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ISOLATION OF SURFACE LECTINS OF GH_3 CELLS FROM WHOLE CELLS AND ISOLATED PLASMA MEMBRANES *

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Lectins localized in the plasma membranes seem to be of special importance for the intercellular interaction mechanisms. We describe the isolation of mannose-binding proteins by Triton X-100 extraction and affinity chromatography on agarose-bound mannose. The isolation procedure was performed with whole GH_3 cells as well as with isolated plasma membranes. For the isolation of plasma membranes of GH_3 cells a mechanical pump was used for the disruption. After differential centrifugation an enriched plasma membrane fraction was achieved by discontinuous sucrose gradient centrifugation. The whole fractionation procedure was controlled by total balance sheets for the marker enzymes of the different cell organelles. The plasma membrane fraction was further characterized by gel electrophoresis and electron microscopy. The SDS gel electrophoresis patterns of the proteins, resulting from the Triton X-100 extraction and the affinity chromatography, are nearly identical for whole cells as well as for the enriched plasma membrane fraction. Therefore we presume these mannose-specific proteins to be plasma membrane bound, showing the molecular properties of integral proteins and having a molecular weight of M_r 67 000, 57 000, 47 000.

Introduction

The GH_3-cell line, derived from a pituitary tumor of a female Wistar-Furth rat, displays differentiated functions such as growth hormone and prolactin secretion [1]. Epidermal growth factor (EGF) is able to induce the expression of specific genes in absence of a proliferative response [2] and changes the phenotype. The serum-free, hormone-supplemented culture conditions, established by Hayashi and Sato [3], for GH_3 cells render possible the studies of the mechanism of hormonal interactions with the plasma membrane which stimulate cell proliferation.

This cell line can also be cultured in a monolayer-like manner as well as in suspension. In both cases the cells grow in aggregates of a few cells which are in close contact. Such cell-cell interactions are believed to be mediated by surface carbohydrates and surface lectins [4] which were found in pro- and eucaryotic cells. One of the first surface lectins was isolated from the plasma membrane of liver cells by Ashwell and Morell [5] and Kawasaki and Ashwell [6]. Beside cell-cell recognition surface lectins are presumably involved in cell adhesion processes.

In the present study we describe the isolation of mannose-binding proteins from GH_3 cells using agarose-bound mannose. These proteins were isolated from Triton X-100 extracts of whole cells as well as of the isolated plasma membrane fraction. The isolation of plasma membranes is performed by differen-

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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tial centrifugation after cell disruption with a mechanical pump. Biochemical markers and marker enzymes were used to characterize the different subcellular fractions and the plasma membrane.

Materials and Methods

Cell culture. GH_3 cells (American Type Culture Collection) were grown in a 1 : 1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Boehringer, Mannheim, F.R.G.) and Ham's nutrient mixture F12 (Boehringer), supplemented with 15% horse serum (Boehringer) and 3% newborn calf serum (Gibco, Nunc, Wiesbaden, F.R.G.). 5 μg/ml tylosin (Serva, Heidelberg, F.R.G.) 2 μg/ml amphotericin (Sigma, Munich, F.R.G.), 25 μg/ml ampicillin (Serva), 50 μg/ml streptomycin (Calbiochem, Giessen, F.R.G.) and 40 U/ml Penicillin G (Calbiochem) were added to the culture medium. Cells were grown in suspension in spinner bottles (Bellco, Tecnomara, Hüttenberg, F.R.G.) or as monolayers in flasks (Lux, 75 cm^2, Seromed, Munich, F.R.G.) at 37°C and 95% humidity. For the described experiments exponentially growing cells of the passages 30 to 35 and 50 to 60 were used. Cells were harvested by centrifugation at 900 $g_{av} \cdot$ min at 4°C. The cells were washed twice in cold (4°C) phosphate buffered saline.

Cell disruption and cell fractionation. For cell disruption GH_3 cells were resuspended in the fractionation buffer (A5) (0.15 M NaCl, 4.1 mM KCl, 0.5 mM $MgCl_2$, 10 mM Hepes, 3 mM sucrose, pH 7.2), adjusted to a concentration of $(2–8) \cdot 10^6$ cells/ml. The cell disruption was performed by an air-driven mechanical pump [7] (Stansted Cell Disrupter, Stansted Fluid Power Ltd., Stansted, Essex, U.K.), back pressure 20 lb/$inch^2$, flow pressure 30 lb/$inch^2$, valve 516 at 4°C.

