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Biochemistry

Epitopic structure of Tn glycophorin A for an anti-Tn antibody (MLS 128)

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ABSTRACT **Glycophorin A was digested with glycoprotease (*Pasteurella haemolytica*) and the digest was fractionated by a combination of high-pressure column chromatographies to produce the glycopeptides GPA-1 to GPA-6. Sequence analysis of the glycopeptides revealed that two serine residues (Ser-14 and Ser-15) are not glycosylated, Thr-17 and Ser-19 being glycosylated instead, in disagreement with the accepted structure. The glycopeptides thus obtained were treated with sialidase and β-galactosidase. The Tn antigenicity, as assayed by the binding to a monoclonal anti-Tn antibody (MLS 128), was found exclusively in the glycopeptides including three (cluster I) or four (cluster II) consecutive residues of GalNAc-Ser/Thr, whereas the glycopeptide (GPA-2) containing two nonconsecutive GalNAc-Ser/Thr residues had practically no Tn antigenicity. The immunoreactivities of GPA-1 and GPA-3, containing both clusters I and II, and GPA-4, containing cluster II, were 63% (calcd. 67%), 81% (calcd. 86%), and 50% (calcd. 50%), respectively, of the immunoreactivity of GPA-5 or GPA-6, containing cluster I (the average being taken as the basis), based on the reactivity per GalNAc residue. These results indicate that clusters I and II react with the antibody to the same extent. The structure consisting of three consecutive glycosylated Ser/Thr residues may be essential for Tn antigenicity in the light of previous results for ovine submaxillary mucin.**

Dausset *et al.* (1) discovered the Tn antigen on erythrocytes of a patient with hemolytic anemia in 1959 and found it to be responsible for the polyagglutination of erythrocytes due to an anti-Tn antibody (Tn syndrome). Since then it has been believed that GalNAc-Ser/Thr is involved in Tn antigen (2), but the exact epitope structure is unknown. It is known that the Tn antigen is also a tumor-associated antigen (3, 4).

A monoclonal antibody, designated MLS 128, which was raised against LS 180 cells, a human colorectal cancer cell line, was identified as an antibody directed to the Tn antigen, as reported previously (5). MLS 128 reacted with ovine submaxillary mucin (OSM) as well as mucin type glycopeptides prepared from LS 180 cells. Other anti-Tn antibodies that have been elicited so far are indistinguishable from MLS 128 with respect to the reactivity with the Tn antigens (5, 6).

Among glycopeptides prepared from protease digests of asialo-OSM, Leu-**Ser**-Glu-**Ser**-**Thr**-**Thr**-Glu-Leu-Pro-Gly was a minimum glycopeptide with Tn antigenicity, where the bold type denotes amino acid residues to which an α-GalNAc residue is attached, indicating that three consecutive GalNAc-Ser/Thr residues are essential for the epitopic structure (6). Since Tn glycophorin A contains two such sequences, cluster I at residues 2–4 and cluster II at residues 10–15 (7), it seemed to be a suitable material for studying Tn antigenicity.

EXPERIMENTAL PROCEDURES

Establishment of Monoclonal Antibodies. Monoclonal antibody MLS 128 (IgG3, κ) was established as described previously (5). The other anti-Tn antibodies, NCC-LU-35 and CA 3239, were kindly provided by S. Hirohashi of the National Cancer Institute (Tokyo) and F. Springer of Northwestern University (Evanston, IL), respectively.

Reactivity of Glycophorin A and Glycopeptides with Monoclonal Antibodies. A binding assay using microplates was used to minimize the amount of glycopeptide required. A polyvinyl chloride plate (96 wells; Costar) was coated with glycophorin A and glycopeptides according to Fukui *et al.* (8), and then the uncoated surfaces were coated with 1% bovine serum albumin (BSA) in 20 mM phosphate-buffered saline (PBS), pH 7.2. An appropriate amount of MLS 128, NCC-LU-35, or CA 3239 in PBS containing 0.1% BSA was added to each well and the plate was left standing overnight at 4°C, and then the wells were washed three times with PBS containing 1% BSA.

