The GA733-2 antigen (GA733) is a homotypic calcium-independent cell adhesion molecule (CAM) present in most normal human epithelial cells and gastrointestinal carcinomas. Because oligomerization of some CAMs regulates cell adhesion and signal transduction, the correlation between GA733 oligomeric state and cell-cell adhesion was investigated. Sedimentation equilibrium studies showed that full-length (-FL) GA733 exists as dimers and tetramers in solution, whereas the GA733 extracellular domain (-EC) is a monomer. The $K_d$ of GA733-FL is less than 10 nM for the monomer-dimer association, whereas the dimer-tetramer association is about 1000-fold weaker ($K_d \approx 10 \mu M$). Chemical cross-linking of purified GA733-FL in solution resulted in a major product corresponding to GA733 dimers, and minor amounts of trimers and tetramers. However, GA733-EC cross-linked under the same conditions was consistently a monomer. Chemical cross-linking of dissociated colon carcinoma cells produced predominantly GA733 dimers, whereas cross-linking of cells in monolayers yielded some tetramers as well. GA733-FL retained its cell-cell adhesion function as shown by inhibition of cell aggregation, whereas monomeric GA733-EC was inactive. These data show that GA733 exists predominantly as high affinity noncovalent cis-dimers in solution and on dissociated colon carcinoma cells. The lower affinity association of dimers to form tetramers is most likely the head-to-head interaction between GA733 cis-dimers on opposing cells that represents its cell-cell adhesion activity.

Cell-cell adhesion is a dynamic process essential for the normal development and function of multicellular organisms and is known to play an important role in tumor progression and metastasis. The GA733-2 (Ep-CAM/EGP40/17-1A antigen/KSA/ESA) cell surface glycoprotein was initially defined by the CO17-1A monoclonal antibody (mAb)$^1$ (1) and GA733 mAb (2).

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The abbreviations used are: mAb, monoclonal antibody; CAM, cell adhesion molecule; BS$_2$, bis(sulfosuccinimidyl) suberate; $C_{12}E_8$, octaethylene glycol dodecyl ether; $C_{16}E_2$, pentaethylene glycol octyl ether, DSG, disuccinimidyl glutarate; EC, extracellular domain; FL, full-length; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; mEGP, mouse epithelial glycoprotein; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; pAb, polyclonal antibody; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; FACs, fluorescence-activated cell sorting; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

More recently, it was shown to function as a Ca$^{2+}$-independent homotypic cell adhesion molecule (CAM) (3). However, it appears to belong to a novel class of CAMs distinct, by its structural features, from the four known superfamilies of CAMs (immunoglobulin-type, selectins, cadherins, and integrins) (for review see Refs. 4 and 5). The sequence of GA733-2 indicates no significant homology with other known proteins except with GA733-1 (Trop-2), previously cloned by Linnenbach et al. (6). The amino acid sequences of GA733-2 and GA733-1 are ~50% identical.

GA733-2 (referred to hereafter as GA733) is synthesized as a 32.8-kDa nonglycosylated precursor, but migrates in SDS-PAGE gels as a 40-kDa band. The extracellular portion of GA733 contains three potential N-linked glycosylation sites and consists of a cysteine-rich N-terminal region followed by a cysteine-poor region. GA733 has a short transmembrane domain and a cytoplasmic tail of 26 amino acids with potential sites of phosphorylation on tyrosine and serine residues.

GA733 is present in most epithelial cells and is overexpressed in the vast majority of gastrointestinal tumors (7–10). Moreover, emerging evidence correlates the increase of GA733 expression with other types of cancer lesions, notably in cervical squamous epithelia (11). The increase of cell proliferation in cervical squamous epithelia, which are normally GA733-negative, correlates with the expression of GA733. An increase of the number of positive cells as well as in levels of GA733 protein was observed during progression of cervical lesions from cervical intraepithelial neoplasia grade I to grade III (11).

The significance of increased GA733 expression in cancer lesions remains mysterious. Surprisingly, a recent study from our group showed that GA733 inhibits invasion of tumor cells in vitro when transfected into CT-26 mouse colon carcinoma cells (12). In the same study, the mouse homologue of GA733, mEGP (a homotypic CAM with 82% amino acid sequence identity with GA733) expressed by transfected mouse CT-26 cells (that are mEGP-negative) significantly decreases cell growth in vitro and inhibits metastasis in vivo. Although the mechanism(s) responsible for these two phenomena remain unclear, the strong intercellular adhesion profile of CT-26-mEGP cells (clumping and clustering observed in vitro) may inhibit cell

Oligomeric State of the Colon Carcinoma-associated Glycoprotein GA733-2 (Ep-CAM/EGP40) and Its Role in GA733-mediated Homotypic Cell-Cell Adhesion*
dispersion and limit their migration capacity (12). The same mechanism was proposed to explain decreased metastases in nude mice by human breast carcinoma cells overproducing another homotypic adhesion molecule, E-cadherin (13). Alternatively, the decrease of cell growth may be due to the activation of certain mEGP-associated signal transduction pathways that down-regulate cell proliferation, when activated by increased homotypic engagement of mEGP. Although very little is known about the biochemical properties of GA733 and its murine homologue, mEGP, other than their role as homotypic CAMs, it is likely that they participate in “inside-out” and “outside-in” cell signaling pathways controlling cell growth and adhesion. This hypothesis is supported by a recent report showing that GA733-1 (Trop-2) is a calcium signal transducer (14).

