Isolation, Partial Characterization, and Molecular Cloning of a Human Colon Adenocarcinoma Cell-surface Glycoprotein Recognized by the C215 Mouse Monoclonal Antibody*

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The monoclonal antibody C215 (IgG2a) was obtained by the immunization of BALB/c mice with the human colon adenocarcinoma cell line COLO 205 and used in the targeting of colorectal carcinomas. The partial characterization and purification of the C215 target molecule from solubilized COLO 205 membranes indicated that it is an integral membrane glycoprotein of the non-mucin type. The denatured antigen appeared as a major 40-kDa form in Western blots after SDS-polyacrylamide gel electrophoresis and migrated as a monomeric 36-kDa species after the reductive cleavage of intramolecular disulfide bridges. Using a five-step procedure, the antigen was purified 4,300-fold from COLO 205 tumors raised in nude mice to a homogeneity of 95% when assessed by capillary electrophoresis. Removal of N-linked carbohydrate by peptide:N-glycosidase treatment did not affect the visualization of the purified antigen in immunoblots but resulted in a faster migration in the SDS gels. The amino acid sequence was partially determined. Seventeen contiguous NH₂-terminal amino acids were identified and coincided exactly with residues 96-112 of the GA733-2 protein cloned by Szala et al. (Szala, S., Froehlich, M., Scollon, M., Kasai, Y., Stepienska, Z., Koprowski, H., and Linnenback, A. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3542–3546). Therefore, the predicted amino acid sequence of this protein was used to prepare overlapping synthetic peptides that cover the entire extracellular domain in order to identify the C215 epitope. A likely epitope, close to the NH₂-terminus and corresponding to the first distinct hydrophilic stretch after the putative signal sequence, was identified in a peptide enzyme-linked immunosorbent assay. Moreover, GA733-2 cDNA was used for the cloning of the C215 protein from COLO 205 cells and the subsequent transfection to K36.16 mouse T cell leukemia cells. The transfected cells were C215 reactive in fluorescence-activated cell sorter analysis, and a 42 kDa band was visualized in Western blots under both non-reducing and reducing conditions. Our findings indicate a close relationship between the C215 antigen and other members of the GA-733 family, some of which are currently being used as targets in clinical trials with monoclonal antibodies. The mammalian expression system described here will enable further studies into the biological role of this protein and the construction of animal models in order to develop optimal therapeutic strategies.

Disseminated colorectal adenocarcinoma has been considered suitable as a potential target for immunotherapy with monoclonal antibodies (mAbs) able to recognize cell-surface antigenic structures. We have developed a therapeutic approach where antibody-targeted bacterial superantigens will direct T cell attack on tumors (1). Under ideal conditions, the target molecule should be expressed at high levels of density on the surface of the tumor cells. It should not be easily shed or internalized and should concomitantly have a low degree of expression in normal tissues. By immunizing mice with the human colon adenocarcinoma cell line COLO 205, a panel of mAbs was developed (2); of these the C215 antibody was selected for further studies since its corresponding target molecule met with most of the requirements listed above: (i) it recognizes an integral membrane glycoprotein of the non-mucin type; (ii) when assessed by FACS analysis, the antigen is expressed at high levels in human colon adenocarcinoma cell lines, and (iii) the C215 antibody binds to freshly isolated tumor cells in 80–90% of surgical specimens obtained from human colon adenocarcinomas (3). Preliminary studies have indicated structural and immunochemical similarities between the C215 antigen and a number of carcinoma-associated antigens, such as the antigen defined by the CO17-1A antibody (4-6), the adenocarcinoma-associated antigen KSA (7), and the epithelial carcinoma marker EGP (8, 9) defined by KS 1/4, the GA733 gene family antigens GA733-1 and GA733-2 (10, 11), and the epithelial glycoprotein (EGP) identified by HEA 125 (12). These antigens are expressed to a varying extent in epithelial neoplasms such as gastric, pancreatic, and mammary cancers as well as in lung carcinomas of both the non-small cell and small-cell histopathological type (8, 13), and have been used as targets...
for murine antibody therapy in several clinical trials (14–17). Recently, a significant increase in survival as well as a reduction in the number of distant recurrences was reported in patients with colorectal cancer treated with the mAb CO17-1A, despite the fact that the protein recognized by this antibody is also expressed to a certain extent in normal epithelial cells (13, 14).

