Comparison of the Structural Characteristics of Cell-CAM 105 from Hepatocytes with Those of an Altered Form Expressed by Rat Transplantable Hepatocellular Carcinomas

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ABSTRACT

The structural features of the adult rat hepatocyte (ARH) forms of cell-CAM 105, a M, 105,000 cell adhesion molecule, were compared using a variety of immunochemoical and biochemical techniques with altered forms of more basic pl present on two transplantable hepatocellular carcinomas (THC 1682c and THC AS-30D). Immunoprecipitation analysis with polyclonal (anti-gp105-2) and monoclonal (MAb) antibodies specific for cell-CAM 105 (MAb 362.50) demonstrated that ARH and THC cell-CAM 105 were indistinguishable in several respects including: (a) binding to wheat germ agglutinin; (b) labeling with NaIO4/NaBH4; (c) susceptibility to digestion with endoglycosidases (endoglycosidase H and F and peptide N-glycosidase F N-glycanase); (d) rate of turnover on the cell surface; and (e) differential resistance of upper and lower forms to trypsin digestion in the presence or absence of calcium. Digestion with Clostridium perfringens or Vibrio cholerae neuraminidase did not equalize pl but instead decreased the size and increased the pl of both ARH and THC cell-CAM 105. Comparison of two-dimensional tryptic peptide maps, however, revealed five unique peptides in the THC AS-30D map and one peptide in the THC 1682c map, peptides which were only apparent in maps of deglycosylated ARH cell-CAM 105. Based on these results, it was concluded that there were significant differences in the glycosylation of ARH and THC cell-CAM 105. Biosynthetic labeling with 32PO4 and 18SO4 showed that both ARH and THC molecules were phosphorylated but not sulfated. Comparison of 2IP-labeled peptides produced by digestion with V-8 protease revealed significant differences in the phosphorylation of the upper and lower forms from ARH and showed that the pattern of phosphorylation on THC cell-CAM 105 most closely resembled ARH upper form. Pulse-chase analysis of ARH cell-CAM 105 further indicated that only a subpopulation of the molecules labeled with 35S-methionine were phosphorylated.

INTRODUCTION

In a previous report (1) we described the production of a xenoantisemur (anti-gp105) reactive with a family of wheat germ agglutinin binding glycoproteins on ARH. We subsequently showed that the two most acidic components recognized by this antisemur were identical to cell-CAM 105, a M, 105,000 glycoprotein involved in the intercellular adhesion of rat hepatocytes (2). In the companion paper to the present report, we demonstrated by immunoprecipitation analysis with a MAb specific for cell-CAM 105 (MAb 362.50) that 3/3 THC lines expressed an altered form of this molecule with a more basic pl on two dimensional gels than its counterpart from ARH. In the present report, we have used immunochemoical techniques to analyze further the structural characteristics of cell-CAM 105 with the goal of determining the structural basis for the altered mobility displayed by the THC forms of this molecule. Since our preliminary findings (3) and those of Odin et al. (4) indicated that cell-CAM 105 from ARH was heavily glycosylated and phosphorylated, we were particularly interested in determining if THC and ARH cell-CAM 105 were distinguished by differences in these modifications. Our results show that THC cell-CAM 105 closely resembles its ARH counterpart in phosphorylation, peptide structure, and sensitivity to digestion with trypsin in the presence and absence of calcium but appears to possess qualitative and/or quantitative differences in glycosylation. We further demonstrate that there is a rapid turnover of a phosphorylated population of cell-CAM 105 and that significant differences exist in the phosphorylation of the two forms expressed by ARH.

MATERIALS AND METHODS

Isolation and Maintenance of Normal Hepatocytes and THC Lines. Normal adult rat hepatocytes were obtained by a modification (1) of the collagenase perfusion technique of Bonney et al. (5). THC 1682c was derived from a primary hepatocellular carcinoma induced in male ACI rats maintained on a choline-deficient diet containing 0.2% ethionine (6). AS-30D THC was derived from an azo-dye-induced hepatocellular carcinoma (7). Procedures for maintenance of THC lines have been described previously (1).

