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Surface Membrane Glycopeptides Correlated with Tumorigenesis†

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ABSTRACT: Glycopeptides were removed by trypsin from the surface membranes of clones of (1) hamster embryo cells transformed after treatment with the chemical carcinogen, dimethylnitrosamine, (2) variants of these cells with suppression of the transformed phenotype, and (3) revertants of these variant cells back to the transformed state. The glycopeptides were examined by gel filtration after Pronase digestion. In all cases, the gel filtration profiles of the fucose-containing glycopeptides were similar to those obtained from secondary hamster embryo cells. In contrast, the profiles derived from the tumors formed after inoculation into animals of all of these cell lines showed the appearance of a specific group of glycopeptides which was not found in the original cells. Other properties characteristic of the transformed phenotype which were examined were not consistent with either the appearance of these glycopeptides or with tumor cells. These results suggest a correlation between the surface membrane glycopeptides and tumor formation.

Glycoproteins from surface membranes of virus transformed cells have been compared to those obtained from control cells by a number of investigators (Wu *et al.*, 1969; Meezan *et al.*, 1969; Buck *et al.*, 1970; Sakiyama and Burge, 1972; Warren *et al.*, 1972a). The appearance of specific glycopeptides was demonstrated by chromatography on Sephadex G-50 (Buck *et al.*, 1970) or DEAE-cellulose (Glick, 1971), following transformation by RNA or DNA viruses (Buck *et al.*, 1971). None of these reports has been concerned with the relationship of these glycopeptides to tumorigenesis.

The interaction of carcinogens with cells leads in some cases to their malignant transformation and results in the hereditary expression of properties which include the ability to form tumors *in vivo* and to multiply *in vitro* under conditions which inhibit the multiplication of nontransformed cells. Once the transformed properties have been hereditarily expressed, they can again be hereditarily suppressed. That is, cells hereditarily transformed by viral (Pollack *et al.*, 1968; Rabinowitz and Sachs, 1968, 1972) or nonviral carcinogens (Rabinowitz and Sachs, 1970; Hitotsumachi *et al.*, 1972) can produce variants with a suppression of the properties of transformed cells. Variants from hamster embryo cells transformed after treatment with chemical carcinogens acquire, like normal cells (Hayflick, 1965), a limited life span *in vitro* (Rabinowitz and Sachs, 1970). Some of the variants subsequently escape from the limited life span and again revert to the transformed state.

In the present study we have examined the surface glycopeptides of the following cell types: (1) hamster embryo cells transformed after treatment with the chemical carcinogen, dimethylnitrosamine (DMNA),[1] (2) variants of the cells with suppression of the malignant transformed properties, (3) revertants of these variant cells back to the transformed state, and (4) cells derived from tumors of all of these cell types. The properties of these cells make it possible to examine the glycoproteins of the surface membranes in relation to the formation of tumors. This study describes the appearances of specific glycopeptides in all of the tumor cells examined.

Materials and Methods

Description of Cells and Cell Culture. Minced whole embryos from Syrian golden hamsters were the source of secondary hamster embryo cells. Transformed cells were obtained after treatment of the hamster embryo cells with DMNA and subsequent subculture until a cell line was established (Huberman *et al.*, 1968). In the present experiments, a cell line D (Rabinowitz and Sachs, 1970) was used and will be designated "DMNA cells." Variants of these cells were produced as described by induction of variant formation at low cell density (Hitotsumachi *et al.*, 1972). For this induction, transformed cells were seeded at 2000 cells/50-mm petri dish, in Eagle's medium with a fourfold concentration of amino acids and vitamins and 10% fetal calf serum, and the cultures were incubated at 37° for 2 days and at 24° for 4 days. The cells were then seeded on X-irradiated (4000 R) rat embryo feeder layers in 35-mm petri dishes at a dilution of one cell per plate, and variants in plates with single colonies were isolated. The variants which are reported in this study are variants 11 and 13. Subsequent subculturing of these variants produced cells which either had a limited life span or reverted back to the malignant transformed state (Rabinowitz and

† From the Department of Genetics, Weizmann Institute of Science, Rehovot, Israel. *Received August 10, 1973.* Supported by Contract No. NC1-69-2014 with the Virus Cancer Program of the National Cancer Institute, the National Institutes of Health, and American Cancer Society Grants PRA-68 and BC-109.

