Chemical characterization of cell-CAM 105, a cell-adhesion molecule isolated from rat liver membranes

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Cell-CAM 105, a glycoprotein that is involved in recognition and adhesion between isolated rat hepatocytes *in vitro*, was purified to homogeneity by a combination of immunoaffinity chromatography, gel-exclusion chromatography and ion-exchange chromatography. Electrophoretic, compositional and enzymic analyses were performed and the glycoprotein was shown to consist of two peptide chains, of apparent M_r 110000 and 105000 respectively, that are glycosylated to similar extents. Carbohydrate analyses demonstrated the presence of sialic acid, galactose, mannose, fucose and glucosamine, but no galactosamine, indicating that only *N*-linked oligosaccharides occurred. The total content of carbohydrate amounted to 33%. Peptide mapping indicated that the two peptide chains were structurally very similar. After incubation of cultured hepatocytes with [32 P] P_i , phosphorylated cell-CAM 105 could be isolated. Both peptide chains were labelled and phospho-amino-acid analysis demonstrated that serine residues had become phosphorylated. A significant feature of cell-CAM 105 was a susceptibility to autolytic degradation that was difficult to inhibit. The major degradation products had apparent M_r 90000 and 70000, respectively. The effect of purified cell-CAM 105 on cell-cell adhesion of re-aggregating hepatocytes was studied. A significant inhibition was observed, indicating that the protein is directly involved in intercellular adhesion of these cells.

INTRODUCTION

The importance of cell recognition and adhesion for a variety of cellular phenomena of developmental biology, physiology and pathology is well documented. Although much knowledge about these phenomena has been collected over the years, the molecular mechanisms of cell adhesion are still not well understood. By employing immunological techniques, several laboratories have recently been able to identify cell-surface molecules that are involved in various types of cellular adhesion reactions (Damsky *et al.*, 1984; Edelman, 1983; Öbrink, 1986). These molecules have been denoted 'cell adhesion molecules' (CAM) and their isolation has made possible studies of cell-adhesion phenomena at the molecular level. An important part of such investigations is a detailed biochemical characterization of the CAMs.

One such CAM is cell-CAM 105, which has been identified in our laboratory (Ocklind & Öbrink, 1982). Cell-CAM 105 is involved in the cell-cell adhesion *in vitro* of re-aggregating hepatocytes isolated from young adult rats. It is an integral cell-surface membrane glycoprotein with an apparent M_r of 105000 and is found, in addition to the liver, also in some simple epithelia (Ocklind *et al.*, 1983). A major reason for choosing adult hepatocytes for these studies has been to examine if specific recognition mechanisms occur in terminally differentiated cells as compared with embryonic cells. That this might be the case has been demonstrated in recent investigations where we have observed changes of the expression of cell-CAM

both in developing foetal liver and regenerating liver (Odin & Öbrink, 1986), and in transplantable hepatocellular carcinomas (Hixson *et al.*, 1985). In the foetal liver, cell-CAM 105 does not appear before day 16 of gestational age and at birth it has only reached a third of the amount present in the adult liver. In the regenerating liver there was a transient decrease in the surface-bound cell-CAM 105 that correlated in time with the cell division and restoration phases. The transplantable hepatocellular carcinomas exhibited an altered expression of this protein, which was manifest as an apparent loss of the protein from the surface of the tumour cells.

In the present paper we report on the purification and chemical characterization of cell-CAM 105.

MATERIALS AND METHODS

Purification of cell-CAM 105

Cell-CAM 105 was purified from rat liver plasma membranes (200–400 mg of membrane protein in each preparation) that were isolated by zonal centrifugation in a Beckman Ti-15 zonal rotor and washed with Ca-medium and EGTA-medium as previously described (Ocklind & Öbrink, 1982). The membranes were solubilized (5 mg of membrane protein/ml) in 1% Triton X-100 in 0.15 M-NaCl/0.01 M-Tris/HCl (pH 7.4)/2 mM-PMSF/Trasylol (1000 k.i.u.) for 30 min at 4 °C. The solubilized membranes were centrifuged at 100000 *g* for 60 min at 4 °C and the supernatant was recirculated on an immuno-

Abbreviations used: cell-CAM, cell-cell adhesion molecule; DTT, dithiothreitol; f.p.l.c., fast protein, peptide and polynucleotide liquid chromatography; k.i.u., kallikrein-inhibitory unit; NP-40, Nonidet P40; PBS, phosphate-buffered saline (for composition, see the text); PMSF, phenylmethanesulphonyl fluoride.

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affinity column overnight in the cold. Immunoaffinity columns were prepared by coupling 200 mg of the IgG fraction of anti-(cell-CAM₆) antiserum [or a mixture of 100 mg of IgG from anti-(cell-CAM₇) and 100 mg of IgG from anti-(cell-CAM₈)] to 40 ml each of CNBr-activated Sepharose 4B (Pharmacia) as recommended by the manufacturer. The specificities of anti-(cell-CAM₇) and anti-(cell-CAM₈) antisera are described in the Results section and in Figs. 4(b) and 4(c) below. Cell-CAM 105 of equal purity was obtained from either column. The column was washed with 100 ml of 0.5 M-NaCl/0.01 M-Tris/HCl (pH 7.4)/1 mM-EDTA/1% Triton X-100, followed by 150 ml of 0.5 M-NaCl/0.01 M-Tris/HCl (pH 7.4)/1 mM-EDTA/0.1% Triton X-100 and was then eluted with 0.05 M-diethylamine/0.15 M-NaCl/1 mM-EDTA/0.1% Triton X-100, pH 11.5. The eluted material was neutralized immediately with 1 M-Tris/HCl, pH 7.0 and concentrated approx. 20 times by pressure dialysis on a PM 10 Amicon filter (Amicon Corp.). The concentrated material was then chromatographed on two Superose 12 gel-exclusion columns (Pharmacia) in tandem. The Superose columns were connected to a Pharmacia f.p.l.c. system and were equilibrated and eluted with 0.07 M-NaCl/0.01 M-Bistris (pH 6.8)/0.1% Triton X-100. The fractions containing cell-CAM 105 were pooled and directly applied to a Mono Q column (Pharmacia) connected to the same f.p.l.c. system. The Mono Q column was equilibrated with 0.07 M-NaCl/0.01 M-Bistris (pH 6.8)/0.1% Triton X-100 and was developed by a NaCl gradient or by stepwise elution with increasing concentrations of NaCl as demonstrated in Fig. 3 (below). All NaCl solutions contained 0.01 M-Bistris, pH 6.8 and 0.1% Triton X-100. The fractions containing cell-CAM 105 were pooled and either used immediately or stored at -70 °C.