Immunochemoical Protocols. The preparation and characterization of monoclonal (MAb 362.50) and polyclonal antibodies (anti-gp105-2) specific for cell-CAM 105 have been described.4 Surface labeling of cells with 125I (Amersham, Arlington Heights, IL, 500 mCi/ml) was performed by the lactoperoxidase-glucose oxidase procedure of Keski-Oja et al. (8). Cell-surface sialic acid residues were labeled as previously described (1) with H using the NaIO4/NaBH4 procedure described by Gahmberg & Andersson (9). Procedures for immunoprecipitation of radiolabeled antigens using S. aureus (IgGsorb, Enzyme Center Inc., Boston, MA) have been described (1, 2). Comparison of the reactivity of two different antisera was determined by immunodepletion analysis (10). One dimensional SDS-PAGE gels were run according to the method of Laemmli (11). Two-dimensional gel analysis was performed as described by O’Farrell (12). Staining, destaining, drying of gels, and autoradiography were carried out as described previously (1, 2, 10). SDS-PAGE gels containing 35S- or 3H-labeled material were impregnated with Enhance (Dupont NEN Research Products, Wilmington, DE) and visualized by fluorography (1). Apparent molecular weights were calculated from protein standards (β-galactosidase, M, 116,000; phosphorylase b, M, 97,000; bovine serum albumin, M, 68,000; ovalbumin, M, 43,000) run concurrently with radioactively labeled samples.

Isolation of wheat germ agglutinin binding glycoproteins on Sepharose-4B (Pharmacia, Piscataway, NJ) using the cyanogen bromide coupling method of Cuatrecasas (13). Biopsy liver samples were homogenized in a glass homogenizer in 100 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, 1 mM PMSF, 0.1% SDS. After centrifugation for 20 min at 100,000 g, the supernatant was dialyzed against PBS overnight and concentrated to 10 ml by centrifugation in an Amicon filter unit. Cell-CAM 105 further indicated that only a subpopulation of the molecules expressed by ARH.

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1 Supported by Grant CA-42715 from the NIH.
2 To whom all requests for reprints should be addressed.
3 The abbreviations used are: PBS, phosphate buffered saline (140 mM NaCl, 4 mM KCl, 2 mM KH2PO4, pH 7.4); SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; THC, transplantable hepatocellular carcinoma; ARH, adult rat hepatocyte; MAb, monoclonal antibody; HEPEs, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pl, isoelectric point.

* Hixson, D. C., and McEntire, K. D. Detection of an altered form of cell-CAM 105 with the goal of determining the structural basis for the altered mobility displayed by the THC forms of this molecule. Since our preliminary findings (3) and those of Odin et al. (4) indicated that cell-CAM 105 from ARH was heavily glycosylated and phosphorylated, we were particularly interested in determining if THC and ARH cell-CAM 105 were distinguished by differences in these modifications. Our results show that THC cell-CAM 105 closely resembles its ARH counterpart in phosphorylation, peptide structure, and sensitivity to digestion with trypsin in the presence and absence of calcium but appears to possess qualitative and/or quantitative differences in glycosylation. We further demonstrate that there is a rapid turnover of a phosphorylated population of cell-CAM 105 and that significant differences exist in the phosphorylation of the two forms expressed by ARH.

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(Staphylococcus aureus) was removed by centrifugation at 10,000 x g for 1 min and the supernatant was diluted with an equal volume of the same buffer without dithiothreitol and containing 250 mM iodoacetamide. After incubation for 30 min at 20°C, cell-CAM 105 was precipitated for 30 min by addition of an equal volume of ice-cold acetone. Precipitated material was collected by centrifugation for 5 min at 10,000 x g, resolubilized in urea sample buffer [9.5 M urea, 2% NP-40, 2% ampholines (1:1 mixture of pH 3.5–5.5 and pH 5–7 ampholines, LKB Instruments, Rockville, MD), 5% 2-mercaptoethanol] and resolved by two-dimensional gel electrophoresis as described by O’Farrell (12). Gel slices containing reactive components visualized by autoradiography were rehydrated in 0.05 M NH₄HCO₃, pH 8.6, macerated with a glass stirring rod, and resuspended in 3 ml of 0.05 M NH₄HCO₃ buffer containing 50 µg/ml TPCK trypsin (Sigma), 0.01 M HCl and 1 mM CaCl₂. After digestion for 18 h at 37°C, an additional 100 µg of trypsin was added and digestion was continued for 4 h more. Gel pieces were removed by filtration and the peptide containing filtrate was lyophilized, redissolved in 50–100 µl of electrophoresis buffer, (30% formic acid) and spotted on a thin-layer chromatography plate (Eastman Chromagram, Eastman Kodak, Rochester, NY). The plate was moistened with 30% formic acid and electrophoresed at constant voltage (150 V) for 3 h. After drying, the plate was placed in a chromatography tank and developed with a buffer composed of butanol:acetic acid:pyridine:water at a ratio of 58:10:45:36. The plate was then dried and the resulting peptide map visualized by autoradiography. Approximate amounts of radioactivity spotted for maps of radiiodinated cell-CAM 105 from ARH upper form, ARH lower form and both THC 1682c, and THC AS-3D combined upper and lower forms were 30,000, 12,000, and 10,000 cpm, respectively.