The present study aimed to further characterize and isolate the C215 antigen and to establish its relationship to other carcinoma antigens. The partial amino acid sequence of the C215 antigen was determined and revealed complete homology with one of the members of the GA733 gene family, the GA733-2 antigen (11). Therefore, GA733-2 cDNA was used in order to construct an expression vector for transfection into K36.16 mouse T cell leukemia cells. This mammalian expression system for the C215 antigen will help in further elucidating the biological function of this protein and is currently being used in the construction of animal models aimed at studying the localization and anti-tumoral effects of hybrid molecules between the C215 antibody and T cell activating superantigens.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials—**Sephadex G-25 medium, prepackaged disposable PD-10 columns, Protein G-Sepharose Fast Flow, Sepharose-6B, ConA-Sepharose, Mono Q HR 5/5, Mono S HR 5/5, Fast Desalting column HR 10/10, Superose 6-HR 10/30, and the SDS-LMW marker kit were purchased from Pharmacia LKB Biotechnology, Bromma, Sweden. Bio-Gel HTP, Bio-Gel HPHT (7.8 × 100 mm), and the goat anti-mouse IgG AP and horseradish peroxidase immunodetection systems were obtained from Bio-Rad. Fluorescein isothiocyanate (FITC) and pl markers were purchased from Sigma, St. Louis, MO. The SelectiSpher-10 (ConA) HPLC column was obtained from Perstorp Biolytica, Lund, Sweden. Pronase from Streptomyces griseus, pepstatin, phenylmethylsulfonyl fluoride, leupeptin, and glycopeptidase F (PNGase F, from Flavobacterium meningosepticum) were purchased from Boehringer Mannheim Scandinavia AB, Bromma, Sweden. The Rainbow colored protein M, markers, enhanced chemiluminescence (ECL) Western blotting analysis system, and the Hyperfilm-ECL were purchased from Amersham Sweden AB, Solna, Sweden. The fluorochrome-labeled rabbit anti-mouse serum and normal goat serum were obtained from Dakopatts AB, Hägersten, Sweden. Sepharose-6B and Superose 6B and Centriprep-10 devices from Amicon Division, W. R. Grace A.B., Helsingborg, Sweden, were used for concentration. Protein measurements were performed using the detergent-compatible bicinecunic acid protein assay (18) from Pierce Europe B.V., Oud-Beijerland, The Netherlands, with BSA as standard. All chemicals used were of at least analytical quality.

**Human Tumor Cell Line and Production of COLO 205 Tumors in Nude Mice—**The COLO 205 human colon adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in vitro as described previously (2). Colon adenocarcinoma tissue was produced by the subcutaneous implantation of 5 × 10⁶ COLO 205 cells into the flank of 8-week-old female BALB/c nu/nu mice (Bomnholtgard, Ry, Denmark). Tumors were excised after 8 weeks and immediately frozen in liquid nitrogen and stored at −70 °C pending further use.

**Colon Adenocarcinoma Antibodies—**The C215 mAb (IgG2α) was raised against COLO 205 cells and purified by immunofluorescence chromatography from the supernatant as described previously (2, 19). The fluorochrome C215 antibody conjugate was prepared as described Ref. 2. The K5 1/4 (IgG2α) and the CO17-1A (IgG2α) mAbs were kindly supplied by Dr. Ralph A. Reisfeld, The Scripps Research Institute, La Jolla, CA, and Dr. Jan-Erik Frödin, Radiumhemmet, Karolinska Hospital, Stockholm, Sweden.

