Characterization of cell-surface glycopeptides from mouse cerebral cortex that inhibit cell growth and protein synthesis

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Glycopeptides were isolated from the cell surfaces of mouse brain cortical tissue that inhibited both cell division and protein synthesis by cells in culture. The protein-synthesis inhibition appeared to affect most cells exposed and was equally effective against glycoprotein and protein synthesis. The inhibition of protein metabolism was independent of mRNA synthesis and uptake of labelled precursors into intracellular pools, indicating that it was directed at intracellular translational events. Fractionation of chloroform/methanol-extracted preparations of this brain cell-surface substance on Bio-Gel P-100 revealed the material to be quite heterogeneous, although inhibitory activity was found only in fractions of mol.wt. 25 000–30 000 and 6000–10 000. Biochemical analysis of these fractions demonstrated that they were 6% carbohydrate and 94% amino acid by weight. The 25 000–30 000-mol.wt. glycopeptides were shown to inhibit cell growth at concentrations of 2 µg/ml in cultured cells and to inhibit protein synthesis by 50% at concentrations of 3 µg/ml. The 25 000–30 000-mol.wt. brain-cell-surface-substance glycopeptides were further purified by ultrafiltration and affinity chromatography with *Ulex europaeus* agglutinin, resulting in a 400-fold increase in specific biological activity. The inhibitor was not lethal to cells and was not species- or tissue-specific.

The cell surface is recognized to play an important part in a number of fundamental aspects of neoplasia, including uncontrolled cell growth, decreased contact inhibition, invasiveness and loss of sensitivity to hormones (Burger, 1971; Clarkson & Baserga, 1974; Wallach, 1975; Nicolson *et al.*, 1977). The alterations in cell-surface architecture found in neoplastic cells include membrane components' mobilities (Mannino & Burger, 1975; Wallach, 1975; Nicolson *et al.*, 1977), glycolipid, glycosaminoglycan and glycoprotein content (Hakamori, 1973; Mannino & Burger, 1975; Warren *et al.*, 1977), and changes in membrane-associated enzymes (Hagopian & Eylar, 1969; Roseman, 1970; Roth *et al.*, 1971*a,b*; Ossowski *et al.*, 1973; Unkeless *et al.*, 1973; Weiser & Podolsky, 1978; Pietras & Szego, 1979).

Burger (1971) has shown that if mouse 3T3 fibroblasts in confluent cultures, which do not normally divide, are briefly treated by mild proteinase digestion, the cells will undergo one round of mitosis and stop. Continuing this work from a somewhat different perspective, Koch (1974) and Fisher & Koch (1976) treated HeLa cells under

conditions similar to those used by Burger, recovered the post-digestive supernatant fluid, concentrated the macromolecules by ethanol precipitation and heated the preparations to denature the added proteinase. Their preparations could inhibit whole-cell and cell-free protein synthesis by other HeLa cells. When similar preparations were made from partially purified HeLa cell membranes, protein-synthesis inhibition was again observed. These experiments suggest the existence of a macromolecule or class of macromolecules on the cell surface that is directly involved in growth regulation.

Further support for this possibility is provided by the work of Whittenberger and Glaser (1977), who found that cell membranes isolated from confluent cultures of mouse 3T3 cells could inhibit DNA synthesis of subconfluent 3T3 cells. When the membranes were added to simian-virus-40-transformed 3T3 (SV3T3) cells, no effect on rates of DNA synthesis was observed. Complementary experiments in which membranes from SV3T3 cells were added to 3T3 or SV3T3 cells showed little or no inhibition of DNA synthesis. In more recent

work, Whittenberger *et al.* (1978) have extracted proteins and glycoproteins from plasma membranes of 3T3 cells and have shown that these extracts bear the inhibitory activity of the intact membrane.

We have reported (Kinders *et al.*, 1979) the isolation of macromolecules from the cell surface of the mouse cerebral cortex; these molecules are effective inhibitors of cellular protein synthesis. Inhibition of protein synthesis occurred without altering the uptake of radioactive precursors. Mild proteinase treatment of cerebral-cortex cells were required to release inhibitor; active inhibitor could not be obtained from cytoplasm or from cells eluted with buffer containing 1 mM-EDTA or buffer alone (Kinders *et al.*, 1979). We have also shown that tumour cells, which are refractory to density-dependent growth regulation, are relatively resistant to the activity of the inhibitor, which is consistent with a role of these cell-surface macromolecules in the conveyance of density-dependent regulatory information. We now report the partial purification and the biochemical characterization of the inhibitor.