Gel electrophoresis and immunoblotting

Analytical one-dimensional SDS/polyacrylamide-gel electrophoresis and immunoblotting analysis were performed as previously described (Ocklind *et al.*, 1984). Two-dimensional electrophoresis was done as described by O'Farrell (1975). Preparative SDS/polyacrylamide-gel electrophoresis was carried out on 3 mm-thick 7%-(w/v)-polyacrylamide gels in the buffer system described by Blobel & Dobberstein (1975). The gels were developed by silver staining (Morrissey, 1981) or staining with Coomassie Brilliant Blue (Weber & Osborn, 1975) or by soaking in 4 M-sodium acetate (Gerton *et al.*, 1982; Higgins & Dahmus, 1979). The band corresponding to cell-CAM 105 was cut out, the gel slice was equilibrated for 2 × 20 min in electrode buffer (0.048 M-glycine/6 mM-Tris/HCl/0.1% SDS, pH 8.3) and electro-eluted in this buffer at 400 V for 4 h and 200 V for 18 h in an apparatus similar to that described by Gerton *et al.* (1982). Equilibration of the gel slices in electrode buffer before electroelution was important for obtaining good elution of the protein.

Labelling with ¹²⁵I

Purified cell-CAM in 0.15 M-NaCl/0.01 M-Tris/HCl/1% octyl glucoside, pH 7.5, was labelled with ¹²⁵I (carrier-free; The Radiochemical Centre, Amersham, Bucks., U.K.) by Iodo-Beads (Pierce Chemical Co.) as described by the manufacturer. Detergent change from Triton X-100 to octyl glucoside was accomplished by adsorbing the cell-CAM 105 to a column of wheat-germ agglu-

tinin (The Separation Central, Biomedical Center, Uppsala, Sweden) coupled to Sepharose 4B and washing the column with octyl glucoside-containing solution before eluting the protein with *N*-acetylglucosamine. When to be used in the radioimmunoassay the iodinated cell-CAM 105 was purified on an immunoaffinity column of anti-(cell-CAM) antibodies coupled to Sepharose 4B. The bound protein was eluted with 0.05 M-diethylamine (pH 11.5)/0.15 M-NaCl/1 mM-EDTA/0.1% Triton X-100.

The iodination of cells was accomplished as follows. Rat hepatocytes were isolated by collagenase perfusion (Öbrink, 1982), seeded in collagen-coated (Öbrink, 1982) 60 mm dishes (3 × 10⁶ cells per dish) and cultured for 12–20 h at 37 °C in RPMI medium (Department of Microbiology, University of Uppsala, Uppsala, Sweden) supplemented with 10% (v/v) calf serum. The cells were then washed with PBS (0.14 M-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.4) and were labelled with ¹²⁵I (0.5 mCi/dish) by the lactoperoxidase technique (Hubbard & Cohn, 1972). The dishes were washed with PBS and the cells were solubilized in 0.15 M-NaCl/0.01 M-Tris/HCl/1% Triton X-100/1 mM-PMSF/Trasylol (150 k.i.u.), pH 7.4, for 30 min at 4 °C. The lysates were centrifuged for 30 min at 20000 rev./min (25700 *g*) in a Beckman rotor 65 and the supernatants were used for immunoprecipitation experiments.

Peptide mapping

Fractions containing cell-CAM 105 obtained after separation on the Mono Q column or by preparative SDS/polyacrylamide-gel electrophoresis and electroelution were labelled with ¹²⁵I. Peptide mapping of the labelled proteins was done by digestion with *Staphylococcus aureus* V8 proteinase (Miles Laboratories) and analytical one-dimension SDS/polyacrylamide-gel electrophoresis essentially as described by Cleveland *et al.* (1977).

Immunoprecipitation and immunodepletion analyses

¹²⁵I-labelled solubilized cell-surface components or purified cell-CAM were immunoprecipitated as described previously (Ocklind & Öbrink, 1982). Before immunoprecipitation of the pure labelled cell-CAM 105, bovine serum albumin (1%) was added to prevent non-specific adsorption. Briefly, the labelled components were treated with preimmune serum and then precipitated with antiserum, IgG or affinity-purified IgG. After both the pre-precipitation and the immunoprecipitation the immunocomplexes were sedimented by the addition of formaldehyde-fixed staphylococci (Kessler, 1975). The precipitated components were analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis or by two-dimensional electrophoresis after solubilization in 4% (w/v) SDS/6 M-urea/0.0625 M-Tris/HCl, pH 8.8, or 9.5 M-urea/2% NP-40/2% (v/v) Ampholines (LKB)/5% β-mercaptoethanol respectively.