Deglycosylation Procedures. Digestion with endoglycosidase H (Miles Scientific) was accomplished essentially as described by McIntyre et al. (16). Briefly, S. aureus-bound immune complexes were boiled for 5 min, in 50 µl of 0.05 M Tris HCl, pH 6.8, containing 1% SDS. After a 1-min centrifugation at 10,000 x g, the supernatant was collected and diluted with an equal volume of 0.15 M citrate phosphate buffer, pH 5.25. Endoglycosidase H was then added at concentrations ranging from 0.025 to 0.25 units/ml. Following a 22-h incubation at 37°C, digestion was terminated by addition of 1/10 volume of 20% SDS and heating for 5 min at 80°C. For 2-D PAGE analysis, proteins were precipitated by addition of three volumes of ice-cold acetone and redissolved in urea sample buffer. For digestion with N-glycanase (peptide N-glycosidase F, Genzyme Corporation, Boston, MA), endoglycosidase-F (Genzyme), endoglycosidase-D (Miles), Fibrochelae neuraminidase (CALBIOCHEM, La Jolla, CA) and Clostridium perfringens neuraminidase (Sigma), immune complexes were eluted by boiling for 3 min in 50 µl of 0.5% SDS, 0.1 M 2-mercaptoethanol. After removal of S. aureus by centrifugation, the supernatant was diluted with 50 µl of one of the following buffers: (a) 0.15 M sodium phosphate, pH 8.6, containing 2.7% NP-40, 100 KIU aprotinin, 12 mM EDTA and 1–2 units N-glycanase or 0.015–0.8 units endoglycosidase-D; (b) 0.25 M sodium acetate buffer, pH 6.0 containing 3% NP-40, 100 KIU aprotinin, 1.5–4.5 milliunits endoglycosidase-F; (c) PBS, pH 7.0 containing 0.5% NP-40, 100 KIU aprotinin, 0.1 mM CaCl₂ and 10–20 milliunits Vibrio cholerae or 10–25 units of Clostridium perfringens neuraminidase. After digestion for 20 h at 37°C, samples were either treated as described above for endoglycosidase-H or were diluted with 1/4 volume of 5X SDS sample buffer (310 mM Tris-HCl, 10% SDS, 10% 2-mercaptoethanol, 30% glycerol, pH 6.8) boiled 3 min and resolved by one-dimensional SDS-PAGE.

Serine or threonine-linked oligosaccharides were removed by beta elimination in 0.5 M NaOH. Immune complexes adsorbed to S. aureus were eluted in 50 µl of 0.5 M NaOH for 10 min at 4°C. After being neutralized by addition of 50 µl of 0.5 M HCl, samples were diluted with 1/4 volume of 5X SDS-sample buffer and boiled for 3 min.

Alkaline Phosphatase Digestion. Digestion of immunoprecipitated cell-CAM 105 with alkaline phosphatase (Sigma, Type III) was accomplished essentially as described by Ottaviano & Gerace (17).

RESULTS

Glycosylation of Cell-CAM 105 from ARH and THC. Both ARH and THC 1682c cell-CAM 105 were labeled by treatment
with NaIO₄/NaB₃H₄, a method which incorporates ³H into the C7-C9 side chain of sialic acid residues (9) (Fig. 1F). Digestion with Clostridium perfringens (data not shown) or Vibrio cholerae neuraminidase of ARH (Fig. 1, A and B) and THC (Fig. 1, C and D) cell-CAM 105 immunoprecipitated from extracts of radioiodinated cells produced a decrease in M₅, (Fig. 1E, Lane 2) and a shift to a more basic pI. Even after extended digestion (18 h), THC cell-CAM 105 still displayed a more basic pI than its ARH counterpart (Fig. 1).