Materials and methods

Preparation of the inhibitor

Cerebral-cortex slices were prepared from 3–6-week-old Swiss albino outbred mice by hand dissection and gently minced by pipetting in ice-cold Dulbecco's Modified Eagle's medium containing 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid] buffer (pH 7.1). The cells were pelleted by centrifugation at 1000 *g* for 1 min and resuspended in HKM buffer (20 mM-Hepes/120 mM-KCl/5 mM-MgCl₂, pH 7.1) containing 0.02 unit of proteinase/ml (from *Streptomyces griseus*). Higher concentrations (0.04–0.08 unit/ml) of proteinase resulted in significantly lower yields of biological inhibitory activity. The brain-cell suspensions (300 mg wet wt./5 ml) were incubated at 37°C for 15 min and the cells were then pelleted by centrifugation for 3 min at 1200 *g*. The supernatant fluid was collected and macromolecules were precipitated at –20°C for 20 min by the addition of 2 vol. of ethanol. The insoluble material was collected by centrifugation at 4000 *g* for 20 min. The resulting pellet was resuspended in deionized water by sonication and re-precipitated with 2 vol. of ethanol at –20°C. The precipitate was collected by centrifugation, resuspended in deionized water and extracted with 2 vol. of chloroform/methanol (2:1, v/v). The aqueous fraction was recovered, freeze-dried to dryness, weighed and stored desiccated at –20°C. The biological activity was retained for several months, and re-solubilized samples could be dialysed without a loss of activity.

Purification of the inhibitor

Freeze-dried material was re-suspended in HKM buffer, insoluble material was removed by centrifugation and the soluble fraction was placed on a Bio-Gel P-100 column (1.1 cm × 100 cm) that had previously been equilibrated with HKM buffer at 4°C. Column calibration was carried out as described by Henn & Ackers (1969) with 2 mg portions of bovine serum albumin, horseradish peroxidase, chymotrypsinogen and ribonuclease A. Elution volumes (2 ml fractions) were used to calculate the partition coefficient for these standard globular proteins [$\sigma = (\text{elution volume} - \text{void volume}) / (\text{total column volume} - \text{void volume})$]. Stokes' radii of the standard proteins were calculated from the data of Sober (1968). The elution volumes of the biologically active materials were used to determine the estimated molecular weights of the inhibitors from the Stokes' radii, where $r = (3M\bar{v}/4\pi N)^{1/3}$. The cell-surface components were eluted with HKM buffer, and 2 ml portions were collected and monitored for absorption at 223 and 280 nm. Fractions possessing biological activity were identified by the protein-synthesis-inhibition assay with BHK-21 cells. The biological activity was reproducibly eluted in two peaks of apparent mol.wts. of 25 000–30 000 and 6000–10 000 on a column calibrated with the standard globular protein markers. Active high-molecular-weight fractions were either dialysed against deionized water containing 0.05 ml each of chloroform and toluene per litre as bacteriocidal agents, at 4°C for 48 h and freeze-dried to dryness, or alternatively, concentrated 10-fold in HKM buffer on an Amicon DM 5 filter at 20 psi (138 kPa) N₂ at 4°C. The biologically active material retained by the DM 5 membrane was washed through a 2 ml *Ulex europaeus* agglutinin-agarose (UEA-I) affinity chromatography column that had been equilibrated with HKM buffer at 25°C. The cell-surface components were eluted from the column with HKM buffer containing 0.25 M-fucose and the fractions were dialysed for 48 h at 4°C in HKM buffer, as described above, and tested for biological activity.

Tissue-culture and cell-growth assays

BHK-21 (baby hamster kidney) cells were grown as monolayer cultures as previously described (Kinders *et al.*, 1979). For cell-growth assays, BHK-21 cells were plated into plastic culture vessels in complete medium containing 7% foetal calf serum. After 24–36 h, the medium was removed and fresh medium (controls) or fresh medium containing various concentrations of partially purified inhibitor was added. Quadruplicate samples were taken every 12 h (approximately one generation time), and cells were harvested with 0.25% trypsin in Hanks BSS medium, washed, dissociated by gentle pipetting, and

counted in a haemocytometer. A portion of each sample was also tested for cell viability by Trypan Blue exclusion.

