Characterization of plasma-membrane glycoproteins from functional domains of the rat hepatocyte

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(Received 19 October 1984/Accepted 28 November 1984)

Plasma-membrane glycoproteins from the three different functional domains of the rat hepatocyte were radioactively labelled by oxidation with NaIO_4 , followed by reduction with NaB^3H_4 . Analysis of the radioactively labelled glycoproteins by polyacrylamide-gel electrophoresis revealed the presence of at least 12 major sialoglycoproteins in each different region of the hepatocyte surface. The M_r -110000 component was homogeneously distributed over the plasma membrane, whereas the M_r -90000 polypeptide was only located at the sinusoidal face. These radiolabelled glycoproteins were solubilized in 1% Triton X-100, and the soluble fraction was subjected to affinity chromatography on Sepharose-conjugated wheat-germ agglutinin (WGA). The labelled glycoproteins were poorly bound to WGA. Membrane glycoproteins were also labelled by the galactose oxidase/ NaB^3H_4 method. The results show that the polypeptides with apparent M_r 170000 from the sinusoidal, 230000 from the canalicular and 170000 from the lateral membranes were specifically labelled. When the membranes were treated with neuraminidase and galactose oxidase/ NaB^3H_4 , the electrophoretic patterns showed changes in the apparent M_r values of the glycoproteins, owing to loss of sialic acid, and a clear increase in labelling in the sinusoidal and canalicular membranes compared with the lateral membranes. When these labelled membranes were solubilized in 1% Triton X-100 and subjected to affinity chromatography on Sepharose-conjugated *Ricinus communis* agglutinin and/or *Lens culinaris* agglutinin, the results showed that the former columns efficiently bound the radiolabelled glycoproteins, whereas the latter columns bound poorly. The results show that there is a differential distribution of glycoproteins along the hepatocyte's surface.

The plasma membrane of liver parenchymal cells has three separate domains that maintain the functional polarity of the cell and which can be recognized morphologically. The functional differentiation is due to an uneven distribution of enzymes, receptors and other molecular components of the plasma membrane, most of them being glycoproteins in nature. The oligosaccharide chains on glycoproteins serve as recognition signals for biological functions (Ashwell & Morell, 1980; Gahmberg, 1981; Olden *et al.*, 1982).

Abbreviations used: SDS, sodium dodecyl sulphate; WGA, wheat-germ agglutinin; RCA_I, and RCA_{II}, *Ricinus communis* agglutinins I and II; LCA, *Lens culinaris* agglutinin.

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The sinusoidal ('basal') plasma membrane, covered by microvilli and comprising about 70% of the total area of the hepatic cell, transports amino acids, bile salts and hormones from the blood to the inside of the cell. At this surface there are receptors for peptide hormones, lipoproteins, asialoglycoproteins and transferrin (Evans, 1981). These membranes are also involved in the secretion of plasma proteins, lipids and carbohydrates, which are essential for extrahepatic metabolism.

The flattened plasma membrane derived from the lateral region comprises about 15% of the total area, is involved in the intercellular adhesion of cells and is in connection through specialized regions such as gap junctions, desmosomes and zonula occludens with neighbouring cells (Wisher & Evans, 1977).

The highly specialized ('apical') plasma membrane of the bile canaliculus constitutes 10–15% of the total surface of the hepatic cells and makes up the bile canaliculus. This part of the membrane is presumably involved in the formation and secretion of bile (Evans, 1980).

Up to now little is known about the identity or function of the major glycoproteins, and we think that a necessary preliminary step in studying the function of the different glycoproteins is the isolation and characterization of the plasma-membrane glycoproteins derived from the three different functional domains of the hepatocyte.

The combination of surface-labelling techniques and affinity chromatography using lectins with SDS/polyacrylamide-gel electrophoresis of highly purified plasma-membrane subfractions of liver cells showed that the hepatocyte surface contains at least 12–15 major glycoproteins in each region of its plasma membrane. Although most glycoproteins seem to be common in the three domains, the results in this paper clearly demonstrate a distinct distribution for specific glycoproteins.