The fractionation was performed as shown in Fig. 1. All isolation steps were performed at 4°C. The homogenate (HOM) was pelleted at $4.5 \cdot 10^3$ $g_{av} \cdot$ min, resulting in the pellet 17 P (nuclear fraction) and the supernatant 17 U. The latter was centrifuged at $1.05 \cdot 10^5$ $g_{av} \cdot$ min to sediment the large granule fraction (95 P). The supernatant (95 U) was further processed by centrifugation at $4.5 \cdot 10^5$ $g_{av} \cdot$ min, yielding the 'crude membrane fraction' (14 P) and the supernatant 14 U. From this supernatant the ribosomal fraction was sedimented at $1.62 \cdot 10^7$ $g_{av} \cdot$ min

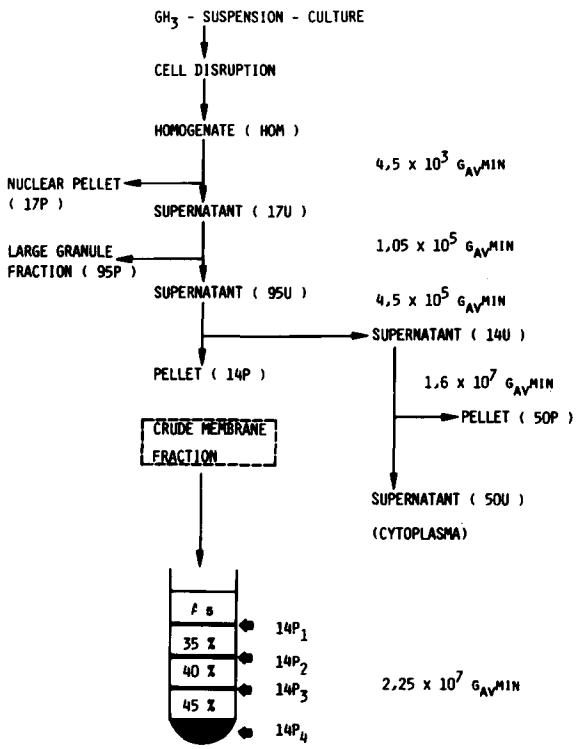

Fig. 1. Flow diagram for the plasma membrane preparation.

(50 P). The supernatant (50 U) from this centrifugation step contains the soluble cytoplasmic components.

Membrane fraction isolation. For further purification of the fraction 14 P (crude membrane fraction) a discontinuous sucrose gradient in A5-buffer was used with the following three density steps: 1.2025 g/cm^3 (3 ml), 1.1764 g/cm^3 (2 ml), 1.1513 g/cm^3 (5 ml). Before adjusting the density of the third step of the gradient the crude membrane fraction was added. A5-buffer was laid over this discontinuous gradient which then was centrifuged at $2.25 \cdot 10^7$ $g_{av} \cdot$ min. The centrifugation resulted in three bands on the density interphases and one pellet. The four fractions in which the crude membranes separate were designed according to the density behaviour 14 P_1, 14 P_2, 14 P_3 and 14 P_4. The single bands were collected and the sucrose was removed by washing in A5-buffer at $1.4 \cdot 10^7$ $g_{av} \cdot$ min. The material was used immediately for further biochemical characterization.

Isolation of surface lectins. This procedure was

performed on whole GH_3 cells, after washing in phosphate-buffered saline as described above, or on the fraction $14\,P_1$ of the cell fractionating procedure. The cells were resuspended ($(1-3)\cdot 10^7$ cells/ml) after determination of cell viability (94–97%) by trypan blue exclusion in isolation buffer (IPT_1) (0.14 M NaCl, 0.02 M NaH_2PO_4, 0.02% NaN_3, 1% Triton X-100, pH 7.2). Using the membrane fraction $14\,P_1$ the protein concentration was adjusted to 200 μg/ml. After incubation for 1 h at 4°C the extract was sonicated 5 s and centrifuged ($1\cdot 10^6$ $g_{av}\cdot$ min). The supernatant containing the solubilized proteins was used immediately thereafter or stored at 4°C for three days.

