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Adhesive properties of carcinoembryonic antigen glycoforms expressed in glycosylation-deficient Chinese hamster ovary cell lines✪

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Carcinoembryonic antigen (CEA) is an oncofoetal cell surface glycoprotein that serves as an important tumour marker for colorectal and some other carcinomas. Its immunoglobulin-like structure places CEA within the immunoglobulin superfamily. CEA functions in several biological roles including homotypic and heterotypic (with other CEA family members) cell adhesion. Cell–cell interaction can be modulated by different factors, e.g., post-translational modifications such as glycosylation. The purpose of this study was to examine whether changes in carbohydrate composition of CEA oligosaccharides can influence homotypic (CEA–CEA) interactions. In order to modulate glycosylation of CEA we used two different glycosylation mutants of Chinese hamster ovary (CHO) cells, Lec2 and Lec8. Lec2 cells should produce CEA with nonsialylated N-glycans, while Lec8 cells should yield more truncated sugar structures than Lec2. Parental CHO (Pro5) cells and the glycosylation deficient mutants were stably transfected with CEA cDNA. All three CEA glycoforms, tested in a solid-phase cell adhesion assay, showed an ability to mediate CEA-dependent cell adhesion, and no qualitative differences in the adhesion between the glycoforms were observed. Thus, it may be assumed that carbohydrates do not play a role in homotypic adhesion, and the interactions between CEA molecules depend solely on the polypeptide structure.

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Abbreviations: CEA, carcinoembryonic antigen; CHO cells, Chinese hamster ovary cells; $PBS^{(-)}$, phosphate-buffered saline lacking Ca^{+2} and Mg^{+2}.

Carcinoembryonic antigen (CEA) is an oncofoetal glycoprotein present in normal foetal gastrointestinal tissues and in some tumours of epithelial origin (Gold & Freedman, 1965; Chu *et al.*, 1972; Fritsche & Mach, 1977; Nap *et al.*, 1988; Jothy *et al.*, 1993). The elevated levels observed in colon, lung, breast and ovarian adenocarcinomas in comparison with normal adult tissues suggest that CEA could play an important role in cancer progression and embryogenesis (von Kleist *et al.*, 1986; Wagner *et al.*, 1992; Eidelman *et al.*, 1993; Jessup *et al.*, 1999; Krop-Watorek *et al.*, 1999).

The CEA molecule has an immunoglobulin (Ig)-like structure composed of a single N-terminal IgV-like domain, and six internal (A1B1, A2B2, A3B3), IgC2 type-like domains (Beauchemin *et al.*, 1987; Oikawa *et al.*, 1987a; Schrewe *et al.*, 1990). This places CEA among members of the immunoglobulin supergene family (IgSF) (Oikawa *et al.*, 1987b; Zimmermann *et al.*, 1987). Moreover, CEA is a cell surface molecule (Hefta *et al.*, 1988; Takami *et al.*, 1988) and functions *in vitro* as a homophilic (CEA–CEA) and heterophilic (CEA–CEA family members), calcium-independent, cell adhesion molecule (Benchimol *et al.*, 1989; Oikawa *et al.*, 1991; Zhou *et al.*, 1993).

CEA is a glycoprotein more extensively glycosylated than other IgSF members. Its molecular mass of about 180 kDa in 50–60% is attributed to carbohydrates (Coligan *et al.*, 1976; Hammarström *et al.*, 1975; Chandrasekaran *et al.*, 1983). The amino-acid sequence of CEA contains 28 potential N-glycosylation sites (Paxton *et al.*, 1987), most of which are occupied by oligosaccharide chains; CEA N-glycans are mostly tetraantennary complex chains, accompanied by mono-, di- and triantennary oligosaccharide structures, and by approx. 10% of high-mannose chains. The antennae of complex chains are composed primarily of repeating (-3Galβ1-4GlcNAcβ1-) units. Some of the N-glycans are sialylated on the nonreducing terminal galactose residues and/or contain sulfate groups (Yamashita *et al.*, 1987). In CEA preparations of various origins considerable differences in the carbohydrate composition and oligosaccharide structures have been shown (Chandrasekaran *et al.*, 1983; Garcia *et al.*, 1991; Fukushima *et al.*, 1995). However, the biological significance of this heterogeneity remains unknown.