Materials and methods

Reagents

The sources of the chemicals were as follows: sucrose, acrylamide, *NN'*-methylenebisacrylamide (Eastman Kodak Co.); 2,5-diphenyloxazole (New England Nuclear); NaIO_4 and D-galactose (Merck); SDS and galactose oxidase (type V) (Sigma Chemical Co.); Triton X-100 (BDH Chemicals); methyl α -mannoside (Calbiochem); *N*-acetylglucosamine (Fluka).

NaB^3H_4 (8.6–13.7 Ci/mmol) and ^{14}C -labelled protein standards were obtained from Amersham International. *Vibrio cholerae* neuraminidase (1 unit/ml) was obtained from Koch–Light Laboratories. All other substances were of highest purity available.

Animals

Male Sprague–Dawley rats weighing 200–250 g maintained on a standard rat diet and under controlled lighting were used for all studies.

Preparation of plasma-membrane subfractions

A plasma-membrane subfraction enriched in sinusoidal membranes was prepared essentially as described by Wisher & Evans (1975). The minced livers were suspended in 5 vol. of ice-cold 1 mM- NaHCO_3 , pH 7.4, containing 0.5 mM- CaCl_2 and homogenized in a loose-fitting Dounce homogenizer (ten strokes). After filtration through a muslin cloth to remove connective tissue, the homogenate was diluted 1:3 with buffer and centrifuged at 1000g for 10 min at 4°C. The combined super-

natants obtained after three centrifugations of the suspended pellet were removed by aspiration and centrifuged at 52000g for 8 min. The supernatant was kept and the pellet resuspended by gentle homogenization (see below). The previous step was repeated once. To obtain a microsomal pellet, the combined supernatants were centrifuged at 52000g for 30 min. The microsomal pellet was resuspended and homogenized in 72% sucrose (w/v) with five up-and-down strokes. The suspension was adjusted to a final volume of 15 ml and loaded at the bottom of SW 27 rotor tubes and overlaid with 39% (15 ml) and 8% (5 ml) sucrose (w/v) solutions in 5 mM-Tris/HCl, pH 7.5. All sucrose concentrations were adjusted with the aid of a refractometer. The discontinuous gradient was centrifuged at 94000g for 16 h. The microsomal crude membrane fraction appeared as a whitish band at the interface between the 39%- and 8%-sucrose layers.

This membrane fraction was homogenized in 8% sucrose/5 mM-Tris/HCl, pH 7.5, by 20 up-and-down strokes with a tight-fitting Dounce homogenizer. Plasma membranes were subfractionated on a discontinuous sucrose gradient (49%, 37% and 8%, w/v) by centrifugation for 150 min at 94000g. The subfraction of plasma membrane derived from the sinusoidal membranes gave a band at the 8%/37%-sucrose interface. The resulting pellets were resuspended in 10 mM-Tris/HCl, pH 7.5, and used for radiolabelling or stored at -70°C until needed.

For the isolation of the canalicular and lateral plasma-membrane subfractions we used the same experimental protocol as Wisher & Evans (1975), but starting with the nuclear pellet after low-speed centrifugation. The pellet was resuspended into 8% sucrose/5 mM-Tris/HCl, pH 7.5, and in the flotation step the discontinuous gradient was made with 72%, 43% and 8% sucrose solutions. In the second gradient the canalicular plasma membranes were recovered at the 8%/37%-sucrose interface and the lateral plasma membranes at the 37%/49%-sucrose interface.

SDS/polyacrylamide-gel electrophoresis

This was performed as described by Laemmli (1970), with 8%-acrylamide slab gels. The treatment of the gels for fluorography was as described by Bonner & Laskey (1974). Densitometric scans were obtained with a Joyce-Loebl double-beam recording microdensitometer.

Radioactive labelling of plasma membranes

Freshly isolated plasma membranes (1–1.5 mg) were labelled by two different methods. In the first, NaIO_4 was added (final concn. 1 mM) to the plasma membranes suspended in 0.01 M-sodium phos-

phate/0.15M-NaCl, pH 7.4, (P_i /NaCl) and incubated at 0°C for 10 min in the dark (Gahmberg & Andersson, 1977). After washing, the plasma membranes were reduced with 0.4 mCi of NaB^3H_4 for 30 min at room temperature. After washing by centrifugation at 52000g for 30 min in P_i /NaCl, the final pellet was stored at -70°C. In the second method, the plasma membranes, in Dulbecco's P_i /NaCl, were oxidized with galactose oxidase with or without neuraminidase for 30 min at room temperature, and after washing the membranes were reduced with 0.4 mCi of NaB^3H_4 (Gahmberg & Hakomori, 1973).