**Solubilization of Tumors and Cultivated Cells—**The tumor tissue (21 g) was sliced with a scalpel while frozen and then homogenized in 6 × 10⁴ ml ice-cold buffer (0.25 M sucrose, 50 mM Tris-HCl, 10 mM KCl, 1.5 mM MgCl₂, pH 7.4) containing 1% (v/v) Nonidet P-40 and protease inhibitors (i.e. 0.2 mM EDTA, 0.2 μM phenylmeth-yalufonyl fluoride, added from a 0.2 mM stock solution in isopropl alcohol, 1 μM peptatin, and 1 μM leupeptin). The slices were homogenized for 6 × 10⁴ s (with a cooling period of 50 s between each homogenization) with an Ultra-Turrax homogenizer and were left in an ice-bath for 15 min. The solubilized tumors were centrifuged at 105,000 × g for 60 min at 2 °C in a Beckman L5-65 ultracentrifuge, using a Ti-70 rotor at 38,000 revolutions/min (Beckman Instruments, Inc., Palo Alto, CA). The resulting Nonidet P-40 extract was finally clarified by filtering through a 0.2-μm cellulose acetate membrane.

Extracts from cultivated cells were prepared by adding the solubilization buffer to the cell pellet and disintegrating the cells using a Dounce glass homogenizer.

**Chromatographic Procedures—**All chromatographic steps were run at ambient temperature using the FPLC system (Pharmacia LKB) equipped with high performance P-3500 pumps, a Superloop for the application of sample volumes up to 50 ml, and UV monitoring at 280 nm. Gel-exclusion chromatography was performed using Sephrose 6B and Superose 6, with a 50,000 m exclusion range for globular proteins of 1 × 10⁴ → 4 × 10⁴ and 5,000 → 5 × 10⁴, respectively. Ion-exchange chromatography was run on the anion and cation exchangers Mono Q and Mono S. SDS-hydroxylaplatite chromatography was run under denaturing and/or reducing conditions on Bio-Gel HP T or Bio-Gel HPHT according to Moss and Ransbuhl (20). Lectin-affinity chromatography was carried out on ConA-Sepharose 4B or the SelectiSpher-10 (ConA) HPLC column.

**Purification Procedure—**The COLO 205 tumor extract was allowed to pass over a Protein G-Sepharose (1.6 × 2.5 cm) in order to remove endogenous mouse IgGs, which might otherwise interfere with the dot-blot analysis (see below). The non-retained fraction was then chromatographed on Sepharose-6B (5.0 × 85 cm) under non-denaturing conditions, i.e. in a buffer containing 0.1% (v/v) Nonidet P-40. Immunoreactive fractions were pooled and dialyzed against sodium phosphate (10 mM at pH 6.8 or 7.0, respectively) (v/v) Nonidet P-40 and 1% (v/v) SDS. SDS was added to a final concentration of 1% (v/v), the sample was denatured by boiling for 15 min and applied to Bio-Gel HTP (1.6 × 30 cm). Retained SDS-protein complexes were eluted with a linear sodium phosphate gradient from 0.01 to 0.40 M. Fractions pooled from the Bio-Gel HTP step were concentrated and filtered through PD-10 equilibrated in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.0, containing 1 mM CaCl₂, 1 mM MgCl₂, and 0.1% (v/v) Nonidet P-40. This sample was injected into the ConA-HPLC column and retained glycosylated proteins were eluted with a buffer of 0.4 M with respect to α-methyl D-mannoside. The buffer was altered in the methyl mannoside eluate to 10 mM sodium phosphate, pH 6.8, containing 20 mM dithiothreitol and 1% (v/v) SDS, by filtration through the fast desalting column. The sample was reduced by incubation for 40 min at 60 °C and finally chromatographed on the hydroxyapatite-HPLC column by elution with a linear sodium phosphate gradient from 0.01 to 0.40 M in the presence of 0.1% SDS and dithiothreitol.