For determination of the amount of MLS 128 bound to the plate, ^{125}I-labeled staphylococcal protein A was added. For NCC-LU-35 and CA 3239 (IgM type), ^{125}I-labeled rabbit anti-mouse IgM antibody (IgG) was used. Incubation was carried out at room temperature for 2 hr, after which the wells were washed three times with PBS containing 1% BSA. Finally, each well was cut out from the plate and radioactivity was measured in a γ counter (Aloka; Tokyo).

Digestion of Glycophorin A with Glycoprotease. Glycophorin A was incubated with a glycoprotease (*Pasteurella haemolytica* A1) preparation isolated as described previously (9). The incubation was in 50 mM Hepes buffer, pH 7.2, at 37°C for 48 hr at a substrate-to-enzyme ratio of 320:1 (wt/wt). The specific activity of the enzyme preparation was 8 mg of glycophorin A degraded per hr per mg of protein, and the preparation was free from measurable sialidase or other proteolytic enzyme activity.

Fractionation of Glycopeptides by HPLC. The digests of glycophorin A were applied to a gel filtration column (Diol 60 from YMC (Kyoto)]. Elution was carried out with 30% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fraction a (Fig. 2) was further loaded on a lentil lectin column (HONEN), which was washed with 50 mM Tris·H_2SO_4, pH 7.2, and then eluted with a linear gradient from 0 to 0.2 M methyl α-D-mannoside in the above buffer. The void volume fraction (a1) was fractionated by HPLC on a reverse-phase YMC Pack AP 303 column. Elution was performed with a linear gradient of 0–50%

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Abbreviations: OSM, ovine submaxillary mucin; BSA, bovine serum albumin.

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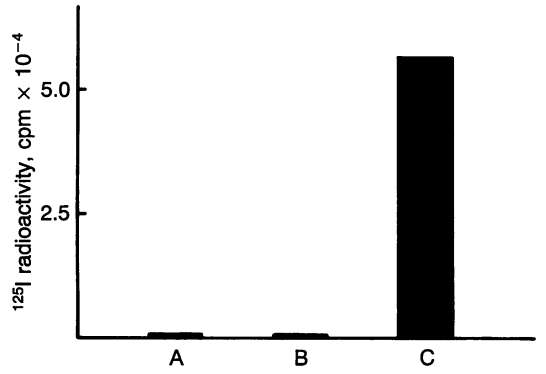

FIG. 1. Tn antigenicity of glycophorin A treated with sialidase and β-galactosidase. One microgram of glycophorin A was coated on an assay plate and then treated with sialidase and β-galactosidase successively. Tn antigenicity was examined at each step. An appropriate amount of each Tn antibody was used, and the activities for MLS 128 were determined by means of binding of ^{125}I-labeled staphylococcal protein A. A, intact glycophorin A; B, asialoglycophorin A; and C, asialoagalactoglycophorin A.

acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The eluted fraction (a2) was purified by gel filtration as described above. Fractions b and c were further fractionated on the reverse-phase column as described above.

Amino Acid and Amino Sugar Analyses. Amino acid analysis was performed on samples hydrolyzed in 6 M HCl at 110°C for 24 hr. The hydrolysates were analyzed with a Hitachi L8500 amino acid analyzer. Amino sugars were determined essentially according to the method of Gardell (10).