Solubilization of plasma membranes and lectin affinity chromatography

To the radiolabelled plasma-membrane subfractions was added an equal volume of 1% Triton X-100 in P_i /NaCl. The suspension was vortex-mixed for 15 s and, after standing for 30 min at 0°C, centrifuged for 5 min at 7000 rev./min in a Beckman Microfuge. The soluble material was subjected to affinity chromatography on carrier-bound lectins (Gahmberg *et al.*, 1980). Lentil lectin (LCA) was isolated as described by Sage & Green (1972), Ricinus communis lectins (RCA_I and RCA_{II}) as described by Nicolson & Blaustein (1972), and wheat-germ lectin (WGA) as described by Marchesi (1972). Purified lectins were coupled to Sepharose 4B (Pharmacia) by using 2 mg of lectin/ml of packed gel, activated with CNBr in the presence of 0.1 M concentration of the appropriate sugar hapten (Cuatrecasas, 1979).

Columns were equilibrated with P_i /NaCl containing 0.1% Triton X-100 and, after washing with

the same buffer, the bound material was eluted with P_i /NaCl containing 0.1% Triton X-100 and 0.1 M-D-galactose (RCA), N-acetylglucosamine (WGA) or α -methyl mannoside (LCA). Fractions were collected, and 10 μ l samples of each were monitored for radioactivity. All steps were performed at 4°C.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and discussion

Isolation of subfractions of rat liver plasma membrane

The sinusoidal, canalicular and lateral plasma-membrane subfractions were isolated and biochemically characterized. The specific activities of the marker enzymes are shown in Table 1. Alkaline phosphatase and Mg^{2+} -dependent ATPase are considered as markers for the canalicular plasma membranes, whereas adenylate cyclase is enriched in the sinusoidal subfraction. To measure the degree of contamination of other intracellular membranes, we assessed for some contamination markers: glucose-6-phosphatase (endoplasmic reticulum), acid phosphatase (lysosomes) and succinate dehydrogenase (mitochondria) (Table 1). It is evident that the plasma-membrane fractions were appreciably purified.

Radiolabelling of cell-surface glycoproteins by the $NaIO_4/NaB^3H_4$ method

The plasma-membrane sialoglycoconjugates of liver cells were effectively labelled by the

Table 1. Enzyme activities in plasma-membrane subfractions prepared from rat livers

Plasma-membrane fractions were prepared as described in the Materials and methods section. Enzymic activities, measured as described in the cited references, are expressed as μ mol of product formed/h per mg of protein (means $\pm$ S.D. for the numbers of experiments in parentheses), except for adenylate cyclase, which is expressed as pmol/h per mg of protein. Abbreviation: n.d., not determined.

Enzyme activity	Homogenate	Sinusoidal	Lateral	Canalicular
$Na^+ + K^+$ -dependent ATPase (Sulakhe <i>et al.</i> , 1971)	0.74 ± 0.30 (3)	7.47 ± 1.67 (9)	8.28 ± 1.58 (5)	10.97 ± 4.31 (4)
Mg^{2+} -dependent ATPase (Sulakhe <i>et al.</i> , 1971)	4.75 ± 1.58 (5)	39.27 ± 8.22 (11)	67.65 ± 8.90 (5)	128.43 ± 35.89 (5)
Alkaline phosphatase (Pekarthy <i>et al.</i> , 1972)	0.33 ± 0.08 (3)	1.14 ± 0.14 (6)	2.13 ± 0.57 (4)	6.17 ± 0.25 (4)
5'-Nucleotidase (Ipata, 1968)	2.84 ± 0.03 (3)	23.02 ± 0.41 (4)	34.87 ± 1.82 (3)	64.17 ± 1.97 (3)
Adenylate cyclase (basal) (Wright, 1977)	n.d.	3.24 ± 0.96 (3)	15.18 ± 7.26 (3)	13.68 ± 10.08 (3)
Adenylate cyclase (F^- stimulated) (Wright, 1977)	n.d.	43.44 ± 9.8 (3)	36.18 ± 6.36 (3)	17.94 ± 3.66 (3)
Glucose-6-phosphatase (Swanson, 1955)	4.04 ± 0.58 (3)	4.17 ± 0.29 (4)	1.15 ± 0.02 (3)	1.98 ± 0.04 (4)
Acid phosphatase (Brown <i>et al.</i> , 1976)	6.30 ± 0.42 (3)	8.20 ± 0.92 (5)	9.30 ± 0.42 (3)	8.40 ± 0.60 (4)
Succinate dehydrogenase (Earl & Korner, 1965)	0.93 ± 0.01 (3)	0.28 ± 0.16 (5)	0.96 ± 0.16 (3)	0.56 ± 0.15 (3)

