Size and Configuration of Glycoprotein Fragments Cleaved from Tumor Cells by Proteolysis*

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SUMMARY

Mucin-type glycoprotein material was fractionated by gel filtration after cleavage from viable TA3-Ha mouse mammary adenocarcinoma ascites cells by a modified trypsin. The macromolecular species present in each fraction were characterized by electron microscopy of metal-contrasted replicas as highly asymmetric rods. Investigation of one fraction gave close agreement for length by electron microscopic measurements and for a length calculated for a single polypeptide backbone model from a molecular weight value by short column sedimentation equilibrium, amino acid composition, and percentage of protein. This result supports a single, extended chain configuration for this fraction. With this value of molecular weight per unit length, approximate molecular weights of particles of the other fractions were calculated. Lengths of polypeptide chains varied from 50 nm (55,000 daltons) to greater than 700 nm, suggesting an average molecular weight for one fraction of over 500,000.

The presence of carbohydrate-containing macromolecules at cell surfaces has been readily demonstrated by histochemical staining (1), and it has been concluded that all animal cells possess glycoproteins at their surfaces (2). Glycoproteins at the surfaces of mammalian cells have been implicated in diverse and vital cellular functions. These have been reported to include ectobiological activities of cells, such as contact inhibition of growth (3), cellular adhesion (4), transplantation rejection (5), tumor-specific antigenic activity (6), and suppression of antigenic activity (7). Cell surface glycoproteins may also participate in active transport (8) and contain blood-specific groupings (9) and recognition sites for viruses (10) and plant agglutinins (11). Despite the current interest in glycoproteins of the cell surface, however, little information has been reported regarding the structure, function, or configuration of any particular cell surface glycoprotein (12).

Gasic and Gasic (13) reported the presence, at the surface of the TA3 mammary adenocarcinoma ascites cell, of a heavy coat of sialic acid-containing material (13), which was later shown to be glycoprotein in character (14). Sanford (15) reported that neuraminidase treatment of this non-strain-specific subtype TA3-Ha reduced its transplantability in the allogeneic C3H mouse (15). In vitro studies later demonstrated that C3H mouse serum possessed a factor toxic to neuraminidase-treated TA3-Ha cells, but non-toxic to cells which had not been incubated with the enzyme (16). Thus, at the TA3-Ha cell surface, glycoproteins appear to be directly implicated in malignancy. In an investigation of the glycoproteins at the surface of this cell, Codington et al. (17) reported the isolation, after proteolysis of viable cells followed by gel filtration, of a glycoprotein fraction, glycoprotein Fraction I, in amounts as great as 0.5% of the cell dry weight. Fractionation of tritium-labeled material on a column of Bio-Gel A-5M suggested the presence of two components, a finding which was supported by sedimentation equilibrium studies (17). Chemical data suggested that these components possessed closely related structures.2

Although suggestions regarding the configuration and sizes of glycoproteins at the surfaces of mammalian cells have been made (18), the visualization by electron microscopy of individual surface macromolecules has not yet been reported (19). Furthermore, to our knowledge, no work has yet been described regarding the configuration of any glycoprotein after isolation from the cell surface; information which might suggest its configuration on the living cells. Recent electron microscopic methods permit the gathering of meaningful statistical information regarding the absolute length and width of macromolecules in an extended configuration (20, 21), including appropriate correction for small measurements such as width, due to the thickness of metal deposits during shadow-casting contrast enhancement.2

This paper describes the configuration, as determined by electron microscopy, of glycoprotein molecules of the TA3-Ha cell surface isolated after passage of glycoprotein Fraction I through

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1 J. F. Codington, and R. W. Jeanloz, in preparation.
2 H. S. Slayter, in preparation.
a column of Sepharose 4B. It discusses the molecular weights of isolated fragments of the native molecules in relation to those obtained by sedimentation equilibrium.

METHODS

Isolation of Glycoprotein Fractions—TA3-Ha cells from strain A/Ha male mice were harvested and washed under sterile conditions, as previously described (17). Glycoprotein material was removed from viable cells (2 to $4 \times 10^6$ cells per batch) by five to six successive, 20-min incubations with TPCK-trypsin $^3$ (18 $\mu$g per ml) at 4°. Supernatant solutions were lyophilized and stored in the cold. Material from 2 to $5 \times 10^6$ cells was pooled and fractionated on columns of Bio-Gels P-4, P-30, and P-100, as described earlier (17). Material eluted with the void volume of a Bio-Gel P-100 column was designated “glycoprotein Fraction I.” The material utilized in this investigation was derived from cells of later generations than the material previously described (17).