Digestion of cell CAM 105 from ARH (Fig. 1, E3) and THC 1682c (data not shown) with a preparation of endoglycosidase-F that was free of peptide:N-glycanase activity produced only a small shift in M₅, 2000–3000 and no apparent changes in pI. Digestion of ARH cell-CAM 105 with both Vibrio cholerae neuraminidase and endoglycosidase-F produced a greater change in molecular weight than that observed with either enzyme alone (Fig. 1E, Lane 4). No change in molecular weight or in pI was observed following digestion with endoglycosidase-D or endoglycosidase-H (data not shown).

Digestion with N-glycanase produced a 45% reduction in molecular weight of both the upper and lower forms of cell-CAM 105 from ARH and THC 1682c (Fig. 2). Two-dimensional gel analysis of partially digested cell-CAM 105 from ARH showed a stairstep pattern comprised of a minimum of seven distinct spots of increasing pl and decreasing molecular weight (Fig. 3). The partially digested forms of THC 1682c cell-CAM 105 were only poorly resolved (Fig. 3B).

No change in the mobility of cell-CAM 105 was observed following incubation in 0.5 M NaOH (data not shown).

Resistance to Trypsin Digestion. Since L-CAM and several other closely related CAMs show a calcium-dependent sensitivity to trypsin digestion (22), it was of interest to determine if structural differences in ARH and THC would be manifested by a differential sensitivity to trypsin in the presence or absence of calcium. As shown in Fig. 4, both ARH and THC cell-CAM 105 displayed a similar sensitivity to trypsin with the only change being a loss of upper form. Removal of calcium with EGTA did not increase sensitivity to trypsin digestion of either ARH or THC cell-CAM 105.

Peptide Maps of ARH and THC Cell-CAM 105. Upper and lower forms of cell-CAM were distinguished in one-dimensional V-8 peptide maps by the presence of an extra peptide in the map of the upper form (Fig. 7), a finding in agreement with our previous results (2). V-8 peptide maps of upper and lower forms of cell-CAM 105 from ARH, THC 1682c, and THC AS-30 (data not shown) were very similar and did not reveal any significant differences in primary structure.

One-dimensional peptide mapping techniques are relatively insensitive to sequence alterations since they only measure the spacing of cleavage sites and are largely unaffected by the actual sequence between these sites. To better resolve subtle differences in peptide structure, two-dimensional peptide maps were prepared from cell-CAM 105 immunoprecipitated from extracts of radioiodinated ARH, THC 1682c and THC AS-30D and purified by 2-D PAGE. As shown in Fig. 5, A and B and Fig. 6A, the two-dimensional peptide maps of the upper and lower forms from ARH were virtually identical. No major peptides unique to the upper form could be resolved.

Peptide maps prepared from ARH cell-CAM 105 which had been deglycosylated by digestion with N-glycanase (Fig. 6B) were similar to maps prepared from fully glycosylated cell-CAM 105. However, two major spots at the origin and at least four other peptides were no longer apparent. Further, maps of N-glycanase-treated cell-CAM 105 contained five new peptides common to both forms and two peptides unique to the upper and three unique to the lower form.

In tryptic peptide maps prepared from THC 1682c, 11 of 12 peptides visible in autoradiograms had counterparts in maps

**Fig. 1.** Analysis of sialic acid on cell-CAM 105. A-E, cell-CAM 105 from ³H-labeled ARH or THC lysates was eluted from *S. aureus* after immunoprecipitation, digested with enzyme as described and subjected to 2-D PAGE (A-D) or SDS-PAGE (E) and autoradiography. A, ARH, no enzyme; B, ARH, neuraminidase; C, THC 1682C, no enzyme; D, THC 1682C, neuraminidase; E, no enzyme (1); neuraminidase (2); endoglycosidase F (3); both neuraminidase and endoglycosidase F (4); F, cell-CAM 105 immunoprecipitated with MAb 362.50 from extracts of THC 1682C (Lane 1, 6-month exposure) and ARH (Lane 2, 4-day exposure) labeled with NaIO₄/NaB₃H₄. In B and D, the position of the undigested forms are indicated by dotted lines.
STRUCTURE OF CELL-CAM 105 ON NORMAL AND MALIGNANT HEPATOCYTES

Fig. 2. N-Glycanase digestion. Cell-CAM 105 was immunoprecipitated from detergent lysates of 125I-labeled ARH (1, 2, 5, 6) and THC 1682c (3, 4, 7, 8) cells. The upper (1-4) and lower (5-8) forms were separated by 2-D PAGE, localized by autoradiography, excised from gels and digested with N-glycanase as described. Lanes 1, 3, 5, 7, undigested; Lanes 2, 4, 6, 8, digested. N and T, ARH and THC lysates.