The affinity chromatographic isolation of the mannose-binding proteins was performed by agarose-bound D-mannose (Selectin 10, Pierce Chemical Company, Karl OHG, Geisenheim, F.R.G.). After equilibration with IPT_2 buffer (IPT-buffer with only 0.1% Triton X-100) at 15°C, 1–5 ml of the Triton X-100 extract were pumped through the agarose-bound mannose column (1.5 ml/h). The efflux was monitored at 280 nm, using Uvicord S (LKB, Bromma, Sweden) and collected in fractions of 1 ml. After an extensive washing step the bound proteins were eluted with D-mannose (0.5 M) in IPT_2-buffer at 24°C. To check the specificity of the elution *N*-acetylgalactosamine (0.5 M) was used in control experiments. Fractions containing the sugar-binding proteins were pooled and precipitated with ice-cold acetone. Triton X-100 was removed by repeated chloroform-extraction as described [8]. For SDS-polyacrylamide gel electrophoresis the precipitates were resuspended in SDS-sample buffer (20 mM Tris-HCl, 20 mM EDTA, 4.3% SDS, 5% 2-mercaptoethanol, 10% sucrose, pH 8.2 and pyronine G as tracking dye). Electrophoresis was performed according to Laemmli [9] using a gel gradient from 4 to 30% acrylamide.

Enzymatic determinations. The activity of alkaline nitrophenyl phosphatase (EC 3.1.3.1) was determined as described by Ey and Ferber [10]. Alkaline phosphodiesterase I (EC 3.1.4.1) was assayed as described by Brown et al. [11]. The activity of β-*N*-acetylglucosaminidase (EC 3.2.1.30) was determined according to the procedure described by Kornfeld and Siemers [12], the activity of acid phosphatase (EC 3.1.3.2) was determined according to Fishman and Lerner [13]. Succinate-tetrazolium oxidoreductase (EC 1.3.99.1) was assayed according to Pennington [14]. The activity of lactate dehydrogenase (EC 1.1.1.27) was assayed as described in Ref. 15 and glucose-6-phosphatase (EC 3.1.3.9) as described in Ref. 16.

Chemical determinations. Protein was determined by the ninhydrin method according to Kabat and Mayer [17] using bovine serum albumin as standard. DNA and RNA were assayed according to Munro and

TABLE I

CHEMICAL ANALYSIS OF FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION

n.d., not determined.

Fraction	Protein		DNA		RNA	
	mg/ml	mg total	mg/mg protein	mg total	mg/mg protein	mg total
HOM	3.5	446.8	0.025	11.4	0.025	11.4
17U	3.0	377.2	0.01	4.1	0.024	9.0
17P	5.6	44.7	0.09	4.1	0.034	1.5
95U	2.8	336.4	0.1	3.9	0.02	6.7
95P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14U	2.7	316.7	0.004	1.4	0.015	4.7
50U	2.4	266.1	0.005	1.5	0.001	0.2
50P	3.7	11.1	0.008	0.09	0.26	2.9

TABLE II

ENZYMATIC ANALYSIS OF FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION

Specific activities are given in nmol/min per mg protein; total activities in μmol/min per mg protein, except for lactate dehydrogenase, for which specific activities and total activities are given in μmol/min per mg protein and mmol/min per mg protein, respectively. n.d., not determined; ∅ not measurable activities.