Several clinical trials targeting GA733 by anti-idiotypic antibodies and recombinant protein vaccines have shown induction of humoral and cellular immunity in colorectal cancer patients (15–20). More importantly, CO17-1A mAb against GA733 enhances survival of patients with colon carcinoma in a phase II randomized control study (21, 22). Furthermore, a recombinant adenovirus expressing GA733 significantly enhanced survival of mice bearing established CT-26-GA733 tumors (23). The inhibitory effect of GA733 on cancer cell growth and invasion, its increased expression in human cancer lesions, and the beneficial treatment with mAbs against GA733 in experimental animals and patients appear to be paradoxical. Clearly, further characterization of the biochemical and functional properties of this protein and its role in tumor progression is needed.

Recent studies have suggested that, in addition to mediating homotypic associations between opposing cell membranes, cis interactions between some types of receptors on the same cell surface can play an important role in regulating cell adhesion, signal transduction, and migration (24). For example, it has been shown that C-CAM (25), PECAM-1 (26), and ICAM-1 (27, 28) exist as cis-dimers in the cell membrane. Furthermore, dimeric forms of recombinant soluble ICAM-1 have significantly higher affinities for its purified ligand, LFA-1 (CD11a/CD18), whereas the monomeric form shows no binding (27, 28), illustrating the central role CAM oligomerization can play in cell-cell adhesion.

The molecular organization of GA733 within normal epithelia and colon carcinoma cells remains unknown, despite its use with some success as a target for passive and active immunotherapy of colon cancer in human clinical trials (15–22). Elucidating the cis-oligomerization of GA733 on the cell membrane and the mechanism of GA733-mediated cell adhesion should contribute to a better understanding of GA733 function and may lead to substantial improvements of GA733 targeting in immunotherapy of colon cancer.

In this study, we investigated the oligomeric state of membrane-associated and soluble forms of GA733 and their ability to block cell-cell adhesion. We show that GA733-FL, which inhibits cell aggregation, self-associates to form high affinity cis-dimers in solution and in intact colon carcinoma cell membranes, whereas the soluble recombinant extracellular domain (GA733-EC) is a monomer and does not inhibit cell aggregation.

MATERIALS AND METHODS

Reagents and Antibodies—Chemicals were from Sigma Chemical Co. (St. Louis, MO). The membrane-impermeable bis(sulfosuccinimidyl) suberate (BS3) and membrane-permeable disuccinimidyl glutarate (DSG) cross-linkers were purchased from Pierce (Rockford, IL). GA733 mAb, which recognizes the extracellular segment of GA733 (2), was produced and affinity-purified using a protein A-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) as described previously (29). GA733 polyclonal antibody (GA733 pAb) was obtained from rabbits immunized with GA733-EC and purified on a Protein A-Sepharose column.

Protein Purification—Recombinant GA733-FL and GA733-EC were produced in insect cells using the baculovirus system as described previously (30). GA733-FL protein concentrations were determined by spectrophotometry at 280 nm in a 1-cm path length using an extinction coefficient of 0.926 × 10^3 g/ml cm^-1 calculated from the GA733-EC sequence according to Pace et al. (33). HPLC Gel Filtration—For analytical ultracentrifugation experiments, recombinant proteins were further purified by HPLC gel filtration to remove trace contaminants and aggregates. GA733-EC was concentrated as described previously, injected into two TSK columns, G3000SWXL and G2000SWXL (Toso-Haas, Japan), connected in series, and separated at 0.8 m/min using PBS. GA733-FL was concentrated in PBS containing 0.5 mM octylxylene dodecyl ether (C12E10) and 10 mM pentaerythrole octyl ether (C8E10) (Sigma). GA733-FL protein concentrations were determined using the molar absorption coefficient of 9.3 × 10^5 M^-1 cm^-1.

Mass Spectrometry—Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was carried out on a Voyager DE-PRO mass spectrometer (PE Biosystems, Framingham, MA). Recombinant protein solutions (2 mM) were mixed 1:1 with a saturated solution of sinapinic acid in 33% acetonitrile and 0.1% triethylammonium acetate (TEAA) containing 0.15 mM PMSF and 0.5 mM C12E8. The concentration of GA733-EC was determined by spectrophotometry at 280 nm in a 1-cm path length using an extinction coefficient of 0.926 × 10^3 g/ml cm^-1 calculated from the GA733-EC sequence according to Pace et al. (33).