Immunodepletion analysis was performed on purified ¹²⁵I-labelled cell-CAM 105 by sequential precipitation with affinity-purified anti-(cell-CAM₈) antibodies until no detectable radioactive components were precipitated. The remaining supernatant was then analysed both by immunoprecipitation with anti-(cell-CAM₇) antiserum and by SDS/polyacrylamide-gel electrophoresis of the whole supernatant.

The electrophoretic polyacrylamide gels were fixed, dried, and exposed to Fuji RX-medical films (Fuji Photo Film Co.).

Radioimmunoassay

A solid-phase radioimmunoassay based on formaldehyde-fixed staphylococci (Kessler, 1975) and highly purified ^{125}I -labelled cell-CAM 105 was developed. Briefly, antibody titration curves were constructed whenever new ^{125}I -cell-CAM 105 was prepared, and the amount of antibody binding 70% of the ^{125}I -cell-CAM was used in the radioimmunoassay. A 100 μl portion of the appropriate dilution of antiserum [anti-(cell-CAM₈)] was mixed with 100 μl of sample or standard and incubated at room temperature for 60 min. Then a 100 μl portion of ^{125}I -cell-CAM was added and the incubation was continued for 15 h. Finally, 100 μl of a 5% (v/v) suspension of staphylococci were added. After 10 min the samples were centrifuged for 20 min at 3000 rev./min (2000 g) in an MSE Multex centrifuge, the pellets were washed twice and then counted for radioactivity in a Packard Autogamma spectrometer. Purified cell-CAM 105 calibrated by amino acid analysis was used as a standard.

Antibodies

Two antisera, anti-(cell-CAM₇) and anti-(cell-CAM₈), were produced by immunizing rabbits with purified cell-CAM 105 by using a previously described scheme (Ocklind & Öbrink, 1982). The cell-CAM 105 used in these immunizations was purified from Triton X-100-solubilized plasma membranes by immunoaffinity chromatography on anti-(cell-CAM₂) antibodies (Ocklind & Öbrink, 1982), followed by preparative SDS/polyacrylamide-gel electrophoresis and electroelution of the 105 kDa component. The IgG fraction of the antisera was isolated by affinity chromatography on protein A-Sepharose (Ocklind & Öbrink, 1982). Contaminating anti-albumin antibodies were removed by passage through a column of rat serum albumin covalently linked to CNBr-activated Sepharose 4B. Anti-(cell-CAM) antibodies were affinity-purified by chromatography on 1 mg of cell-CAM 105 coupled to 3 ml of Sepharose 4B. Bound immunoglobulins were eluted with 3 M-KSCN in 0.05 M-phosphate buffer, pH 6.0.

Compositional analyses

Amino acid analysis was performed with a LKB 4151 Alpha Plus amino acid analyser as described by Spackman *et al.* (1958) after hydrolysis of the samples *in vacuo* with 6 M-HCl at 110 °C for 24 h. Hydroxyproline and hydroxylysine were determined on a Biotronik LC 5001 amino acid analyser. Cysteine and methionine were determined as cysteic acid and methionine sulphone respectively after performic acid oxidation and hydrolysis (Moore, 1963). Tryptophan was determined after hydrolysis with 4 M-methanesulphonic acid/0.2% 3-(2-aminoethyl)indole at 110 °C for 24 h (Moore, 1972).

Hexosamines were determined on the LKB amino acid analyser after hydrolysis *in vacuo* with 2 M-HCl at 110 °C for 16 h. Sialic acid was determined by the periodate/resorcinol procedure of Jourdian *et al.* (1971). Neutral sugars were determined after hydrolysis of the samples in 2 M-trifluoroacetic acid at 100 °C for 3 h. Monosaccharides were separated on a Bio-Rad HPX-87P carbohydrate column eluted with water. The effluent was analysed for

carbohydrate by using an automated version of the orcinol method (Lohmander, 1986).

Inorganic phosphate was determined as described by Ames (1966).

Protein determination

Protein was determined by the procedure of Lowry *et al.* (1951) or that of Bradford (1976), with bovine serum albumin as a standard.

Phosphorylation

Rat hepatocytes were isolated by collagenase perfusion as described previously (Öbrink, 1982). The cells were seeded (2×10^6 cells/dish) in collagen-coated (Öbrink, 1982) 60 mm-diameter culture dishes and cultured for 12–20 h at 37 °C in RPMI medium supplemented with 10% (v/v) calf serum. The cells were then washed with phosphate-free F-10 medium and were incubated at 37 °C for 3 h in phosphate-free F-10 medium plus [^{32}P]P_i [2 ml of medium and 1 mCi of ^{32}P (carrier-free; The Radiochemical Centre, Amersham, Bucks., U.K.) per dish]. The medium was removed, the cells were washed with 0.15 M-NaCl, 0.01 M-Tris/HCl, pH 7.4, and were then lysed at 4 °C for 30 min in a medium (1 ml/dish) containing 0.15 M-NaCl, 0.05 M-Tris/HCl, pH 7.4, 1% Triton X-100, 0.01 M-EDTA, 5 μM -Na₃VO₄, 0.03 M-Na₄P₂O₇, 0.05 M-NaF, 1 μM -PMSF and 150 k.i.u. of Trasylol. The lysate was centrifuged for 3 min at 15000 rev./min (11300 g) in an Eppendorf 3414 S centrifuge and the supernatant was immunoprecipitated with affinity-purified anti-(cell-CAM₈) antibodies after pretreatment with preimmune serum and formaldehyde-fixed staphylococci. The immunoprecipitate was subjected to SDS/polyacrylamide-gel electrophoresis; the labelled band was localized by autoradiography, cut out from the gel and hydrolysed *in vacuo* in 6 M-HCl at 110 °C for 1 h. Phospho-amino acids in the hydrolysate were separated and identified by high-voltage electrophoresis at pH 3.5 (Hunter & Sefton, 1980).