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[1] Abbreviations used are: DMNA, dimethylnitrosamine; TBS, 0.15 M NaCl–0.02 M Tris-HCl (pH 7.5).

Sachs, 1970). As soon as the first foci of the transformed cells appeared in the variant cell populations, the cells were cloned on feeder layers and colonies were isolated. The reverted variants reported in this study are revertant 1 of variant 11 (11-1).

To further define the cell types which were used: the term variant of the DMNA cells corresponds to revertant as reported by Yamamoto *et al.* (1973). In a similar manner, the reverted variants reported here correspond to the cells termed *in vitro* re-revertant.

When a sufficient number of the variant cells and the variants which had reverted back to the transformed state were available, they were examined for surface membrane glycopeptides and the expression of the transformed properties. The ability to overcome contact inhibition of cell replication as measured by saturation density and to form colonies in soft agar and liquid medium with high cloning efficiency (Macpherson and Montagnier, 1964) was tested as described (Rabinowitz and Sachs, 1968). The cultures were examined for *Mycoplasma* and no detectable contamination was observed by the method of Chanock *et al.* (1962).

Tumor Formation and Culture of Tumor Cells. To determine tumor formation, the cells were inoculated subcutaneously into 5- to 7-week-old hamsters in 0.2 ml of Eagle's medium. The animals were examined for the development of palpable tumors twice weekly up to 60 days for all of the cells examined with the exception of the variant cells with the suppression of transformed properties which were examined for 90 days. Animals were scored as positive for tumors only when tumors grew progressively. The latency period for tumor development was taken as the time when tumors first became palpable.

The tumors were collected, trypsinized, and seeded at 10^6 cells/50-mm petri dish. At least 80% of the cells was viable. In order to avoid changes that may occur during prolonged culture, the glycopeptides and transformed properties were examined 48 hr after seeding of the tumor cells. At this time, the cells were reseeded and treated similarly to the other cells examined. In some cases portions of the cells were inoculated again into hamsters at cell concentrations given for individual experiments.

Preparation of Fucose-Containing Glycopeptides. Secondary hamster embryo cells, DMNA cells, all variants, and the cells derived from the hamster tumors were reseeded and cultured for 72 hr in the presence of L-[1-^{14}C]fucose (50.8 mCi/mmol) or L-[^{3}H]fucose (general label, 4.3 Ci/mmol) obtained from New England Nuclear Corp., Boston, Mass. Cultures were seeded at 5×10^5 cells/50-mm petri dish (Falcon & Co.) with 5 μCi of L-[^{14}C]- or 8 μCi of L-[^{3}H]fucose in 5 ml of Eagle's medium.

All cells were in exponential growth and were washed five times with TBS[1] and harvested from the monolayer cultures by treatment with 1 mg of trypsin (4× crystallized, Worthington Biochemicals, Freehold, N. J.) in 1 ml of TBS per 3–4 × 10^6 cells. After 5 min at room temperature an equivalent amount of purified soybean trypsin inhibitor (Worthington Biochemicals) was added. The cells were centrifuged at 600*g* for 5 min and the supernatant solution containing the surface glycopeptides was centrifuged at 40,000*g* for 30 min and lyophilized. The material removed from the cell surface by this procedure is called the "trypsinate" and in all cases represented 20–30% of the total radioactivity of the cells.

Characterization of Fucose-Containing Glycopeptides. The radioactive glycopeptides (trypsinates) to be compared were dissolved in water, combined, and digested exhaustively with Pronase (Calbiochem). The Pronase digests were chromatographed on Sephadex G-50 fine. All details of these procedures have been described previously (Buck *et al.*, 1970). Fractions of 0.6 ml which were eluted from the Sephadex G-50 column were diluted to 1 ml with the separation buffer (0.1 M Tris-HCl (pH 8.9) containing 0.1% sodium dodecyl sulfate, 0.01% EDTA, and 0.1% mercaptoethanol) and mixed with 10 ml of Triton X100–toluene scintillation fluid. The radioactivity in the fractions was determined on a Packard TriCarb scintillation counter.