A sample of 8.04 mg of tritium-labeled (22) glycoprotein Fraction I was eluted with 0.05 $\mu$g pyridine acetate (pH 5.3) from a column (2.6 x 87 cm) of Sepharose 4B (Pharmacia, Uppsala, Sweden) at a rate of 2.5 ml per cm$^2$ per hour. Fractions of 6.0 ml were collected, and appropriate aliquots (0.23 ml) were mixed with Aquasol (10 ml, New England Nuclear Corp., Boston, Mass.) and counted for radioactivity in a Packard Tri-Carb scintillation counter. Fractions were pooled as follows: A, 144 to 192 ml; B, 193 to 258 ml; and C, 259 to 330 ml. Each sample was lyophilized.

Analytical Determinations—Amino acid analyses were performed on a Technicon amino acid AutoAnalyzer or by gas-liquid chromatography on a Perkin-Elmer gas chromatograph, model 900, after hydrolysis of nitrogen-flushed samples in 5.7 M distilled hydrochloric acid at 110° for 20 hours. Samples for gas-liquid chromatography were prepared by a modification of the method of Roach and Gehlke (23) and injected into a column of Ttabsorb (Regis Chemical Co., Chicago, III.).

Carbohydrate components were determined by gas-liquid chromatography after methanolysis (1.0 $\mu$g hydrochloride in methanol at 80° for 20 hours) followed by conversion to the per trimethylsilylated methyl glycosides (17). Sialic acid was also determined by the thiobarbituric acid procedure (24), following acid hydrolysis.

Molecular Weight by Sedimentation Equilibrium—A sample of 150 $\mu$g of Fraction C in 200 $\mu$l of 6 $\mu$g guanidine hydrochloride (Hoech, Inc.)-10 mM EDTA was dialyzed at 4° against the same solution for 32 hours and analyzed on the model E analytical ultracentrifuge by the method of Yphantis (25), at an equilibrium speed of 20,000 rpm and a temperature of 21.2°. The partial specific volume ($\beta = 0.66$) was calculated from published values for carbohydrate and amino acid components. An analysis of Fraction A was run under similar conditions at a speed of 10,124 rpm.

Electron Microscopy—Electron microscopy was carried out on a Siemens 1A electron microscope at 80 kv with a 70-um objective aperture and a magnification of 27,000. Calibration was based upon photographs of indanthrene olive crystals. Micrographs were recorded sufficiently close to focus to make metal grain at the 2-nm level clearly resolved on the original plates.

Plates at the 2-nm level clearly resolved on the original plates. The correctness of this extrapolation is borne out by sedimentation equilibrium studies on Fraction A, which check closely with the value obtained by extrapolation (see “Discussion”).

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$^3$ TPCK-trypsin in trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone in order to eliminate chymotryptic activity (14).

$^4$ L. W. Labaw (1964) Proceedings of the 22nd Annual Meeting, Electron Microscopy Society of America.
RESULTS

Elution of 8.0 mg (dry weight) of glycoprotein Fraction I (17), from a column of Sepharose 4B (Fig. 1), gave a shoulder followed by a major peak. The effluent was cut into three fractions, A, B, and C, as shown by the dotted lines. The three fractions exhibited no significant difference in amino acid composition. Fraction C (3.6 mg), present in larger quantity than Fraction A (1.1 mg), was chosen for extensive investigation.

A completely reliable figure for protein content based upon amino acid analysis was not possible for Fraction C, since an accurate measure of the extent of degradation of the components by acid hydrolysis was not possible with the small amount of material available. More reliable values, based upon quantitation of carbohydrate components by gas-liquid chromatography and N-acetylneuraminic acid by the thiobarbituric acid procedure, gave, by difference, an average value of 23 ± 5% protein for Fraction C.

Sedimentation equilibrium analysis (25) of Fraction C in 6 M guanidine hydrochloride-10 mM EDTA suggested sample monodispersity based upon a straight line plot of half the radius squared (r^2/2) versus the natural logarithm of the concentration (ln C), as illustrated in Fig. 2. Extrapolation of apparent weight and number average molecular weights, \( \sigma_w \) and \( \sigma_n \), to infinite dilution gave molecular weight values, respectively, of 99,700 and 101,500; and the value obtained from the slope of the curve (Fig. 2) was 107,000. A similar analysis of Fraction A indicated that the principal species has a molecular weight of 463,000. A second smaller, and minor, component was found to have a molecular weight of 169,000, which represents an average of the smaller species present (see “Discussion”).