Fig. 3. 2-D PAGE analysis of a partial N-glycanase digest of 125I-labeled cell-CAM 105 isolated by immunoprecipitation from ARH (A, 2-week exposure) and THC (B, 4-week exposure) cells. The position of the undigested forms are indicated by dotted lines. Arrow heads, completely or partially digested forms from THC and ARH, respectively. The 43KD component is nonspecifically bound to S. aureus.

Fig. 4. Trypsin digestion. 1-4, control digests; 5-8, trypsin digests of radiiodinated ARH (A) or THC 1682 (B) cells that were digested before (1, 2, 5, 6) or after (3, 4, 7, 8) lysis in 1% NP-40. Digestions were carried out in either 2 mM Ca2+ (1, 3, 5, 7) or 2 mM EGTA (2, 4, 7, 8). Cell-CAM 105 was immunoprecipitated from digested extracts with anti-gpl05-2 prior to 2-D PAGE and autoradiography.

Biosynthetic Labeling. Immunoprecipitation analysis showed that both the upper and lower forms of cell-CAM 105 from primary cultures of ARH or THC were labeled after a 4-h pulse with 1 mCi of 32P04 but were not labeled after incubation for 4 h in the presence of 1–3 mCi of 35SO4 (data not shown). Not unexpectedly, these phosphorylated forms of cell-CAM 105 displayed a slightly more acidic pI than their 35S-labeled counterparts. Comparison of V-8 peptide maps of labeled upper and lower forms of cell-CAM 105 isolated from ARH cultured for 6 h with 32P04 revealed differences in phosphorylation (Fig. 7, Lanes 2 and 4). The V-8 peptide map of the combined upper and lower forms of 32P-labeled cell-CAM 105 from THC 1682c cultured for 14 h in medium containing 32P04 (Fig. 7, Lane 3) was very similar to the map from the upper form on ARH (Fig. 7, Lane 2). The specific activity was insufficient to allow visualization by autoradiography of separate 32P-peptide maps from THC 1682c upper and lower forms.

Pulse-Chase Analysis. Steady state labeling with both [35S]-methionine and [35S]cysteine for 6 or 14 h showed that approximately three-fourths of the 35S-labeled cell-CAM 105 was present as the lower molecular weight form (Fig. 8, Panels 6–10). Chase for up to 10 h after a 2-h pulse with [35S]methionine and [35S]cysteine did not reveal any significant conversion of the upper to lower form. Instead, labeled upper and lower forms appeared to decrease at similar rates. This decrease was estimated by densitometric analysis to be 30% after a 10 h chase.

As shown in Fig. 8, Panels 1 and 2, both forms were labeled after a 6–14 h incubation with 32P04. Densitometric analysis indicated that after a 2-h pulse with 32P04, more than 90% of the labeled cell-CAM 105 was present as the upper form. Chase for 4 h resulted in a 30% decrease in 32P-labeled upper form that was paralleled by a comparable increase in lower form. After a 12-h chase, less than 1% of the upper form remained and the lower form had decreased back to a level similar to that observed at 2 h. The ratio of the lower to upper form after 12 h of chase, however, was eight times greater than that observed at 2 h.

Pulse iodination analysis indicated that after 16 h in culture, the cell surface associated cell-CAM 105 had decreased by 50% on both ARH and THC 1682c cells (data not shown).

Alkaline Phosphatase Digestion. The more acidic pI of 32P-labeled cell-CAM 105 suggested that differential phosphorylation might contribute to the differences in pI of the ARH &
THC forms of this molecule. However, no change in pI was observed after digestion of 125I-labeled ARH and THC cell-CAM 105 with alkaline phosphatase.

DISCUSSION

In a previous study, we demonstrated that THC and primary hepatocellular carcinoma expressed decreased levels of an altered form of cell-CAM 105 that was distinguished from its normal counterpart by a more basic pI on two dimensional gels (3). More recently we have shown that this altered form of cell-CAM 105 is also expressed by primary hepatocellular carcinomas (4), an observation which suggests that the appearance of an altered form of cell-CAM 105 is a primary event rather than a secondary change resulting from adaptation or selection during the establishment or maintenance of THC lines. In the current investigation, we have attempted to elucidate the structural basis for this alteration in pI.