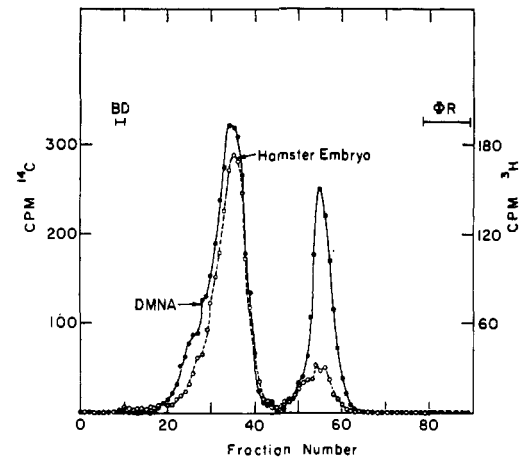

FIGURE 1: Chromatography on Sephadex G-50 of trypsinates from cells transformed after treatment with dimethylnitrosamine (●—●) and secondary hamster embryo cells (O- -O) grown in the presence of L-[^{3}H]- or [^{14}C]fucose, respectively. The trypsinates were combined and digested with Pronase before chromatography. All details are described under Materials and Methods: BD, fractions in which Blue Dextran 2000 was eluted; ΦR, fractions where Phenol Red was eluted.

Results

Surface Glycopeptides from Clones of DMNA Cells. Glycopeptides removed by trypsin (trypsinate) from the surface of the transformed DMNA cells, grown in the presence of L-[^{3}H]fucose, were compared to those obtained from secondary hamster embryo cells grown in the presence of L-[^{14}C]fucose. Chromatography of these Pronase-digested trypsinates showed the presence of one major group of glycopeptides, which was eluted from Sephadex G-50 columns in fractions 30–40, Figure 1. The trypsinate from the DMNA cells always showed a slight shift toward more rapidly migrating glycopeptides when compared to those of control cells. This slight shift was reproducible when the radioactive labels were reversed and was observed in all of the clones of DMNA cells which were examined. The radioactivity which was eluted from the gel in fractions 51–60 (Figure 1) was dialyzable and was not examined further. Free fucose is eluted in these fractions.

Surface Glycopeptides from Tumors Formed by DMNA Cells. Cells derived from tumors produced at 16 days after the incubation of 10^6 DMNA cells into animals were cultured for 48 hr and subsequently made radioactive by growth in the presence of L-[^{3}H]fucose. Glycopeptides were removed by trypsin from the surface of these cells. Figure 2a shows the patterns obtained when the radioactive Pronase-digested glycopeptides were chromatographed on Sephadex G-50 with a comparable trypsinate from secondary hamster embryo cells made radioactive by growth in the presence of L-[^{14}C]fucose. The appearance of a specific group of fucose-containing glycopeptides (fractions 20–28) was always observed in the tryp-