Treatment with neuraminidase and endoglycosidase F

Digestion with neuraminidase from *Vibrio cholerae* (Calbiochem-Behring Corp.) was performed with 0.05 unit of enzyme/mg of cell-CAM 105 at 37 °C for 24 h in 1 ml of 2 mM-CaCl₂/0.2 mM-EDTA/50 mM-sodium acetate, pH 5.0 (Hoffman *et al.*, 1982).

Digestion with endoglycosidase F (New England Nuclear) was carried out according to the recommendations of Elder & Alexander (1982), essentially as described by Sorkin *et al.* (1984). Cell-CAM 105 obtained from the Mono Q column (component III) was freeze-dried and washed with ethanol to remove Triton X-100 (Ocklind & Öbrink, 1982). A 30 μg portion of cell-CAM 105 was dissolved in 50 μl of 0.1% SDS/1 mM-Tris/HCl, pH 7.4. DTT was added to a concentration of 0.1 M and the sample was boiled for 3 min. The following additions were then made: (1) 200 μl of a solution containing 0.1 M-sodium phosphate, pH 6.1, 1% NP-40, 50 mM-EDTA, 1.35 mM-PMSF and 1000 k.i.u. of Trasylol/ml; (2) 3.75 units of endoglycosidase F. The solution was then incubated at 37 °C and, at various times, samples were removed, diluted with an equal volume of sample buffer [0.062 M-Tris/HCl (pH 8.8)/0.01% Bromophenol Blue/10% (v/v) Glycerol/4% (w/v) SDS] and frozen at -70 °C. The samples were thawed, reduced

Fig. 1. Purification of cell-CAM 105: immunoaffinity chromatography

Triton X-100-solubilized plasma-membrane components were bound to, and eluted from, anti-(cell-CAM)-Sephacryl as described in the Materials and methods section. The fractions were analysed for cell-CAM 105 by radioimmunoassay (●) and by A_{280} (○). The fractions containing cell-CAM 105 were pooled, concentrated, and fractionated further on Superose 12 as described in Fig. 2. A portion of the pooled fractions was analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions and developed by silver staining (inset); the M_r values of standard proteins are indicated.

with DTT, subjected to SDS/polyacrylamide-gel electrophoresis and developed by immunoblotting with anti-(cell-CAM₀) antiserum and 125 I-protein A.

Cell aggregation

Aggregation of hepatocytes isolated by collagenase perfusion (Öbrink, 1982) was measured as previously described (Ocklind & Öbrink, 1982). For investigations of its effect on hepatocyte aggregation, pure cell-CAM 105 was treated in the following way. After Mono Q chromatography the protein was dialysed against distilled water, freeze-dried and washed with ethanol to remove Triton X-100 (Ocklind & Öbrink, 1982). The protein was dissolved in 0.1% SDS/0.15 M-NaCl/0.01 M-Tris/HCl, pH 7.4 and dialysed extensively, first against 0.15 M-NaCl/0.01 M-Tris/HCl, pH 7.4, and then against medium A (137 mM-NaCl/4.7 mM-KCl/0.6 mM-MgSO₄/1.2 mM-CaCl₂/10 mM-Hepes, pH 7.4) in order to remove SDS from the solution. Treated in this way, cell-CAM 105 stayed in solution in the absence of detergent. The protein was then added to the hepatocytes in the aggregation assay.

RESULTS

Purification of cell-CAM 105

In order to monitor and optimize the purification of cell-CAM 105, it was necessary to develop a quantitative assay for the determination of the intact detergent-solubilized protein. Thus a solid-phase radioimmunoassay based on formalin-fixed immunoglobulin-binding staphylococci and 125 I-labelled cell-CAM 105 was developed. Originally, cell-CAM 105 purified solely by immunoaffinity chromatography was used for 125 I labelling. Later it was replaced by cell-CAM 105 purified by the complete established procedure described below. We also moni-

Fig. 2. Purification of cell-CAM 105: gel-exclusion chromatography

The pooled material from the immunoaffinity chromatography was chromatographed on two Superose 12 columns in tandem as described in the Materials and methods section. The fractions were analysed for cell-CAM 105 by radioimmunoassay (●) and A_{280} (○). The major u.v.-absorbing peak represents Triton X-100 micelles. The fractions were also analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions and developed by silver staining (inset: the M_r values for standard proteins are indicated), which illustrated that the 105 kDa protein was well separated from the 70 kDa component and other smaller components. The fractions indicated by the horizontal bar were pooled and subjected to ion-exchange chromatography as described in Fig. 3. Fraction numbers are indicated both in the chromatogram and in the inset.

tored the purification by SDS/polyacrylamide-gel electrophoresis, the gels being developed by silver staining. The 105 kDa protein stained very poorly with Coomassie Brilliant Blue.

Initial attempts to purify cell-CAM 105 by conventional chromatographic procedures were without success, but the introduction of immunoaffinity chromatography resulted in a highly purified 105 kDa protein (Fig. 1). However, smaller components with apparent M_r values of about 90 000 and 70 000 appeared irregularly. They were most likely degradation products, since the same components appeared on prolonged storage of the purified 105 kDa protein as a result of autodegradation (results not shown). In order to remove them, gel-exclusion chromatography was introduced. Both Sephadex G-200 and Sephacryl S-300 worked, but the most efficient column was Superose 12 of the f.p.l.c. system (Fig. 2). The 105 kDa protein obtained from Superose 12 chromatography was then chromatographed on a Mono Q column of the f.p.l.c. system (Fig. 3). When the column was developed with a shallow salt gradient, a slight charge heterogeneity of cell-CAM 105 was revealed. Cell-CAM 105 was invariably eluted as three peaks, but the relative proportions of these peaks varied from preparation to preparation. Peak III, which was eluted at the highest salt concentration, contained the major component and accounted for 50–70% of cell-CAM 105. Peak I was sometimes almost absent. The purification scheme is summarized in Table 1.