The oligosaccharide chains of many cell surface glycoproteins are directly involved in recognition phenomena, including intracellular targeting, interactions with other cells and circulating proteins, reactions with anti-carbohydrate antibodies and binding of micro-organisms (Boehm *et al.*, 1996; Feizi, 1994; Fieger *et al.*, 2000; Wyss *et al.*, 1995; Rudd *et al.*, 1999). Moreover, glycosylation may modulate interactions dependent on the polypeptide chain. For example, a neural cell adhesion molecule has been reported to show enhanced adhesion with decreased polysialylation (Hoffman & Edelman, 1983) and the intercellular adhesion molecule 1 with a truncated carbohydrate structure shows increased adhesion to the Mac-1 integrin (Diamond *et al.*, 1991). It is known that defined fragments of the CEA polypeptide chain are involved in CEA–CEA interaction (Zhou *et al.*, 1993; Boehm *et al.*, 1996; Taheri *et al.*, 2000). However, the question remains whether modified CEA oligosaccharide chains may affect the CEA-related homotypic adhesion.

In order to study the role of glycosylation it is useful to compare the same protein at different glycosylation levels. One approach to obtain stable glycosylation variants is to express the protein in cells with defined glycosylation defects. The aim of this report is to examine whether changes in glycosylation of CEA affect its homophilic cell adhesion. To this end, using a solid phase cell adhesion assay, we tested stably expressed CEA in wild-type Chinese hamster ovary (CHO) cells (C5 and Pro5) and in glycosylation-defective variants of CHO cells (Lec2 and Lec8). The Lec2 and Lec8 mutants are unable to transport CMP-sialic acid and UDP-galactose, respectively, into the Golgi compartment

(Deutscher *et al.*, 1984; Deutscher & Hirschberg, 1986). Therefore, these cells should produce CEA glycoforms with nonsialylated (Lec2), or more truncated (Lec8) N-glycans.

To date, we are aware of only one other attempt to study the role of oligosaccharide chains in CEA homotypic cell adhesion (Charbonneau & Stanners, 1999). These authors concluded that carbohydrates do not determine specificity of CEA adhesion, but taking into account the unusually high degree of glycosylation, they do not exclude modification of the strength of the adhesion. However, these conclusions were based on a cell aggregration assay in solution, while in our studies we used a solid-phase cell adhesion assay, which in our opinion is more accurate and better reflects the natural conditions occuring in tissues.

MATERIALS AND METHODS

Cell lines and cell culture conditions. Wild-type Chinese hamster ovary (CHO) cells (Pro5) and the glycosylation-defective mutants (Lec2 and Lec8) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.); C5 and CEA-expressing C5 cells, C5/CEA, were from Dr. Shinzo Oikawa (Institute for Biomedical Research, Suntory Ltd., Osaka, Japan). CHO cells were cultured in alpha-Minimal Essential Medium (α-MEM) supplemented with 10% foetal calf serum (FCS) (Gibco-BRL, Grand Island, NY, USA), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Irvine, Great Britain). The cell cultures were maintained at 37°C in a humidified atmosphere at 5% CO_2 in air.

Transfection procedure and positive clone selection. For construction of stable transfectants, CHO Pro5, Lec2 and Lec8 cells (1×10^6) were co-transfected with 20 μg of the eukaryotic expression vector pSG5CEA and 2 μg of the pSV2neo plasmid by the DNA-calcium phosphate precipitation method (Graham & Van der Eb, 1973) using the Cell Phect Transfection Kit (Pharmacia, Sweden). The full-length cDNA of CEA was isolated from the pdKCR-neo plasmid, kindly provided by Dr. Shinzo Oikawa (Institute for Biomedical Research, Suntory Ltd., Osaka, Japan) (Oikawa *et al.*, 1987a). Control cells were transfected with 2 μg of the pSV2 neomycin-resistance plasmid only. After 48 h the medium was replaced with fresh complete α-MEM medium containing 0.5 mg/ml of geneticin G418 (Gibco-BRL, Paisley, Scotland). The medium was replaced approximately every 2 days for 14 days. The neomycin-resistant colonies were isolated, cloned by limiting dilution, expanded, and screened for CEA expression by the fluorescence activated cell sorter assay and Western blotting.