Although our analyses were often hampered by the low level of expression on THC cells, we were able to demonstrate a close similarity between many of the structural properties of ARH and THC cell-CAM 105. Both were expressed on the cell surface in two forms which differ in size, pI, relative abundance, and sensitivity to digestion with V-8 protease and trypsin. Both ARH and THC cell-CAM 105 also demonstrated a similar shift in pI and size following digestion with neuraminidase and an essentially identical size and pI following treatment with N-glycanase. The large difference in the pI of the N-glycanase and neuraminidase-treated THC cell-CAM 105 most likely results from the asparagine to aspartic acid conversion which occurs during the cleavage of N-linked oligosaccharides by N-glycanase (18).

By applying two-dimensional peptide mapping techniques to the analysis of native and deglycosylated forms of cell-CAM 105, we were able to gain additional insight into the structural basis for differences between the upper and lower forms of ARH cell-CAM 105 and for the altered mobility displayed by THC cell-CAM 105. Although the close similarity of V-8 peptide maps indicated that there were no major differences in sequence in cell-CAM 105 from ARH and THC which could be resolved by one-dimensional mapping techniques, comparison of maps prepared from the upper and lower forms on ARH did reveal a V-8 peptide unique to the upper form. In contrast, two-dimensional tryptic peptide maps of the upper and lower forms were essentially identical, a finding at odds with results from trypsin digestion studies on native molecules which showed that trypsin produced a selective loss of the upper form. This apparent discrepancy could result, if the peptide unique to the upper form was completely digested to low molecular weight fragments or was glycosylated and thus essentially immobile under the conditions chosen for chromatography. This latter possibility was supported by results from two-dimensional peptide analysis of the N-glycanase digested upper and lower forms of ARH cell-CAM 105. Comparison of these maps revealed the loss of several components present in maps of undigested cell-CAM 105 including the two prominent components at the origin. In addition, there were at least 10 new components, five that were held in common, two that were unique to the upper, and three to the lower form. It seems likely that at least some of these new components were derived from glycopeptides, perhaps contained in the two prominent spots near the origin (peptides 17 and 18 in Fig. 7), whose sensitivity to trypsin degradation and/or mobility in the chromatographic step were greatly increased by the absence of N-linked oligosaccharide chains.

Consistent with results from one-dimensional V-8 protease maps, the two-dimensional peptide maps of cell-CAM 105 from ARH, THC 1682c, and THC AS-30D showed a high degree of homology. It was also apparent, however, that there were a number of components in THC AS-30D maps and at least one component in the THC 1682c map that were not present in ARH maps. Comparison with maps of N-glycanase-treated cell-CAM 105 revealed components homologous to the THC 1682c component and to four of the five of the THC AS-30D peptides. Based on these findings, it was concluded that the new peptides present in THC maps were most likely due to variations in digestion patterns resulting from differences in glycosylation rather than from changes in primary amino acid sequence, a conclusion consistent with results from glycosidase digestion studies with cell-CAM 105 from THC 1682c and the large degree of microheterogeneity displayed by the molecule from THC AS-30D.

Differential glycosylation of ARH and THC cell-CAM 105

Fig. 5. Two-dimensional peptide map of ARH cell-CAM 105. Upper and lower forms of immunoprecipitated 125I-labeled cell-CAM 105 from ARH and THC were separated by 2-D PAGE, localized by autoradiography, excised from the gel and digested with trypsin as described by McIntyre et al. (11). A, upper form, ARH (2-week exposure); B, lower form, ARH (2-week exposure).
Fig. 6. Comparison of the THC and ARH cell-CAM 105 tryptic peptide maps with maps from N-glycanase-digested ARH-cell-CAM 105. A, composite diagram of upper and lower peptide maps of ARH cell-CAM 105; B, composite diagram of N-glycanase treated upper and lower ARH maps; C, diagram of THC 1682C map; D, diagram of THC AS-30D map. O, Peptides held in common by both upper and lower ARH forms. □, Peptide present in map prepared from a mixture of upper and lower ARH forms. ▪, Unique peptides present in maps from both upper and lower N-glycanase treated forms. □, Peptides unique to the N-glycanase treated lower form. □, Peptides unique to N-glycanase treated upper form. ▪, Peptide unique to the THC AS-30D map. Autoradiograms used for diagrams in B-D were exposed for 2 (B) or 4 (C and D) weeks.
linked to the C-4 position of the 0-mannosyl residue in the acetylglucosamine residues (A'-acetylglucosamine residues general had less sialic acid and were enriched with bisect N-...tumor cell oligosaccharides in cell-CAM 105. One possibility is suggested by the work of Yamashita et al. (19). These investigators have reported that those of the rat liver enzyme. Tumor cell oligosaccharides present on -y-glutamyl transpeptidase pu...digested THC counterpart. Additional alterations which coun...digested THC cell-CAM 105 relative to its un...glycosidase digestion indicated that the THC and ARH forms of...the THC 1682C labeled with 32PO4 for 14 h. For comparison, the V-8 peptide maps of the upper (Lane 1) and lower (Lane 5) 125I-labeled ARH cell-CAM 105 are included.