**Gel Electrophoresis and Western Blotting—**SDS-electrophoresis was run in 12.5% vertical slab gels using the buffer systems of Laemmli (21), isoelectric focusing in PhastSystem (Pharmacia LKB) according to the instructions of the manufacturer. Gels were stained for protein in Amido Black or Coomassie Blue R-250. Transfer to nitrocellulose was performed using the semi-dry electrotransfer described previously by Kyse-Andersen (22) or the PhastTransfer system from Pharmacia LKB. Membranes were blocked for 30 min in 3% (w/v) BSA in Tris-buffered saline (TBS, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.5). Visualization was performed by incubation with the primary antibody overnight and development using either the goat anti-mouse IgG AP immunodetection system or the anti-mouse IgG horseradish peroxidase system together with ECL for increased sensitivity. After the blocking step, 0.05% (v/v) Tween-20 in TBS was used for washing and antibody buffer purposes. In order to reduce nonspecific binding, 1% normal goat serum was added during incubation with the first antibody. The quantitation of developed blots was carried out by scanning at 653 nm in the Ultrascan XL densitometer (Pharmacia LKB).

**Dot-blot Analysis—**C215 reactivity in appropriately diluted fractions from the various chromatographic steps was determined in a dot-blot assay. To test for the sensitivity and optimal concentration of the primary antibody, a serially diluted crude COLO 205 tumor extract was immobilized on nitrocellulose, using the Microfold membrane filtration device from Whatman and Hechter & Schenkman (23). Blocking and immunodetection were performed in the same way as described in the case of Western blotting at four concentrations.
of the primary antibody, 0.2, 1, 2, and 10 μg/ml (Fig. 1).

**Capillary Electrophoresis**—A homogeneity test was performed using capillary electrophoresis on a Beckman P/ACE System 2000 (Beckman Instruments, Inc., Fullerton, CA). The analysis was performed with an uncoated fused-silica capillary (75 μm × 57 cm, 50 cm to the detector) in 20 mM CAPS buffer, pH 11.0. The injections were performed by pressure for 3 s, and the separation was conducted at 25 °C and 20 kV with monitoring at 214 nm.

**Release of N-Linked Carbohydrates**—The samples, dissolved in 25 mM Tris-HCl, 0.2% (w/v) SDS, pH 6.8, were dialyzed 1:1 in 20 mM sodium phosphate, 50 mM EDTA, pH 6.8, and 2-mercaptoethanol and the dialyzed samples were added up to a final concentration of 1 and 0.5% (v/v), respectively. The sample was divided into two equal parts, one incubated for 18 h at 37 °C together with 20 IU/ml of PNGaseF, and the other treated identically but with the enzyme omitted.

**Amino-terminal Sequencing**—Amino-terminal peptide sequencing was performed with an automated solid-phase Prosequencer 6600, equipped with on-line phenylthiodyantoin-amino acid analysis and Maxima data handling system (Millipore Corporation, Milford, MA). A 3-μg sample was immobilized on a diisothiocyanate-membrane (Millipore Corporation) according to Ref. 23, and 20 cycles of sequencing were performed.

**Amino Acid Synthesis**—Peptides were synthesized using t-Boc amino acids (Bechem AG, Buchendorf, Switzerland) and a p-methylbenzhydrylamine resin (Fluka AG, Buchs, Switzerland) according to the multiple solid-phase peptide synthesis method (24). Removal of the protecting groups from the formyl-trypthophane and methionine sulfide residues was achieved by cleavage with 25% hydrogen fluoride (25). The peptides were then cleaved from the resin by treatment with liquid hydrogen fluoride using a multi-vessel apparatus (26). The homogeneity of the peptides was estimated by reversed-phase HPLC on a C18 column.

**Peptide ELISA**—The synthetic peptides were diluted in 0.1 M Tris-HCl, pH 8.8, and coated overnight on half-area (50 μl/well) microtiter plates (Costar, Cambridge, MA) at a concentration of 20 μg of peptide/ml. After washing in PBS, 0.05% Tween 20 (PBS-Tween), the plates were blocked for 60 min at 37 °C with 3% BSA in PBS. The primary antibody was diluted in sterile-filtered normal goat serum (Sigma) and allowed to react for 2 h with the immobilized peptides. The detection was performed by incubation for 60 min at 37 °C with biotinylated goat anti-mouse Ig (γ-chain specific; Southern Biotechnology, Atlanta, GA), diluted 1:1,000 in PBS, 1% goat serum, and for 30 min with avidin-horseradish peroxidase (Dakopatts), diluted 1:1,000 in PBS, 0.1% BSA. The plates were washed five times with PBS-Tween after each step. Finally, development was carried out with 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid), diluted 1:50 in 0.1 M citrate buffer, pH 4, and 0.9% H₂O₂, with the absorbance at 405 nm recorded after 60 min.