Fluorescence activated cell sorter (FACS) assay. Cells were detached (from culture dish) with 0.2% EDTA in Hanks' balanced salt solution, pH 7.4 and washed twice in a Tris/BSA (bovine serum albumin) buffer (50 mM Tris/HCl, 0.1% BSA, 150 mM NaCl, pH 7.8). Tris/BSA buffer at 4°C was used in all subsequent cell treatments as follows: cells (0.5×10^6) were incubated for 1 h with goat anti-CEA polyclonal antibody (50 μg/ml), washed three times, and incubated for an additional hour with fluorescein-isothiocyanate-conjugated rabbit $F(ab')_2$ fragment of anti-goat Ig antibodies (1:100, Becton-Dickinson, San Jose, CA, U.S.A.). Labelled cells were washed, resuspended in 0.5 ml Tris/BSA buffer and the intensity of fluorescence was measured using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, U.S.A.) using WinMDI 2.0.4 software for data processing. Five thousand cells were acquired for each data file. Dead cells were detected by low forward and right angle scatter and excluded from the analysis.

Electrophoresis and Western blotting. Untransfected and CEA-expressing cells (2×10^6) were solubilized in a lysis buffer (50 mM Tris/HCl, pH 8.0, containing 0.5% NP-40,

150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl-fluoride, 2 μg/ml aprotinin and 2 μg/ml leupeptin) (Sigma, St. Louis, MO, U.S.A.), vigorously mixed by Vortex and after 15 min incubation on ice centrifuged at 20 000 r.p.m. for 10 min. The supernatants were analysed for protein concentration by the method of Lowry (Lowry *et al.*, 1951) with bovine serum albumin as a standard. Samples containing 50 μg of protein were separated by SDS/PAGE (Laemmli, 1970) using a 7.5% gel. The electrophoretically separated proteins were transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany) (Towbin *et al.*, 1979). The blots were blocked for 1 h with 1% casein in Tris-buffered saline (TBS; 50 mM Tris/HCl, 200 mM NaCl, pH 7.5) and overlaid subsequently with polyclonal rabbit anti-CEA antibodies (1:500, Dako, Glostrup, Denmark) and with goat anti-rabbit immunoglobulins conjugated with phosphatase (1:500, Bio-Rad, Hercules, CA, U.S.A.). Following a 1 h incubation at room temperature, the blots were developed with phosphatase substrates: BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) in 0.1 M AMP buffer (2-amino-2-methyl-1,3-propandiol) (Sigma, St. Louis, MO, U.S.A.) with 5 mM $MgCl_2$, pH 9.5. The following prestained electrophoretic standards (Bio-Rad, Hercules, CA, U.S.A.) were used: myosin, 194 kDa; β-galactosidase, 116 kDa; bovine serum albumin, 85 kDa; ovalbumin, 48 kDa.

Solid-phase cell adhesion assay. The cells for isotopic labelling were cultured in a 10 cm tissue culture dish (Falcon, Lincoln Park, NJ, U.S.A.), washed with α-MEM and labelled with $Na_2{}^{51}CrO_4$ (Centre of Isotopic Research, Polatom, Świerk, Poland) (100 μCi/1.5 ml α-MEM) for 2 h at 37°C, washed once with medium and cultured in fresh α-MEM for an additional hour. The ^{51}Cr-labelled cells were then washed twice with phosphate-buffered saline lacking Ca^{+2} and Mg^{+2} (PBS $^{(-)}$; 0.01 M NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.4), detached with 0.2% EDTA in PBS$^{(-)}$, pelleted and resuspended in 3 ml α-MEM by three passes through a 26-gauge needle and were added in 0.1 ml aliquots/well to a monolayer of unlabelled cells cultured in 0.5 ml of α-MEM in 24-well tissue culture plates (Falcon, Lincoln Park, NJ, U.S.A.). The cells were allowed to adhere for 1 h at 37°C (5% CO_2) and nonadherent cells were washed off twice with PBS$^{(-)}$. The adherent cells were lysed in 1% NP-40 and the radioactivity was measured in a gamma-counter (Beckman Gamma-5500B, Palo Alto, CA, U.S.A.). Three independent cell adhesion assays were carried out in triplicate.