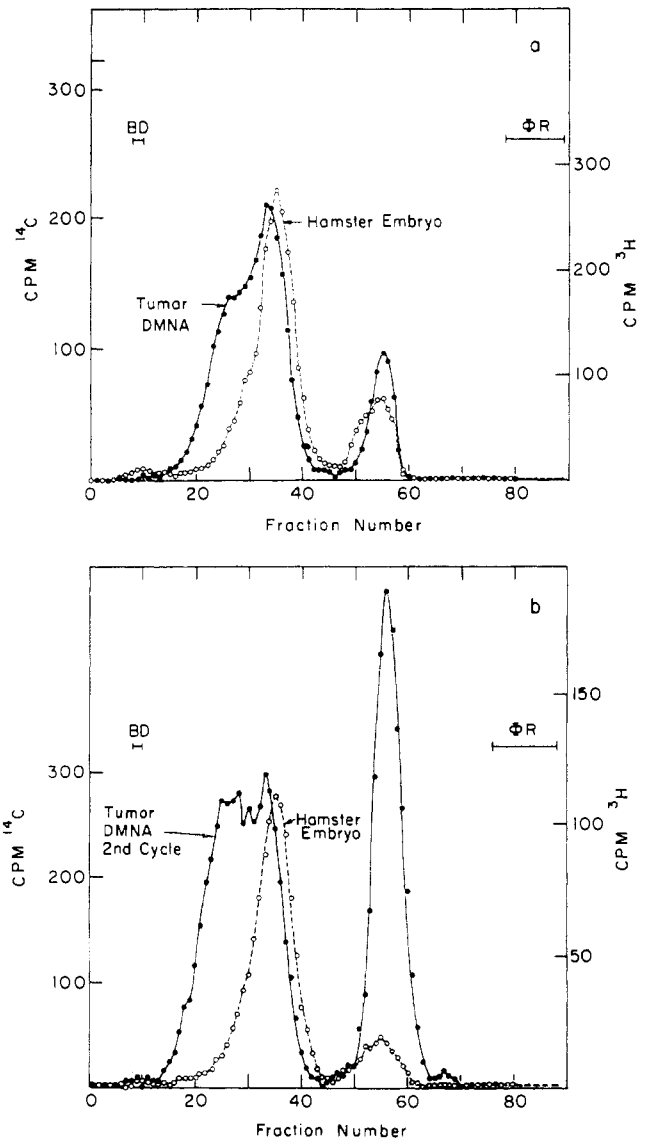

FIGURE 2: Chromatography on Sephadex G-50 of Pronase-digested trypsinates from (a) cells derived from a tumor of the dimethylnitrosamine cells shown in Figure 1 (●—●); (b) tumor cells (2nd cycle) derived from the tumor cells shown in Figure 2a (●—●); and secondary hamster embryo cells (○- -○). The hamster embryo cells and the tumor cells were made radioactive by growth in the presence of L-[^{14}C]- and [^{3}H]fucose, respectively. All conditions are described in the legend to Figure 1 and under Methods and Materials.

sinates from these tumor cells. These glycopeptides were not observed in the trypsinates from the control hamster cells (Figure 2a) or the DMNA cells in culture from which these tumors were derived (Figure 1).

Cells (10^5) from this tumor (Tumor DMNA, Figure 2a) were inoculated into animals giving rise to second cycle tumors. The distribution by gel filtration of the fucose-containing glycopeptides derived from these tumor cells (Tumor DMNA, second cycle) is compared in Figure 2b to the glycopeptides from control hamster cells. The glycopeptides which migrated rapidly on the gel, fractions 20–28, were more abundant in the trypsinate from these tumor cells than in the comparable trypsinates from the tumor cells of the first cycle (Figures 2a and 2b). Similar results were obtained in the analysis of several tumors, first and second cycles.

Table I shows the incidence of tumors and the average

TABLE I: Tumor Formation in Adult Animals.

Cell Type	Tumor Incidence[a] for No. of Cells Inoculated per Animal			
	10^2	10^3	10^4	10^5
DMNA, line D	3/4[a]	4/4	4/4	4/4
	(45)[b]	(37)	(19)	(8)
Tumor DMNA	3/4	4/4	4/4	4/4
1st cycle	(35)	(27)	(17)	(7)
Tumor DMNA	4/4	4/4	4/4	ND[c]
2nd cycle	(18)	(14)	(10)	
Variant 11	ND	0/4	0/4	0/4[d]
Tumor variant 11[e]	2/4	4/4	ND	ND
1st cycle	(27)	(25)		
Tumor variant 11[f]	2/4	4/4	ND	ND
2nd cycle	(23)	(16)		
Variant 11	ND	0/4	3/4	4/4
reverted			(38)	(20)
Tumor variant 11[f]	3/4	4/4	4/4	4/4
reverted	(19)	(18)	(14)	(8)

[a] Number of animals with tumors/numbers of animals inoculated. [b] Numbers in parentheses are the average latency period in days. [c] ND = not done. [d] Two of six animals formed tumors in 46 days and six of six animals formed tumors in 37 days when 10^6 and 10^7 cells, respectively, were inoculated per animal. [e] Derived after inoculation of 10^7 cells into animals. [f] Derived after inoculation of 10^5 cells.

latency period of these cells. It should be noted that the first and second cycle tumors were more tumorigenic than the parental line, D, of the DMNA cells.