Fig. 8. Pulse-chase analysis. 1-5, cell-CAM 105 immunoprecipitated from detergent lysates of ARH cells labeled in culture with 32PO4 (/-5) for 6 and 14 h (1 and 2), or pulsed for 2 h, (3) and chased for 4 (4) or 12 h (5). 6-10, show ARH cell-CAM 105 immunoprecipitated from lysates of ARH labeled with both [35]Smethionine and [35]Scysteine for 2, 4, or 12 h (6, 9, and 10) or pulsed for 2 h and chased for 2 (7) or 10 h (8) followed by 2-D PAGE and fluorography. 5, 2-month exposure. Other panels: 2-week exposure.

was also consistent with the changes in pl and size produced by neuraminidase digestion. The shift following digestion to a pl characteristic of untreated THC molecules suggested that the more acidic pl exhibited by ARH molecules resulted at least in part from a higher degree of sialylation. However, differential sialylation alone could not explain the smaller size of the neuraminidase-digested ARH cell-CAM 105 relative to its undigested THC counterpart. Additional alterations which counteracted the decrease in size (increase in mobility) associated with a lower sialic acid content also had to be present on THC cell-CAM 105. One possibility is suggested by the work of Yamashita et al. (19). These investigators have reported that the oligosaccharides present on γ-glutamyl transpeptidase purified from AH 66 hepatoma cells were very different from those of the rat liver enzyme. Tumor cell oligosaccharides in general had less sialic acid and were enriched with bisect N-acetylglucosamine residues (N-acetylglucosamine residues linked to the C-4 position of the β-mannosyl residue in the trimanosyl core). Since the enzyme responsible for addition of these residues is apparently not present in normal liver, it was suggested that its induction may be a transformation related event.

The additional increase in pi produced by neuraminidase digestion of THC cell-CAM 105 further suggested that the sialic acid residues on ARH and THC cell-CAM 105 differ in their susceptibility to neuraminidase. Given the large number of oligosaccharide chains present on cell-CAM 105, it would take only a few residual sialic acid residues to produce a pl that was significantly more acidic than that of a fully desialylated THC molecule. Alternatively, there may be additional acidic saccharide residues on ARH molecules which are not removed by neuraminidase. Although more detailed structural analysis could decide between these alternatives, such studies will be limited by the sensitivity of available techniques and the low level of expression on THC cells.

Even after extended treatment with a preparation of endoglycosidase F specified to be essentially free of N-glycanase activity, we observed a decrease of only 2000–3000 in apparent molecular weight. This finding coupled with the 45% decrease in molecular weight following N-glycanase digestion and the total resistance to digestion with endoglycosidase-D or endoglycosidase-H indicated that, with the exception of 1–2 endoglycosidase-F sensitive bi-antennary complex chains (assuming an average molecular weight of 2000–3000 per chain), essentially all the oligosaccharides present on both THC 1682c and ARH cell-CAM 105 are in the form of complex triantennary or tetraantennary chains. This conclusion is based on the resistance of tri- and tetra-antennary complex chains and some hybrid structures with bisecting N-acetyl glucosamine residues to endoglycosidase-F (18) and on the low activity of endoglycosidase-D and endoglycosidase-H with complex sialylated oligosaccharide chains (20). Partial digestion with N-glycanase (Fig. 3A) produced a stair step pattern of seven distinct spots on two-dimensional gels, demonstrating the presence of a minimum of six oligosaccharide chains on ARH cell-CAM 105. Partially digested THC cell-CAM 105 was not sufficiently well resolved to distinguish intermediate forms. However the identical decrease (45%) in apparent molecular weight following N-glycanase digestion indicated that the THC and ARH forms of this molecule were glycosylated to similar extents. The retention of the differences between the molecular weight of upper and lower forms on both ARH and THC further suggested that the differential in the mobility of the two forms of this molecule resulted from actual differences in the size of the peptide moieties rather than alterations in size or mobility related to variation in glycosylation.