Fig. 1. Fluorograms of surface-labelled glycoproteins from rat liver cells separated by SDS/polyacrylamide-gel electrophoresis (a), (b), (g) and (j) are sinusoidal membranes; (c), (d), (h) and (k) are canalicular membranes; (e), (f), (i) and (l) are lateral membranes. The plasma membranes were treated with NaB^3H_4 alone (b, d, f), with $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ (a, c, e), with galactose oxidase/ NaB^3H_4 (g, h, i), or with neuraminidase-galactose oxidase/ NaB^3H_4 (j, k, l). St, ^{14}C -labelled standard proteins: the numbers indicate their apparent M_r values ($\times 10^{-3}$).

$\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method. NaIO_4 at low concentrations specifically oxidizes sialic acid, and tritiated 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid is the major product formed after reduction with NaB^3H_4 (Gahmberg & Andersson, 1977).

At least 10–12 major glycoproteins were detected by electrophoresis in the three different subfractions of plasma membranes labelled by this procedure (Fig. 1). When the densitometric scans were compared, some glycoprotein bands were observed in all fractions, which indicates that these molecules are homogeneously distributed all along the plasma membrane. Some components were found only in one or two, but not in all, domains. The M_r -250000 and the M_r -90000 components were found in the sinusoidal plasma membrane, that of M_r 170000 was present at the canalicular domain and those of M_r 200000, 180000 and 60000 were enriched in the lateral region (Fig. 2).

Affinity chromatography of cell-surface glycoproteins labelled by the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method

The plasma-membrane subfractions labelled by

Fig. 2. Comparison of sialoglycoproteins from the three functional domains of rat hepatocytes

Densitometric scans of plasma-membrane subfractions of rat liver labelled by the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method and isolated by SDS/polyacrylamide-gel electrophoresis: (a) sinusoidal, (b) canalicular and (c) lateral plasma membranes. Key: *, specific major components of each fraction; GP160 etc., glycoprotein with apparent M_r of 160000 etc.

the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ technique were solubilized in 1% Triton X-100 and subjected to affinity chromatography on Sepharose-bound WGA. The solubilization of the radiolabelled portion with 1% Triton X-100 was different in the three subfractions; in the sinusoidal plasma membrane 80–90% of the ^3H label was solubilized, in the canalicular membranes 60–70%, and in the lateral membranes 30–40%.

As shown in Fig. 3, the sialoglycoconjugates labelled by the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method did not bind effectively to Sepharose–WGA, and the amount of radioactive material bound and recovered from the columns was low. The peaks of radioactivity were pooled, dialysed and freeze-dried. The different samples were then separated by gel electrophoresis in the presence of SDS. The results are shown in Fig. 4.

The glycopolypeptides with apparent M_r values of 250 000, 180 000 and 110 000 were common to the

three different plasma-membrane regions of the hepatocyte, whereas the M_r -150 000 component was found only at the canalicular and lateral surfaces, and the M_r -160 000 and 90 000 proteins were the major components of the sinusoidal front.