Protein-synthesis assay

Protein synthesis was measured with $(4-5) \times 10^6$ BHK-21 cells suspended in 100 μ l of Dulbecco's Modified Eagles medium containing 25 mM-Hepes buffer, pH 7.1. Either 25 μ l of HKM buffer (controls) or 25 μ l of HKM buffer with various concentrations of purified material was added to each tube. The cells were then incubated at 37°C for 45–60 min, after which 10 μ l of Dulbecco's Modified Eagle's/Hepes medium containing 2.0 μ Ci of [35 S]-methionine or 1.0 μ Ci of [14 C]fucose was added to each tube. After incubation for an additional 60 min, the reaction was terminated with cold 10% trichloroacetic acid and the amount of radioactivity in the cellular acid-soluble pools and acid-insoluble macromolecules was determined in a Packard model 3200 scintillation system (Hughes & Johnson, 1978). Cellular protein concentrations were measured by the colorimetric method of Lowry *et al.* (1951), with bovine serum albumin as a protein standard. At each stage of purification, the ability of the fractions to inhibit cellular protein synthesis was compared with that of the original chloroform/methanol-extracted material in parallel assays. All assays, at each concentration, were carried out in triplicate.

RNA-synthesis assay

BHK-21-cell mRNA synthesis was measured by a modification of the assay described for protein synthesis. After preincubation with inhibitor at various concentrations for 60 min, 2.0 μ Ci of [3 H]-uridine was added to each reaction mixture. After 15 min incubation the reactions were terminated with cold (0°C) 10% trichloroacetic acid. The insoluble material was collected on millipore HA WP filters and washed with cold (0°C) 5% trichloroacetic acid four times; then the amount of radioactivity was determined.

Carbohydrate analysis

Carbohydrate analysis was carried out with two independent preparations of the high-molecular-weight material that were recovered from the Bio-Gel P-100 column and shown to possess protein-synthesis inhibitory activity. The fractions from the Bio-Gel column were dialysed for 72 h against water as described above and then freeze-dried. Weighed samples were hydrolysed with 4 M-HCl for 2 h, the acid was removed by vacuum evaporation and the samples were then reduced with NaB 3 H $_4$. Carbohydrate content was determined by the phenol/sulphuric acid procedure (Dubois *et al.*, 1956) with glucose as standard. The samples were then N-

acetylated, deionized on a mixed-bed ion-exchange resin and electrophoresed in borate buffer (Taka-saki & Kobata, 1974). Each sample contained two standards: 1.16 μ g of 2-deoxyribose added before NaB 3 H $_4$ reduction, from which the relative concentrations of monosaccharides were calculated, and [14 C]sorbitol, added just before electrophoresis, from which mobilities were compared.

Amino-acid analysis

Bio-Gel P-100-purified material was dialysed against deionized water for 72 h as described above and then freeze-dried. The recovered materials were weighed and then hydrolysed in 6 M-HCl at 110°C for 24 h, with 45 μ g of norleucine added as an internal standard. The hydrolysed material was freeze-dried and diluted in buffer (FEMTO system 2; Dionex Laboratories) to a concentration of 1 μ g of amino acids/ μ l, on the assumption that the material was at least 6% carbohydrate (based on the previous carbohydrate analysis). The samples were analysed in duplicate on a Dionex MBN/SS High-Pressure Ninhydrin Microanalyzer.

Materials

Tissue-culture media, vitamins, antibiotics, trypsin and foetal calf serum (virus-screened) were purchased from Grand Island Biological, Grand Island, NY, U.S.A. Agarose-bound *U. europaeus* agglutinin I, peanut agglutinin, and concanavalin A were purchased from Vector Laboratories, Burlingame, CA, U.S.A. All other enzymes, buffers and reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A. Ultrafiltration membranes were purchased from Amicon Corp., Lexington, MA, U.S.A. [35 S]Methionine (sp. radioactivity 400 Ci/mmol) and [14 C]fucose (sp. radioactivity 50 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. and [3 H]uridine (sp. radioactivity 60 Ci/mmol) was from Amersham, Arlington Heights, IL, U.S.A.