HPLC Gel Filtration—For analytical ultracentrifugation experiments, recombinant proteins were further purified by HPLC gel filtration to remove trace contaminants and aggregates. GA733-EC was concentrated as described previously, injected into two TSK columns, G3000SWXL and G2000SWXL (Toso-Haas, Japan), connected in series, and separated at 0.8 m/min using PBS. GA733-FL was concentrated in PBS containing 0.5 mM C12E10 and 10 mM pentaerythrole octyl ether (C8E10) (Sigma). GA733-FL protein concentrations were determined using the molar absorption coefficient of 9.3 × 10^5 M^-1 cm^-1.

Mass Spectrometry—Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was carried out on a Voyager DE-PRO mass spectrometer (PE Biosystems, Framingham, MA). Recombinant protein solutions (2 mM) were mixed 1:1 with a saturated solution of sinapinic acid in 33% acetonitrile and 0.1% trifluoroacetic acid and allowed to air dry on the sample target before analysis. Several 2-fold serial dilutions of the protein samples were tested, and protein A was used as an external and internal standard.

Analytical Ultracentrifugation—Experiments were performed in an Optima XL-I analytical centrifuge. Sedimentation equilibrium runs were performed with three initial concentrations of the protein sample. Absorbance or fringe displacement data was collected every 4–6 h until equilibrium was reached, as determined by comparison of successive
Sedimentation equilibrium experiments were performed using either the interference optics (for GA733-EC) or the absorbance optics (for GA733-FL) to measure the protein concentration gradient. For experiments with GA733-EC, three cells were assembled with double-sector 12-mm centerpieces and sapphire windows. The cells were loaded with 110 μl of HPLC gel filtration buffer as the reference, and 110 μl of sample at concentrations of 2.0, 1.0, or 0.5 mg/ml. A blank scan of distilled water was taken before the run, to correct for the effects of window distortion on the fringe displacement data. Experiments were performed at 4 °C or 30 °C, at 23,000–30,000 rpm. Fringe displacement data was collected every 4–6 h until equilibrium was reached.

Sedimentation equilibrium experiments on GA733-FL were performed in the presence of either C8E5 or C12E8 nonionic detergents. For experiments using C8E5, affinity-purified GA733-FL was concentrated in PBS containing 0.15 mM PMSF and 10 mM C8E5, and dialyzed against the same buffer for 24 h at 4 °C. For experiments in the presence of C12E8, GA733-FL was chromatographed using HPLC gel filtration as described above. Fractions containing purified GA733-FL were then concentrated to 0.1–0.4 mg/ml and dialyzed for at least 24 h at 4 °C against PBS containing 0.15 mM PMSF and 0.5 mM C12E8 in 22% D2O (v/v). In the presence of detergent, the protein is part of a protein-detergent complex that has a buoyant molecular mass, MDet(1 – ρd) containing contributions from the protein and the bound detergent (35, 36).

\[ M_{Det}(1 - \rho_d) = M_p(1 - \rho_p) + \delta_{Det}(1 - \rho_{Det}) \]  
(Eq. 1)

where \(M_p\) is the molecular weight of the protein, \(\rho_p\) and \(\rho_{Det}\) are the partial specific volumes of the protein and detergent respectively, \(\delta_{Det}\) is the number of grams of detergent bound per gram of protein, and \(\rho\) is the density of the solvent. Because the partial specific volume of C12E8 is known (0.973 cm^3/g (37)), the density of the solvent can be adjusted to 1/\(\rho_{Det}\) with D2O. In this case the second term of the equation equals zero, effectively removing the contribution of the bound detergent. The density of the dialysis buffer described above was calculated to be 1.0278 g/cm^3 at 20 °C using the SEDNTERP program. For experiments according to Schaegger and Von Jagow (39).

Western blotting was performed after electroblotting proteins to polyvinylidene difluoride Immobilon-P membranes (Millipore, Bedford, MA), using a Trans-Blot cell (Bio-Rad, Hercules, CA) at 80 mA for 2 h, as described by Mozdzanowski et al. (40). Membranes were blocked overnight with 5% nonfat dry milk and probed either with GA733 mAb or GA733 pAb, followed by an appropriate secondary antibody and detection was performed using either the 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (Promega, Madison, WI) or ECL chemiluminescence reagents (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Cell Aggregation Assay—Cells were detached either with EDTA containing 1% BSA (PBS-B) were incubated for 20 min on ice with 20 μg of GA733 mAb or GA733 pAb, followed by an appropriate secondary antibody and FITC (Calbiochem, San Diego, CA) was added to the cells for another 20 min on ice (dilution 1:40). Excess antibody was removed by washing three times with PBS-B. Cells were analyzed on an EPICS XL flow cytometer (Coulter Corp., Hialeah, FL) using forward scatter and side scatter to exclude dead cells. For the negative control condition, the cells were labeled with the secondary antibody-FITC only. Data analysis was performed using the WinMDI software available on the web from the Scripps Institute.
RESULTS

Purification and Characterization of Recombinant GA733-EC and GA733-FL—Recombinant proteins were expressed and purified as described under “Materials and Methods”. Both recombinant proteins exhibited moderate covalent cross-linking to dimers and higher oligomers during expression in insect cells and affinity purification, presumably resulting from transglutaminases in the insect cells. Fig. 1A shows a Coomassie Blue stained gel of GA733-EC after immunoaffinity chromatography and after HPLC gel filtration. The retention time of GA733-EC (MW = 28 kDa) in the HPLC column falls between those of protein standards with molecular weights of 44 and 17 kDa (Fig. 2A), suggesting that GA733-EC is possibly a monomer. After HPLC gel filtration, the minor amount of cross-linked dimers was efficiently separated from the uncross-linked GA733-EC as shown in Fig. 1A.