Electron micrographs of Fractions A, B, and C are shown in Fig. 3. The particles found in these three fractions are best characterized as highly asymmetric rods, reasonably monodisperse with respect to length for a given fraction. Distributions of length are plotted in Fig. 4. Fraction C shows a peak length of 50 to 80 nm, Fraction B of 140 nm, and Fraction A an average value of approximately 350 nm.

The measured particle widths for Fractions A, B, and C are virtually identical within the precision of the measurements and are included in Table I. After correction for replicating metal, the width is 2.5 nm. The accuracy of this measurement is indeterminate for such a small dimension, but the precision indicates no difference in width.

When Fraction C is subjected to excessive hydrolysis by TPCK trypsin (see Fig. 5), no significant change in the distributions of lengths is found after removal of particles trypsin-sized or smaller by gel filtration, compared with the starting material. Furthermore, the results indicate that more than 80% of the column charge was eluted in the void volume, as expected for Fraction C.

Table I also summarizes the length measurements from electron micrographs. The number and weight averages were calculated from the data, in order to provide a more meaningful comparison with the sedimentation equilibrium data, since the distributions are skewed toward the longer lengths, Fraction A showing greater polydispersity than the others. When the method of sedimentation equilibrium is applied to heterogeneous preparations of polymers, it yields a molecular weight average (25). In a high speed, short column equilibrium sedimentation run, such as the one used here, the number average molecular weight is considered to be the best figure to use in comparing the sedimentation data with electron microscopic results. The linearity of the plot \( r^2/2 \) versus \( \ln C \) indicates homogeneity. However, it is quite possible that due to the shortness of the cell column, larger molecular weight components might be pelleted.

The electron microscopic measurements, on the other hand, would include those larger particles, which would then tend to raise a weight average inordinately, compared with the sedimentation figure. Since molecular weight is proportional to length for a rodlike molecule, we can best compare a sedimentation molecular weight with a number average length (25). The ratio of weight average length (\( L_{av} \)) to number average length (\( L_{C} \)) is a measure of heterogeneity; and, as expected for a fractionated polymer sample, this ratio is about 1.2 to 1.3, indicating substantial homogeneity.

DISCUSSION

In a previous report (17), fractionation on polyacrylamide gel columns of fragments of large glycoprotein molecules removed by proteolysis from the TA3-Ha cell surface produced a fraction which appeared to consist of two components (17). Elution of this material from a column of Sepharose 4B (Fig. 1) fractionated the glycoprotein material on the basis of length of the polypeptide backbone. As viewed by electron microscopy, all fractions appeared similar, except for the rod lengths (Fig. 3).
FIG. 4. Histograms of measurements of lengths of fractionated fragments from electron micrographs. A, Fraction A; B, Fraction B; C, Fraction C.

TABLE I
Summary of length measurements from electron micrographs

| Fraction | Peak lengths | Lw | Lw/Lx | Peak width | WnLx |
|----------|--------------|----|-------|------------|------|
| A        | 350          | 320| 390   | 1.2        | 2.5  |
|          | 310          |    |       |            |      |
|          | 255          |    |       |            |      |
|          | 225          |    |       |            |      |
| B        | 140          | 170| 220   | 1.2        | 2.5  |
|          | 80           | 75 | 120   | 1.3        | 2.5  |
| C        | 45           | 73 | 89    | 1.2        |      |
|          | 75           |    |       |            |      |
| C'       | 50           | 72 | 87    | 1.2        |      |
| Redigest |             |    |       |            |      |
|          | 75           |    |       |            |      |

| a Number average width. |
| b Principal peak. |
| c Different lot of Fraction C. |