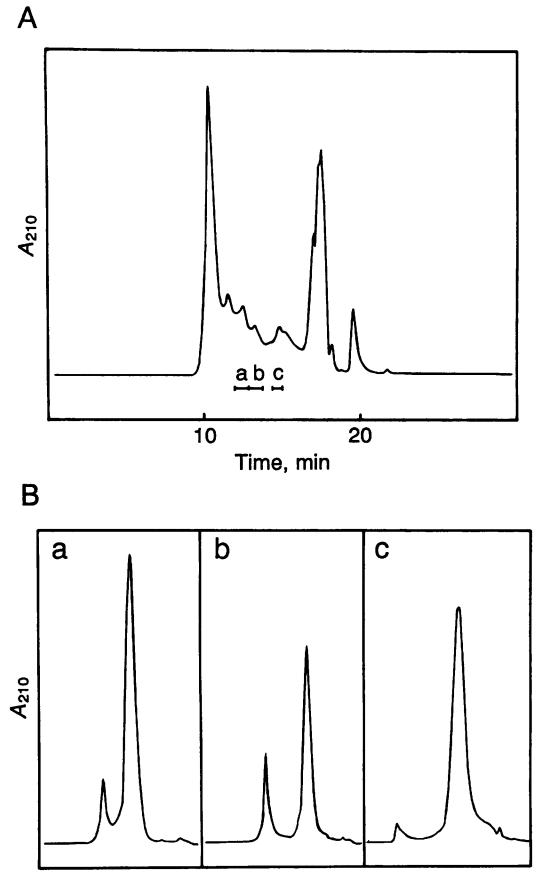

FIG. 2. Gel filtration of glycoprotease digest of glycophorin A. (*A*) Digest of glycophorin A was subjected to HPLC on a gel filtration column (YMC Diol 60). (*B*) Fractions a, b, and c obtained in *A* were subjected to rechromatography on the same column.

Determination of Amino Acid Sequences. The amino acid sequences of the glycopeptides were determined by the Edman degradation method, using a pulse liquid protein sequencer equipped with a 120 phenylthiohydantoin analyzer (Applied Biosystems 477A).

Treatment of Glycophorin A and Glycopeptides with Sialidase and β-Galactosidase. Assay plates coated with glycophorin A or glycopeptides were treated with 5 milliunits of sialidase (*Vibrio cholerae*) in 50 mM sodium acetate buffer, pH 5.6, containing 10 mM $CaCl_2$ and 0.1 M NaCl at 37°C for 2 hr, and then with 5 milliunits of sialidase (*Arthrobacter ureafaciens*) in 0.2 M sodium acetate buffer, pH 5.0, at 37°C for 2 hr (1 unit catalyzes release of 1 μmol of sialic acid per min). The plates were further treated with 10 milliunits of β-galactosidase (bovine testis) in 50 mM sodium acetate buffer, pH 4.6, containing 0.15 M NaCl at 37°C for 24 hr. Complete release of sialic acid and galactose residues was confirmed by following the progression of the antibody binding to saturation.

RESULTS

Treatment of Glycophorin A with Sialidase and β-Galactosidase. O-Glycosidic carbohydrate chains of glycophorin A have been identified as Siaα2-3Galβ1-3(Siaα2-6)GalNAc-

