Transformation-specific cell killing by a cancer-associated galactosyltransferase acceptor and cellular binding

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(Received 2 March 1982/Accepted 22 July 1982)

Cancer-associated galactosyltransferase acceptor (CAGA glycoprotein), a small glycoprotein purified from human malignant effusion that selectively kills transformed cells, was tritiated by reductive methylation in the presence of NaB^3H_4 . CAGA-glycoprotein-sensitive cells (baby-hamster kidney cells transformed by polyoma virus and chick-embryo fibroblasts infected with Ts68 temperature-sensitive mutant of Rous sarcoma virus grown at 37°C, the permissive temperature) bound 3–5-fold more ^3H -labelled CAGA glycoprotein than did their CAGA-glycoprotein-resistant non-transformed counterparts. The Rous-sarcoma-virus-infected chick-embryo fibroblasts grown at non-permissive temperature (41°C) bound an intermediate amount of ^3H -labelled CAGA glycoprotein; however, this intermediate amount appeared to be sufficient to induce inhibition of cell growth when the infected chick-embryo fibroblasts treated at 41°C were switched to 37°C. Binding of ^3H -labelled CAGA glycoprotein was time- and temperature-dependent and was not inhibited by monosaccharide. Binding was completely inhibited by the oligosaccharide liberated by endoglucosaminidase H treatment or by exhaustive Pronase digestion of intact CAGA glycoprotein. However, the isolated oligosaccharide failed to demonstrate the growth-inhibition characteristics of the intact glycopeptide. Binding of ^3H -labelled CAGA glycoprotein was unaffected by co-incubation with the peptide core released by endoglucosaminidase H treatment. ^3H -labelled CAGA glycoprotein bound to intact cells could be removed by trypsin treatment up to 4 h after addition of the glycoprotein but not thereafter. This time course paralleled the decreasing reversibility of growth inhibition. However, all ^3H -labelled CAGA glycoprotein was found in the supernatant when cells were first disrupted by sonication followed by trypsin treatment for up to 12 h. ^3H -labelled CAGA glycoprotein linked to Sepharose 4B failed to cause growth inhibition in CAGA-glycoprotein-sensitive cells. These findings suggest that binding of CAGA glycoprotein occurs via its oligosaccharide moiety. Binding appears to be a necessary but not sufficient condition to induce cell killing. Growth inhibition appears to depend on internalization of the glycoprotein and the presence of a transformation-specific cell process.

Abbreviations used: BHK cells, baby-hamster kidney fibroblasts; BHKpy cells, baby-hamster kidney fibroblasts transformed by polyoma virus; CEF (41°C) and CEF (37°C) cells, chick-embryo fibroblasts grown at 41°C or 37°C; CEF-RSV (41°C) and CEF-RSV (37°C) cells, chick-embryo fibroblasts infected with Ts68 temperature-sensitive mutant of Rous sarcoma virus grown at non-permissive (41°C) or permissive (37°C) temperature; CAGA glycoprotein, cancer-associated galactosyltransferase acceptor; CAGA-O oligosaccharide, CAGA-glycoprotein oligosaccharide prepared by endoglucosaminidase H treatment; CAGA-O(Asn) glycopeptide, CAGA-glycoprotein glycopeptide prepared by Pronase treatment.

Although many of the mechanisms controlling normal cell growth and differentiation remain unclear, a variety of factors have been found to influence these processes. In particular, numerous reports have suggested that glycoconjugate structures both on the cell surface and in the environmental substrate are important determinants of cell adhesion (Yamada & Olden, 1978; Schubert & LaCorbiere, 1980), intercellular interaction (Ocklind *et al.*, 1980), differentiation (Fukuda *et al.*, 1980) and cell growth (Hatten, 1981; Stanley & Sudo, 1981). In addition, it has been suggested that other agents that influence normal and malignant cell

growth, such as growth factors (e.g. epidermal growth factor) and peptide hormones (e.g. insulin), exert their effects by interaction with cell-surface glycoprotein receptors (Ronnelt & Lane, 1981; Hedo *et al.*, 1981). It is therefore possible that many of the alterations in growth and differentiation found in transformed and malignant cells are mediated via alteration in glycoconjugate structures. Numerous studies have demonstrated changes in the glycoprotein content of transformed cells when compared with their non-transformed counterparts, but the function of the oligosaccharide moieties of glycoproteins remains uncertain (see, e.g., Buck *et al.*, 1970, 1971; Smets *et al.*, 1976; Blithe *et al.*, 1980; Sherblom & Carraway, 1980).

We have previously described the purification from human malignant effusions of a low-molecular-weight glycoprotein (M_r approx. 3600) (termed cancer-associated galactosyltransferase acceptor, CAGA glycoprotein) that may serve as an acceptor substrate for a transformation-associated galactosyltransferase (Podolsky *et al.*, 1978). The glycoprotein has been shown to contain mannose and *N*-acetylglucosamine residues as well as a peptide backbone (Podolsky & Weiser, 1979). This glycoprotein was found to cause profound inhibition of cell growth, leading to cell death, when added to media of transformed cells in culture (Podolsky *et al.*, 1978; Podolsky & Isselbacher, 1980). In contrast, non-transformed cells were unaffected by addition of CAGA glycoprotein. In addition, administration of CAGA glycoprotein to hamsters inoculated with tumorigenic cells was found to prevent tumour development or cause actual tumour regression.