Radiolabelling of cell-surface glycoproteins by the galactose oxidase/ NaB^3H_4 method

Plasma-membrane glycoproteins of the hepatocyte, like those of other cells, can be labelled by the galactose oxidase/ NaB^3H_4 method. Resolution of the radiolabelled glycoproteins by SDS/polyacrylamide-gel electrophoresis revealed the presence of most of the glycoproteins labelled by the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method, and only that of M_r 170 000 at the sinusoidal subfraction, that of M_r 230 000 at the canalicular and that of M_r 170 000 at the lateral plasma membranes appeared specifically labelled in these patterns (Figs. 1 and 5). On the other hand, it was observed that the M_r -60 000 component of

Fig. 3. Affinity chromatography of radiolabelled cell-surface glycoproteins on Sepharose-conjugated lectins. Radiolabelled membranes were treated with P_i/NaCl containing 1% Triton X-100, the insoluble material was removed by centrifugation, and the supernatant was applied to columns of Sepharose–RCA, –WGA and –LCA. The samples for RCA or LCA chromatography were labelled by the neuraminidase–galactose oxidase/ NaB^3H_4 method, and that for WGA was labelled by the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method. The columns were eluted with 0.1% Triton X-100 containing 0.1 M-galactose (RCA), α -methyl mannoside (LCA) or *N*-acetylglucosamine (WGA). (a) Sinusoidal, (b) canalicular and (c) lateral plasma membranes. The arrow indicates the addition of competing sugar.

Fig. 4. Polyacrylamide-gel electrophoresis of glycoproteins from the three different membrane subfractions eluted from lectin affinity columns

Proteins from the three functional domains of rat liver radiolabelled by the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method (a, b, c) were chromatographed on Sepharose-WGA columns; galactose oxidase/ NaB^3H_4 -labelled components after treatment with neuraminidase were chromatographed on Sepharose-RCA (d, e, f) or Sepharose-LCA (g, h, i) columns. In all cases the bound material was specifically eluted and separated by polyacrylamide-gel electrophoresis. (a), (d) and (g), sinusoidal membranes; (b), (e) and (h), canalicular membranes; (c) (f) and (i), lateral plasma membranes. St, ^{14}C -labelled standard proteins; the numbers indicate their M_r values ($\times 10^{-3}$).

Table 2. Lectin binding of plasma-membrane glycoproteins

Isolated plasma membranes were solubilized in 1% Triton X-100 and subjected to affinity chromatography. The bound material was eluted from the columns and treated as described in the Materials and methods section. The values presented are the estimated M_r values of the major components revealed by fluorography ($\times 10^{-3}$), determined from calculation on two to four slab gels.

$10^{-3} \times$ Apparent M_r	Sinusoidal membrane			Canalicular membrane			Lateral membrane		
	WGA	RCA	LCA	WGA	RCA	LCA	WGA	RCA	LCA
250	+	+		+			+		
230					+				
200					+				
180	+			+	+		+		
170			+			+			+
160	+	+							
150				+	+		+		
130						+			
110	+	+	+	+	+	+	+	+	+
90	+	+	+			+			
80		+	+		+	+		+	+
70					+				
65									
60									
58		+							
55					+			+	+
50									
48		+			+			+	+
42									
40									
32									

* Minor component.

Fig. 5. Comparison of galactoproteins from the three functional domains of the rat hepatocyte

Densitometric scans of the plasma membrane subfractions from rat liver labelled by the galactose oxidase/ NaB^3H_4 method and isolated by SDS/polyacrylamide gel electrophoresis: (a) sinusoidal, (b) canalicular and (c) lateral plasma membranes. Key: *, specific major components in each region; OR, origin; BF, buffer front.

the lateral plasma-membrane subfraction was not labelled by this method.

Some components were relatively more intensively labelled by galactose oxidase/ NaB^3H_4 than with $\text{NaIO}_4/\text{NaB}^3\text{H}_4$. This was true for the M_r -110000 and -90000 components of the sinusoidal and the M_r -70000, -65000, -60000, -50000, -48000 and -42000 components of the canalicular plasma membranes. This indicates that these glycoproteins contain terminal galactosyl and/or *N*-acetyl-galactosaminyl residues. In order to expose penultimate galactosyl/*N*-acetyl-galactosaminyl residues, we treated the isolated plasma membranes with neuraminidase and galactose oxidase/ NaB^3H_4 . The labelled membranes were then separated by gel electrophoresis (Fig. 1).