RESULTS

Characterization of CEA expressed in wild type cells and the glycosylation mutants

CEA was stably expressed in the wild type (Pro5) CHO cells and the glycosylation-defective mutants (Lec2 and Lec8) transfected with recombinant CEA cDNA. (The C5 CHO cells expressing CEA were used for comparison). The presence of CEA on the cell surface was evaluated by cytofluorimetric analysis using goat anti-CEA polyclonal antibodies. Subclones with the highest CEA expression (C5/CEA, Pro5/CEA 4.24, Lec2/CEA 12.14 and Lec8/CEA 11.1) were selected for further studies (Fig. 1). (For simplicity the numbers of the selected subclones are omitted in the text and in the figures). It had previously been shown that the Lec2 cells are defective in sialylation, while in the Lec8 cells galactosylation is inhibited (Deutscher *et al.*, 1984; Deutscher & Hirschberg, 1986). Thus, CEA expressed in these glycosylation mutants should contain truncated, instead of fully glycosylated, N-glycans (Fig. 2). Taking into account that over 50% of the CEA molecule consists of carbohydrates, the molecular masses of the CEA glycoforms expressed in Lec2 and Lec8 cells should be decreased. This was confirmed by SDS/PAGE and Western blotting analysis (Fig. 3). Untransfected control cells did not expressed CEA while all

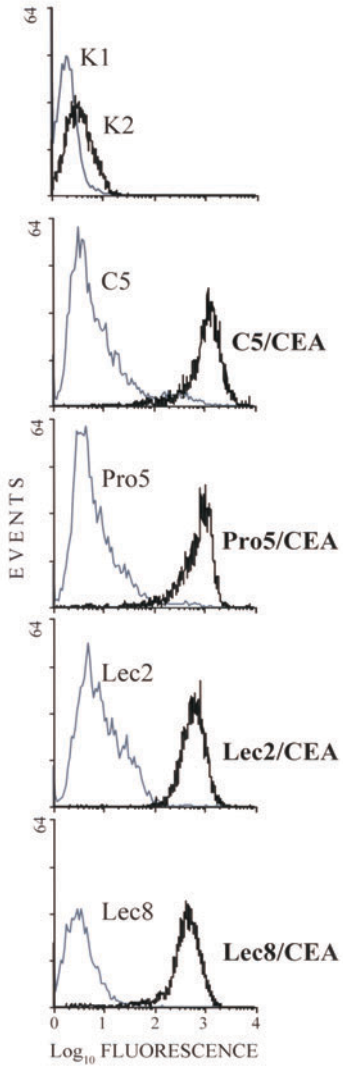

Figure 1. Flow cytofluorometric profiles of untransfected cells (C5, Pro5, Lec2 and Lec8) and their CEA cDNA transfectants.

K1 and K2 represent control cells treated respectively: with Tris/BSA buffer or with second antibody only. The cells (0.5×10^6) were incubated as described in Materials and Methods. The fluorescence intensity of the labelled cells was measured using a FACScan flow cytometer. Five thousand cells were acquired for each data file. The results shown are representative of four independent experiments.

types of transfected cells showed two major bands, as it is usually observed in CEA preparations obtained from different sources. This heterogeneity presumably results from the presence of high-mannose and less branched oligosaccharides instead of mature complex-type chains. The slowest-migrating bands of C5/CEA and Pro5/CEA cells, most likely representing the fully glycosylated CEA, corresponded to a molecular mass of about 180 kDa, similar to that of CEA molecules isolated from the liver metastasis of colon adenocarcinoma (not shown). As expected, the CEA glycoforms synthesized by transfected Lec2 and Lec8 cells migrated faster as about 160 kDa and about 140 kDa molecules, respectively. Thus, the diminished molecular mass of CEA in Lec2/CEA and Lec8/CEA cells suggests that CEA synthesized in these glycosylation-defective mutants is underglycosylated.