A major problem in the purification of cell-CAM 105

Fig. 3. Purification of cell-CAM 105: ion-exchange chromatography

The pooled material from gel-exclusion chromatography was chromatographed on a Mono Q column as described in the Materials and methods section. The fractions were analysed for cell-CAM 105 by radioimmunoassay (●). —, [NaCl] profile. (a, b) Separation of two different preparations by NaCl gradients of various shapes. Cell-CAM 105 was separated into three fractions (I, II and III) under these conditions. The inset in (b) represents analysis by SDS/polyacrylamide-gel electrophoresis under reducing conditions and silver staining of fraction III. The migratory position corresponding to 105 kDa is indicated. (c) Elution of cell-CAM 105 subjected to neuraminidase digestion.

was its susceptibility to proteolytic degradation. To decrease the degradation during the preparation, a variety of proteinase inhibitors, including PMSF, Trasylol, leupeptin, pepstatin, iodoacetamide, EDTA and high salt, were tested. Of these, PMSF and Trasylol were the most effective ones, and they were added routinely in the preparation as described in the Materials and methods section. However, even in the presence of these inhibitors, some degradation was occasionally observed and therefore the purification scheme was designed to be as rapid as possible. This was facilitated by the introduction of the f.p.l.c. system, which allowed the time for each chromatographic step to be decreased to only about 1 h.

The purity of cell-CAM 105 was assessed by SDS/polyacrylamide-gel electrophoresis, by two-dimen-

sional gel electrophoresis and by immunodepletion analysis. All purity tests were performed on the material eluted in peak III on the Mono Q column. Cell-CAM 105 consists of an A- and a B-chain (see below), and on SDS/polyacrylamide-gel electrophoresis and silver staining, only a doublet band, corresponding to the A- and B-chains, of apparent M_r 105000, was detected (Fig. 3). It has been found, however, that rat hepatocytes have several cell-surface glycoproteins migrating with apparent M_r ~ 105000 on SDS/polyacrylamide-gel electrophoresis that can be separated by two-dimensional electrophoresis (Hixson *et al.*, 1985). We therefore analysed the purified cell-CAM 105 with two-dimensional electrophoresis to see if any of these proteins were present as contaminants. However, it was not possible to obtain a good isoelectric focusing of cell-CAM 105 at a protein concentration high enough to be detected by silver staining. Therefore the purified protein was labelled with ^{125}I before being subjected to two-dimensional electrophoresis and was detected by autoradiography. The only components detected in this way were the A- and B-chains of cell-CAM 105 (Fig. 4a). As a final test of the homogeneity of cell-CAM 105, we performed immunodepletion analysis (results not shown) with affinity-purified anti-(cell-CAM₆) antibodies. These antibodies only detected the A- and B-chains of cell-CAM 105 (Fig. 4b). The purified protein was labelled with ^{125}I and was then immunoprecipitated repeatedly with the affinity-purified antibodies. The precipitates were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Only the 105 kDa doublet was observed in the first and second precipitates, and after the third precipitation, no labelled band at all could be precipitated. The remaining supernatant was then precipitated with anti-(cell-CAM₇) antiserum, which in addition to the A- and B-chains of cell-CAM 105 also recognizes another 105 kDa cell-surface protein (component 3 in Hixson *et al.*, 1985; Fig. 4c). No labelled band was precipitated by the anti-(cell-CAM₇) antiserum. In addition, the supernatant remaining after the serial precipitation with anti-(cell-CAM₆) was analysed directly by SDS/polyacrylamide-gel electrophoresis, but no labelled bands were detected. These experiments clearly demonstrated that the only macromolecular components present in the purified material were the A- and the B-chains of cell-CAM 105.

Heterogeneity of cell-CAM 105

Cell-CAM 105 exhibited a slight charge heterogeneity and could be separated into three fractions when the Mono Q column was eluted with a shallow salt gradient (Fig. 3b). This heterogeneity was presumably due to a variation in the amount of sialic acid, since neuraminidase treatment resulted in elution of all the material at a much lower salt concentration (Fig. 3c). In order to compare the peptide portions of the components eluted in peaks I–III, they were labelled with ^{125}I , digested with *S. aureus* V8 proteinase and subjected to SDS/polyacrylamide-gel electrophoresis. An identical peptide pattern for all three components was observed (Fig. 5). This supports the conclusion that the heterogeneity resides in the carbohydrate portions of cell-CAM 105.

Subunit composition of cell-CAM 105

Analyses of purified cell-CAM 105 (component III) by high-resolution SDS/polyacrylamide-gel electrophoresis

Table 1. Purification of cell-CAM 105 from rat liver membranes

Protein was determined by the method of Bradford (1976), and cell-CAM 105 was determined by radioimmunoassay. Purification was assessed by dividing the ratio 'mg of cell-CAM 105/mg of protein' for each fraction with the corresponding ratio of 'Solubilized membranes'. The amount of protein in the 'Mono Q-purified' fraction was set equal to the amount of cell-CAM 105 (0.94 mg) in this fraction, since the analysis by the Bradford (1976) method gave a lower value than the radioimmunoassay.

Fraction	Protein (mg)	Cell-CAM 105 (mg)	Yield (%)	Purification (fold)
Solubilized membranes	190	1.6	100	1
Immunoaffinity-purified	1.8	1.41	88	93
Superose 12-purified	—*	0.98	61	
Mono Q-purified				
Peak I + II	0.09	0.10	59	119
Peak III	0.21	0.84		

* The high concentration of Triton X-100 made the protein determination in this fraction difficult.