The removal of sialic acid by treatment of the plasma-membrane fractions with neuraminidase yielded glycoproteins exhibiting M_r values somewhat different from those labelled by $\text{NaIO}_4/\text{NaB}^3\text{H}_4$. These shifts in apparent M_r must be due to loss of sialic acid, which accentuates the anomalous electrophoretic behaviour of glycoproteins. As demonstrated previously, the electrophoretic mobility depends on the charge as well as the mass of the glycoproteins (Gahmberg & Andersson, 1982). The results in Fig. 1 show that, whereas in the sinusoidal and the canalicular plasma membranes there was clearly an increased labelling, this was not seen in the lateral plasma membranes.

Affinity chromatography of cell-surface glycoproteins labelled by neuraminidase-galactose oxidase/ NaB^3H_4 treatments

The three different plasma-membrane subfractions were solubilized in 1% Triton X-100, and the soluble components subjected to affinity chromatography on Sepharose-bound RCA or LCA lectins. The bound and eluted components (Fig. 3) were studied by gel electrophoresis in the presence of SDS (Fig. 4).

The RCA used in these experiments was a mixture of RCA_I and RCA_{II} lectins, having affinity for D-galactose and *N*-acetylgalactosamine. In Figs. 3 and 4 it was demonstrated that the Sepharose-RCA columns efficiently bound radiolabelled glycoproteins.

The LCA lectin has affinity for α -D-mannose and α -D-glucopyranoside residues. Most of the radiolabelled glycoproteins did not bind to Sepharose-LCA columns (Figs. 3 and 4). The results of the affinity chromatographies are summarized in Table 2.

We conclude: (a) that several cell-surface glycoproteins of the hepatocyte contain sialic acid, but also terminal galactose/*N*-acetylgalactosamine, (b) that the sialoglycoproteins labelled by the $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ method bind poorly to WGA, and (c) the most prominent component is that of M_r 110000 in the sinusoidal plasma membranes, and that of M_r 90000 is only present at the sinusoidal aspect.

The M_r -110000 component is possibly the dipeptidyl aminopeptidase IV isolated and characterized by Kötting *et al.* (1978). This has similar properties to our M_r -110000 glycoprotein. It is a major component and binds efficiently to WGA. Furthermore it seems to be homogeneously distributed all over the hepatocyte-surface.

The M_r -90000 component could be the transferrin-receptor subunit, a prominent cell-surface glycoprotein located in sinusoidal membranes (Van Agthoven *et al.*, 1984). Another possibility is

Fig. 6. Comparison of glycoproteins from the sinusoidal and canalicular domains separated by affinity chromatography on Sepharose-LCA and polyacrylamide-gel electrophoresis

Densitometric scans of sinusoidal (M-L) and canalicular (N-L) plasma-membrane glycoproteins labelled by the neuraminidase-galactose oxidase/ NaB^3H_4 method, eluted from Sepharose-LCA columns and separated by polyacrylamide-gel electrophoresis. Key: GP110, glycoprotein with apparent M_r of 110000; GP90, glycoprotein with apparent M_r of 90000; OR, origin; BF, buffer front.

that it is a structural component of coated pits (Steer & Klausner, 1983; Murphy *et al.*, 1983), which also mainly are located at the sinusoidal surfaces. When neuraminidase-galactose oxidase/ NaB^3H_4 -labelled membrane proteins were subjected to affinity chromatography on LCA, it was seen that, whereas in the sinusoidal subfraction the most prominent band was that of M_r 90000, in the canalicular membranes there was a change in the efficiency of binding to LCA (Fig. 6). This could mean that the carbohydrate structure of this glycoprotein is different in the canalicular and the sinusoidal membranes. It has been shown that the carbohydrate of specific polypeptides changes during cellular and membrane differentiation (Atienza-Samols *et al.*, 1980; Gahmberg *et al.*, 1984).

This study was supported by National Cancer Institute grant no. 2 R01CA26294-04 (to C. G. G) and by the Comissió Interdepartamental de Recerca i Innovació Tecnològica grant no. EE83/1-24. C. E. is grateful to the Finnish Ministry of Education for support.

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