CARBOHYDRATE STRUCTURE AND SEROLOGICAL BEHAVIOUR OF "ANTIFREEZE" GLYCOPROTEINS FROM AN ANTARCTIC FISH.

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SUMMARY

The carbohydrate moiety of the "antifreeze" glycoprotein from Trematomus borchgrevinki was found to be ß-D-galactosyl 1-3 N-acetyl galactosamine by gas-liquid chromatography. The glycoprotein inhibited anti-T antibody from human serum and Arachis hypogoea lectin, but was inactive against Vicia graminea. Native "antifreeze" glycoprotein did not inhibit the agglutinins from Helix pomatia or Cepaea hortensis, although after Smith degradation showed a strong inhibition towards them. Inhibition of the latter agglutinin demonstrates the carbohydrate-protein linkage to be α -linked. The presence of the Thomsen-Friedenreich antigen (T-antigen) on the "antifreeze" glycoprotein and its relation to tumour cell surfaces is briefly discussed.

A group of glycoproteins have been found in the sera of Antarctic fish which depress the freezing point of the blood, without any significant effect on the osmolarity, enabling them to live at temperatures below the freezing point of the blood of most marine fish (1), (2). The structure of the glycoproteins from the Antarctic fish Trematomus borchgrevinki is composed of a repeating diglycosyl-tripeptide, Ala-Ala-Thre-O-disaccharide (3), in which the disaccharide contains the two sugars, galactose and N-acetyl galactosamine (4).

Shier et al. (5) and Vandeheede et al. (6) have proposed the structure as being ß-D-galactosyl (1-4 and 1-3)-N-acetyl galactosamine respectively, Vandeheede et al. by using chromagen formation in an alkaline medium and Shier et al. from NMR analyses.

The disaccharide ß-D-galactosyl 1-3 N-acetyl galactosamine is found linked to protein in a variety of glycoproteins, although it is usually masked by neuraminic acid or L-fucose. Glycoproteins in which this structure is found include submaxillary glycoproteins (7), rabbit brain glycopeptides (8), casein (9), tumour cells (10), as well as human erythrocyte membrane glycoproteins (11), erythrocytes of other species and the milk fat globule membrane (unpublished

results). It is also found as a constituent of human brain gangliosides (12) and the finding by Kim and Uhlenbruck (13) that these gangliosides possessed the T-antigen, was the first demonstration that this disaccharide is the chemical structure associated with T-activity.

The occurence of this disaccharide in so many different and varied tissues prompted us to examine the linkage found in "antifreeze" glycoproteins, by a different method than had been previously used, in order to clarify the glycoprotein structure.

MATERIALS AND METHODS

The "antifreeze" glycoproteins were generous gifts of Drs. A. L. De Vries (Scripps Institute, La Jolla, California) and W. T. Shier (Salk Institute, San Diego, California) which had been isolated by De Vries as previously described (4). The Smith degraded glycoprotein was also obtained from Dr. W. T. Shier.

Crystalline ß-D-galactosyl 1-3 N-acetyl galactosamine from human brain gangliosides (12) was a gift from Prof. W. Gielen (Pharmakologisches Institut der Universität Köln). Dowex ion exchange resin 50W x 8 (200-400 mesh) was obtained from Serva Ltd. (Heidelberg), neuraminidase (500U/ml) from Behringwerke (Marburg) and all other chemicals of analytical grade from Merck Ltd. (Darmstadt).

For alkaline borohydride treatment, glycoprotein samples (0.5 - 1.0 mg) were soluted in 0.05M NaOH containing 1.0M $NaBH_4$ (2ml) and incubated under nitrogen for 24h at 50°C in the dark. After this time, trehalose (30µg) was added as an internal standard and excess borohydride destroyed by neutralising the solution with Dowex 50W x 8 (H^+ form) until no more gas was evolved. The supernatant was removed and the resin washed three times with water. The supernatant and washings were combined and lyophilised, the resulting boric acid being removed by repeated evaporation with methanol. The disaccharide from brain gangliosides was treated in an identical manner.

Samples were dried overnight under vacuum and trimethylsilylated for 2h by the method of Sweeley et al. (14) using hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS). Aliquots of the trimethylsilylated material (1-2 µl) were chromatographed on glass columns (6ft x 0.25in) packed with 3.1% silicone gum rubber SE-30 on Gas-Chrom Q (100-120 mesh) and 3% OV-17 on Gas-Chrom Q (Serva Ltd.). The chromatograph (Hewlett-Packard HP 7620A) was run isothermally at 250°C with a nitrogen flow rate of 45ml/min.

Haemagglutination tests were performed as previously described (15), (16). Arachis hypogoea and Vicia graminea lectins, human anti-T, Helix pomatia and Cepaea hortensis agglutinins were also prepared as previously described (15), (16).

RESULTS AND DISCUSSION

Alkaline borohydride treatment of "antifreeze" glycoprotein released a disaccharide which, when compared by gas-liquid chromatography to the reduced disaccharide from brain ganglioside, gave a single peak that was identified as ß-D-galactosyl 1-3 N-acetyl galactosaminitol. Alkali-labile oligosaccharides from human erythrocyte membrane glycoprotein, after removal of neuraminic acid, gave identical retention times to the standard disaccharide on both columns.

Compared to an internal standard of trehalose, the disaccharide gave retention times of 1.66 and 1.87 on SE-30 and OV-17 respectively. No other peaks were detected, establishing that the disaccharide found in "antifreeze" glycoproteins consists only of the ß 1-3 linked type.

Serological tests, using "antifreeze" glycoproteins to inhibit the haem-agglutination reaction between anti-T antibody, specific agglutinins and desialised human erythrocytes, are summarised in Table 1.