Fig. 4. Two-dimensional electrophoretic separation of cell-CAM 105

Two-dimensional electrophoresis was performed as described in the Materials and methods section and developed by autoradiography. (a) ^{125}I -labelled purified cell-CAM 105 (peak III from the Mono Q chromatography). Only the A- and B-chains were detected, although they were not as well separated from each other as demonstrated in (b). (b) Triton X-100-solubilized components from ^{125}I -labelled hepatocytes immunoprecipitated by affinity-purified anti-(cell-CAM₈) antibodies. The A-chain (higher M_r and more basic) and the B-chain (lower M_r and more acidic) were clearly separated. (c) Triton X-100-solubilized components from ^{125}I -labelled hepatocytes immunoprecipitated by anti-(cell-CAM₇) antiserum. In addition to the A- and B-chains of cell-CAM 105, this antiserum also precipitated a minor component that was more basic.

Fig. 5. Peptide mapping of cell-CAM 105

All fractions were labelled separately with ^{125}I , digested with *S. aureus* V8 proteinase [50 $\mu\text{g}/\text{ml}$ in 0.125 M-Tris/HCl (pH 6.8)/0.5% SDS at 37 °C for 3 h] and subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions and autoradiography. Lanes III–I, mapping of fractions III–I of cell-CAM 105 obtained on Mono Q chromatography. Identical patterns were obtained from all three fractions. The weaker bands seen in fraction I reflect the fact that this was the minor fraction obtained in the separation. Lanes A and B, mapping of the A- and B-chains isolated from pure cell-CAM 105 (fraction III) by SDS/polyacrylamide-gel electrophoresis under reducing conditions. The two peptide chains gave almost identical peptide patterns.

or by two-dimensional electrophoresis revealed two peptide chains (Figs. 3 and 4). They were invariably present both under reducing and non-reducing conditions and were named the 'A-chain' and the 'B-chain'. The non-reduced chains migrated somewhat faster (having apparent M_r values of about 90000) than the reduced

Table 2. Amino acid, sugar and phosphate composition of cell-CAM 105

Constituent	Composition	
	(mol/100 mol of amino acids)	($\mu\text{g}/100 \mu\text{g}$ of amino acids)
Asx	10.5	
Thr	6.8	
Ser	11.2	
Glx	12.6	
Pro	5.2	
Gly	12.3	
Ala	5.2	
Cys	1.8	
Val	5.7	
Met	1.2	
Ile	4.2	
Leu	7.4	
Tyr	3.3	
Phe	3.7	
Lys	3.7	
His	1.5	
Arg	3.8	
Trp	0.2	
Galactose	5.8	9.8
Fucose	0.5	0.8
Mannose	8.3	14.1
N-Acetyl-glucosamine	7.7	16.0
N-Acetyl-neuraminic acid	3.4	10.0
Phosphate	1.2	1.1

chains (having apparent M_r values of about 105000) (results not shown). The apparent pI values of the two chains separated by two-dimensional electrophoresis were 4.1 (the B-chain) and 4.3 (the A-chain) respectively.

The A- and B-chains were analysed by peptide mapping. Purified cell-CAM 105 was subjected to SDS/polyacrylamide-gel electrophoresis. The gel was silver-stained, the A-chain and the B-chain were cut out and recovered by electroelution. The isolated chains were labelled with ^{125}I , digested with *S. aureus* V8 proteinase and analysed by SDS/polyacrylamide-gel electrophoresis. An almost identical peptide pattern was obtained (Fig. 5), indicating that the two peptide chains of cell-CAM 105 are structurally closely related.

Compositional analyses and phosphorylation

The amino acid analysis of cell-CAM 105 (Table 2) showed a very low content of tryptophan but relatively large amounts of serine, glycine, asparagine/aspartic acid and glutamine/glutamic acid. No hydroxyproline or hydroxylysine was detected. Amino-sugar analysis demonstrated a large amount of glucosamine, but a complete lack of galactosamine, indicating that cell-CAM 105 contained only N-linked oligosaccharides. The protein contained both sialic acid and the neutral sugars usually found in glycoproteins (Table 2). The carbohydrate analyses demonstrated that cell-CAM 105 is highly glycosylated, the total carbohydrate amounting to $50 \mu\text{g}/100 \mu\text{g}$ of protein or making up 33% of the relative mass of the glycoprotein.

Cell-CAM 105 contained a significant amount of

Fig. 6. Phosphorylation of cell-CAM 105

Cultured hepatocytes were incubated with $[\text{}^{32}\text{P}]\text{P}_i$, solubilized by Triton X-100 and the labelled components were isolated by immunoprecipitation. The immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions and revealed by autoradiography. Lane 1, components precipitated by anti-(cell-CAM₈) antibodies; lane 2, components precipitated by preimmune serum. The 105 kDa band in lane 1 was cut out, hydrolysed, and analysed by high-voltage electrophoresis (lane 3) after addition of standard phospho-amino acids; lane 3, an autoradiogram of the high-voltage electrophoresis of the hydrolysed, labelled, cell-CAM 105. The standard amino acids were monitored by ninhydrin staining. Labelled phosphoserine, but not phosphothreonine or phosphotyrosine, was detected. The two labelled spots that did not migrate as free phospho-amino acids probably represent incompletely hydrolysed peptides.

phosphate (Table 2). To further characterize the phosphorylation of cell-CAM 105, cultured hepatocytes were incubated with $[\text{}^{32}\text{P}]\text{P}_i$, lysed with Triton X-100 and immunoprecipitated with anti-(cell-CAM) antibodies. As illustrated in Fig. 6, both the A- and the B-chains became phosphorylated under these conditions. On phospho-amino acid analysis after acid hydrolysis of the labelled band, phosphoserine residues were found (Fig. 6). Under these conditions no labelled phosphothreonine or phosphotyrosine residues were detected, but two labelled spots, probably representing incompletely hydrolysed peptides, were seen.