Fraction	Alkaline phosphatase		Alkaline phosphodiesterase		β-Glucosaminidase		Acid phosphatase		Succinate-tetrazolium oxidoreductase		Lactate dehydrogenase		Glucose-6-phosphatase	
	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.
HOM	15.0	6.7	14.2	6.3	12.1	5.4	13.0	5.8	6.6	2.9	4.9	2.2	1.11	500.2
17U	13.3	5.0	13.4	5.0	12.9	4.8	13.9	5.2	4.7	1.8	4.5	1.7	0.78	295.79
17P	21.3	0.9	22.2	0.9	19.9	0.9	13.7	0.6	14.5	0.6	1.1	0.05	1.07	48.09
95U	4.2	1.4	3.7	1.2	0.6	0.2	9.4	3.2	∅	∅	5.5	1.8	0.83	281.41
95P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14U	2.0	0.6	1.6	0.5	∅	∅	8.6	2.7	∅	∅	5.3	1.7	0.70	223.17
50U	1.3	0.3	0.9	0.2	∅	∅	8.2	2.2	∅	∅	5.8	1.5	0.74	198.4
50P	13.7	0.1	13.6	0.1	∅	∅	3.3	0.04	0.14	∅	3.9	∅	0.59	6.67

TABLE III

ENZYMATIC ANALYSIS OF FRACTIONS OBTAINED BY PURIFICATION OF THE CRUDE MEMBRANE FRACTION (14P)

For specifications see Table II.

Fraction	Alkaline phosphatase		Alkaline phosphodiesterase		β-Glucosaminidase		Acid phosphatase		Succinate-tetrazolium oxidoreductase		Lactate dehydrogenase		Glucose-6-phosphatase	
	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.	Total act.
$14P_1$	135.9	0.1	150.9	0.1	57.8	0.05	57.8	0.05	∅	∅	0.3	∅	1.12	1.02
$14P_2$	57.7	0.06	48.7	0.06	30.4	0.03	20.9	0.02	1.7	∅	0.1	∅	0.30	0.36
$14P_3$	54.2	0.03	43.8	0.03	15.1	∅	12.1	∅	19.5	0.01	0.2	∅	7.07	4.44
$14P_4$	32.0	0.02	23.8	0.01	15.6	∅	9.2	∅	∅	∅	0.2	∅	∅	∅

Fleck [18]. The cholesterol and phospholipids were determined according to Ref. 19.

Electron microscopy. Electron microscopy was performed as described in Ref. 19.

Results

Surface lectins were isolated by affinity chromatography on agarose-bound mannose from whole cells as well as from isolated plasma membranes.

Cell fractionation and plasma membrane purification

Electron microscopy, SDS-polyacrylamide gel electrophoresis and chemical and enzymatic markers were used for characterization of the different fractions resulting from the isolation procedure (Fig. 1). Total balance sheets of the complete fractionation procedure were performed for protein, DNA, RNA (Table I) and the following marker enzymes (Table II): alkaline nitrophenyl phosphatase and alkaline phosphodiesterase for the plasma membrane, acid phosphatase and β-glucosaminidase for lysosomal material, succinate-tetrazolium oxidoreductase for mitochondria, glucose-6-phosphatase for endoplasmic reticulum and nuclear membranes and lactate dehydrogenase for cytoplasma.

The crude membranes (14 P), further separated by discontinuous sucrose gradient centrifugation, resulted in one fraction ($14\,P_1$) which shows the highest enrichment of all the plasma membrane markers (Tables III and IV). Electron micrographs demonstrate that this fraction consists of membrane vesicles (Fig. 2a). Ribosomes can be observed in fraction $14\,P_2$ plus $14\,P_3$, indicating that this material is derived from the endoplasmic reticulum. This correlates well with the protein patterns of the SDS-polyacrylamide gel electrophoresis which shows a large amount of low molecular weight-proteins in the fractions $14\,P_3$ and $14\,P_4$ which characterizes the presence of ribosomes (Fig. 3). This is in agreement with the RNA determination (Table IV). The highest content of cholesterol, another plasma membrane marker (Table IV), appears also in $14\,P_1$. From these data one can conclude that the fraction $14\,P_1$ represents a plasma membrane fraction. This fraction was used for the isolation of the surface lectins.