Results

BHK-21 cells were assayed with both [35 S]-methionine and [14 C]fucose to determine if preparations of the brain cell-surface substance, concentrated by ethanol and extracted with chloroform/methanol, could inhibit both protein and glycoprotein synthesis. The cellular incorporation of both radioactive methionine and fucose into acid-insoluble macromolecules was clearly affected by the inhibitor and the degree of inhibition was comparable for the two (Table 1). The mechanism of this inhibition was independent of cell uptake of the radiolabelled precursors, since the specific radioactivity of the intracellular acid-soluble pools was not decreased by the presence of brain cell-surface

Table 1. *Inhibition of BHK-21-cell protein and glycoprotein synthesis*

BHK-21 cells were preincubated with the inhibitor for 45 min at 37°C, washed, and then re-incubated with either [³⁵S]methionine or [¹⁴C]fucose as described in the Materials and methods section. The specific radioactivity of both the acid-insoluble and acid-soluble cell fractions was determined, and the values represent the means for triplicate determinations ± s.d.

Inhibitor (μg)	[³⁵ S]Methionine				[¹⁴ C]Fucose			
	Acid-insoluble protein		Acid-soluble intracellular pools		Acid-insoluble glycoprotein		Acid-soluble intracellular pools	
	(c.p.m./mg of protein)	(% of control)	(c.p.m./mg of protein)	(% of control)	(c.p.m./mg of protein)	(% of control)	(c.p.m./mg of protein)	(% of control)
0 (controls)	27990 ± 3640	100	233 700 ± 3260	100	31 840 ± 780	100	79 560 ± 4800	100
1.5	23 790 ± 1400	85	249 100 ± 1990	106	27 740 ± 1800	87	77 100 ± 5200	97
5.0	17 740 ± 1720	63	220 930 ± 7600	95	20 380 ± 1130	64	79 740 ± 14 700	100
10.0	10 620 ± 2120	38	290 040 ± 1070	124	16 640 ± 2340	52	93 780 ± 23 400	118

Table 2. *Inhibition of mRNA synthesis in BHK-21 cells by brain cell-surface substance*

BHK-21 cells were preincubated with two different preparations of brain cell-surface substance for 60 min and then [³H]uridine was added. After 15 min of further incubation the acid-insoluble RNA was collected on filters as described in the Materials and methods section. The results represent the means for two independent assays, each performed in triplicate, ± s.d.

Inhibitor (μg)	[³ H]Uridine incorporation (c.p.m./mg of protein)
0 (control)	18 370 ± 2430
Preparation 1	
5.0	20 100 ± 2000
10.0	23 380 ± 2900
Preparation 2	
10.0	19 000 ± 1030
20.0	19 470 ± 1900

substance (Table 1), despite the significant decrease in protein and glycoprotein synthesis. During the total 105 min incubation period, no change in cell viability, as determined by Trypan Blue exclusion, could be measured.

We considered that the intracellular action of brain cell-surface substance may be primarily directed toward transcriptional events. However, BHK-21 cells incubated with brain cell-surface substance and [³H]uridine showed no inhibition of uridine incorporation into rapidly labelled RNA (Table 2). Two independent preparations of the inhibitor were examined and although cellular protein synthesis was inhibited by 62% of controls, the incorporation of [³H]uridine remained unaffected. These observations suggested that the primary point of action of inhibition by brain cell-surface substance was most likely at the trans-

Fig. 1. *Outline of the purification of brain cell-surface substance from mouse brain cortex cell surfaces*

Typically, 500 mice yielded approx. 130 μg of material after *U. europaeus* agglutinin I affinity chromatography. Three independent preparations of brain cell-surface substance have now been carried to this stage of purification. The numbers in parentheses indicate the fold purification.

lational level and independent of mRNA transcription. In the light of these observations and the future experimental direction of using cell-free procedures to identify the mode of action of the brain cell-surface substance, further purification and chemical characterization of this naturally occurring

Fig. 2. Absorption profiles of a preparation of brain cell-surface substance fractionated on a Bio-Gel P-100 column

Chloroform/methanol-extracted material (25 mg) was re-suspended in HKM buffer and filtered on a Bio-Gel P-100 column as described in the Materials and methods section. Each fraction was monitored for absorption at 223 nm (●), 280nm (○) and for protein-synthesis inhibitory activity. The column was calibrated with standard globular-protein markers as described in the Materials and methods section. The high-molecular-weight material was eluted at an apparent mol.wt. of 25 000 and the low-molecular-weight material at an apparent mol.wt. of 6000–10 000. The bars indicate those fractions that were active in growth and protein-synthesis inhibition. No other fractions inhibited protein synthesis or cell growth. High indicates high-molecular-weight material and Low represents low-molecular-weight material.

metabolic inhibitor were deemed necessary. The general purification procedure that has allowed over a 400-fold increase in specific biological activity is outlined in Fig. 1.