The mechanism whereby the glycoprotein leads to selective killing of transformed cells *in vitro* and *in vivo* and the relationship to its action as a galactosyltransferase substrate remain uncertain. In the present paper we describe results of binding studies with ^3H -labelled CAGA glycoprotein. The glycoprotein was found to bind specifically through its carbohydrate moiety. However, studies in chick-embryo fibroblasts (CEF cells) infected with a temperature-sensitive Rous sarcoma virus (RSV) mutant (Ts68) demonstrated that specific binding of CAGA glycoprotein is necessary but not sufficient to cause cell killing. Instead, cell killing appears to require internalization of the bound glycoprotein, the presence of the CAGA-glycoprotein peptide core and the presence of the transformed state of the cell.

Experimental

Cell culture and inhibition assays

Baby-hamster kidney fibroblasts (BHK cells) and BHK cells infected with polyoma virus (BHKpy cells) were maintained in Dulbecco's modified

Eagle's medium (GIBCO Laboratories, Grand Island, NY, U.S.A.) containing 10% (v/v) heat-inactivated (65°C, 20 min) foetal calf serum as well as penicillin and streptomycin. Cells from second to ninth passage were grown to semi-confluence for harvesting or use in CAGA-glycoprotein growth-inhibition assays. Primary CEF-cell cultures were prepared from pathogen-free 10-day chick embryos as previously described (Inui *et al.*, 1980) and maintained at 41°C. Secondary cultures were infected on day 3 after seeding with a temperature-sensitive RSV mutant (Ts68) maintained in this laboratory (2 plaque-forming units/cell by focus plaque assay). Infected cells were either maintained at 41°C (non-permissive temperature) or transferred to 37°C (permissive temperature) 24 h after infection. Cells from semi-confluent dishes were either harvested in the presence of EDTA and trypsin or used for CAGA-glycoprotein growth-inhibition assay.

CAGA-glycoprotein growth-inhibition assays were performed on semi-confluent dishes (35 mm and 60 mm) with purified glycoprotein (see below) added in sterile deionized water (25–100 μl /dish). Inhibition was scored at 24 h by microscopic evaluation and cell counts. Viability was assessed by incorporation of [^3H]thymidine (10 μCi /dish; specific radioactivity 80 Ci/mmol; New England Nuclear Corp.) after addition of the labelled precursor 2 h before harvesting; at the end of that time cells were harvested by brief trypsinization and then washed twice in phosphate-buffered saline (0.154 M-NaCl/10 mM-sodium phosphate buffer, pH 7.2). This was followed by the addition of ice-cold trichloroacetic acid to 10% (w/v) final concentration. The resulting precipitate was collected on glass-fibre filters and washed twice with 10% trichloroacetic acid followed by ice-cold 100% ethanol, and incorporated radioactivity was determined in 10 ml of toluene/1,4-bis-(5-phenyloxazol-2-yl)benzene scintillant (New England Nuclear Corp.). Viability was also determined by the ability to exclude Trypan Blue (1%, w/v) after 5 min.

Preparation of CAGA glycoprotein and ^3H -labelled CAGA glycoprotein

Purified CAGA glycoprotein was prepared from malignant human effusions as previously described (Podolsky & Weiser, 1979). Tritiated CAGA glycoprotein was prepared by reductive methylation by using a modification of a described method (Gregoriadis & Ryman, 1978). CAGA glycoprotein (20 mg) was dissolved in 1.2 ml of 0.2 M-sodium borate buffer, pH 9.0, and cooled on ice. NaBH_4 (0.5 mg) containing NaB^3H_4 (3.3 mCi; New England Nuclear Corp.) was added in a volume of 10 μl . Subsequently 1.0 μl portions of 37% (v/v) formaldehyde were added at 5 min intervals over

30 min. The reaction mixture was then applied to a column (1.5 cm $\times$ 30 cm) of Bio-Gel P-2 (100–200 mesh) equilibrated in deionized water; the column was developed at a rate of approx. 15 ml/h, 4 ml fractions being collected; radioactivity was counted in 10 μ l samples dissolved in 10 ml of Aquasol scintillation fluid (New England Nuclear Corp.). Fractions containing ^3H -labelled CAGA glycoprotein (material in excluded volume) were pooled, dried under N_2 and redissolved in 1.0 ml of 0.1 M-acetic acid to destroy any remaining hydride. The ^3H -labelled CAGA glycoprotein (specific radioactivity 2.6×10^7 c.p.m./mg) was freeze-dried and stored at -20°C before use.

The homogeneity of the labelled glycopeptide was compared with that of the unlabelled species by chromatography on Bio-Gel P-4. ^3H -labelled or unlabelled CAGA glycoprotein (10 mg) was dissolved in 2.5 mm-pyridine/acetate buffer, pH 5.5, and applied to a column (1.5 cm $\times$ 100 cm) of Bio-Gel P-4, which was developed at a rate of 7.0 ml/h, 2.5 ml fractions being collected. The column was monitored for absorbance at 280 nm, and hexose was determined by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956). Radioactivity of 100 μ l samples was counted in 10.0 ml of Aquasol II (New England Nuclear Corp.).