Affinity purification of GA733-FL was more problematic and several procedures gave poor yields and resulted in more extensive cross-linking of the protein (up to 50% as determined by SDS-PAGE) with the presence of several low and high molecular weight contaminants. Extensive washes of protein bound to the mAb column using buffer with 0.05% Triton X-100 substantially reduced the amount of most contaminants. However, a 220-kDa band, identified as myosin, was difficult to eliminate totally (Fig. 1B). The amount of cross-linked protein could be reduced to about 5% (Fig. 1B) by using 100 mM Tris buffer with 10 mM EDTA, 10 mM EGTA throughout the affinity purification as described under “Materials and Methods”. HPLC gel filtration of GA733-FL in the presence of 0.5 mM C12E5 lead to a high protein recovery (Fig. 1B) compared with the use of other detergents (10 mM C12E5, 0.1% Triton X-100, 0.1% Tween 20, 1% n-octyl glucoside) (data not shown). As shown in Fig. 1B, myosin and minor low molecular weight contaminants were efficiently separated from GA733-FL on the HPLC gel filtration column, whereas cross-linked and uncross-linked GA733-FL coeluted.

MALDI mass analysis of GA733-EC after HPLC gel filtration chromatography is shown in Fig. 3A. Two broad peaks were observed, with average molecular masses of about 28,331 and 29,348 Da. Since the sequence molecular weight of the extracellular domain is 27,372 Da after removal of the signal peptide, these results suggest that the molecule is heterogeneously glycosylated, resulting in two major populations with about 960 and 1,977 Da carbohydrate, with substantial mass heterogeneity within these peaks as reflected by their shape. Attempts to obtain GA733-FL mass spectra with affinity-purified, detergent-free protein or protein after HPLC gel filtration in 0.5 mM C12E5 were not successful. The data shown in Fig. 3B was obtained using affinity-purified protein concentrated in the presence of 10 mM C12E5 and shows two peaks with average masses of 34,291 and 35,962 Da. Comparison of these masses with the expected sequence mass for GA733-FL (32,675 Da) suggests heterogeneous glycosylation, which contributes ~1,616 and 3,287 Da to the mass, although some of this mass difference might be contributed by tightly bound detergent or lipid molecules, or other uncharacterized post-translational modifications.

Sedimentation Equilibrium Analyses of GA733 Recombinant Proteins—To systematically evaluate the oligomeric state of GA733-EC, sedimentation equilibrium experiments were performed at 4 °C and 30 °C, and the concentration versus radius data was fitted with various models using nonlinear regression (38). At both temperatures, the data were described well by a single ideal species model; representative 30 °C data is shown in Fig. 4A. The estimated molecular mass obtained by the fitting program was 28,705 Da, in excellent agreement with the average molecular mass of the two-glycosylated species observed using mass spectrometry (28, 839 Da). The data clearly demonstrates that the extracellular domain is monomeric up to a concentration of at least 6 mg/ml.

Sedimentation equilibrium analyses of GA733-FL were initially performed on samples in the presence of 10 mM C12E5. This detergent is a useful alternative to C12E8 for sedimentation equilibrium as it has a partial specific volume of 0.993 cm3/g (43), eliminating the need for density correction of the solvent with D2O (44). Affinity purified samples could not be further purified by HPLC gel filtration due to poor sample recovery in this detergent, and therefore the affinity purified protein was used directly for sedimentation equilibrium analyses. Table I summarizes the analyses of GA733-FL using different conditions. Various models were used to fit the sedimentation equilibrium data. For all six experiments performed in C12E5, the data was fitted well with models where the predominant species was a dimer. However, in four experiments there was also a small proportion of a large molecular weight species (n = 8 to 40) that was not in chemical equilibrium with the dimer and apparently represented irreversible aggregates. The presence of this large species interfered with unambiguous identification of other species that may have been present. However, in one experiment with no detectable aggregate, the data was fitted well by a reversible monomer-dimer-tetramer model, with estimated Kd’s of 98 nM for the monomer-dimer association and 49 μM for the dimer-tetramer association.