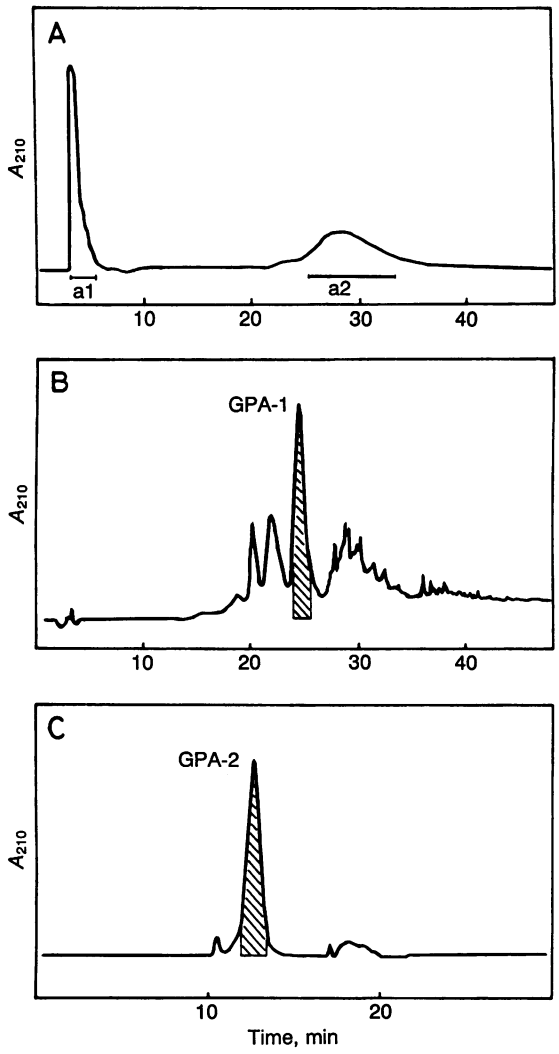

FIG. 3. Separation of glycopeptides. (*A*) Fraction a was applied to a column of lentil lectin and separated into void volume (a1) and eluted (a2) fractions. (*B*) Fraction a1 was further fractionated by reverse-phase column (YMC Pack AP 303) chromatography. (*C*) Fraction a2 was purified by gel filtration as in Fig. 2.

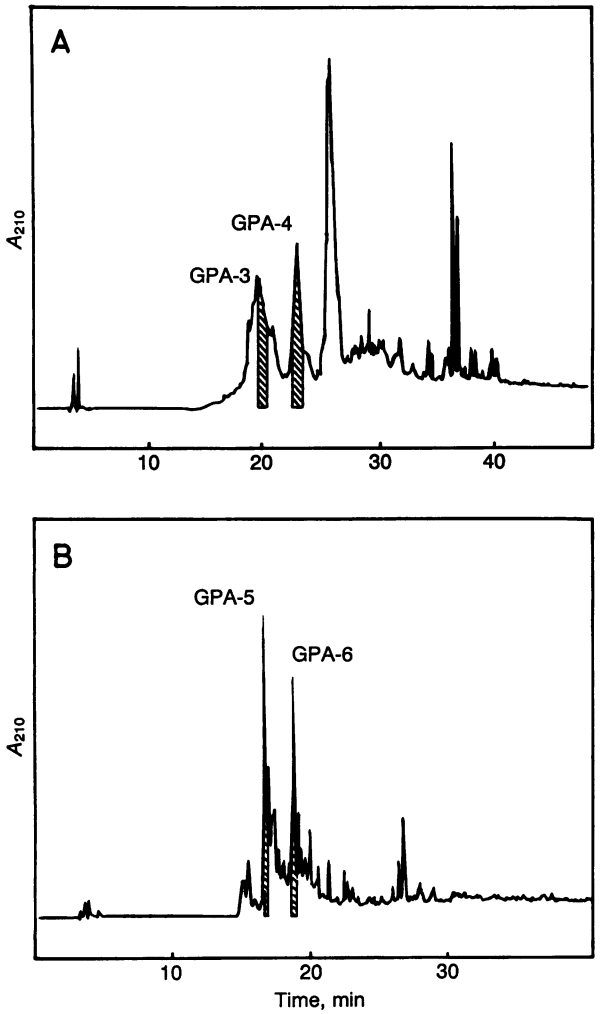

FIG. 4. Elution profiles of fractions b and c on reverse-phase column chromatography. GPA-3 and -4 (*A*) and GPA-5 and -6 (*B*) were obtained from fractions b and c, respectively.

Ser/Thr (11). Tn antigenicity should be induced on treatment with sialidase and β-galactosidase. Thus, intact glycophorin A was coated on an assay plate and treated with sialidase and β-galactosidase as described in *Experimental Procedures*. As shown in Fig. 1, MLS 128 reacted only with asialo agalactoglycophorin A. The other anti-Tn antibodies NCC-LU-35 and CA 3239 showed the same reactivity as MLS 128 (data not shown).

Glycophorin A is of two types, designated as MM and NN, which differ in two amino acids—i.e., residues 1 and 5. Since both types of glycophorin A showed the same reactivity with MLS 128 (data not shown), we used MN glycophorin A as the starting material.

Protease Digestion and Fractionation of Digests. To determine the epitopic structure for MLS 128, glycophorin A was digested with glycoprotease as described in *Experimental Procedures*. It should be noted that the glycoprotease is a neutral metallo-protease produced by *P. haemolytica* and has the ability to degrade sialylated mucin-type glycoproteins, but not all the cleavage sites for glycophorin A have yet been identified (9).