Binding of ^3H -labelled CAGA glycoprotein

Cells were harvested and suspended in phosphate-buffered saline at a concentration of 1×10^7 /ml. Assay mixtures contained 100 μ l of cell suspension, 0–100 μ l of CAGA glycoprotein solution (2.0 mg/ml) containing ^3H -labelled CAGA (3×10^5 c.p.m./100 μ l) and 0–100 μ l of phosphate-buffered saline. Mixtures were incubated in plastic Microfuge tubes at 37°C for various times; assays were terminated by addition of 1.0 ml phosphate-buffered saline (at room temperature) and cells were collected by centrifugation (at 4000 g for 2 min). The supernatant was discarded and cell pellets were washed three more times with phosphate-buffered saline (1.0 ml) and pelleted by centrifugation. The cell pellets were then resuspended in 100 μ l of phosphate-buffered saline and bound radioactivity was determined in 10.0 ml of Aquasol II.

Inhibition by monosaccharides or oligosaccharides of the binding of ^3H -labelled CAGA glycoprotein was assessed by addition of 100 μ l of phosphate-buffered saline containing sugar (2.0 mm) or buffer alone to the binding-assay mixture at the time of addition of ^3H -labelled CAGA glycoprotein.

Sensitivity of bound ^3H -labelled CAGA glycoprotein to proteinase treatment was determined by modification of the above assay system. Cells [BHKpy, CEF-RSV (41°C) and CEF-RSV (37°C)] were incubated with ^3H -labelled CAGA glycoprotein (2×10^6 c.p.m./ 5×10^5 cells) at 37°C for 15 min. Subsequently cells were pelleted and washed twice

with 1.0 ml of phosphate-buffered saline and resuspended in medium without CAGA glycoprotein, and incubation was continued at 37°C . At various intervals, cells were pelleted and resuspended in 1.0 ml of phosphate-buffered saline containing 5 μg of trypsin [1-chloro-4-phenyl-3-tosylamido-butan-2-one ('TPCK')-treated; Worthington Biochemical Corp.]/ml and incubated at 37°C with gentle shaking for 20 min. Cells were pelleted by centrifugation, and the solubilized radiolabel was determined by counting the radioactivity of a 200 μ l sample of supernatant in 10.0 ml of Aquasol. The trypsin was omitted from control assays.

Treatment of CAGA glycoprotein with endoglycosidase and with proteinase

Endoglucosaminidase H was obtained from Miles Laboratories and further purified in this laboratory by previously described methods (Tarentino & Maley, 1974) to eliminate residual contaminating exoglycosidase activities. Endoglucosaminidase H activity was verified by assay with [^{14}C]acetyl-ovalbumin glycopeptide IV, prepared in this laboratory by published methods (Tai *et al.*, 1975). ^3H -labelled CAGA glycoprotein (10 c.p.m./10 mg) was incubated with 16.0 munits of endoglucosaminidase H in 0.2 M-sodium citrate buffer, pH 5.5, at 40°C for 16 h under toluene (1 unit of endoglucosaminidase H is defined as the amount hydrolysing 1 μmol of [^{14}C]acetyl-ovalbumin glycopeptide IV/min at pH 5.0 at 37°C). The reaction mixture was subsequently applied to a column (1.5 cm $\times$ 100 cm) of Bio-Gel P-4 and developed in 2 mm-pyridine/acetate buffer, pH 5.5, at a rate of 10 ml/h; approx. 2.5 ml fractions were collected and assessed for absorbance at 280 nm as well as for hexose, determined by the method of Dubois *et al.* (1956) on 200 μ l samples. Further samples (100 μ l) were diluted in 5.0 ml of Aquasol for determination of radioactivity. Peaks containing radioactivity (which coincided with absorbance at 280 nm) or hexose were pooled separately and freeze-dried. ^3H -labelled CAGA glycoprotein was similarly incubated with endoglucosaminidase D (9.8 munits) purified in this laboratory by the method of Glasgow *et al.* (1977). Intact ^3H -labelled CAGA glycoprotein was exhaustively digested with Pronase (GIBCO; 75 500 PUK units/mg) (1 PUK unit of Pronase is defined as the amount liberating 1 μmol of tyrosine from casein/min at pH 7.5 at 37°C); 20 mg (10^7 c.p.m.) of CAGA glycoprotein was dissolved in 2.5 ml of 50 mm-Tris/HCl buffer, pH 7.6, containing 10 mm- CaCl_2 ; Pronase (2.5 mg) was then added and the mixture was incubated at 45°C ; a drop of toluene was added to retard bacterial growth. After 24 h an additional 1.5 mg of Pronase was added and incubation was continued. After an additional 24 h, the mixture was applied to a column (2.5 cm $\times$

100 cm) of Bio-Gel P-4 and developed with 20 mM-pyridine/acetate buffer, pH 5.5, at a rate of 10 ml/h; 2.5 ml fractions were collected and monitored for radioactivity and hexose as above. Hexose-containing fractions were pooled, freeze-dried and redissolved in 2.5 ml of 50 mM-Tris/HCl buffer, pH 7.6, containing 10 mM-CaCl₂. After addition of 2.5 mg of Pronase, digestion was again continued for 24 h at 45°C before re-chromatography on Bio-Gel P-4 as above. Again hexose-containing fractions were pooled, and after these had been freeze-dried a third Pronase digestion performed for 24 h followed by chromatography on Bio-Gel P-4 as above. Hexose-containing fractions, which were devoid of any radiolabel, were pooled and freeze-dried. Carbohydrate and amino acid compositions were determined by g.l.c. as previously described (Podolsky & Weiser, 1979).