In the presence of 0.5 mM C12E5, the small proportion of aggregates and minor contaminants in the affinity-purified GA733-FL could be removed by HPLC gel filtration. In contrast to the experiments on the affinity purified protein, three sedimentation equilibrium experiments with GA733-FL further purified by HPLC gel filtration, consistently fitted well to models containing only dimer and tetramer species, as shown in Fig. 4B and 4C. Models that included monomer or oligomers other than tetramer (e.g., trimer, hexamer, octamer) gave significantly poorer fits as judged by the size of the variance and randomness of the residuals. When the six data sets shown in Fig. 4B and 4C (representing three different loading concentrations of protein and two rotor speeds) were fitted simultaneously, the data was fitted well by a model describing a reversible dimer-tetramer association, with an estimated Kd of 10 μM. When the data sets were fitted individually, allowing a
different equilibrium constant for each set, the randomness of the residuals improved slightly, and $K_d$'s ranged between 5 m$M$ and 14 m$M$, but the improvement in the variance was not significant at a 67% confidence level. Thus the data is consistent with a predominantly reversible association of GA733-FL dimers to tetramers with a $K_d = 10$ m$M$. Two other experiments on different preparations of GA733-FL were also fitted well by the same model and returned a $K_d$ for the dimer-tetramer association of $\approx 7$ m$M$. The slight improvement in the fit with individual equilibrium constants suggests that a very small amount of one or more species is unable to associate or dissociate. This is most likely due to the small proportion of covalently cross-linked dimer present in most samples of GA733-FL. The association of GA733-FL monomers to dimers appears to be very strong, with no detectable dissociation observed in all samples in $C_{12}E_{10}$, and very little dissociation observed in $C_{8}E_{5}$ with $K_d = 98$ nM. The absence of detectable monomer in the $C_{12}E_{10}$ experiments is consistent with a $K_d < 10$ nM for the monomer-dimer association. The slightly weaker association in $C_{8}E_{5}$ ($K_d = 98$ nM) could be a detergent effect, but is more likely due to the presence of contaminating low molecular weight proteins in these less pure samples.

**Chemical Cross-linking of GA733 Recombinant Proteins in Solution**—The oligomeric states of GA733 recombinant proteins, GA733-EC and GA733-FL, in the presence of various detergents were evaluated using chemical cross-linking. Fig. 5 shows a representative cross-linking experiment of GA733 recombinant proteins in the presence of 0.5% Triton X-100. GA733-FL was affinity-purified and eluted in the presence of 0.5% Triton X-100, as described under “Materials and Methods”, to minimize artifactual cross-linking during purification. Triton X-100 was added to the GA733-EC preparations after purification to have consistent conditions for both proteins. Both membrane permeable (DSG) and membrane impermeable (BS3) cross-linkers were evaluated (see intact cell experiment below) and an optimal concentration of 0.1 mM was determined in preliminary experiments. As seen in Fig. 5, cross-linking of GA733-FL produces predominantly dimers with additional bands at the trimers and tetramers positions. On the other hand, no cross-linking of GA733-EC was observed under similar conditions, confirming that it is a monomer in both the presence and absence of detergents. Similar results to those shown for Triton X-100 were obtained using BS3 and DSG in the presence of other detergents (0.1% Triton X-100, 0.5 mM $C_{12}E_{10}$, 10 mM $C_{8}E_{5}$, 0.1% Tween-20, 0.5% CHAPS, 1% n-octyl glucoside), as well as with the thiol-cleavable cross-linkers (0.1
and 0.2 mM dithiobis [succinimidyl propionate], 0.1 and 0.2 mM 3, 3'-dithiobis [sulfosuccinimidylpropionate]) (data not shown).

**GA733 Oligomeric State in Intact Colon Carcinoma Cells**—To characterize the molecular organization of GA733 in intact cell membranes, chemical cross-linkers (BS3 and DSG) were added to colon carcinoma cells either on monolayers or in single cell suspension. Two human colon carcinoma cell lines expressing high levels of GA733 were evaluated: 1) a homogeneous clone of Caco-2 epithelial cells (C2BBe1; passages 45–76), which forms tight and polarized monolayers, with an apical brush border morphologically comparable to that of the human colon (45, 46) and; 2) the colorectal adenocarcinoma cell line Colo-205 (47), which is characterized by reduced cell-cell and cell-substrate adhesion. When cultured in tissue culture flasks, Colo-205 cells exhibit a minimally adherent morphology, form small aggregates, and a substantial proportion of the cells grows in suspension.

Treatment of intact Caco-2 cells with the membrane-impermeable BS3 cross-linker at different concentrations, followed by protein extraction, immunoprecipitation, and Western blots (Fig. 6, A and B) resulted in appearance of multiple cross-linked species with a major band at ~80 kDa in GA733 mAb immunoprecipitates. When Caco-2 cells in monolayer are treated with high cross-linker concentrations (Fig. 6A), a 160-kDa band corresponding to GA733 tetramers is observed. When single cell suspensions are cross-linked using the same conditions (Fig. 6B), a strong dimer band is still observed but the tetramer band is substantially reduced. Quantification of tetramer/dimer ratios using densitometry showed a ratio of 0.4 versus 0.1 for cells in monolayer and single cell suspensions, respectively, when 0.5 mM BS3 was used. Similarly, tetramer/dimer ratios were 0.8 versus 0.2 at 2 mM BS3 for cells in monolayer and single cell suspension, respectively. The non-cross-linked controls (Cont) only showed the monomeric GA733 band (Fig. 6). Similar cross-linking results were obtained for cells in monolayers and cell suspensions using the membrane-permeable DSG cross-linker (data not shown), suggesting that GA733 was not cross-linked to cytoplasmic proteins under these conditions.