The digest was fractionated by HPLC on a gel filtration column (YMC Diol 60) to produce fractions a, b, and c *inter alia* (Fig. 2*A*). Other fractions were either undigested glycophorin A or peptides derived from the nonglycosylated region. Fractions a, b, and c were subjected to rechromatography on the same column, and a major peak for each fraction

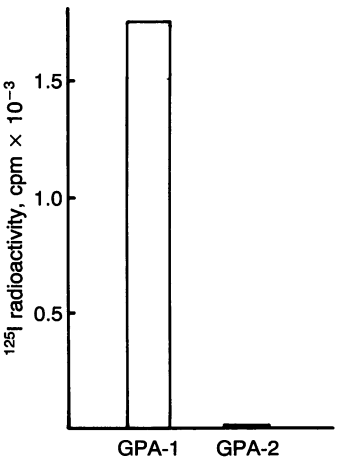

FIG. 5. Reactivities of GPA-1 and -2 with MLS 128. The plates were coated with GPA-1 and -2 in amounts corresponding to 50 ng of GalNAc. After successive treatments with sialidase and β-galactosidase, the plates were incubated with MLS 128 and then with ^{125}I-labeled protein A. The bound radioactivity was determined.

was obtained (Fig. 2*B*). By sequence determination and amino sugar analysis, fraction a was found to be a mixture of glycopeptides containing both O- or O-plus N-glycosidic carbohydrate chains. To separate these glycopeptides, fraction a was applied to a column of lentil lectin. As shown in Fig. 3*A*, fraction a was separated into void volume (a1) and eluted (a2) fractions. Fraction a1 was further purified by reverse-phase column chromatography (Fig. 3*B*). The major peak, designated GPA-1, was identified as a glycopeptide composed of 21 amino acids from the N terminus to Ile-21 by sequence determination.

The eluted fraction (a2) from the lentil lectin column was further purified by gel filtration (Fig. 3*C*). The purified glycopeptide was designated GPA-2 and identified as a glycopeptide composed of 15 amino acids from Ser-22 to Ala-36 with O-glycans attached to Ser-22 and Thr-25.

Upon further fractionation of fractions b and c (Fig. 2) by reverse-phase column chromatography, two glycopeptides, designated GPA-3 and GPA-4, were obtained from fraction b, and GPA-5 and GPA-6 were obtained from fraction c (Fig. 4 *A* and *B*). GPA-3 corresponded to the region from the N terminus to Val-16, and GPA-4 to that from Met-8 to Ile-21. GPA-3 contained both clusters I and II, like GPA-1, whereas GPA-4 contained only cluster II. GPA-5 and GPA-6 were glycopeptides extending from the N terminus to Ala-7, differing in the N-terminal and 5th amino acids, and contained only cluster I. Obviously they were derived from MM and NN type glycophorins A, respectively (Fig. 4*B*).

Structures and Immunoreactivities of the Isolated Glycopeptides. The amino acid sequences of the glycopeptides were determined by the Edman degradation method, in which glycosylated amino acids were not detected. Thus, the positions of the glycosylated amino acids were deduced from the data reported by Siebert and Fukuda (12). We found some differences with respect to the O-glycosylated sites of GPA-1 or GPA-3 from the structure proposed by Tomita and Marchesi (7). They reported that all the residues from Thr-10 through Ser-15 were O-glycosylated, but that the Thr-17 and Ser-19 residues were nonglycosylated. In our sequence determination, however, both Ser-14 and Ser-15 were quantitatively recovered as unsubstituted serine residues, but neither Thr-17 nor Ser-19 was recovered as such. In addition, amino sugar analysis showed that GPA-1 contained nine galactosamine residues, consistent with the proposed structure (7). Since the absence of carbohydrate on Ser-14 and Ser-15 and the presence of carbohydrate on Thr-17 and