Preparation of CAGA-Sepharose 4B

CNBr-activated Sepharose 4B was purchased from Pharmacia; 2 g was swollen and washed with 1 mM-HCl. Unlabelled (20 mg) and labelled CAGA glycoprotein (3×10^6 c.p.m.) were dissolved in 0.1 M-NaHCO₃, added to moist gel, and incubated with shaking at room temperature for 2 h followed by

incubation at 0–4°C overnight. The gel was washed several times with 0.1 M-NaHCO₃ and then suspended in 1 M-ethanolamine for 2 h at room temperature. The prepared gel was then washed successively with three cycles of 0.1 M-sodium acetate buffer, pH 4.0, alternating with 0.1 M-sodium borate buffer, pH 8.0. The gel was finally washed and suspended in either distilled water or phosphate-buffered saline.

Results

The CAGA glycoprotein was labelled by exhaustive reductive methylation. The tritiated preparation was found to co-chromatograph with unlabelled CAGA glycoprotein on Bio-Gel P-4 (Fig. 1) as well as on DEAE-cellulose (results not shown). The label was incorporated into the peptide backbone, as demonstrated by chromatography on Bio-Gel P-4 after treatment of the tritiated glycoprotein with endoglucosaminidase H (see below).

The labelled CAGA glycoprotein was used to study the binding of the glycopeptide to transformed and non-transformed cells. As demonstrated in Fig. 2, CAGA-glycoprotein-binding sites on both BHK and BHKpy cells were apparently saturated at

Fig. 1. Chromatography of ³H-labelled CAGA glycoprotein on Bio-Gel P-4

³H-labelled CAGA glycoprotein was prepared as described in the text and twice chromatographed on Bio-Gel P-4 to remove unchanged NaB³H₄. Freeze-dried ³H-labelled CAGA glycoprotein (1×10^5 c.p.m./10 μl) and unlabelled CAGA glycoprotein (10 mg/200 μl) were applied together to a column (1.5 cm × 100 cm) of Bio-Gel P-4 equilibrated in 2.5 mM-pyridine/acetate buffer, pH 5.5. The column was developed at a flow rate of 7 ml/h, 2.5 ml fractions being collected under continuous u.v. monitoring at 280 nm (—). Hexose was determined by the phenol/H₂SO₄ method (Dubois *et al.*, 1956) at 490 nm (-----). Samples (100 μl) for determination of radioactivity were dried, redissolved in water and their radioactivities counted in 10 ml of Aquasol II (—). The elution positions of molecular-weight standards are indicated: OVI and OVV [ovalbumin glycopeptides I and V, prepared by the method of Tai *et al.* (1975); *M_r* 1980 and 1260 respectively]; Raf, raffinose (*M_r* 540).

concentrations of 5 $\mu\text{g}/\text{ml}$. Binding was competitively inhibited by unlabelled glycoprotein, indicating that the labelled preparation retained binding characteristics identical with those of the naturally occurring glycopeptide. Binding was found to be relatively rapid, with maximal binding occurring within 15 min (Fig. 3). However, the transformed BHKpy (CAGA-glycoprotein-sensitive) cells bound 4–5 times more ^3H -labelled CAGA glycoprotein than did the non-transformed BHK cell line (Table 1).

Studies on the effect of CAGA glycoprotein on cell growth, morphology and [^3H]thymidine incorporation were performed with secondary CEF cells, as well as CEF cells infected with a temperature-sensitive RSV mutant (Ts68) grown at 37°C and 41°C. As shown in Table 2, CAGA glycoprotein selectively killed the infected (transformed) cells grown at 37°C (permissive temperature) but did not affect either uninfected CEF cells or infected CEF cells at 41°C. However, when infected CEF-RSV cells initially treated with CAGA

Fig. 2. Binding of ^3H -labelled CAGA glycoprotein to BHK and BHKpy cells

BHK cells (○) or BHKpy cells (●) ($1 \times 10^6/100 \mu\text{l}$ of phosphate-buffered saline) were incubated at 37°C for 30 min with various concentrations of ^3H -labelled CAGA glycoprotein (in $100 \mu\text{l}$ of phosphate-buffered saline; specific radioactivity $3 \times 10^7 \text{ c.p.m./mg}$) in Microfuge tubes. Assays were stopped by addition of 1.0 ml of phosphate-buffered saline. Cells were pelleted and washed three times, then resuspended in $100 \mu\text{l}$ of phosphate-buffered saline, and their radioactivity was counted in 5.0 ml of Aquasol. Binding of ^3H -labelled CAGA glycoprotein to BHKpy cells in the presence of a 10-fold excess of unlabelled CAGA glycoprotein was also measured (△).

Fig. 3. Binding of ^3H -labelled CAGA glycoprotein to BHK and BHKpy cells as a function of time

BHK cells (○) or BHKpy cells (●) ($1 \times 10^6/100 \mu\text{l}$ of phosphate-buffered saline) were incubated at 37°C with $100 \mu\text{l}$ of ^3H -labelled CAGA glycoprotein ($20 \mu\text{g}/\text{ml}$; $3 \times 10^7 \text{ c.p.m./mg}$) for various lengths of time. Bound ^3H -labelled CAGA glycoprotein was determined as detailed in the Experimental section and the legend to Fig. 2.