Similarly, Colo-205 colon carcinoma cells were cross-linked

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**Fig. 3.** Mass spectra of GA733 recombinant proteins. MALDI-TOF mass analysis of GA733-EC after HPLC gel filtration (A) and affinity-purified GA733-FL concentrated in 10 mM C8E5 (B). For both proteins, two major species are observed, consistent with heterogeneous glycosylation.
FIG. 4. Representative sedimentation equilibrium experiments of GA733-EC and GA733-FL. Sedimentation equilibrium data for three loading concentrations of GA733-EC (A) at 23,000 rpm and 30 °C. The three data sets were fitted simultaneously using the nonlinear regression program NONLIN as described under "Materials and Methods." The data were fitted with a model describing monomers. The raw data (circles) and the global fit of an ideal single species model (lines) are shown. In the upper panels, the residuals of the fitted curve to the data points for the three protein concentrations, from highest to lowest (top to bottom) are shown. Sedimentation equilibrium data of GA733-FL at 14,000 rpm (B) and 19,000 rpm (C), at 20 °C. The six data sets were globally fitted with a model describing high affinity dimers with no detectable dissociation to monomers and much weaker self-association to tetramers. The lower panels show the concentration versus radius data for three loading concentrations of GA733-FL (circles). The solid lines represent the calculated fit. The upper panels show the residuals of the fitted curves for the data points for the three protein concentrations, from highest to lowest (top to bottom).

TABLE I
Sedimentation equilibrium experiments performed with GA733-FL in the presence of C_{8}E_{8} and C_{12}E_{8}.

| Run | HPLC | Detergent | Speed (x 10^{3} rpm) | Best model | K_{d} |
|-----|------|-----------|----------------------|------------|------|
| 1   | No   | 10 mM C_{8}E_{8} | 23                   | M→D + agg. (n = 20) | 228 |
| 2   | No   | 10 mM C_{8}E_{8} | 20                   | M→D→T     | 98  |
| 3   | No   | 10 mM C_{8}E_{8} | 17                   | D→T + agg. (n = 20) | 49  |
| 4   | No   | 10 mM C_{8}E_{8} | 17                   | D + agg. (n = 9) | 21  |
| 5   | No   | 10 mM C_{8}E_{8} | 15                   | D→T + agg. (n = 16) | 10  |
| 6   | No   | 10 mM C_{8}E_{8} | 15/21                | D→T + agg. (n = 8) | 6   |
| 7   | Yes  | 0.5 mM C_{12}E_{8} | 14/19               | D→T       | 7   |
| 8   | Yes  | 0.5 mM C_{12}E_{8} | 14/19               | D→T       | 10  |
| 9   | Yes  | 0.5 mM C_{12}E_{8} | 19                   | D→T       | 24  |

*Abbreviations used: M, monomer; D, dimer; T, tetramer; agg., aggregates; ↔, reversible equilibrium.

either in situ, in a tissue culture flask (Fig. 6C) or in single cell suspensions (Fig. 6D). Addition of BS^{3} to nondetached cells resulted in a decrease in the intensity of the GA733 monomer band in a concentration-dependent manner and the appearance of one major band with the molecular weight of a GA733 dimer (Fig. 6C). GA733 pAb also recognized minor bands with approximate molecular sizes of 120 and 160 kDa, presumably corresponding to GA733 trimers and tetramers. Compared with adherent Caco-2 cells, the ratio of tetramers to dimers for Colo-205 was very low, i.e. 0.07 at 0.5 mM BS^{3} and 0.12 at 2 mM BS^{3}. When single cell suspensions were cross-linked using the same conditions, dimers but not tetramers were observed (Fig. 6D). Cross-linking was also performed using DSG at the same concentrations and produced similar results both in detached and nondetached cells (data not shown).

GA733 in polarized monolayers of Caco-2 was less extensively cross-linked compared with Colo-205 (compare Fig. 6A with 6C), even when high concentrations of cross-linkers were used. The decreased cross-linking efficiency on Caco-2 cells is likely due to reduced accessibility of the reagent to GA733 protein complexes in these cells. GA733 is known to be predominantly present at the cell-cell boundaries of epithelial cells and carcinoma cells (lateral membranes) and absent from the exposed apical surface (3, 48, 49), which is consistent with the observed reduced accessibility of the cross-linkers to GA733 molecules in Caco-2 cells compared with Colo-205 cells in these experiments.