Adhesive properties of CHO cells expressing fully glycosylated and underglycosylated CEA

To examine whether incomplete glycosylation of CEA may influence its adhesive properties, a solid phase cell adhesion assay was performed (Fig. 4). All CEA transfectants tested (C5/CEA, Pro5/CEA, Lec2/CEA and Lec8/CEA) were able to adhere (50–70%) to monolayers of all types of transfected cells (A). The adhesion levels of control cells, i.e., untransfected cells, to the monolayers of untransfected cells were below 30% (not shown). However, the adhesion levels of CEA-transfected cells to untransfected cells varied from 5% to 35% (B, CEA-independent adhesion); the real CEA-dependent adhesion levels (C) were obtained by subtracting these values (B) from total adhesion of CEA transfectants (A). Higher adhesion of the C5/CEA cells to all monolayers, compared with the adhesion of the remaining cell lines, was the most readily observable difference. Since the C5/CEA and Pro5/CEA cells expressed similarly glycosylated CEA (Fig. 3), the observed divergence of their adhesive properties may result from higher CEA expression in C5/CEA cells (Fig. 1). It is noteworthy that the CEA-unrelated adhesion of CEA transfectants to nontransfected cell monolayers was distinctly higher if at least one of the cell-types in the cell–cell interaction was Lec2 or Lec8

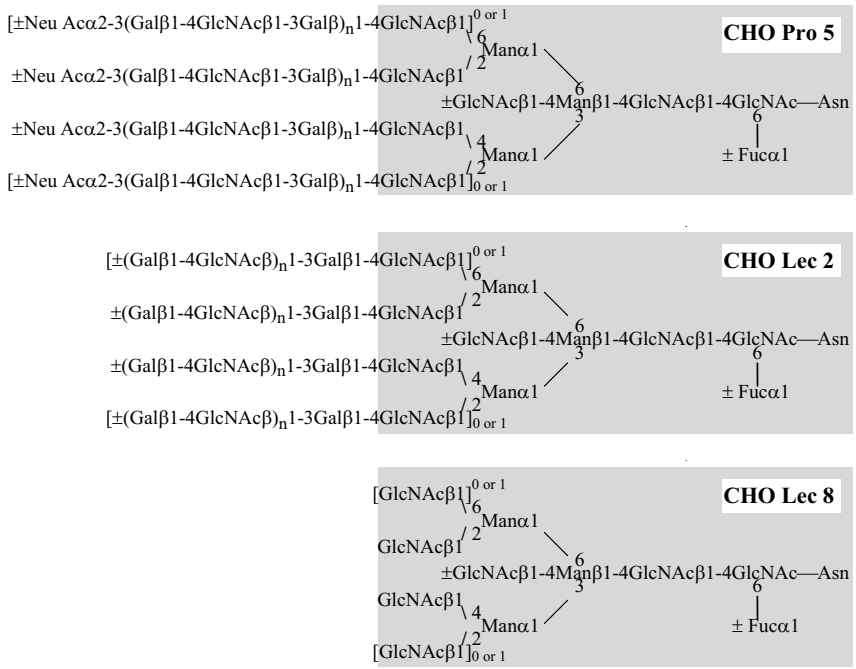

Figure 2. Putative structures of CEA complex N-glycans expressed in wild type CHO Pro5 and glycosylation-defective Lec2 and Lec8 cells.

The structures are based on data obtained with CEA from liver metastases of colorectal carcinoma (Yamashita *et al.*, 1987). ± Denotes that the residue may be either present or absent in this position. Shadowed boxes show the common oligosaccharide structure expressed on CHO Pro5, Lec2 and Lec8 cells.

