li, Y.-T. & Li, S. C. (1982) *Adv. Carbohydr. Chem. Biochem.* **40**, 235–286

Lowry, P. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Morgan, W. T. J. & Elson, L. A. A. (1934) *Biochem. J.* **28**, 988–995

Morgan, W. T. J., Soh, C. P. C. & Watkins, W. M. (1979) in *Glycoconjugates* (Shauer, R., Boer, P., Buddecke, E., Kramer, M. F., Vliegenthart, J. F. G. & Wiegands, W., eds.), pp. 582–583, Thieme, Stuttgart

Morton, J. A., Pickles, M. M. & Terry, A. M. (1970) *Vox Sang.* **19**, 474–482

Race, R. R. & Sanger, R. (1975) in *Blood Groups in Man*, 6th edn., pp. 395–405, Blackwell, Oxford

Reinhold, W. N. (1972) *Methods Enzymol.* **25**, 244–249

Serafini-Cessi, F. (1977) *Biochem. J.* **166**, 381–386

Serafini-Cessi, F. & Conte, R. (1982) *Vox Sang.* **42**, 141–144

Serafini-Cessi, F. & Dall'Olio, F. (1982) in *Lectins-Biology, Biochemistry, Clinical Biochemistry* (Bøg-Hansen, T. C., ed.), Vol. II, pp. 367–379, Walter de Gruyter, Berlin and New York

Soh, C. P. C., Morgan, W. T. J., Watkins, W. M. & Donald, A. S. R. (1980) *Biochem. Biophys. Res. Commun.* **93**, 1132–1139

Tamm, I. & Horsfall, F. L. (1952) *J. Exp. Med.* **95**, 71–97

Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975

Watkins, W. M. (1978) *Proc. R. Soc. London Ser. B* **202**, 31–53