Trypsin and EDTA Treatments Have No Effect on GA733 Expression at the Surface of Colon Carcinoma Cells—Before testing the ability of GA733 recombinant proteins to inhibit cell-cell aggregation, we evaluated whether EDTA or trypsin/EDTA treatments had any effect on GA733 expression at the cell surface. Nose et al. (50) showed that trypsin/EDTA treatment, but not trypsin/CaCl_{2} treatment affected the membrane expression of cadherins, which were temporarily removed from the cell surface most likely by internalization. Furthermore, Litvinov et al. (3) reported that GA733 was internalized in the absence of calcium on some human mammary carcinoma cells.
To test whether total cellular GA733 levels were affected by trypsin and/or EDTA treatments, single cell suspensions of Caco-2 cells (dissociated with trypsin/EDTA) and Colo-205 cells (dissociated with EDTA alone) were lysed as described under “Materials and Methods” and GA733 cellular protein levels were compared with the corresponding cell line lysed in monolayers (without dissociation) by Western blot using GA733 mAb. As shown in Fig. 7A, quantitative Western blot reveals a single major GA733 band at the expected molecular mass (~40 kDa), the intensity of which is not appreciably affected by trypsin/EDTA (Caco-2) or EDTA (Colo-205) treatments.

To further confirm that the GA733 molecules detected by Western blots are on the surface of detached colon carcinoma cell lines, flow cytometry analysis using GA733 mAb was performed. Both dissociated Caco-2 and Colo-205 cells were highly and homogeneously positive for GA733 expression (Fig. 7B).

GA733-FL Inhibits Cell Aggregation, Whereas GA733-EC Has No Effect—Recent work from our group showed that GA733-FL was active and GA733-EC was inactive in solid-phase binding assays, where the recombinant protein was immobilized on nitrocellulose-coated plates (12). Although its validity was previously demonstrated for GA733 (12) and other adhesion molecules (51), the solid-phase binding assay is not fully representative of adhesion of two living cells. Furthermore, a truncation mutant of GA733 lacking the 26-amino acid cytoplasmic tail was not able to mediate aggregation when transfected into L cells, although adhesion of this transfectant to solid-phase-adsorbed GA733 remained unaffected (48).

Therefore, cell-cell aggregation assays were performed to further confirm the biological activity of the GA733 recombinant proteins analyzed above. Specifically, the inhibitory effect...
of GA733 recombinant proteins on cell-cell aggregation was correlated with their oligomeric states using Caco-2 and Colo-205 cells. Cell-cell aggregation is observed as early as 30 min, and the size of the cell aggregates increases progressively with time. An optimal aggregation time of 2 h was chosen for counting particles and was used for most experiments.

The photographs in Fig. 8A, show representative fields of Caco-2 cell aggregation under different conditions. In the absence of added protein, the cells were extensively aggregated after 2 h (Fig. 8A, b). Aggregation was greatly reduced in the presence of GA733-FL (c) but was unaffected by addition of GA733-EC (d). Similar results were observed when Colo-205 cells were tested (data not shown). As shown in Fig. 8B, addition of affinity purified GA733-FL to the cells inhibits aggregation, whereas GA733-EC has no effect, indicating that dimers, but not monomers, are capable of blocking cell-cell adhesion. Fig. 8C shows the concentration-dependent effects of GA733-FL and GA733-EC on the inhibition of Caco-2 cell aggregation. Even at concentrations of ~1 μM, GA733-EC does not significantly inhibit cell aggregation, whereas 50% inhibition by GA733-FL occurred at less than 0.5 μM. Cell-cell adhesion is a complex and poorly understood process involving multiple CAM systems. In addition, other proteins that may interact with GA733, including cytoskeletal proteins, may affect GA733-mediated cell-cell adhesion in vivo. Nonetheless, the inhibition of cell-cell adhesion by GA733-FL in the low μM range as shown in Fig. 8C is consistent with the Kd ~10 μM measured for the dimer-tetramer association using the analytical ultracentrifuge.

DISCUSSION

Intercellular interactions mediated by cell surface CAMs are known to be involved in a wide variety of dynamic processes, including cell movement, proliferation, and differentiation. The control of these different processes plays critical roles in embryogenesis, wound healing, maintenance of normal tissue morphogenesis, and tumor progression. GA733 is a transmembrane glycoprotein that mediates homotypic Ca2+-independent cell-cell adhesion. GA733 targeting has shown promising results in the immunotherapy of colon cancer (21, 22), and recent data demonstrated an inhibitory effect of GA733 on tumor invasion in vitro (12). Yet, the precise function of GA733-mediated cell-cell adhesion, its role in normal epithelial cells, and its involvement in colon cancer are poorly understood.