For Fraction C, calculation of the length of a single polypeptide chain of an average amino acid residue of 93 and a length of 0.36 nm per amino acid residue in an extended polypeptide chain (26) gives a figure of 90 nm for a glycoprotein of 23% protein and a molecular weight of 100,000, a value arrived at from sedimentation equilibrium data. Since Sample C appears homogeneous by this method (Fig. 2), any error in the molecular weight would probably lie in inaccuracies in the value for the partial specific volume \( \bar{v} \); but, since the proportion of both carbohydrate and amino acids is known with reasonable accuracy, errors due to discrepancies in this parameter should not exceed a few percent. The calculated rod length (90 nm) is about 5% less than the measured number average molecular length of 94 nm (Table I). Evidence against a double chain configuration appears convincing. If the configuration were an \( \alpha \) helix with a 0.15-nm (26) displacement per peptide bond, then the peptide chain would be 2.42 times shorter per amino acid residue, or about 37 nm, which is far too short in comparison with measured values. If it were a two-chain non-helix, it would be one-half as long, or 45 nm. If the particles were in a two-chain helical configuration, the length would be substantially less. Thus, in addition to the apparent impossibility for substantial amounts of helix formation, due to steric conditions (since one glycosidic side chain is found for every three amino acids), the data argue for an extended polypeptide chain, especially in view of the close agreement between the observed polypeptide length and values calculated from molecular weight determinations.

Available evidence suggests that the glycoprotein fragments of Fractions A, B, and C are not associations of noncovalently bonded smaller particles, attached to one another in an end to end manner. Elution from a column of Sepharose 4B, with pyridine acetate solution, fractionated the material mainly according to chain length (Fig. 4). If a reassociation of particles in the fraction containing the smallest rods (Fraction C) had occurred in the absence of pyridine acetate, one would have expected to see some long particles in electron micrographs, such as in Fraction A; but none were observed. More convincing, however, is the fact that in an experiment in which electron micrographs were made of material in salt-free aqueous solution, conditions which should favor noncovalent interactions, the average particle was even shorter than that of Fraction C.

Since we have determined that a length of 94 nm corresponds to a molecular weight of 100,000, as determined by sedimentation equilibrium, it is possible to calculate the approximate...
molecular weights of the other two glycoprotein fractions, A and B, from their measured lengths. The relative proportion of protein in the three fractions does not differ widely, and on the assumption that the gross composition of Fractions A and B is the same (23% protein) as that of Fraction C, the peak material of Fraction A, with an average length of 320 to 360 nm, would have a molecular weight between 300,000 and 500,000. Fraction B would have a molecular weight of 150,000 to 200,000. When Fraction A was subjected to sedimentation equilibrium measurement, a nonideal, two-component system was encountered (27). The value for the principal high molecular weight component of this system obtained from a two species plot was 450,000. A second component of molecular weight 170,000 was found as well, which probably represents an average of the species present in the considerable spread seen in the electron microscope length distribution.

A comparison of the measured width of these particles, 2.5 nm, with that obtained previously for the rod of myosin (2.0 nm), which was shown to be double-stranded (20), suggests that carbohydrate chains are attached to amino acids along the length of the protein core. A width of only 2.5 nm suggests that the carbohydrate chains would be, on the average, not more than about 3–4 residues in length, if they were oriented perpendicular to the particle axis. These results are in agreement with chemical data which indicate that carbohydrate chains, 2 to 5 residues in length, are, on the average, attached to every third amino acid along the length of the protein core (28).

Fraction C, subjected to excessive TPCK-trypsin degradation, undergoes no further diminution in length below about 50 nm. Thus, it may be significant also that in a different preparation purified on Bio-Gel P-100, the glycoprotein Fraction I shows a principal peak at about 50 nm. It, therefore, appears that there is a minimum length beyond which the bulk of this material, represented in Fraction C, cannot be degraded by TPCK-trypsin. Such a cleavage mode could conceivably be due to two factors, namely, the presence of linkages susceptible to cleavage by the enzyme (i.e. containing arginine), and the attachment of side chains containing sialic acid, which might block enzyme action (29). The trace amounts of arginine which could be detected in this material are consistent with breakage down to fragments of the size of Fraction C.

It seems probable that the intact molecule or molecules on the cell surface are of greater length than any of the fragments obtained by proteolysis. Measurements on the intact glycoprotein will no doubt soon allow formulation of a clearer picture of the way in which the fragments fit in. The work of Marchesi et al. (9), on the glycoprotein of the erythrocyte surface, indicated a more or less random breakage. However, the TA3 glycoprotein is much more highly saturated with glycosidic side chains, and so is not directly comparable. It is conceivable that a hydrophobic portion of the molecule(s) is not removed by TPCK trypsin, but, as in the case of the major glycoprotein of the erythrocyte membrane (9), remains rooted in the TA3-Ha cell membrane.