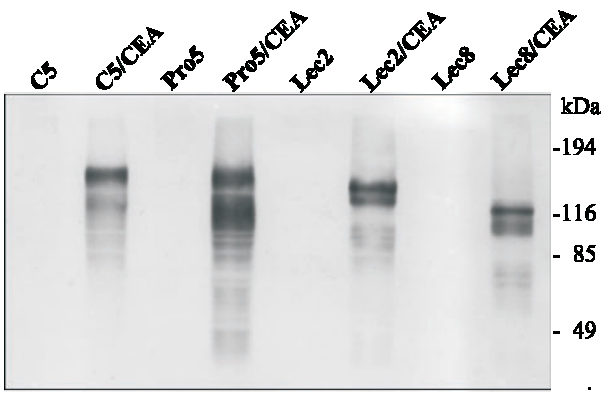

Figure 3. Immunoblot analysis of CEA expression in untransfected and CEA cDNA transfected CHO cells.

Cell lysates were fractionated by SDS/PAGE and immunoblotted as described in Materials and Methods. The apparent molecular mass of the protein standards are indicated on the right.

(Fig. 4B). Thus, it seems that truncated CEA oligosaccharide chains cause an increase of unspecific cell–cell interaction, while specific (i.e., CEA-related) cell adhesion remains unchanged. These data allow the conclusion to be drawn that sialic acid or galactose residues present on the CEA molecule have no influence on CEA-related homotypic cell adhesion.

DISCUSSION

CEA is a highly abundant cell surface glycoprotein mainly expressed on colonic epithelial cells in early embryonic and carcinoma stages. It functions as a calcium independent, homophilic cell adhesion molecule. Since sugar residues of some glycoproteins are involved in adhesion events (Hoffman & Edelman, 1983; Diamond *et al.*, 1991; Ono *et al.*, 2000; Horstkorte *et al.*, 2001) glycosylation of CEA could have important consequences in embryo- and carcinogenesis. The CEA molecule is heavily glycosylated (Yamashita *et al.*, 1987) and it was shown that carbohydrate

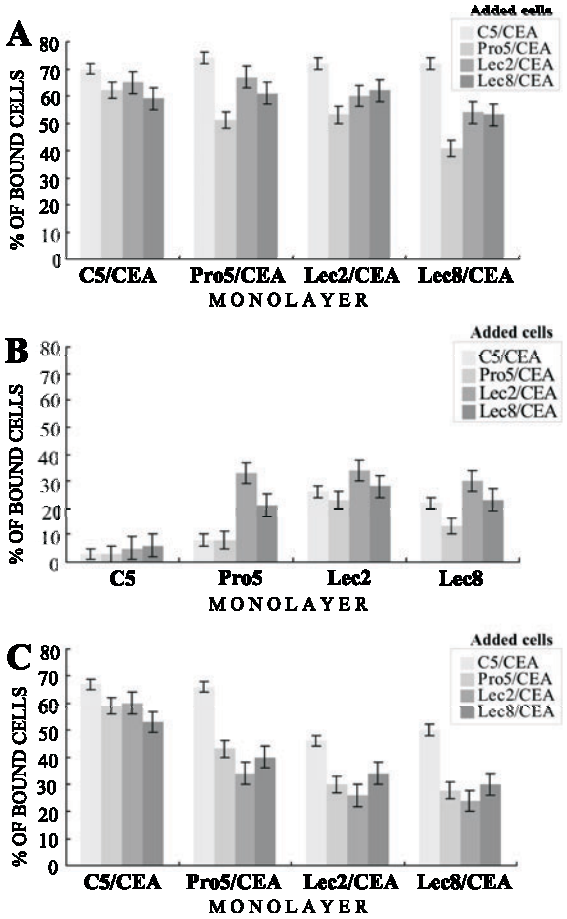

Figure 4. Solid-phase cell adhesion assay of fully glycosylated (C5 and Pro5) and underglycosylated (Lec2 and Lec8) CHO cells transfected with CEA cDNA.