**Polymerase Chain Reaction-based cDNA Cloning**—The total cytoplasmic RNA from 15 × 10⁶ COLO 205 cells was isolated by lysing in an ice-cold buffer solution consisting of 10 mM Tris-HCl, 0.15 M NaCl, 0.1% SDS, pH 7.4. After the removal of the nuclei and cell debris by centrifugation, the RNA was extracted with phenol and precipitated with ethanol.

**Reverse transcription** was performed with 0.5 μg of cytoplasmic RNA, using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Superscript) and random hexamer primers according to the manufacturer's recommendations. The resulting single-stranded cDNA was subjected to 35 cycles of polymerase chain reaction (PCR) on a Perkin Elmer thermal cycler using Taq DNA polymerase supplied in the GeneAmp RNA-PCR kit (Perkin Elmer-Cetus) according to the recommendations of the supplier. The primers designed for the amplification of the entire coding region of the GAP733-2 cDNA had the following sequences: GA-1, 5'-ATATAAGCTTCCACATGGCGCCCGAGGTTCT-3' and GA-2, 5'-CCGCGAGTCTTATGCTTATGTTACCCTATG-3'. The primer GA-1 hybridizes to nucleotides 108–127 in the GAP733-2 cDNA (11) and encodes a HindIII restriction site at the 5'-end. The primer GA-2 hybridizes to nucleotides 1033–1052 in the GAP733-2 cDNA (11) and encodes a BglII restriction site at the 5'-end. The resulting amplified DNA was analyzed by electrophoresis on a 1% NuSieve agarose gel and found to consist of a single product of the expected size (960 base pairs). The amplified DNA was digested with HindIII and BglII and cloned into the mammalian expression vector pKGE328. This vector contains a transcription unit consisting of the SV40 early promoter/enhancer, mouse dihydrofolate reductase gene flanked by HindIII and BglII, and a polyadenylation sequence derived from the SV40 early region, inserted in a pML2-based vector (27). After the transformation of Escherichia coli, plasmid DNA was prepared from a single colony, and the sequence of the insert GA733-2 cDNA in the resulting vector, pKGE796, was verified byideoxy-nucleotide sequencing (28).

**Transfection of Cells**—The mouse T-cell leukemia cell line K36.16 (29) was transfected using diocoadimaleglycylglycylspermin (Transfectam<sup>®</sup>, Sepracor, BFP s.a., Villeneuve la Garenne, France) according to Behr et al. (30). Briefly, 10 μg of both the plasmids pKGE796 and pSV2neo (31) were mixed with 30 μg of diocadimaleglycylglyclys- min and 0.5 ml of Dubelcco's modified Eagle's medium without serum and used to transfect one million cells. After the addition of 0.5 ml of Dubelcco's modified Eagle's medium with 0.5% fetal calf serum and incubation at 37 °C for 3 h, the cells were supplied with Dubelcco's modified Eagle's medium containing 10% fetal calf serum and further incubated for 1 day. Subsequently, the cells were incubated with the same medium containing 1.5 mg/ml G418. After a period of 3 weeks, the resulting resistant clones were harvested as a pool.

**Flow Cytometry Analysis**—The expression of the C215-reactive antigenic structures on the K36.16 transfectant cells was analyzed by immunofluorescence using a FACStar<sup>®</sup> Flow Cytometer (Becton Dickinson, Mountain View, CA). For purposes of comparison, the transfected K36.16 cells were also examined for possible reactivity with the CO17-1A and KS 1/4 antibodies.

**Biochemical Characterization of C215 Antigen**—The C215 antigen was readily solubilized in its native form with a non-ionic detergent such as 1% Nonidet P-40. Harsher conditions (i.e. using an ionic detergent such as 1% SDS) did not improve the extraction yield of the C215 antigen (data not shown).