Isolation of the surface lectins

After extracting the proteins of whole cells or of the plasma membrane fraction with Triton X-100, and isolation of mannose-binding proteins by affinity chromatography as shown in Fig. 4, 0.05–0.2 mg protein is obtained. The recovery varied from 40 to 96%, depending on the flow parameter during the application and on the elution of the sample. After removal of Triton X-100 by repeated chloroform extraction a concentration by acetone precipitation is performed. It cannot be excluded that such a treatment induces irreversible aggregation of the isolated proteins. The protein pattern of SDS-polyacrylamide gel electrophoresis shows three main bands (Fig. 3) M_r: 67 000; 57 000; 47 000. Whole cells as well as isolated plasma membranes result in the same protein pattern, except for one protein which appears only in extracts from purified membranes (Fig. 3). Using Sepharose CL-2B as control no detectable unspecific

TABLE IV

CHEMICAL ANALYSIS OF FRACTIONS OBTAINED BY PURIFICATION OF THE CRUDE MEMBRANE FRACTION (14P)

Fraction	Protein		DNA		RNA		Cholesterol	
	mg/ml	mg total	mg/ml protein	mg total	mg/mg protein	mg total	*mol · mg^{-1} *nmol	protein *μmol
$14P_1$	1.8	0.9	0.019	0.01	0.08	0.07	358.8	0.33
$14P_2$	2.3	1.1	0.029	0.03	0.17	0.2	189.9	0.20
$14P_3$	1.2	0.6	0.062	0.004	0.21	0.13	256.3	0.15
$14P_4$	1.2	0.6	0.134	0.08	0.09	0.06	n.d.	n.d.