A common structural property of a number of different cell adhesion molecules is a greatly increased sensitivity to proteolytic degradation in the absence of calcium. Indeed, this property is the basis for calcium-dependent cell adhesion mechanisms (21–23). It was of interest, therefore, to determine if cell-CAM 105 also showed an increased sensitivity to trypsin digestion in the absence of calcium. Our results indicate that both ARH and THC cell-CAM 105 are highly resistant to digestion with 0.01% trypsin in the presence or absence of calcium, the only change being the apparent loss of the upper form. It seems unlikely that this loss is the result of a more rapid conversion to low molecular weight peptides which no longer react with anti-gp105-2 antiserum. With the exception of the peptide domain represented by the extra peptide in the V-8 maps of the upper form, the bulk of the peptide chain on the upper and lower forms has a close similarly in sensitivity to protease digestion (Figs. 6, 7, and 8). On the basis of these findings and...
the identity observed between 2-D peptide maps prepared from untreated and trypsin treated lower form (data not shown), the more likely mechanism is a rapid degradation of the peptide domain unique to the upper form thereby producing an antibody reactive, trypsin-resistant proteolytic fragment indistinguishable by 2-D PAGE from the endogenous lower form.

When ARH and THC were cultured in the presence of $^{32}$PO$_4$ for 4–6 h, cell-CAM 105 from both ARH and THC was rapidly labeled by $^{32}$P, indicating that cell-CAM 105 exists in a phosphorylated form, an observation also made by Odin et al. (4).

More importantly, our results further demonstrated that after a 2-h pulse, more than 90% of the $^{32}$P-labeled cell-CAM 105 is present as the upper form. This was in contrast to radioiodinated or $^{[35]}$S)methionine (2-h pulse) labeled cell-CAM 105 which is present predominantly (greater than 60%) as the lower form. After 4 h of chase, there were reciprocal changes in the $^{32}$P-labeled lower and upper forms suggesting some conversion of upper to lower form had taken place. This conclusion was also supported by the 8-fold increase in the ratio of lower to upper form after 12 h of chase. Chase for 12 h also led to a large decrease (99%) in the $^{32}$P-labeled upper form without a corresponding increase in lower form. Indeed, the $^{32}$P-labeled lower form appeared to decrease to a level similar to that observed before initiating chase with cold $^{32}$PO$_4$.

In contrast to the rapid turnover exhibited by the phosphorylated population of cell-CAM 105, the $^{35}$S-labeled population showed only a 30% decrease after 10 h of chase. A similar rate of turnover was also observed for the radioiodinated cell-surface localized cell-CAM 105. There was no apparent chase of labeled upper onto lower form; rather, both appeared to decrease at the same rate. Taken together with results from $^{32}$P-labeling studies, these observations indicate that only a small portion of the $^{35}$S-labeled molecules are phosphorylated.

Comparison of V-8 peptide maps prepared from $^{32}$P-labeled molecules revealed differences in the phosphorylation of the upper and lower forms from ARH. The close similarity between the $^{32}$P-labeled V-8 peptides from ARH upper form and those from the combined THC 1682c forms suggests that most of the $^{32}$P-labeled THC cell-CAM 105 produced following a 14 h incubation in $^{32}$PO$_4$-containing medium is present as the upper form. This is in contrast to the $^{32}$P-labeled population of ARH cell-CAM 105 which contains comparable amounts of upper and lower forms.

In summary, our results show that the altered mobility of cell-CAM 105 from THC cells results at least in part from differences in glycosylation. Although the exact nature of these differences and their functional significance will require a detailed structural analysis beyond the scope of the present studies, it seems unlikely that these alterations result from some general defect or change in glycosylation systems since as we have previously shown (2), other THC cell surface glycoproteins display a mobility on two-dimensional gels identical to their counterpart on normal hepatocytes. Our results also suggest that phosphorylation and proteolytic conversions from upper to lower form are important posttranslational modifications which modulate the expression and/or function of cell-CAM 105 on ARH. Determining whether similar modifications also play a role in the decrease or loss of cell-CAM 105 on THC cells or whether the altered expression on THC cells results primarily from changes at the transcriptional level will be the subject of future investigations.

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Comparison of the Structural Characteristics of Cell-CAM 105 from Hepatocytes with Those of an Altered Form Expressed by Rat Transplantable Hepatocellular Carcinomas

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