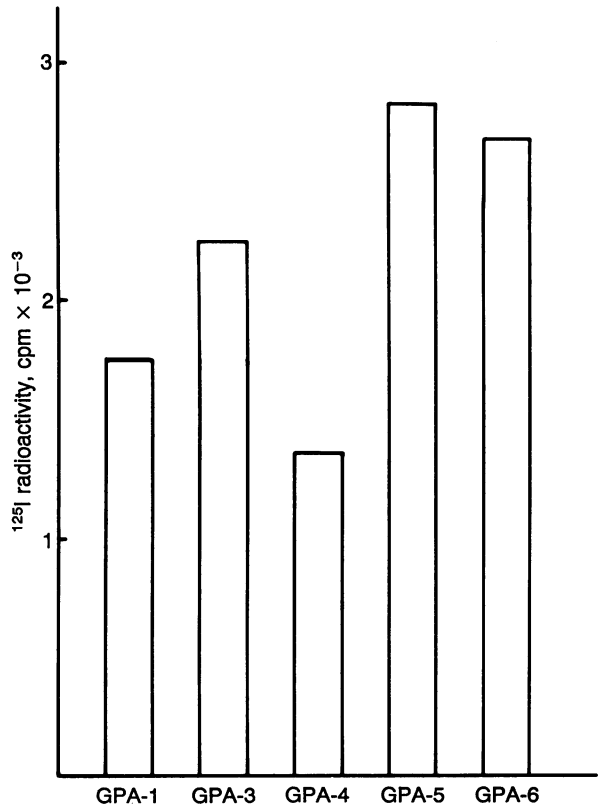

FIG. 6. Reactivities of the glycopeptides containing a cluster structure with MLS 128. The plates were coated with various glycopeptides in amounts corresponding to 50 ng of GalNAc. Binding of MLS 128 to the coated plates was determined as in Fig. 5. (For structures see Fig. 7.)

Ser-19 were confirmed by amino acid sequence determination of intact glycophorin A, it is unlikely that some of the GalNAc residues were released or transferred to different positions during the glycoprotease treatment. Thus, it is now evident that glycophorin A contains two clusters, clusters I and II—i.e., ones based on GalNAc-Ser-(GalNAc)-Thr-(GalNAc)-Thr (cluster I) and GalNAc-Thr-(GalNAc)-Ser-(GalNAc)-Thr-(GalNAc)-Ser (cluster II), respectively. GPA-1 and GPA-2 were then coated on assay plates and treated with sialidase and β-galactosidase. GPA-1 reacted strongly with MLS 128, whereas GPA-2 showed practically no activity, as shown in Fig. 5. These results are consistent with our previous finding for OSM that the Tn antigen is borne not by a simple structure such as GalNAc-Ser/Thr, but by a more complex cluster structure (6).

The glycopeptides GPA-3, GPA-4, GPA-5, and GPA-6 were coated on assay plates and then treated with sialidase and β-galactosidase. All the glycopeptides showed reactivity with MLS 128 (Fig. 6), indicating that both clusters I and II are essential for the Tn antigenicity. It should be noted that there was some difference in the reactivity between GPA-5 and GPA-6, which seems to indicate that amino acids around the cluster structure have some influence on the reactivity, which is detectable in short glycopeptides. GPA-4 had reactivity corresponding to about half that of GPA-5 or GPA-6. This is reasonable, since the activity expressed depends on the amount of GalNAc contained and GPA-4 contained six GalNAc residues, whereas GPA-5 and GPA-6 each contained three.

DISCUSSION

Glycophorin A possesses a large number of oligosaccharides based on the GalNAc-Ser/Thr structure. Some are dispersed along the polypeptide chain and others are bound to consecutive residues to form cluster structures. It has not been determined whether such structures equally express the Tn antigenicity. To determine this, *P. haemolytica* glycoprotease was useful, since this enzyme is capable of fragmenting glycophorin A at several sites, whereas the substrate is resistant to common proteolytic enzymes. In fact, the glycoprotease produced six identifiable glycopeptides, GPA-1 to GPA-6.