When the chloroform/methanol-extracted material was filtered through a Bio-Gel P-100

Fig. 3. Inhibition of cellular protein synthesis by Bio-Gel P-100 fractionated brain cell-surface substance

Portions (25 μ l) of the high- and low-molecular-weight fractions were assayed as described in the Materials and methods section. Values shown are the means and standard deviations of two independent determinations each with the high-molecular-weight inhibitor (○) and the low-molecular-weight inhibitor (●) performed in triplicate.

Table 3. Inhibition of cell division by brain cell-surface substance

Cells were plated in culture dishes and grown as described in the Materials and methods section for at least 24 h before the addition of the brain cell-surface substance. Expt. 1 was carried out in duplicate with 78 cm² dishes, and expts. 2 and 3 were carried out in quadruplicate with 2 cm² dishes. The percentage inhibition of growth was calculated as the difference in cell numbers at 12 h or 24 h after brain cell-surface substance treatment compared with untreated controls.

	Brain cell-surface substance (μ g/ml)	Time of exposure (h)	Average cell number		Inhibition (%)
			Untreated	Brain-cell-surface-substance-treated	
Expt. 1	40	0	8.1×10^5	8.1×10^5	—
		12	1.6×10^6	1.2×10^6	53.6
		24	3.1×10^6	1.25×10^6	81.2
Expt. 2	50	0	7.0×10^4	7.0×10^4	—
		12	1.5×10^5	1.1×10^5	50.0
		24	3.3×10^5	1.25×10^5	78.8
Expt. 3	High-molecular-weight fraction	0	1.3×10^4	1.26×10^4	—
		24	2.6×10^4	1.59×10^4	75.2
		24		1.93×10^4	49.6
	Low-molecular-weight fraction	24		2.00×10^4	44.4
		24		2.06×10^4	39.8

column the elution pattern, monitored at both 223 and 280nm, showed the original brain-cell-surface-substance preparation to consist of species of various molecular weights. Despite the heterogeneity of the brain cell-surface substance at this stage of purification, only two portions of eluted material possessed biological activity as measured by the inhibition of BHK-21 cell protein synthesis (Fig. 2). We have now analysed nine independent preparations of brain cell-surface substance by gel filtration, and the biological activity is eluted consistently in the two areas that represent an apparent mol.wt. of approx. 25 000–30 000 and 6000–10 000 when calculated by an Ackers' plot ($\sigma = 0.76$, $r_s = 2.05 \times 10^{-7}$ cm) as described in the Materials and methods section. At this stage of purification, the specific activity of the brain cell-surface substance was increased by approx. 40-fold, and protein synthesis could be inhibited by 50% with as little as 400 ng or 900 ng of the high-molecular-weight and low-molecular-weight inhibitors respectively (Fig. 3). The difference in the A_{280}/A_{223} ratio between the high-molecular-weight and low-molecular-weight inhibitors was inconsistent from preparation to preparation.

Both high- and low-molecular-weight inhibitors were also effective against cell growth as determined with BHK-21 cells grown in tissue culture. Purification of the growth-inhibitory properties of brain cell-surface substance was monitored in a similar manner to that used for protein-synthesis inhibitory activity. The chloroform/methanol-extracted brain cell-surface substance, at a concentration of 40 μ g/ml in complete media, inhibited cell growth in duplicate cultures by over 50% in the first 12 h of treatment, and by 24 h cell division was inhibited by 80% (Table 3, expt. 1). Both controls and brain-cell-surface-substance-treated cell cultures were fed only at the start of the experiment, when the

brain-cell-surface-substance-containing medium was added (zero time). Expt. 2 (Table 3) was performed with an independently prepared pool of brain cell-surface substance, and the results were comparable with those obtained previously in expt. 1. After the growth-inhibitory activity of the brain cell-surface substance had been tested, the remainder of this second preparation was fractionated on the Bio-Gel column and the high- and low-molecular-weight materials, without further treatment, were added to complete medium, sterilized by filtration, and tested for their ability to inhibit BHK-21 cell growth (Table 3, expt. 3). Concentration of high- and low-molecular-weight fractions of brain cell-surface substance were calculated from parallel samples that were dialysed, freeze-dried and weighed. On the basis of cell-growth inhibition the results demonstrated a 26-fold purification of the low-molecular-weight inhibitor, and an 18-fold purification of the high-molecular-weight inhibitor. Furthermore, after 24 h of exposure, BHK-21 cell division was inhibited by 75% by the high-molecular-weight material, whereas the low-molecular-weight material was somewhat less effective, inhibiting cell division by 44%. (In both cases, the degree of purification is in good agreement with that obtained for the protein-synthesis inhibitory activity of brain cell-surface substance.) Finally, no effect of brain cell-surface substance on cell viability, based on Trypan Blue exclusion, or on cell morphology was ever observed.