Table 1. Binding of ^3H -labelled CAGA glycoprotein to transformed and non-transformed cells

Cells ($10^6/100 \mu\text{l}$) were incubated with $100 \mu\text{l}$ of ^3H -labelled CAGA glycoprotein solution ($20 \mu\text{g}/\text{ml}$; $3 \times 10^7 \text{ c.p.m./mg}$) at 37°C for 30 min. Bound ^3H -labelled CAGA glycoprotein was determined as described in the Experimental section.

Cells	$10^{-3} \times ^3\text{H}$ -labelled CAGA glycoprotein bound (c.p.m./ 10^6 cells)	$10^{-3} \times$ CAGA glycoprotein bound (molecules/cell)
BHK	1.2 ± 0.5	1.52
CEF	1.8 ± 0.4	2.28
CEF-RSV (41°C)	3.8 ± 0.6	4.83
BHKpy	4.8 ± 0.7	6.10
CEF-RSV (37°C)	8.7 ± 0.8	11.05

Table 2. *Effect of ³H-labelled CAGA glycoprotein on cell growth*

Cells were seeded at 1×10^6 /100 mm dish; 100 μ l of CAGA glycoprotein solution (5 μ g/ml) or phosphate-buffered saline was added on day 3 after seeding and incubation was continued for 24 h. Before harvesting of the cells [³H]thymidine (10 μ Ci; specific radioactivity 80 Ci/mmol) was added; 2 h after this addition, cells were harvested and resuspended in 1.0 ml, and radioactivity was determined in a 100 μ l sample dissolved in 10.0 ml of Aquasol. Determinations were made in duplicate on separate experiments.

Cells	Temperature (°C)	³ H-labelled CAGA glycoprotein	$10^{-6} \times$ No. of cells	[³ H]Thymidine uptake (c.p.m./min per 100 μ l)
CEF	41	—	5.7 ± 1.1	2830
		+	6.2 ± 1.3	2140
CEF	37	—	4.6 ± 0.8	1760
		+	4.3 ± 0.7	1840
CEF-RSV	41	—	4.8 ± 1.2	2150
		+	3.9 ± 0.8	1920
CEF-RSV	37	—	5.1 ± 1.4	2170
		+	0.5 ± 0.3	150
CEF-RSV	41 $\rightarrow$ 37*	—	4.6 ± 1.6	2060
		+	0.9 ± 0.4	270

* At 6 h after the addition of CAGA glycoprotein, the medium was changed and cells were shifted to incubation at 37°C.

glycoprotein at 41°C were subsequently shifted to 37°C, cell killing was observed at 24 h, the time necessary for expression of transformation, despite a change to non-CAGA-glycoprotein containing media before the shifting of the temperature (Table 2). Uninfected CEF cells pretreated with CAGA glycoprotein were not affected by a shift to 37°C.

Evaluation of the binding of ³H-labelled CAGA glycoprotein in CEF, CEF-RSV (41°C) and CEF-RSV (37°C) cells disclosed differential binding similar to that observed with BHKpy and BHK cell lines. As shown in Table 1, the transformed cells bound approx. 5 times more glycoprotein than did the uninfected CEF cells. It is noteworthy that the infected non-transformed CEF-RSV (41°C) cells bound an intermediate amount of ³H-labelled CAGA glycoprotein, i.e. about twice that found with CEF cells but half that observed for transformed CEF-RSV (37°C) cells. This intermediate amount of binding of CAGA glycoprotein appeared to be sufficient to initiate cell death, in view of the cytotoxic effect of CAGA glycoprotein observed when these cells were shifted to 37°C. The time course and saturation concentration of the binding of ³H-labelled CAGA glycoprotein to CEF, CEF-RSV (41°C) and CEF-RSV (37°C) cells were similar to observations made with BHK and BHKpy cells. Determination of binding affinity constants by equilibrium dialysis of whole cells indicates a homogeneous set of receptors in each of the cell types studied, and Scatchard-plot analysis suggests equivalent affinity for all cell types (apparent $K_a = 1.7 \times 10^6 \pm 0.4 \times 10^6$ /mol).

The role of the carbohydrate moiety of CAGA glycoprotein on specific cell binding was investi-

gated by competitive inhibition with a variety of monosaccharides. As shown in Table 3, none of the monosaccharides tested resulted in significant inhibition of the binding of ³H-labelled CAGA glycoprotein. Similarly a variety of amino acids failed to inhibit binding. Subsequently, the effects of CAGA-O oligosaccharide and CAGA-O(Asn) glycopeptide were examined. Treatment with endoglucosaminidase H successfully removed carbohydrate from the tritiated peptide backbone. The isolated oligosaccharide moiety and the peptide backbone (with *N*-acetylglucosamine attached) were separated and isolated by chromatography on Bio-Gel P-4 after the endoglucosaminidase H treatment (Fig. 4). The intact glycoprotein was found to be resistant to treatment with endoglucosaminidase D prepared in this laboratory (results not shown). A CAGA-O(Asn) glycopeptide fragment was also prepared, by exhaustive Pronase digestion of the intact glycoprotein followed by chromatography on Bio-Gel P-4 (Fig. 5). The final carbohydrate-containing fractions were found to be devoid of radioactivity. Carbohydrate-containing fractions obtained from Bio-Gel P-4 columns after either endoglucosaminidase H treatment (CAGA-O) or Pronase digestion [CAGA-O(Asn)] were freeze-dried and resuspended in distilled water. Carbohydrate and amino acid compositions of CAGA-O oligosaccharide (estimated M_r 2250 by Bio-Gel P-4/polyacrylamide-gel chromatography) and CAGA-O(Asn) glycopeptide (estimated M_r 2530) were determined by g.l.c. and compared with those of intact CAGA glycoprotein (estimated M_r 3600) as detailed in Table 4. Mannose and *N*-acetylglucosamine were the only carbohydrates detected in any