Surface Glycopeptides from Variants and Tumors of These Cells. Variants of the established line of DMNA cells were selected on the basis of a reduction of transformed properties which were characteristic of the parent clone. These cells showed complete suppression of the transformed phenotype, *in vitro* (Hitotsumachi *et al.*, 1972), and were able to form tumors in 100% of the animals only when inoculated at 10^7 cells per animal (Table I). The saturation density, cloning efficiency, and other properties of the variant cells were more characteristic of the secondary hamster embryo cells than the transformed DMNA cell line (Table II).

When the radioactive trypsinates of these variant cells (variant 11) were examined, the pattern of the fucose-containing glycopeptides obtained by gel filtration was more similar to that of the parental line, DMNA cells (Figure 3a) than the tumor cells (Figure 3b and 3c). The trypsinates from the cells of the tumors produced by variant 11 always showed the presence of a larger amount of more rapidly migrating glycopeptides (Figure 3b). Again, a repeated inoculation of these tumor cells into hamsters gave rise to second cycle tumors which showed an increase in the specific group of fucose-containing glycopeptides (fractions 20–28, Figure 3c). In Figure 3c, the glycopeptides from the cells of the second cycle tumor are compared with those of another variant, variant 13, labeled with L-[^{14}C]fucose. The glycopeptides from the cells of tumors of variant 13 have gel filtration patterns similar to those of the tumors from variant 11.

It should be noted that tumors of these variants, like the variant cells, showed a suppression of the transformed phenotype (Table II); that is, the cloning efficiency and saturation

TABLE II: Comparison of Surface Membrane Glycopeptides with Transformed Properties.

Cell Type	Glycopeptides Group[a] I	II	Tumor Formation[b] (%)	Properties *in Vitro*[c]
Hamster embryo	−	+	0	N
DMNA, line D	−	+	100	T
Tumor DMNA	+	+	100	T
Variant 11	−	+	0	N
Tumor variant 11	+	+	100	N
Variant 11 reverted	−	+	0[d]	T
Tumor variant 11 reverted	+	+	100	T

[a] Fucose-containing glycopeptides were divided into two groups and are defined as eluted from Sephadex G-50 columns, fractions 20–28, group I, and fractions 30–40, group II (Figure 2b). [b] Percentage of animals forming tumors after inoculation of 10^3 cells. See Table I for details. [c] Saturation density and cloning efficiency in liquid medium and soft agar. N denotes that these properties resemble normal cells while T denotes a resemblance to transformed cells (Hitotsumachi *et al.*, 1972). [d] 75% of the animals formed tumors after inoculation of 10^4 cells.

density were more characteristic of secondary hamster embryo cells than of transformed cells.

Surface Glycopeptides from Reverted Variants and Tumors of These Cells. Variants from the DMNA cells on subsequent subculturing reverted back to the malignant transformed state. These reverted variant cells showed a reversion to malignancy and all of the transformed properties, simultaneously. The cells produced tumors after the subcutaneous inoculation of 10^4–10^5 cells into hamsters as compared to the variants which produced tumors only after the inoculation of 10^6–10^7 cells (Table I). In addition, the expression of the transformed properties, *in vitro* by these cells, was similar to that of the DMNA cells (Table II).

The distribution on Sephadex G-50 of the fucose-containing glycopeptides from the trypsinates of these reverted variant cells (variant 11, reverted) was similar to that of the parental variant (Figure 4a) or the DMNA cell line (Figure 1). However, as in all the previous cases, when the radioactive trypsinates from the tumors formed after the inoculation of the reverted variant cells were examined, fucose-containing glycopeptides were seen which were not as apparent in the trypsinates from the cells in culture (fractions 20–28, Figure 4b).

Comparison of Surface Membrane Glycopeptides with Transformed Properties. Some of the properties of the cells which were examined are summarized in Table II. The surface membrane glycopeptides were examined from two or more examples of each cell type shown in Table II. In addition, tumors derived from two examples of each particular cell type were examined from two different animals. In all cases, the glycopeptides were similar to those reported in Table II (see also the figures).

Glycopeptides from group I could be detected to a significant extent only in cells that originated in tumors, that is, cells which were actively involved in tumor formation. Moreover, these particular glycopeptides were more abundant in the more tumorigenic cell populations.