In the present study, we explored the oligomeric state of GA733 and the mechanism of GA733-mediated cell-cell adhesion. Our data show: (i) recombinant GA733-EC is a monomer in solution and is inactive in cell-cell aggregation assays; (ii) recombinant GA733-FL forms high affinity dimers (Kd < 10 nm) and moderate affinity tetramers (Kd ~ 10 μM) in solution and exhibits inhibitory activity in cell aggregation assays in the low micromolar range; and (iii) GA733 in human colon carcinoma cells exists primarily as noncovalent cis-dimers in single cell suspensions. These results demonstrate that GA733 dimerization is essential for GA733-mediated cell-cell adhesion. The monomeric nature of GA733-EC indicates that the cytoplasmic and/or the transmembrane domain of GA733 are needed for this cis-dimer formation. Further studies with truncated recombinant GA733 proteins will be needed to map the specific regions of these domains responsible for controlling dimer formation.

The extent of cross-linking to dimers, in both Caco-2 and Colo-205 colon carcinoma cells, was similar for adherent monolayers versus single cell suspensions. This indicates that GA733 dimerization occurs by cis-interactions within the membranes of individual cells, rather than trans-interactions between GA733 monomers present in two different cells. Other CAMs exhibit similar interactions, e.g. the carcinoembryonic antigen was found to form noncovalent dimers (52), and there are a growing number of other CAMs that require dimer formation for ligand binding. Dimerization of PECAM-1, E-cadherin, VCAM-1, and ICAM-1 seems to be important for their adhesion function and may represent an important regulatory mechanism of signal transduction pathways (26–28, 53, 54). The small amount of GA733 tetramers detected when Caco-2 cells were cross-linked in monolayers are most likely due to the head-to-head association between GA733 dimers on opposing cells, because GA733 is a homotypic CAM (see model in Fig. 9).

The dimer-tetramer association is a moderate affinity interaction (Kd ~ 10 μM), which is consistent with reversible intercellular adhesion.

Minor bands at the trimer and tetramer position are observed when Caco-2 cells are cross-linked in suspension, which could result from the fast cell-cell aggregation kinetics of Caco-2 cells during cross-linking. Alternatively, these bands could represent minor cross-linking of GA733 to other proteins.

The failure to detect large amounts of GA733 tetramers in Colo-205 colon carcinoma cells cross-linked while attached to the culture flask is not surprising. Despite their normal expression of E-cadherin and catenins as well as GA733, Colo-205 cells do not form monolayers in culture, but grow as small aggregates or dispersed cells with minimal cell-cell contacts, and hence the majority of the surface of individual cells is not engaged in adhesion with other cells. Furthermore, these cells exhibit poor adhesion to extracellular matrix proteins, do not form tight interactions with each other, and dissociate easily in PBS during the cross-linking experiments (data not shown).

Other minor bands are also detected at different positions after cross-linking of Caco-2 and Colo-205 cells, especially a band between the monomer and the dimer at ~60 kDa. This band may be the result of one of the following: (i) gel shift of GA733 due to internal cross-linking and side chain modifications; (ii) coincidental random cross-linking with other proteins due to the high density of GA733 at the surface of colon carcinoma cells (about 10⁶ copies of GA733 per cell (55)); or (iii) specific interactions with other membrane proteins.

The oligomerization of cell surface receptors plays a central role in the regulation of cellular functions by modulating signal transduction pathways. GA733-1 (Trop-2) that shares 50% amino acid identity with GA733 was shown to be involved in cell signaling. A recent study (14) showed that monoclonal antibodies against GA733-1 induce calcium fluxes in MCF-7 and OvCa-432 cells. Another study (56) demonstrated that GA733-1 is phosphorylated on serine 303, and that protein kinase C is involved in phosphorylating this protein in vitro. The exact mechanism by which homotypic GA733 engagement might activate signal transduction pathways in epithelial cells and the nature of these pathways remain intriguing questions for future studies.

The inhibitory effects of GA733 expression on growth and invasion of transfected mouse tumor cells are surprising in view of the beneficial effects of mAbs against GA733 for treating colorectal carcinoma patients. It is tempting to speculate on how these apparently conflicting observations might be reconciled. In addition to tumor cell elimination by the immune system, mainly via the antibody-dependent cellular cytotoxicity pathway, mAbs to GA733 in clinical therapy may have two other beneficial effects: (i) inhibit cancer cell dissociation from the primary tumor by increasing cell-cell association; (ii) decrease cell growth by mimicking GA733 self-ligation and activating signal transduction pathways leading to inhibition of cell proliferation and/or induction of apoptosis. We are currently generating a soluble fusion protein, a covalent GA733...
dimer containing the GA733-EC fused to an Fc fragment of an immunoglobulin (EC-Fc), to further study the role of dimerization in homotypic cell-cell adhesion and signal transduction pathways mediated by GA733.

In addition to colon cancer, an increase of GA733 expression was observed in cervical cancer lesions (11) and a very recent paper reported increased GA733 levels in lung cancer (57). Understanding the precise function of GA733 in normal epithelial cells and its role in the biology of tumors should contribute to the development of new strategies for cancer treatment or at minimum substantially improve the existing GA733-based therapy of colorectal tumors.

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