During determination of the amino acid sequences of these glycopeptides, we found that the last two serine residues of the six consecutive possible glycosylation sites in the se-

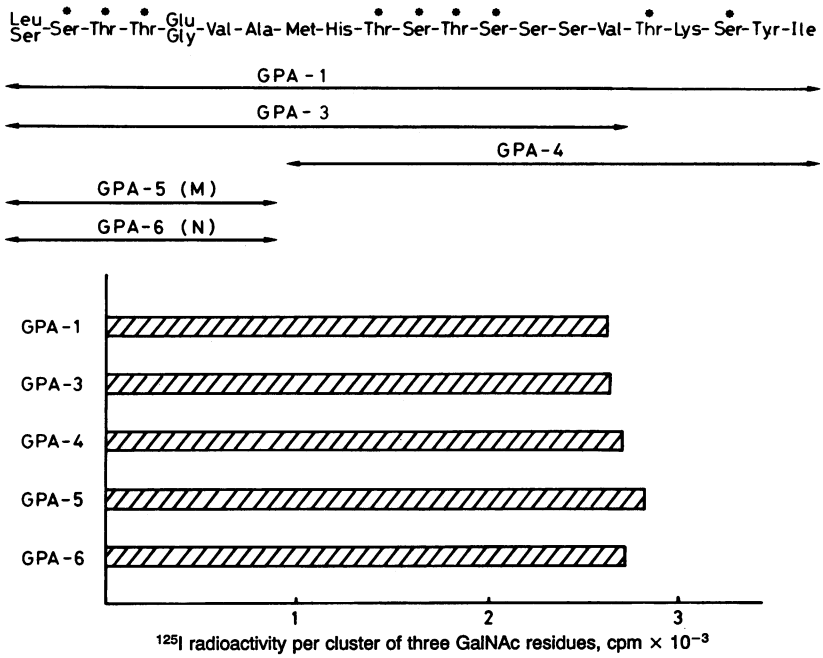

FIG. 7. Structures and immunoreactivities of the glycopeptides. The reactivity, as shown in Fig. 6, is expressed relative to the clusters of three consecutive GalNAc-Ser/Thr residues. * indicates O-glycosylation sites.

quence (Ser-14 and Ser-15) are not glycosylated, Thr-17 and Ser-19 being glycosylated instead. This result was confirmed with the MM and NN types of glycophorin A by direct amino acid sequence determination. The six glycopeptides were then tested in binding studies with MLS 128 after successive treatments with sialidase and β-galactosidase.

If the immunoreactivity is exclusively accounted for by the three consecutive GalNAc-Ser/Thr residue structure, then any three of the four GalNAc residues in GPA-4 could be responsible for the reactivity, and the remainder of the residues need not be involved in the reactivity. By making such assumptions, we could estimate the reactivity of GPA-4 in comparison with that of GPA-5 or GPA-6. Since the number of GalNAc residues was six in GPA-4, and double that in GPA-5 or GPA-6, the specific activity (reactivity per GalNAc) should be 0.5 for GPA-4 versus 1.0, the mean value of GPA-5 and GPA-6. Such estimates were extended to all the glycopeptides, and the reactivities were expressed as percentages relative to the mean value for GPA-5 and GPA-6. The experimental values are in good agreement with those calculated (shown in parentheses)—i.e., 63 (67), 81 (86), and 50 (50) for GPA-1, GPA-3, and GPA-4, respectively.

All the sequence and reactivity data are summarized in Fig. 7, in which the reactivities of the three- and four-consecutive-residue sequences of GalNAc-Ser/Thr are assumed to be equal. Although we have no evidence showing which three GalNAc-Ser/Thr residues of the four consecutive ones are responsible for the reactivity, it is clear that more than three consecutive GalNAc-Ser/Thr residues are not necessary for the Tn antigenicity.

Recently, we found that a major glycoprotein, leukosialin (CD43), of a T-lymphoid cell line, Jurkat, expresses the Tn antigen (13). On the basis of the amino acid sequence and carbohydrate structures reported by Pallant *et al.* (14) and Piller *et al.* (15), leukosialin seems to contain seven cluster structures, each consisting of three or four consecutive GalNAc-Ser/Thr residues. gp120, a coat protein of human immunodeficiency virus, also expresses the Tn antigen (16). Although the antigenic site has not been determined, possible consecutive sites to which GalNAc is bound could be found in the deduced amino acid sequence (17). These glycoproteins and Tn antigen all appear to play significant biological roles.

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