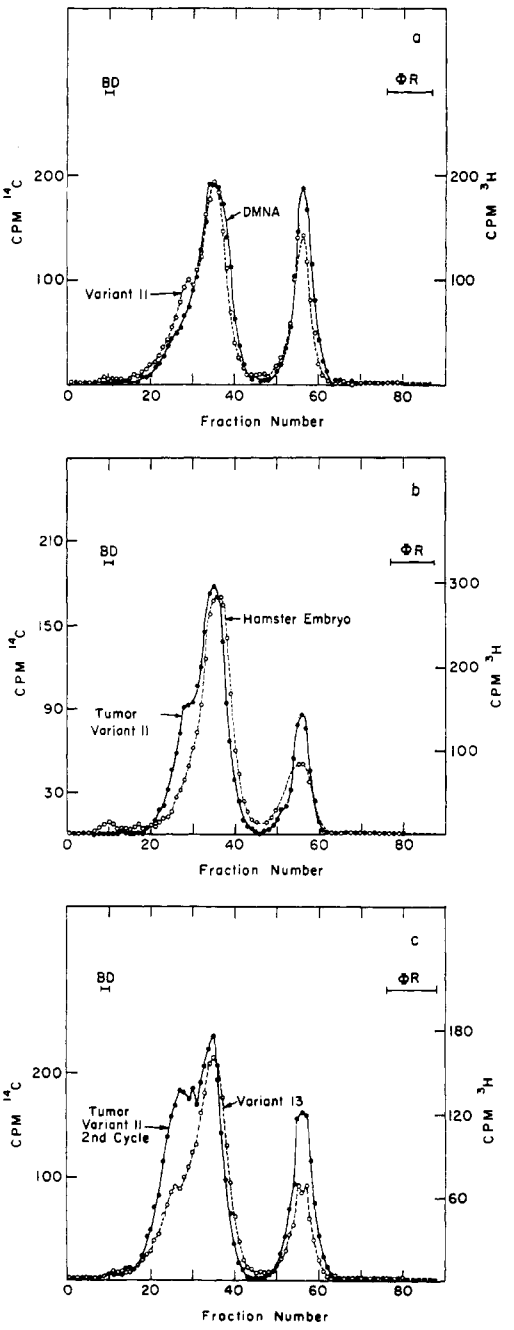

FIGURE 3: Chromatography on Sephadex G-50 of Pronase-digested trypsinates from (a) variant 11 cells (O--O) and the parent dimethylnitrosamine cells (●—●) grown in the presence of L-[^{14}C]- or [^{3}H]-fucose, respectively; (b) tumor cells derived from variant 11 cells (●—●) and secondary hamster embryo cells (O--O) grown in the presence of L-[^{3}H]- or [^{14}C]fucose, respectively; and (c) tumor cells (2nd cycle) derived from the variant 11 cells (●—●) shown in Figure 3b and variant 13 cells (O--O) grown in the presence of L-[^{3}H]- and [^{14}C]fucose, respectively. All conditions are described in the legend to Figure 2.

There appears to be no correlation between the surface glycopeptides when examined by these procedures and the expression of the transformed properties, *in vitro*, such as the ability to overcome contact inhibition of cell replication as measured by the saturation density and to form colonies in soft agar and liquid medium with high cloning efficiency. Cells which contain glycopeptides from group I can show either normal (tumor variant 11) or the transformed (tumor DMNA) phenotype. Other cells which contain only group II glycopeptides can also show either phenotype. No other