Table 3. *Effects of monosaccharides, CAGA-O oligosaccharide and CAGA-O(Asn) glycopeptide on the binding of ³H-labelled CAGA glycoprotein*

Cells ($10^6/100\mu\text{l}$) were incubated with $100\mu\text{l}$ of ^3H -labelled CAGA glycoprotein solution ($20\mu\text{g/ml}$; $3 \times 10^7\text{ c.p.m./mg}$) for 30 min at 37°C . Bound ^3H -labelled CAGA glycoprotein was determined as described in the Experimental section and the legend to Fig. 2. CAGA-O oligosaccharide was prepared by endoglucosaminidase H treatment of CAGA glycoprotein as described in the text ($100\mu\text{g/ml}$). CAGA-O(Asn) glycopeptide is the oligosaccharide-containing peptide isolated after exhaustive Pronase digestion of CAGA glycoprotein as described in the text ($100\mu\text{g/ml}$). CAGA-P peptide is the peptide isolated after endoglucosaminidase H treatment of CAGA glycoprotein as described in the text ($100\mu\text{g/ml}$).

Cells	Addition	³ H-labelled CAGA glycoprotein bound	
		(c.p.m./ 10^6 cells)	(%)
BHKpy	None	4630	100
	Glucosamine	4920	106
	Mannose	4170	90
	Galactose	4820	104
	Fucose	4930	106
	CAGA-O oligosaccharide	840	18
	CAGA-O(Asn) glycopeptide	780	17
	CAGA-P peptide	5900	127
CEF-RSV (37°C)	None	8750	100
	Glucosamine	8140	93
	Mannose	7960	91
	Galactose	8590	98
	Fucose	8930	102
	CAGA-O oligosaccharide	2120	24
	CAGA-O(Asn) glycopeptide	1830	21
	CAGA-P peptide	7760	89

Fig. 4. *Isolation of free CAGA-O oligosaccharide and peptide from ³H-labelled CAGA glycoprotein after endoglucosaminidase H treatment*

^3H -labelled CAGA glycoprotein (10^7 c.p.m. ; 10mg) was incubated with 16.0 munits of endoglucosaminidase H in 0.2 M-citrate buffer, $\text{pH } 5.5$, for 16 h at 40°C . The reaction mixture was then applied to a column ($1.5\text{ cm} \times 100\text{ cm}$) of Bio-Gel P-4 and developed with $20\text{ mm-pyridine/acetate}$ buffer, $\text{pH } 5.5$, at a rate of 10 ml/h , approx. 2.0 ml fractions being collected with constant monitoring at 280 nm (—). Hexose was determined in $200\mu\text{l}$ samples by the phenol/ H_2SO_4 method (-----). Radioactivity was determined of $100\mu\text{l}$ samples dissolved in 10.0 ml of Aquasol II (.....).

of these preparations. CAGA-O oligosaccharide was found to be devoid of amino acid and had a carbohydrate composition similar to that of CAGA glycoprotein, differing by approximately one residue

of *N*-acetylglucosamine/molecule. CAGA-O(Asn) glycopeptide contained a carbohydrate complement similar to that of intact CAGA glycoprotein but appeared to retain only two or three amino acid

Fig. 5. Isolation of CAGA-O(Asn) glycopeptide from ^3H -labelled CAGA glycoprotein after Pronase digestion. ^3H -labelled CAGA glycoprotein (20 mg; 10^7 c.p.m.) was dissolved in 2.5 ml of 50 mM-Tris/HCl buffer, pH 7.6, containing 10 mM- CaCl_2 , and Pronase (2.5 mg) was added before incubation at 45°C ; after 24 h 1.5 mg of Pronase was added and digestion continued for 24 h at 45°C . The reaction mixture was applied to a column (1.5 cm $\times$ 100 cm) of Bio-Gel P-4, which was developed with 2.0 mM-pyridine/acetate buffer, pH 5.5, at 10 ml/h, 2.5 ml fractions being collected with constant monitoring at 280 nm (—). Hexose was determined on 200 μl samples by the phenol/ H_2SO_4 method (-----). Radioactivity assessed of 100 μl samples dissolved in 5.0 ml of Aquasol II (---). Hexose-containing fractions were pooled and subjected to further Pronase digestion as described in the text.

Table 4. Carbohydrate and amino acid compositions of CAGA glycoprotein, CAGA-O oligosaccharide and CAGA-O(Asn) glycopeptide

Carbohydrate and amino acid analysis was performed by g.l.c. on a Varian 6000 gas chromatograph in tandem with a Hewlett Packard 3390A integrator by using previously described methods (Podolsky & Weiser, 1975, 1979). Data are expressed as mol/mol of glycoprotein or oligosaccharide, based on molecular weights detailed in the text as determined by Bio-Gel P-4/polyacrylamide-gel permeation chromatography.