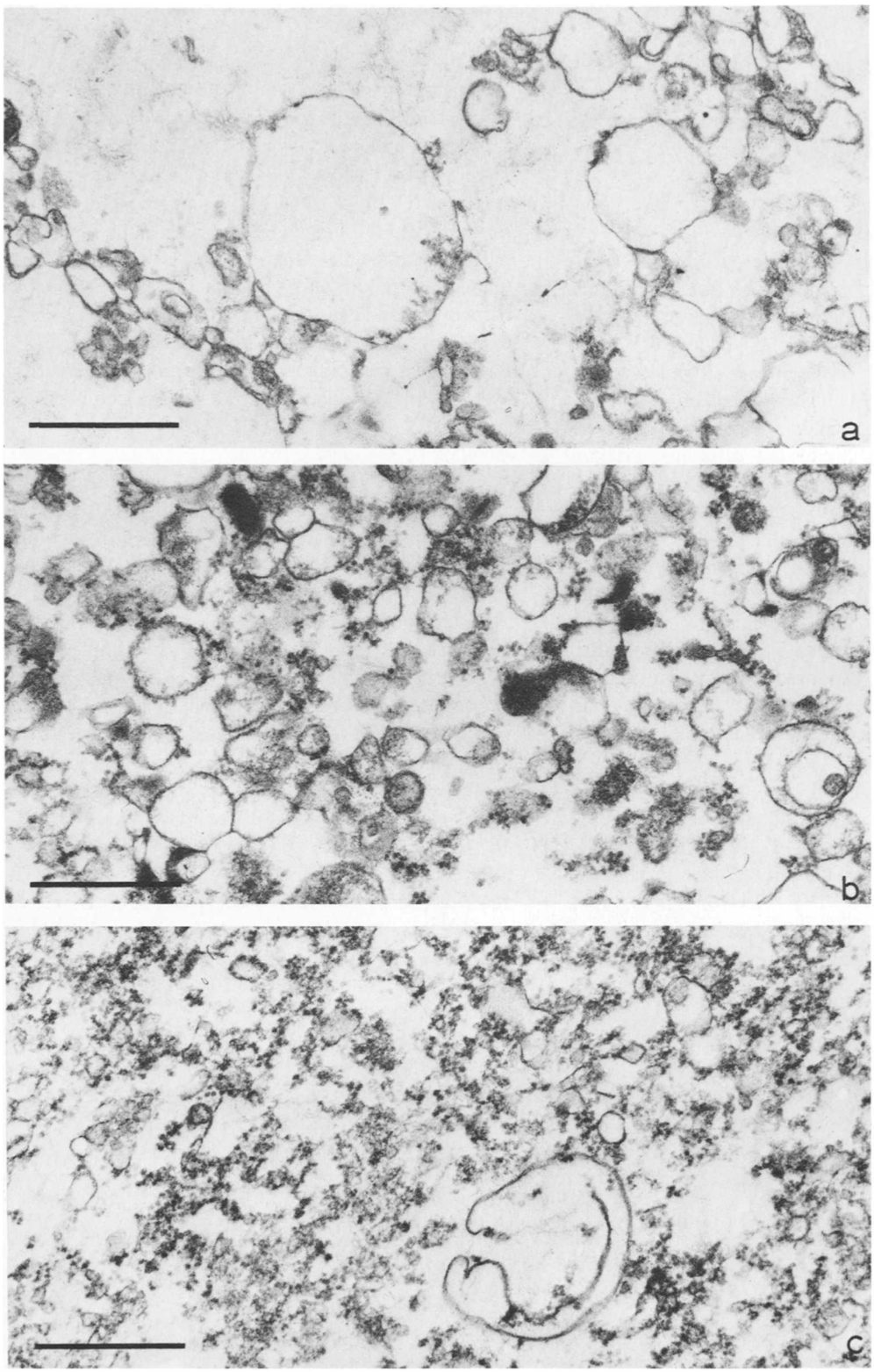

Fig. 2. Electron micrographs of sections of the different fractions of the crude membrane purification. (a) Fraction 14 P_1, representing the plasma membrane enriched fraction; (b) pooled fractions 14 P_2 and 14 P_3, representing mainly membranes of intracellular organelles; (c) fraction 14 P_4, consisting mainly of ribosomes.

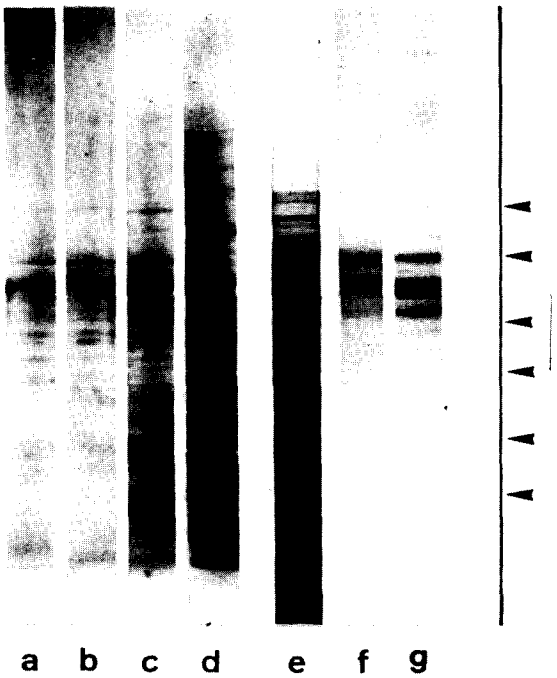

Fig. 3. SDS polyacrylamide gel electrophoresis (linear gradient 4–30%). (a–d) Fractions obtained by the purification of the crude membranes: (a) 14 P_1, (b) 14 P_2, (c) 14 P_3, (d) 14 P_4. (e) Pattern of proteins of the Triton X-100 extract of whole cells not retained by chromatography over the mannose agarose column. (f) Mannose-binding proteins isolated from Triton X-100 extract of whole cells and (g) of purified plasma membranes. Arrows indicate the position of the molecular weight markers (from top to bottom) M_r: 94 000 (phosphorylase *b*), 67 000 (bovine serum albumin), 43 000 (ovalbumin), 30 000 (carbonic anhydrase), 20 100 (soybean trypsin inhibition), 14 400 (α-lactalbumin).

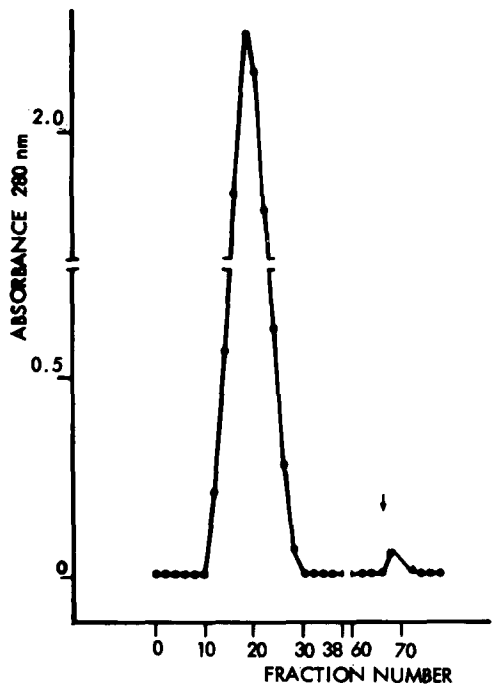

Fig. 4. Affinity chromatography of the Triton X-100 extract of GH_3 cells on agarose-bound mannose. Arrow gives the starting point of the specific elution with 0.5 M mannose.