"Antifreeze" glycoprotein was found to inhibit anti-T antibody, demonstrating the presence of the T-antigen on the glycoprotein, as well as strongly inhibiting the lectin from Arachis hypogoea. The free disaccharide also inhibited both agglutinins (Table 1). In both cases however, Smith degradation of the glycoprotein removed its inhibitory activity.

Vicia graminea possesses a lectin which is reportedly blood group N specific (17) and is known to require the disaccharide ß-D-galactosyl 1-3 N-acetyl galactosamine for activity. As might be expected, Vicia graminea was not

TABLE 1

Inhibition of various agglutinins by "antifreeze" glycoprotein (native and Smith degraded) and the disaccharide ß-D-galactosyl 1-3 N-acetyl galactosamine. +

Inhibitor	Source of agglutinin.				
	A. hypogoea	Anti-T	V. graminea	H. pomatia	C. hortensis
Native 1) glycoprotein.	2^9	2^4	ø	ø	ø
Smith degraded glycoprotein.	ø	ø	ø	2^8	2^{11}
Disaccharide ß-Gal 1-3 GalNAc.	2^5	2^3	ø	ø	ø

1) Glycoprotein concentrations of 5mg/ml and disaccharide concentrations of 2.9mg/ml were used.

+ Inhibition values are expressed as the reciprocal of their inhibition titres against four agglutination doses of agglutinin.

inhibited by "antifreeze" glycoprotein, showing that, although it contained the above disaccharide, it did not possess the other requirements necessary for the expression of N activity (i.e. correct amino acid sequence) (18).

The native "antifreeze" glycoprotein did not inhibit the agglutinin from Helix pomatia or Cepaea hortensis showing that no free galactosamine is linked to the peptide backbone and that all the galactosamine is confined to the disaccharide units. Smith degradation of the glycoprotein however, led to the exposure of receptors for both Helix pomatia and Cepaea hortensis due to the removal of galactosyl units, the peptide linked N-acetyl galactosamine residues being available for agglutinin binding. The agglutinin from Cepaea hortensis is specific for α-linked N-acetyl galactosamine (15) and inhibition of this agglutinin by Smith degraded glycoprotein demonstrates the presence of an α-carbohydrate-protein linkage in the "antifreeze" glycoprotein.

Shier, using "antifreeze" glycoprotein, modified by removal of some of the disaccharide units, was able to demonstrate that antibodies developed against this glycoprotein were capable of enhancing or inhibiting chemically induced mammary adenocarcinoma cell growth, at high and low antigen concentrations respectively (19). These results would suggest that the antigenic determinants present on the "antifreeze" glycoprotein, are also present on the tumour cell surface. Springer has reported the detection of the Thomsen-Friedenreich antigen (T-antigen) on the surface of mouse and human mammary adenocarcinoma (10), (20), which together with our finding (i.e. that the "antifreeze" glycoprotein possesses the T-antigen) is of significance for the hypothesis that tumour cells possess higher amounts of this antigen on their surface. An examination of the relative levels of sialyl transferase between normal and mammary carcinoma cells may prove to be significant.

During the course of this work, W. T. Shier has also found the disaccharide linkage to be 1-3 by an independant method (personal communication).

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REFERENCES

1. De Vries, A. L., Ph. D. thesis, Stanford Univ. (1968).
2. De Vries, A. L., Komatsu, S. K. and Feeney, R. E. (1970), J. Biol. Chem. 245, 2901-08.
3. De Vries, A. L., Vandeheede, J. and Feeney, R. E. (1971), J. Biol. Chem. 246, 305-8.

4. Komatsu, S. K., De Vries, A. L. and Feeney, R. E. (1970), J. Biol. Chem. 245, 2909-2913.
5. Shier, W. T., Lin, Y., De Vries, A. L. (1972), Biochim. Biophys. Acta 263, 406-413.
6. Vandeheede, J. R., Ahmed, A. I. and Feeney, R. E. (1972), J. Biol. Chem. 247, 7885-7889.
7. Gottschalk, A., Bhargava, A. S. and Murty, V. L. N. (1972), in "Glycoproteins" pp. 810-829, Edit. Gottschalk, A. Elsevier Press, Amsterdam, London, New York.
8. Margolis, R. K. and Margolis, R. U. (1973), Biochim. Biophys. Acta 304, 421-429.
9. Jollès, P. (1972) in "Glycoproteins" pp. 782-819, Edit. Gottschalk, A., Elsevier Press, London, Amsterdam, New York.
10. Springer, G. F., Desai, P. R., Bantwala, I. (1975), J. Nat. Cancer Inst. 54, 335-339.
11. Thomas, D. B., and Winzler, R. J. (1969), J. Biol. Chem. 244, 5943-5946.
12. Klenk, E., Hendriks, U. W. and Gielen, W. (1962), Hoppe Seyler's Z. Physiol. Chem. 330, 140-144.
13. Kim, Z., Uhlenbruck, G. (1966), Z. Immunforsch. 130, 88-99.
14. Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W. (1963) J. Amer. Chem. Soc. 85, 2497-2507.
15. Dahr, W., Uhlenbruck, G. and Bird, G. W. G. (1974), Vox Sang. 27, 29-42.
16. Dahr, W., Uhlenbruck, G, and Bird, G. W. G. (1975), Vox Sang. 28, 133-148.
17. Uhlenbruck, G. and Dahr, W. (1971), Vox Sang. 21, 338-351.
18. Lisowska, E. and Duk, M. (1975). Eur. J. Biochem. 54, 469-474.
19. Shier, W. T. (1973), Nature 244, 99-101.
20. Springer, G. F., and Desai, P. R. (1974), Ann. Clin. and Lab. Sci. 4, 294-298.