The method of preparation and physical size of the high- and low-molecular-weight inhibitors suggested that they were derivatives of cell-surface proteins or glycoproteins. Carbohydrate and amino-acid analysis of the active post-Bio-Gel P-100 fractions that had been dialysed and freeze-dried was performed to test this interpretation. Based on total carbohydrate content, the high- and low-molecular-

Table 4. Carbohydrate composition of Bio-Gel P-100-purified brain cell-surface substance

Brain cell-surface substance that had been purified on the Bio-Gel column was dialysed and freeze-dried. Weighed samples were analysed as described in the Materials and methods section. The results shown are means of two independent determinations for each fraction. Calculated values for each carbohydrate are based on the total carbohydrate measured by the phenol/sulphuric acid method, with glucose as a standard.

	Carbohydrate content (% of total wt.)	
	High-molecular-weight fraction	Low-molecular-weight fraction
Pentoses	0	0
Uronic Acids	0	0
N-Acetylglucosamine	0.9	1.2
Glucose	3.6	3.3
Mannose/fucose	0.5	0.5
Galactose	0.6	0.4
N-Acetylgalactosamine	0	1.2

Table 5. *Amino-acid composition of Bio-Gel P-100 purified brain cell-surface substance fractions*

Brain cell-surface substance that had been purified on the Bio-Gel column was dialysed and freeze-dried. Weighed samples were analysed as described in the Materials and methods section.

Amino acid	Amino-acid content (g/100 g)	
	High-molecular-weight fraction	Low-molecular-weight fraction
Lysine	4.44	7.25
Histidine	3.88	6.67
Arginine	17.66	5.16
Aspartate	9.71	5.79
Threonine	3.98	5.76
Serine	11.89	4.89
Glutamate	17.50	8.96
Proline	5.55	6.62
Cystine	Trace	4.15
Glycine	5.21	2.98
Alanine	4.22	4.38
Valine	4.19	5.10
Methionine	1.04	6.29
Isoleucine	2.13	5.63
Leucine	2.54	5.72
Tyrosine	2.50	6.53
Phenylalanine	2.21	6.90
Ammonia	1.33	0.92

weight inhibitors appeared to be similar in that they were approx. 6% carbohydrate by weight (Table 4). This only represents a partial carbohydrate profile of the Bio-Gel fractions since sialic acid was not determined and sialic acid is a common component of mammalian brain glycopeptides (Brunngraber *et al.*, 1973; Margolis *et al.*, 1975). Both were approx. 0.5% fucose/mannose by weight and both contained similar quantities of *N*-acetylglucosamine, galactose and glucose. The dissimilarity was primarily associated with the lack of *N*-acetylgalactosamine in the high-molecular-weight fraction. Except for the unusually high glucose content, which may be a remnant of the starch extender present in the commercially obtained proteinase preparations, the carbohydrate composition was typical of that of a glycoprotein. Fractionation of either high- or low-molecular-weight material on a concanavalin A-agarose column did not retard the inhibitor, indicating that the glucose was probably not associated with an active molecule. Most importantly, there was never any evidence for the presence of uronic acids or pentoses in the three samples analysed, ruling out nucleic acids and most of the proteoglycans as candidates for the inhibitory material. The protein content of the inhibitor was supported by the low level of carbohydrate in the sample, as well as by the $A_{1\text{cm},280}^{1\%}$ of 10.4–11.2, which is typical of proteins.

The amino-acid composition of the high- and low-molecular-weight-inhibitor fractions emphasized the dissimilarities between the preparations

(Table 5). Whereas the low-molecular-weight fractions showed amino-acid compositions of almost uniform values for all the amino acids detected, the high-molecular-weight material was quite diverse. There were high levels of arginine, serine, aspartate and glutamate, with only a trace of cystine in the high-molecular-weight inhibitor. It should be noted that this amino-acid profile is only an estimate, since corrections were not made for hydrolysis and tryptophan content was not measured.