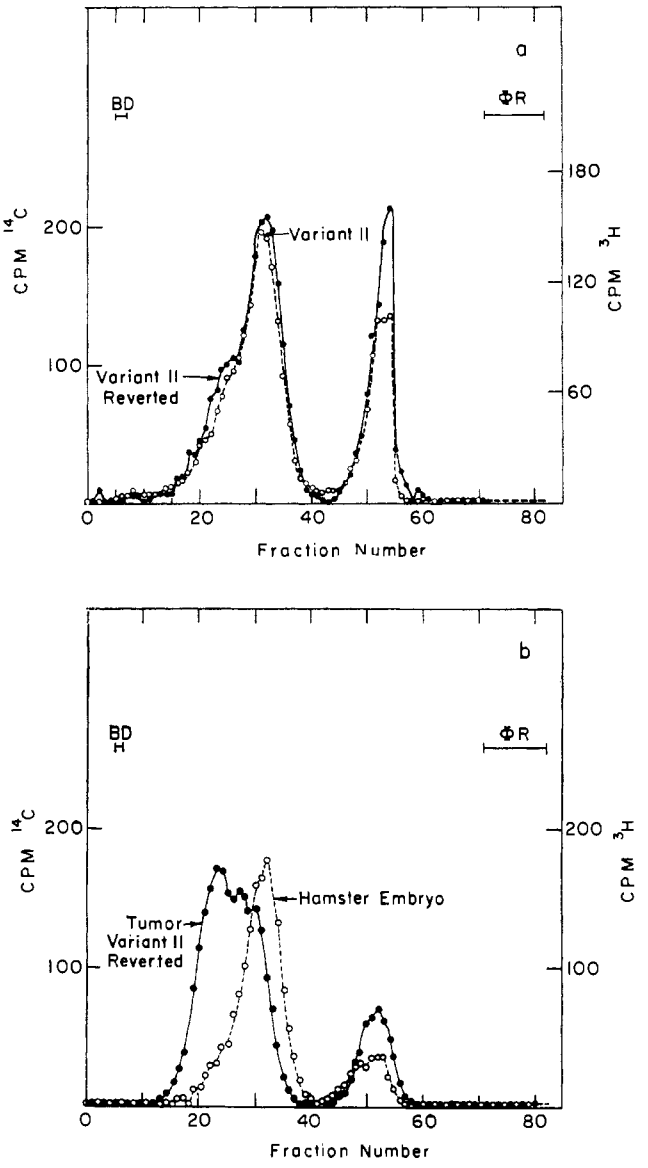

FIGURE 4: Chromatography on Sephadex G-50 of Pronase-digested trypsinates from (a) variant 11 cells which have reverted to transformed properties similar to the original dimethylnitrosamine cells (variant 11 reverted (●—●) and variant 11 (○- -○); and (b) tumor cells derived from variant 11 reverted (●—●) and secondary hamster embryo cells (○- -○). All of the cells were made radioactive by growth in the presence of L-[^{14}C]fucose (○- -○) or L-[^{3}H]fucose (●—●). All conditions are described in the legend to Figure 2.

property examined in these cells corresponded to the expression of the particular glycopeptide pattern with the exception that the cells had to be derived from the hamster tumors. In addition, this characteristic glycopeptide pattern was seen in all tumors whereas the other properties examined were not consistently found in cells derived from tumors (tumor, variant 11).

Discussion

The present study suggests a correlation between surface membrane glycopeptides and the expression of malignancy. A specific group of fucose-containing glycopeptides has been demonstrated in surface membranes of cells derived from tumors produced by hamster cells transformed after treatment with the chemical carcinogen, DMNA, and variants of these cells. Indeed, the more tumorigenic the cell population, the more abundant were these particular glycopeptides (Figures 2 and 3 and Table I).

The appearance of a specific group of surface membrane glycopeptides reported in this study of the tumor cells parallels that observed after virus transformation (Buck *et al.*, 1970, 1971; Warren *et al.*, 1972b). However, we could not detect the presence of these glycopeptides to a significant extent in the transformed DMNA cells. These cells exhibited other properties of the transformed phenotype which were demonstrated by saturation density, cloning efficiency in soft agar and liquid medium (Macpherson and Montagnier, 1964), and lack of limited life span *in vitro* (Hayflick, 1965). The absence of these particular surface membrane glycopeptides was also noted in variants with suppression of the transformed properties as well as cells which had reverted back to the transformed state. The specific glycopeptides, however, were present to a greater extent in all of the tumors from these various cell types which were examined. Thus, the glycopeptide profile appears to correlate to tumorigenesis rather than the expression of transformed properties.

It should be noted that all of the cells in culture which were examined showed very small amounts of this specific group of glycopeptides (Figures 1, 3, and 4, fractions 25–30), but never as much as seen in any of the tumor cells examined or as seen previously in virus transformed cells (Buck *et al.*, 1971). Small amounts of this group of glycopeptides have also been reported when baby hamster kidney cells were arrested in metaphase with Velban (Glick and Buck, 1973). The further comparison of these glycopeptides to those seen in the tumor cells is under investigation.