	Composition (mol/mol)		
	CAGA glycoprotein	CAGA-O oligosaccharide	CAGA-O(Asn) glycopeptide
Carbohydrate			
N-Acetylglucosamine	5.1	3.8	5.4
Mannose	7.1	6.9	7.3
Amino acid			
Alanine	2.2	—	0.6
Valine	1.1	—	—
Glycine	1.3	—	—
Leucine	0.9	—	—
Threonine	1.2	—	—
Serine	0.8	—	—
Asparagine/aspartic acid	2.3	—	2.1
Glutamine/glutamic acid	1.7	—	—
Lysine	0.8	—	—
Tyrosine	1.1	—	—

residues/molecule. The prepared oligosaccharide and glycopeptide competitively and quantitatively inhibited binding of intact ^3H -labelled CAGA glycoprotein to BHKpy, CEF-RSV (41°C) and

CEF-RSV (37°C) cells (Table 3 and Fig. 6). Titration curves of CAGA-glycoprotein growth inhibition by CAGA-O oligosaccharide and CAGA-O(Asn) glycopeptide were essentially identical (Fig.

Fig. 6. Inhibition of the binding of ^3H -labelled CAGA glycoprotein by CAGA-O oligosaccharide and CAGA-O(Asn) glycopeptide

BHKpy cells ($10^6/100\mu\text{l}$) were incubated with $100\mu\text{l}$ of ^3H -labelled CAGA glycoprotein solution ($40\mu\text{g/ml}$; $3 \times 10^7\text{ c.p.m./mg}$) in the presence of CAGA-O oligosaccharide (○) or CAGA-O(Asn) glycopeptide (●) ($100\mu\text{l}$); bound ^3H -labelled CAGA glycoprotein was determined as described in the Experimental section and the legend to Fig. 2.

6). No inhibition was observed when peptide isolated after endoglucosaminidase H treatment was added to binding assays.

To determine whether CAGA glycoprotein was found at the cell surface, we assessed the ability of exogenous trypsin to remove bound ^3H -labelled CAGA glycoprotein from intact cells. Cells [BHKpy, CEF-RSV (41°C) and CEF-RSV (37°C)] were incubated with ^3H -labelled CAGA glycoprotein for 15 min and then changed to CAGA-glycoprotein-free phosphate-buffered saline. At various times trypsin was added to individual assay mixtures and the amount of ^3H -labelled CAGA glycoprotein released into the incubated supernatant was determined. As demonstrated in Fig. 7, nearly all of the ^3H -labelled CAGA glycoprotein was released when trypsin treatment was done within 30 min after the initial incubation with ^3H -labelled CAGA glycoprotein. However, when trypsin was added at longer periods after initial exposure to CAGA glycoprotein there was a corresponding decline in the amount of ^3H -labelled CAGA glycoprotein removed by the proteinase treatment. After 2 h only about half of the ^3H -labelled CAGA glycoprotein was removed by exogenous trypsin; by 4 h less than 10% of the

Fig. 7. Release of bound ^3H -labelled CAGA glycoprotein by trypsin

CEF-RSV (37°C) cells (10^7 in 1.0 ml) were incubated with ^3H -labelled CAGA glycoprotein ($3 \times 10^6\text{ c.p.m. in } 1.0\text{ ml}$) and incubated for 15 min at 37°C . Cells were then washed twice and resuspended in 1.0 ml of phosphate-buffered saline. After various lengths of time, cells were again resuspended in 1.0 ml of phosphate-buffered saline with (●) or without (○) trypsin ($5\mu\text{g/ml}$) and incubated for 20 min at 37°C . Cells were then pelleted and radioactivity was determined in the supernatant ($200\mu\text{l}$ sample in 10.0 ml of Aquasol II).

^3H -labelled CAGA glycoprotein was solubilized by the proteinase treatment. When cells were sonicated 4 h after exposure to ^3H -labelled CAGA glycoprotein all of the label was recovered in the supernatant fraction after centrifugation at $105\,000\text{ g}$ for 60 min. Cell-growth inhibition was further assessed by using CAGA glycoprotein bound to Sepharose 4B. Inclusion of ^3H -labelled CAGA glycoprotein as tracer demonstrated the presence of $1\text{--}2\mu\text{g}$ of CAGA glycoprotein bound/ $25\mu\text{l}$ of settled Sepharose 4B. The gel was used within 48 h of preparation, during which time less than 0.05% of the ^3H -labelled CAGA glycoprotein was found in the supernatant of the gel. Despite the addition of up to $200\mu\text{l}$ of CAGA–Sepharose 4B, there was no demonstrable cell killing in either BHKpy or CEF-RSV (37°C) cell cultures.

The reversibility of CAGA-glycoprotein-induced growth inhibition was also examined. Cells were incubated with the glycoprotein for various times, after which the cells were washed and then further incubated in media lacking CAGA glycoprotein. After 24 h the number of surviving cells was determined by Trypan Blue exclusion. As demonstrated in Fig. 8, the number of recoverable cells declined progressively with increasing time of exposure to CAGA glycoprotein. Although there was little decrease in cell numbers after brief treatment ($>90\%$ viability up to 1 h), with longer exposure

Fig. 8. Effect of duration of exposure to CAGA glycoprotein on reversibility of growth inhibition

Semi-confluent dishes (35 mm) of BHKpy cells were incubated with 100 μ l of phosphate-buffered saline (O) or CAGA glycoprotein (10 μ g) (●). After various lengths of time, the medium was aspirated off and replaced with non-CAGA-glycoprotein-containing media. After 24 h further incubation at 37°C cell counts were determined with a Coulter counter, and viability was assessed by Trypan Blue exclusion.

reversibility appeared to decline precipitously; after 4 h fewer than 10% of cells survived, despite change to CAGA-glycoprotein-free medium.