With this information in hand, we proceeded with further purification, our emphasis being on the high-molecular-weight material because of its greater biological activity in protein synthesis and cell-growth inhibition. Ultrafiltration was employed to concentrate the rather large volume of high-molecular-weight inhibitor obtained from several gel-filtration runs. The active material was retained with minimal adhesion by an Amicon DM-5 membrane. All of the inhibitor is retained above the filter membrane, and we have never been able to detect protein-synthesis inhibitory activity in the filtrate material.

In preliminary experiments we determined that the lectins concanavalin A and peanut agglutinin (agarose-bound) could not adsorb the inhibitor. The high-molecular-weight inhibitor, however, could be adsorbed by DEAE-cellulose, and both the high- and low-molecular-weight inhibitors could be adsorbed from solution by agarose-bound *U. europaeus* agglutinin I and subsequently recovered by elution

with fucose. Active high-molecular-weight inhibitor was therefore further purified by affinity chromatography with agarose-bound agglutinin I columns and eluted with 0.25 M-fucose. The eluted material was dialysed for 48 h against HKM buffer to remove the fucose before protein-synthesis inhibition assays. A titration curve of this material revealed that about 55 ng of inhibitor could decrease BHK-21-cell protein synthesis by 50% (Fig. 4). Despite the loss of activity by handling that might be expected in this purification step, we actually observed an increase in yield of active high-molecular-weight inhibitor in the protein synthesis assay. Purification of the high-molecular-weight inhibitor, based on specific activity of inhibition of protein synthesis, shows that this

Fig. 4. Inhibition of cellular protein synthesis by *U. europaeus*-agglutinin-I-purified brain cell-surface substance

High-molecular-weight brain cell-surface substance that was purified by *U. europaeus* agglutinin I affinity chromatography was tested for its ability to inhibit protein synthesis by the assay described in the Materials and methods section. The values shown are the means and standard deviations for six determinations.

material, which is almost certainly a cell-surface glycopeptide, has been purified more than 400-fold over the initial chloroform/methanol-extracted preparation (Table 6).

Discussion

Several lines of evidence suggest that proteinase activity associated with the cell surface of mitotic, transformed and malignant cells is correlated with their ability to avoid density-dependent growth control (Burger, 1971; Bosmann, 1972; Ossowski *et al.*, 1973; Unkeless *et al.*, 1973; Hynes, 1974, 1976; Roblin *et al.*, 1975; Nicolson *et al.*, 1977). In addition, several experiments have demonstrated that proteinase-sensitive glycoproteins differ in normal and transformed cells (Buck *et al.*, 1970; Warren *et al.*, 1972, 1973; Nicolson *et al.*, 1977). Several investigators have provided evidence that cell-surface proteins and glycoproteins may be signal molecules involved in density-dependent growth regulation (Koch, 1974; Fisher & Koch, 1976; Whittenberger & Glaser, 1977; Whittenberger *et al.*, 1978). The work of Roseman and his colleagues (Roseman, 1970; Roth *et al.*, 1971a,b) has provided a model to explain how surface glycosyltransferases could be involved in density-dependent growth regulation, and by implication, cell-surface glycoproteins.

We have previously reported that the inhibitor activity of brain cell glycopeptides are not cell-type or species specific (Kinders *et al.*, 1979). Instead, the specificity seems to lie in the ability of the tested cell lines to be density regulated in culture or *in vivo*. Primary mouse embryo fibroblasts and mouse brain cells are more sensitive to brain cell-surface substance than are the tissue-culture-adapted BHK-21 or Chinese hamster ovary cell lines. Mouse tumour cell lines are least sensitive or are completely refractory.

In the present study we have demonstrated that proteinase-prepared fragments of brain cortical

Table 6. Purification of the high-molecular-weight inhibitor from mouse cerebral cortex

The biological activity of the brain cell-surface substance at each stage of purification, was determined by the protein synthesis assay. One inhibitory unit (IU) was arbitrarily defined as the amount (μ g) that gave 20% inhibition of protein synthesis.