In a study of similar cell types Yamamoto *et al.* (1973) examined the chromosomes by the Giemsa differential staining technique (Sumner *et al.*, 1971) and showed that all of the tumors studied had detailed karyotypes which differed from those of the cultured cells which were inoculated into the animals. All of these cell types differed from the detailed karyotype of the control secondary hamster embryo cells. From their results it could be suggested that the number of Y chromosomes had to equal the number of 7$_2$ chromosomes (even though rearranged) in order to express the specific group of surface glycopeptides. However, it is possible that a molecular change in the other chromosomes could produce this result, since the alteration of only one enzyme in the biosynthesis or degradation of glycoproteins could conceivably produce the altered Sephadex profile.

Different kinds of studies have suggested the importance of specific surface carbohydrates for the migration of cells *in vivo* (Gasic and Gasic, 1962; Gesner and Ginsberg, 1964; Pricer and Ashwell, 1971) or the ability to form tumors when injected into animals (Shoham *et al.*, 1970). The latter have shown that cells which have been treated with concanavalin A, a carbohydrate-binding protein, lose the ability to form tumors. In a similar manner, transformed hamster cells with a reduced ability to agglutinate with concanavalin A also have a reduced ability to form tumors (Inbar *et al.*, 1972). Our results now demonstrate chemically a change in glycoproteins of the cell surface with tumor formation. The exact chemical nature and meaning of this change will be the subject of further studies.

Acknowledgments

The excellent technical assistance of Messrs. Aryeh Goldenberg and Mordechai Ben-Ezra and Miss Ronnie Koren is gratefully acknowledged.

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Purification and Partial Sequencing of Cyanogen Bromide Peptides from L-Asparaginase of *Escherichia coli* B†

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ABSTRACT: *S*-Aminoethylated asparaginase from *Escherichia coli* B has been fragmented with cyanogen bromide, and the seven expected peptides isolated in homogeneous condition. The total amino acid composition of the peptides agrees to within 4% of that reported for asparaginase itself. Four of the cyanogen bromide peptides have been sequenced by conventional methods; two of them are linked in the native enzyme by an intrachain disulfide bond. The N- and C-terminal peptides have been identified. The C-terminal peptide contains approximately 173 amino acids, accounting for over half of the amino acids per subunit. A Beckman Sequenator was used to establish the sequence of twelve amino acids at the N-terminal end of this large C-terminal cyanogen bromide peptide. The isolation and sequencing of an overlap tryptic peptide permitted the alignment of three of the interior cyanogen bromide peptides with respect to one another. The sequence of 78 residues, about 25% of the asparaginase molecule, has been established. The results presented here are supportive evidence for a four-subunit model in which the subunits are identical.

L-Asparaginase from *Escherichia coli* B is an enzyme of particular interest because of its ability to cause the regression of certain tumors in mice, and its antileukemic activity in some cases of human acute lymphatic leukemia (for reviews, see Adamson and Fabro, 1968, Capizzi *et al.*, 1970, Cooney and Handschumacher, 1970, and Wriston, 1971). The purification of this enzyme has been described (Whelan and Wriston, 1969) and certain properties of the enzyme, including molecular weight, amino acid composition, isoelectric point, and the existence of four identical or nearly identical subunits have been established (Wriston, 1971). There has been little work so far, however, on the primary structure of the enzyme. Arens *et al.* (1970) reported that asparaginase contained only leucine as the N-terminal amino acid, and established the sequence of ten amino acids at the N-terminal end. Greenquist and Wriston (1972) showed that asparaginase also contained only tyrosine as the C-terminal amino acid; established the sequence of four amino acids at the C-terminal end; and reported that the results obtained by preparing two-dimensional peptide maps of tryptic digests of reduced, aminoethylated asparaginase were in agreement with a four-subunit model in which the subunits are identical or nearly so. Here we report the initial results of sequence studies on the enzyme involving cleavage with cyanogen bromide, isolation in homogeneous condition of the expected number of peptides, and the se-

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