Treatment with neuraminidase and endoglycosidase F

In order to investigate how the carbohydrate contributes to the macromolecular properties of this glycoprotein, we subjected it to treatments with neuraminidase and endoglycosidase F respectively. Neuraminidase treatment resulted in a decrease in the apparent M_r from 105000 to 90000 (results not shown). Treatment with endoglycosidase F yielded a more complex pattern. Endoglycosidase F cleaves both high-mannose and complex N-linked oligosaccharides from the core peptide of glycoproteins and should in theory give rise to a peptide without any N-linked oligosaccharides. However, it has

Fig. 7. Digestion of pure cell-CAM 105 with endoglycosidase F

Pure cell-CAM 105 was digested for various lengths of time with endoglycosidase F as described in the Materials and methods section. The protein was analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions and developed by immunoblotting with anti-(cell-CAM₈) antibodies. Lane 1, non-treated cell-CAM 105; lane 2, digestion for 1 h; lane 3, digestion for 4 h; lane 4, digestion for 12 h.

been found that the susceptibility to endoglycosidase F varies between different glycoproteins, since oligosaccharides may be more, or less, shielded from the attack of the enzyme (Elder & Alexander, 1982).

After extensive digestion (4–12 h) of cell-CAM 105 with endoglycosidase F, two components of apparent M_r 58000 and 54000 were obtained (Fig. 7). They probably represented the A-chain and the B-chain (respectively) from which all the *N*-linked oligosaccharides had been removed. After shorter incubation times, also, larger, partially deglycosylated, components appeared. Together with the intact A- and B-chains and the 58000- and 54000- M_r end products, a total of six pairs of bands could be detected, indicating that each chain of cell-CAM 105 carried at least five *N*-linked oligosaccharides. Crucial for the interpretation of these experiments is that the endoglycosidase F was free of proteinase activity. According to the manufacturer there was no proteinase activity in the batches used. In addition we analysed the enzyme directly for proteolytic activity with ^{125}I -labelled cell-CAM 105 as substrate; no such activity was found. Thus we conclude that the degradation of the 58 and 54 kDa components resulted from the glycosidic activity of the enzyme.

Effect of cell-CAM 105 on hepatocyte aggregation

In order to test the biological activity of the purified cell-CAM 105, its effect on hepatocyte intercellular adhesion was investigated. Freeze-dried cell-CAM 105 was dissolved in a low concentration of SDS and the detergent was dialysed away as described in the Materials and methods section. After dialysis the protein stayed in

Fig. 8. Effect of pure cell-CAM 105 on hepatocyte aggregation

Isolated hepatocytes were allowed to aggregate in suspension for 40 min at 37 °C in the absence and presence of fractions I–III (from Mono Q chromatography) of purified cell-CAM 105. The percentage inhibition (ordinate) at various concentrations of cell-CAM 105 (abscissa) is shown. The indicated ranges are the results obtained for duplicate incubations. Δ , Cell-CAM 105, fraction I; $\circ$, cell-CAM 105, fraction II; $\bullet$, cell-CAM 105, fraction III.

solution, presumably in the form of protein micelles (Hjertén *et al.*, 1982). As illustrated in Fig. 8, cell-CAM 105 inhibited aggregation of the hepatocytes to a significant extent, even at concentrations down to 50 ng/ml. Cell-CAM 105 eluted in all three peaks from the Mono Q column seemed to be effective to a similar extent. The inhibitory effect of cell-CAM 105 exhibited a complex concentration-dependence. Above 4 $\mu\text{g/ml}$ an increasing concentration increased the inhibition, but between 1 $\mu\text{g/ml}$ and 50 ng/ml the concentration-dependence was reversed, and instead a slight increase of the inhibitory effect was seen with a decreasing concentration of cell-CAM 105. The reason for this strange concentration-dependence is not known, but the explanation might be sought in the solute behaviour of cell-CAM 105. Cell-CAM 105 most likely occurs as multimolecular aggregates in solution (Hjertén *et al.*, 1982) and, since any molecular assembly has a tendency to dissociate when diluted, the effective concentration of cell-CAM 105 does not necessarily decrease in proportion to the dilution. If each cell-CAM-containing particle has the same inhibitory effect on cell aggregation regardless of its number of cell-CAM molecules, a concentration-dependence similar to that observed in Fig. 8 might be expected.

DISCUSSION

We have purified cell-CAM 105, a cell-surface glycoprotein involved in cell–cell adhesion of adult rat hepatocytes *in vitro*, and have carried out a chemical characterization of this molecule. It was purified from rat liver plasma membranes, where it accounted for 0.3% of the membrane protein, and from 100 g of liver we could isolate about 0.5 mg of highly purified cell-CAM 105. Cell-CAM 105 behaves like an integral membrane protein, since neither variations of salt concentration nor chelators or bivalent cations, but only detergents, could solubilize it from the membranes. The protein consists of two highly glycosylated peptide chains with apparent M_r

values of 105 000 and 110 000 respectively. The two chains are apparently not disulphide-linked, since reduction did not increase the electrophoretic mobility in SDS/polyacrylamide-gel electrophoresis. Instead a decreased mobility was observed, indicating that both chains contain intra-chain disulphide bonds. Peptide mapping of the isolated chains gave similar patterns. This agrees with previous peptide mappings performed on immunoprecipitated A- and B-chains separated by two-dimensional electrophoresis, which indicate that the two chains differ essentially only in one peptide after *S. aureus* V8 proteinase digestion (Hixson *et al.*, 1985). Thus the peptide backbones of the A- and B-chains of cell-CAM 105 seem to be structurally closely related.