The data presented here indicate that these isolated glycoprotein fragments are long rods, about 2.5 nm in diameter, with a single chain polypeptide backbone and a molecular weight range of approximately 55,000 to more than 500,000. It is probable that the molecular weight of the native molecules on the cell surface is in excess of 500,000. The biological function of these extended molecules at the cell periphery has not yet been determined. A plausible role, however, is suggested by the observation that, although the material was found in abundance on the surface of the non-strain specific subtype, TA3-Ha, it was not detected under similar conditions on the cell surface of a subtype of the same tumor, TA3-St, which had retained strain specificity (30). Since strain specificity in TA3 ascites tumor cells has been correlated with the degree of expression of histocompatibility (H-2) antigens (31), it has been suggested (32) that this material may mask H-2 antigens on the TA3-Ha cell surface. The presence of these rodlike molecules may thus contribute to the virulence of the TA3-Ha tumor.

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REFERENCES
1. Rambourg, A. (1971) Int. Rev. Cytol. 31, 57
2. Bennett, H. S. (1965) J. Histochem. Cytochem. 11, 14
3. Abercrombie, M. & Ambrose, E. J. (1962) Cancer Res. 22, 525
4. Martinez-Palomo, A. (1970) Int. Rev. Cytol. 29, 29
5. Sanderson, A. R., Caperswell, P. & Welsh, K. I. (1971) Nature New Biol. 230, 8
6. Gold, P., Gold, M. & Freedman, S. O. (1968) Cancer Res. 28, 1331
7. Aff Velis, C. A. & Peters, J. H. (1970) J. Theor. Biol. 26, 47
8. Higuchi, D. R., Daniels, M. C. & Ellenberger, L. (1967) J. Biol. Chem. 242, 1010
9. Marchesi, V. T., Tillack, T. W., Jackson, R. L., Sognest, J. P. & Scott, R. E. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1445
10. Springer, G. F. (1970) Naturwissenschaften 57, 162
11. Berger, M. M. (1972) in Biomembranes, (Manson, L. A., ed) Vol. 2, p. 247, Plenum Publishing Co., New York
12. Kraemer, P. M. (1971) in Biomembranes, (Manson, L. A., ed) Vol. 1, p. 67, Plenum Publishing Co., New York
13. Gasic, G. & Gasic, T. (1962) Nature 196, 170
14. Codington, J. F., Sanford, B. H. & Jeanloz, R. W. (1970) J. Natl. Cancer Inst. 45, 637
15. Sanford, B. H. (1967) Transplantation 5, 1273
16. Sanford, B. H. & Codington, J. F. (1971) Tissue Antigens 1, 149
17. Codington, J. F., Sanford, B. H. & Jeanloz, R. W. (1970) Biochemistry 9, 2550
18. Winzler, J. R. (1970) in Blood and Tissue Antigens, (Amhoff, D., ed) p. 117, Academic Press, New York
19. Parsons, D. F. & Suhjick, J. R. (1972) Biochim. Biophys. Acta 265, 85
20. Lowey, S., Slatter, H. S., Weeds, A. G. & Baker, H. J. (1969) J. Mol. Biol. 42, 1
21. Slatter, H. S. & Lowey, S. (1967) Proc. Natl. Acad. Sci. U. S. A. 58, 1611
22. Van Lenten, L. & Ashwell, G. (1971) J. Biol. Chem. 246, 1899
23. Roach, D. & Gercke, C. (1968) J. Chromatogr. 44, 269
24. Ward, I. (1960) J. Biol. Chem. 235, 1961
25. Yphantis, D. A. (1964) Biochemistry 3, 297
26. Pauling, L. (1951) Proc. Natl. Acad. Sci. U. S. A. 37, 205, 295, 729, (1953) Proc. Natl. Acad. Sci. U. S. A. 38, 86
27. Roark, D. & Yphantis, D. A. (1969) Ann. N. Y. Acad. Sci. 164, 245
28. Codington, J. F., Sanford, B. H. & Jeanloz, R. W. (1971) Fed. Proc. 30, 1290 Abstr.
29. Gottschalk, A. & Fazekas de St. Groth, S. (1960) Biochem. Biophys. Acta 43, 513
30. Codington, J. F., Sanford, B. H. & Jeanloz, R. W. (1972) Fed. Proc. 31, 465 Abstr.
31. Fribourg, S. (1972) J. Natl. Cancer Inst. 48, 1477
32. Sanford, B. H., Codington, J. F., Jeanloz, H. W. & Palmer, P. D. (1971) J. Immunol. in press
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