(A) Adhesion of CEA transfectants to CEA transfectants monolayers, (B) adhesion of CEA transfectants to untransfected cells monolayers, (C) the difference between the adhesion of CEA transfectants to the monolayers of transfected (A) and untransfected cells (B). The adhesion assay was performed as described in Materials and Methods. Data represent the means of three replicates of three independent experiments. Standard deviation from triplicate assays are indicated at the top of each bar.

moieties of tumour CEA and its counterparts produced by colonic epithelial cells of normal adults (NFA-2) or foetuses (NCA-2) revealed differences induced by development and malignant transformation of normal epithelial cells (Yamashita *et al.*, 1989; Wojciechowicz *et al.*, 2000).

To study a possible role of carbohydrate in CEA–CEA interaction, we prepared stable transfectants expressing CEA molecules in CHO glycosylation-defective mutant cell lines, Lec2 and Lec8. The expected deficiencies in glycosylation of CEA molecules synthesized in these cells were confirmed by a decrease in the molecular size of CEA in Lec2 and Lec8 cells (Fig. 3). The adhesive properties of CEA-expressing fully glycosylated and underglycosylated CHO cells were evaluated by a solid-phase cell adhesion assay. It was found that all tested types of cells expressing CEA glycoforms adhered to monolayers of CEA-expressing transfectants irrespective of their carbohydrate composition. It is noteworthy that CEA-unrelated adhesion of the fully glycosylated cells C5 and Pro5 was distinctly lower than adhesion of the mutant cells Lec2 and Lec8. This may suggest that truncated oligosaccharide chains promote an increase of CEA-unrelated adhesion. However, when CEA was present on the surface of both interacting cells, adhesion levels of all the cell lines tested were similar. In addition, in the mutant cell lines Lec2 and Lec8, other cell surface glycoproteins may also be underglycosylated. This may be a reason of increased "unspecific" interaction between these cells. Thus, these results allow us to conclude that the terminal sialic acid and Galβ1-4GlcNAcβ1-3 (Fig. 2) are not involved in homophilic CEA dependent cell adhesion.

Charbonneau and Stanners (1999) used CHO cells (wild type LR-73, parental Pro5 and its glycosylation mutants Lec2 and Lec8) transfected with a functional cDNA of CEA. In addition, Lec1 cells, which are able to express only oligo-mannose chains, were also transfected. The adhesive properties of these cells were tested by an aggregation assay of cells in suspension and it was demonstrated, in agreement with our results, that all CEA glycoforms are able to mediate cell adhesion. While the Lec1 and Lec2 transfectants showed an increased speed and final extent of aggregation, the Lec8 transfectants showed weaker aggre-

gation than that of wild type transfectants. It is difficult to find a simple explanation why the Lec8 and Lec1 CEA transfectants, both carrying neutral N-glycans of similar size, differ in adhesion.

Modulation of the binding activity of a polypeptide chain by glycosylation occurs even if the carbohydrates do not participate directly in the interaction. The effects of N-glycans on glycoprotein biological functions cannot be generalized, as each glycoprotein, or even each active site, represents an individual case. Glycosylation may be required for the proper exposure or conformation of peptidic epitopes. A decrease of polypeptide reactivity by oligosaccharide chains may be caused not only by masking of the active sites, but also by alterations of the peptide conformation or flexibility. For example, it was shown that apparent "masking" of the antigenic epitope of the influenza virus hemagglutinin is independent of N-glycan size. However, reactivity of the antibodies was observed only after complete removal of the carbohydrate by digestion with peptide-N-glycosidase F (Munk *et al.*, 1992). In the case of CEA, the hydrophobic interaction between CEA N-domains (Kaplan *et al.*, 1998) may be diminished or altered as a result of the presence of oligosaccharides. It is known that recombinant, nonglycosylated CEA N-domain expressed in *Escherichia coli* forms high molecular-mass oligomers in aqueous solutions (Krop-Watorek *et al.*, 1998). Thus, it cannot be ruled out that complete deglycosylation would give a strong increase of CEA–CEA interaction, while truncation of N-glycans gives only limited (Charbonneau & Stanners, 1999) or negligible (our results) quantitative alterations in this interaction.

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