binding of these proteins occurs. The specificity of the binding behaviour of these mannose-binding proteins was examined with the affinity chromatography. Compared to the unfractionated Triton X-100 extract these isolated proteins show a high tendency to form aggregates in aqueous buffers and a lower solubility at room temperature even in the presence of Triton X-100. Using the cytoplasmic fraction (50 U) for affinity chromatography on agarose-bound mannose only small amounts of protein are bound.

Discussion

Although GH_3 cells have acquired more and more the position of a model cell within the last few years, no isolation and purification of plasma membranes has been described until now. Prolactin [21] and growth hormone genes [22] were isolated from GH_3 cells and the secretion of these hormones can be regulated by adding other hormones [23]. The hormone receptors and interactions of these hormones were studied extensively. Furthermore, the surface charges on the plasma membrane are heterogeneous and are distributed in mosaic-like patterns [24].

Spreading phenomena of GH_3 cells can be induced specifically by succinylated concanavalin A [25]. Concanavalin A is a plant-derived lectin, binding the mannose residues of the glycomolecules of the cell membrane. Assuming that this succinylated concanavalin A-induced spreading imitates the cell-cell interactions of the GH_3 cells, mediated by mannose-binding proteins, we were looking for molecules with the same binding specificity in the GH_3 plasma membrane.

Such molecules were isolated by Triton X-100 extraction of whole cells and affinity chromatography on agarose-bound mannose. The SDS gel electrophoresis demonstrates that at least three proteins exist, showing mannose-binding properties. Their specificity was examined by chromatography in the presence of mannose which inhibits the binding of these proteins to mannose-modified agarose. Furthermore, only trace amounts of proteins will be released from the affinity column when *N*-acetylgalactosamine is used as eluent. Applying the cytoplasmic fraction of GH_3 cells to the affinity column nearly no protein was bound. This finding, and the fact that the proteins need detergents for solubilisation, indicate that the mannose-binding proteins must be membrane-bound, or are localized in intracellular membrane vesicles.

In order to localize the mannose-binding proteins we have isolated an enriched plasma membrane fraction. All the markers used for characterization of the plasma membrane are enriched in the 14 P_1 fraction: the cholesterol content, alkaline phosphatase, and alkaline phosphodiesterase. In the case of GH_3 cells these two enzymes are obviously located in the plasma membrane (Wieser, R., Golecki, J.R., Ferber, E. and Brunner, G. submitted). The protein patterns of the SDS gel electrophoresis demonstrate that this fraction seems to lack the ribosomal low molecular weight proteins. Furthermore, electron microscopy shows that this fraction consists of empty vesicles.

Using this plasma membrane fraction for Triton X-100 extraction and affinity chromatography on mannose-bound agarose, a protein pattern nearly identical to the Triton X-100 extract of whole cells is obtained (after SDS gel electrophoresis of the proteins bound to the mannose-modified agarose). These results lead to the conclusion that the mannose-binding proteins isolated from GH_3 cells are located membrane-bound mainly in the plasma membrane.

Preliminary experimental results show that mannose-bearing proteins can also be isolated from the Triton X-100 extract of GH_3 cells, at least seven mannose-bearing proteins can be found. When both types of these proteins, mannose-bearing and mannose-binding, are used as attachment factors in serum-free, hormone-supplemented cell culture, an alteration of the proliferation response of GH_3 cells is observed [26,27]. Therefore, it can be assumed that intercellular-regulated interactions, e.g. growth control or tissue homeostasis, are based on such sugar-specific molecular 'couples': the surface lectin and the corresponding glycomolecule of the opposite cell.

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