Preparation	Activity recovered (IU)	Weight recovered (mg)	Specific activity (IU/mg)	Fold purification
Chloroform/methanol extraction	21 017 (100%)	194.6	109	—
Freeze-dried, re-solubilized	21 017 (100%)	107.3	197	1.8
Bio-Gel P-100 chromatography	13 903 (66%)	3.48	3995	20.0
Ultrafiltration	5440 (26%)	0.43	12 513	115.0
<i>U. europaeus</i> agglutinin I affinity chromatography	5790 (27%)	0.13	44 569	409.0

cell-surface glycoproteins are effective inhibitors of cell division, glycoprotein synthesis and protein synthesis. Several lines of evidence indicate that the high- and low-molecular brain cell-surface substance inhibitors we have isolated are glycopeptides. The inhibitory activities were recovered in the aqueous phase after chloroform/methanol extraction; the $A_{1\text{cm},280}^{1\%}$ was 10.7 for the high-molecular-weight material; and the amino-acid content was approx. 94% by weight and carbohydrate content 6% by weight. In addition, we have determined that increased concentration of proteinases in the preparation of the inhibitor from the brain decreased the yield of active material (based on specific activities). The ability to adsorb the high-molecular-weight inhibitor to DEAE-cellulose at pH 7.1 further characterizes this glycopeptide as acidic (Kinders *et al.*, 1979). Both the high- and low-molecular-weight inhibitors contain carbohydrate, probably an oligosaccharide with a terminal fucose moiety, as indicated by our ability to purify them with the lectin *U. europaeus* agglutinin I, which specifically binds terminal fucose (Horejsi & Kocourek, 1973). This was also demonstrated by the carbohydrate analyses (Table 4). That the inhibitors are fragments of cell-surface glycoproteins is supported by their purification with this fucose-binding lectin. This is consistent with our previously reported data that the inhibitors could not be prepared from cytoplasm and microsomal fraction, or in the absence of proteinase treatment of whole cells (Kinders *et al.*, 1979).

Several important features of the high-molecular-weight glycopeptide have emerged in these studies. The inhibitory activity was effective against both protein and glycoprotein synthesis, and the amount of inhibition attained (62%) indicate that most cells were sensitive. Yet even at high amounts of inhibition, we have never observed any significant difference in cell viability between treated and untreated cells. Measurements of precursor uptake and mRNA synthesis have shown that this non-lethal inhibition is directed against protein synthesis at the translational level. A more striking result was that in the purified high-molecular-weight preparations of the brain-cell-surface-substance glycopeptide, 55 ng of material inhibited protein synthesis by 50% in 5×10^6 BHK-21 cells. This amount of inhibition with the lectin-purified material is in the range associated with hormone action and is consistent with a possible physiological role for the brain-cell-surface-substance inhibitor.

The ability of brain-cell-surface-substance glycopeptide to inhibit growth of exponentially multiplying cells in subconfluent cultures could well be the most interesting ramification of our work. We have successfully inhibited growth of both BHK-21 and primary mouse embryo fibroblast cells in these types

of experiments. The inhibition of BHK-21 cell growth shown in Table 3 occurred in the presence of complete medium containing 7% foetal calf serum, suggesting that the growth inhibition was not readily inactivated or otherwise counteracted by serum enzymes and growth factors. The inhibition of cell growth was never coupled with a decrease in cell viability and occurred at conditions that gave 50% inhibition of protein synthesis in the treated cells in 2h. In addition, the anti-mitotic activity co-purified with the protein-synthesis inhibitory activity. These data suggest that the protein-synthesis inhibition was related to the decreased cell growth. The ability to inhibit cell division, and the lack of any effect on cell morphology observable with phase-contrast microscopy, clearly distinguished the brain-cell-surface-substance glycopeptides from the large external transformation-sensitive protein (Yamada *et al.*, 1976).

There is no reason *a priori* to assume that there is only one growth-inhibiting substance on cell surfaces, and our data do not suggest this since we have specifically selected against lipids and lipoproteins. In addition, we have obtained two very potent inhibitors, which, based on the data presented, may be different glycopeptides and of different surface glycoprotein origin (Tables 4 and 5). Finally, the use of a bioassay to monitor the material eluted from the Bio-Gel column without additional concentrating steps before assay selects for more potent inhibitors, and we may have missed inhibitors in other molecular-weight classes that were too dilute to be detected.

Our ability to purify the high-molecular-weight brain-cell-surface-substance glycopeptide to high-specific activity may allow the determination of the mechanism by which tumour cells evade density-dependent growth regulation and brain-cell-surface-substance-glycopeptide-mediated inhibition. The possible role of cell-surface proteinases (Roblin *et al.*, 1975) and transglycosylation (Roseman, 1970) in cell-cell communication and growth regulation suggests numerous lines of experimentation that are now available with this model system. In addition to our efforts to further purify the brain-cell-surface-substance glycopeptide, we are examining the molecular basis for the responsiveness of normal or cell-culture-adapted cell lines to nanogram concentrations of the inhibitor and the insensitivity of tumour cells to this naturally occurring growth inhibitor.

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