Discussion

In recent years numerous factors have been described that appear to promote or retard the growth of transformed cells *in vitro* and *in vivo*. These factors range from low-molecular-weight tumour promoters and peptides (see, e.g., Becker & Cook, 1981; Heifetz & Prager, 1981) to larger polypeptide hormones and growth factors (see, e.g., Im *et al.*, 1978; Todaro *et al.*, 1980; Sutherland *et al.*, 1981; Donahoe *et al.*, 1981). Reports from this laboratory previously described the presence in human malignant effusions of a low-molecular-weight galactosyltransferase acceptor (cancer-associated galactosyltransferase acceptor; CAGA glycoprotein) that specifically inhibited the growth of transformed cells *in vitro* and at least one solid tumour *in vivo* (Podolsky *et al.*, 1978; Podolsky & Isselbacher, 1980). This inhibitor was purified to apparent homogeneity and found to be a glycoprotein containing, almost exclusively, glucosamine and mannose in its carbohydrate moiety (Podolsky & Weiser, 1979). The specificity of the binding of CAGA glycoprotein to transformed cells and some of the early effects of CAGA glycoprotein that ultimately lead to cell death were investigated in the present studies.

The present studies provide further observations on the association of CAGA-glycoprotein-induced

cell killing with the transformed state. Thus, as shown in Fig. 3, infection of CEF cells with a temperature-sensitive RSV mutant was in itself insufficient to induce sensitivity of the infected cells to CAGA glycoprotein; killing was seen only at the permissive temperature (37°C), and the time course for 'sensitivity' to CAGA glycoprotein was comparable with that required for the expression of transformation.

Binding studies with ³H-labelled CAGA glycoprotein indicated that CAGA-glycoprotein-sensitive cells bound 4–5-fold more glycoprotein than did those resistant to this inhibitor. However, it is evident from the present study that increased binding of CAGA glycoprotein was not the only distinguishing feature between sensitive (transformed) and resistant (non-transformed) cell types. Of particular interest is the observation that CEF-RSV (41°C) cells did not demonstrate CAGA-glycoprotein-sensitivity (i.e. cell death), although they appeared to bind sufficient ³H-labelled CAGA glycoprotein to lead to inhibition of growth since these same cells were killed when shifted to 37°C without any further exposure to CAGA glycoprotein. Thus specific binding of CAGA glycoprotein appears to be necessary, but not in itself sufficient, to make a cell susceptible to the CAGA-glycoprotein-induced cytotoxic effect. It is of further interest that preliminary Scatchard-plot analysis suggests a uniform set of receptors with similar association constants on both CAGA-glycoprotein-sensitive and -insensitive cells, indicating that differential binding may indeed be due primarily to quantitative differences.

Although CAGA glycoprotein appears to bind initially at the cell surface, as determined by sensitivity to limited proteinase treatment, the progressive decline in the amount of CAGA glycoprotein solubilized by such treatment suggests that the bound glycoprotein may be internalized over a period of 4 h, thus making it inaccessible to an exogenous proteinase. Alternatively the progressive resistance to solubilization by trypsin could have resulted from a change in the conformation or distribution of the glycoprotein and its receptor in the cell membrane rendering CAGA glycoprotein resistant to release by the proteinase. The inability of CAGA-Sepharose 4B to induce cell killing supports the concept that CAGA glycoprotein must be internalized; however, this lack of efficacy might also have resulted from the Sepharose-bound CAGA glycoprotein preventing conformational or distributional changes because of the fixed nature of the ligand. It is noteworthy, however, that the 4 h period leading to complete resistance to trypsin-induced solubilization of CAGA glycoprotein is comparable with the time during which the cytotoxic effect of CAGA glycoprotein remained reversible (Fig. 7).

It appears that both oligosaccharide and peptide components are needed for the biological activity of CAGA glycoprotein. Thus results of competitive-inhibition experiments suggest that specific binding is mediated through the oligosaccharide chain, whether prepared as the free oligosaccharide by endoglucosaminidase H treatment or as the asparagine-bound species prepared by exhaustive Pronase digestion. It is noteworthy, however, that the component monosaccharides as such failed to cause significant inhibition of the binding of ^3H -labelled CAGA glycoprotein, suggesting that the CAGA-glycoprotein-binding site requires a more extended carbohydrate structure. However, the oligosaccharide produced by endoglucosaminidase H treatment was incapable of causing growth inhibition and cell death, suggesting that the peptide backbone is needed for full expression of growth inhibition. Although the oligosaccharide plays an important role in the binding of ^3H -labelled CAGA glycoprotein, the relationship between ability of CAGA glycoprotein to function as a galactosyltransferase acceptor substrate and as a growth inhibitor remains unclear.

In summary, these present studies demonstrate that CAGA-glycoprotein-sensitive cells specifically bind the glycoprotein through its oligosaccharide moiety and suggest that the binding of CAGA glycoprotein is followed by internalization of the glycoprotein. These studies also suggest that the explanation of the apparent specificity of the effects of CAGA glycoprotein on the transformed state may be related, at least in part, to events subsequent to these initial processes.

This work was supported by a grant from the National Cancer Institute (CA-31277) and a Searle Scholar Award (to D. K. P.).

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