The protein contains a large amount of carbohydrate, apparently only in the form of *N*-linked oligosaccharides, since no galactosamine was found. Both peptide chains seem to be glycosylated to a similar extent as judged from the change in electrophoretic mobility after treatment with endoglycosidase F. This experiment, which indicated that each peptide chain contains at least five oligosaccharides, also showed that the carbohydrate contributes significantly to the macromolecular properties of cell-CAM 105. The heterogeneity observed in the cell-CAM preparations most likely resides in the carbohydrate portion and, more particularly attributable to variations in the amount of sialic acid. In addition to being a glycoprotein, cell-CAM 105 is also a phosphoprotein on the basis of both composition analysis and the finding that both of the peptide chains could be phosphorylated on serine residues. This property indicates that the protein spans the plasma membrane, since most, if not all, phosphorylated membrane proteins are phosphorylated on their cytoplasmic domains.

A consistent feature of cell-CAM 105 was its ability to undergo a spontaneous autolytic degradation to lower- M_r material. This might be an inherent property of the protein that could be related to its function, although at present we cannot rule out the possibility of contamination of the purified protein with proteinase.

In recent years, several cell-adhesion molecules have been identified and characterized by immunological approaches (Damsky *et al.*, 1984; Öbrink, 1986). Of the vertebrate CAMs, the most well-characterized molecules so far are N-CAM (Thiery *et al.*, 1977; Edelman, 1983), D2 (Jorgensen *et al.*, 1980), BSP-2 (Hirn *et al.*, 1981), Ng-CAM (Grumet *et al.*, 1984), L1 (Rathjen & Schachner, 1984) and J1 (Kruse *et al.*, 1985) of the nervous system and L-CAM (Gallin *et al.*, 1983), uvomorulin (Peyriras *et al.*, 1983), E-cadherin (Yoshida-Noro *et al.*, 1984), Arc-1 (Imhof *et al.*, 1983) and cell-CAM 120/80 (Damsky *et al.*, 1983) found in various epithelia. Several of these cell-adhesion molecules, like cell-CAM 105, consist of more than one peptide chain and these chains are not disulphide-linked. In addition, N-CAM (Hoffman *et al.*, 1982) and uvomorulin (Peyriras *et al.*, 1983) share with cell-CAM 105 the ability to undergo spontaneous autolytic degradation to smaller components. Accordingly it seems possible that all these cell-adhesion molecules could be members of a superfamily of proteins with similar functions. Some of these cell adhesion molecules are closely related. Thus N-CAM, D2 and BSP-2 are homologous molecules isolated from chickens, rats and mice respectively (brink, 1986). Uvomorulin and E-cadherin, which both originate from mice, are probably identical and seem to be homologous

with L-CAM, Arc-1 and cell-CAM 120/80, which have been isolated from chickens, dogs and humans respectively (brink, 1986). Recently we have performed a direct comparison between cell-CAM 105 and uvomorulin, which showed that they are distinct and different proteins with different functions in cell-adhesion reactions (Vestweber *et al.*, 1985). By employing immunohistochemical and radioimmunoassay techniques we have not been able to demonstrate the presence of cell-CAM 105 in the central nervous system (Ocklind *et al.*, 1983; P. Odin & B. brink, unpublished work). Thus we conclude that cell-CAM 105 is distinct from all the other cell-adhesion molecules mentioned above.

Cell-CAM 105 was identified as a cell-adhesion molecule by the ability of specific univalent antibodies directed against it to inhibit cell-cell adhesion of reaggregating rat hepatocytes *in vitro* (Ocklind & brink, 1982). Apart from this, little is known about what role cell-CAM 105 has in cellular recognition and adhesion, or about its mechanism of action. The present finding, namely that purified cell-CAM 105 could inhibit hepatocyte aggregation, indicates that it has a direct role in hepatocyte cell-cell binding. The cell-CAM used in these experiments was without detergent, but remained soluble, probably in the form of giant protein micelles (Hjertn *et al.*, 1982). The peculiar concentration-dependence of this inhibitory effect probably resulted from the dissociation of the cell-CAM aggregates at increasing dilution.

We have recently obtained results indicating that cell-CAM 105 exerts its major function in terminally differentiated cells. The protein does not appear in the foetal-rat liver before day 16 of gestation (Odin & brink, 1986). Furthermore, there is a transient decrease in the amount of cell-CAM 105 on the surface of the hepatocytes during liver regeneration after partial hepatectomy (Odin & brink, 1986). The disappearance and reappearance of cell-CAM 105 was well correlated with the enzymic differentiation of the hepatocytes. Finally, in a collaborative investigation we have recently reported that the expression of cell-CAM 105 is altered in transplantable hepatocellular carcinomas (Hixson *et al.*, 1985). This was manifested as an apparent loss of the molecule from the cell surface in all the 13 different carcinomas that were tested. Although there are several possibilities for the physiological function of cell-CAM 105, these results make it tempting to speculate that the protein is important for the organization of the hepatocytes in the mature liver. The present possibility of obtaining cell-CAM 105 in pure form will facilitate future studies on the biological function of this protein at the molecular, cellular and tissue levels.

This investigation was supported by grants from the Swedish Medical Research Council (project nos. 05200 and 6686), Konung Gustaf V:s 80-rsfond, Pharmacia, Uppsala, Sweden and the University of Uppsala. The excellent technical assistance by Ms. Birgitta Wrmegrd and Miss Anne-Marie Nordgren is gratefully acknowledged. We are grateful to Professor Dick Heinegrd, University of Lund, Sweden, for kindly helping with the analyses of neutral sugars and sialic acids.

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