C215 reactivity in chromatographic fractions was detected under the conditions described in Fig. 1. The gel filtration of crude COLO 205 extract on Superose 6 indicated a non-denatured Mr ~700,000 (Fig. 2a), which is probably due to the presence of glycoprotein associated with colon adenocarcinoma.

**RESULTS**

**Colo 205 extract**

<table>
<thead>
<tr>
<th>Total protein (μg/well)</th>
<th>mAb C215 (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
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<tr>
<td>10</td>
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**Buffer**

<table>
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<tr>
<td>16</td>
<td>8.1</td>
<td>4.1</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>0.51</td>
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<tr>
<td>0.25</td>
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**FIG. 1.** Dot-blot analysis for quantitation of the C215 antigen. Serially diluted COLO 205 tumor extract was applied on nitrocellulose, 0.1 ml/well, and incubated with the C215 antibody at 0.2, 1, 2, and 10 μg/ml after blocking. Development was made with the goat anti-mouse IgG AP system (dilution 1:3,000). The C215 antigen was clearly detected in the crude COLO 205 tumor extract containing 2.5 μg of total protein/ml. A concentration of 1 μg/ml of C215 was used for immunoblotting throughout this study as it yielded minimal background staining with preserved sensitivity. For further details see "Experimental Procedures."
co-elution of the C215 antigen with micelles formed in the presence of the detergent. The removal of the detergent (i.e. by filtration through Extracti Gel™, Pierce) promoted aggregation as the C215 reactivity was eluted with the excluded volume on the same column (data not shown). The immunoreactivity was almost completely retained on ConA-Sepharose, which is consistent with the C215 antigen being a glycoprotein. When the crude COLO 205 tumor extract was subjected to isoelectric focusing under non-denaturing conditions, the C215 reactivity appeared as an intense band at pH 6.1 and as a weaker band at pH 7.1 (Fig. 2b). Moreover, diffuse and extended staining was also observed at lower pH values. This pattern is consistent with the somewhat curious surface-charge properties exhibited by the C215 protein, since most of the immunoreactivity of the crude tumor extract was eluted at physiological pH with the starting buffer both on the Mono Q anion and the Mono S cation exchangers (data not shown). By using the same systems at pH 9 and pH 5, respectively, peaks were eluted with rather large volumes, whereas the removal of the detergent resulted in an increased adsorption to these matrices, probably due to a reduced solubility of the C215 antigen in the absence of the solubilizing agent. In contrast, the chromatography of the crude denatured tumor extract on SDS-hydroxylapatite (Bio-Gel HTP or HPHT) significantly improved the homogeneity of the C215 antigen, with the immunoreactivity eluted as a major fraction at 0.29 M sodium phosphate and as a minor fraction at a slightly lower phosphate concentration, i.e. 0.24 M (data not shown). Therefore, SDS-hydroxylapatite chromatography was preferable to ion-exchange chromatography as a step in the purification of the C215 protein (see below).

The Western blot analysis of the crude COLO 205 extract after gel electrophoresis in 12.5% gels is shown in Fig. 3. Under non-reducing conditions, a major specific band appeared at 40 kDa and a minor at 45 kDa. After the reduction of disulfide bridges, a single specific band at 36 kDa was observed, indicating a monomeric form of the C215 antigen. For comparison, the extract was examined for its reactivity with mAbs CO17–1A and KS 1/4, both having been reported to be closely related to GA733–2 (11). The two bands from the non-reduced sample were visualized with almost the same intensity by KS 1/4, but, in contrast to the C215 antibody, KS 1/4 could not identify its corresponding epitope in the reduced antigen. No specific bands were seen after incubation with CO17–1A under denaturing as well as reducing conditions. The presence of endogenous mouse Ig was indicated by a weak staining for intact IgG and heavy and light chains in negative control strips of denatured and reduced sample, respectively.

**Purification of the C215 Antigen**—The cell surface glycoprotein identified by C215 was isolated from the detergent-solubilized COLO 205 membranes by a procedure designed in light of the results of the biochemical characterization delineated above. The chromatograms from the five-step procedure are shown in Fig. 4. The minute amounts of endogenous mouse IgG were efficiently removed in the immunoaffinity step (Fig. 4a). The non-denatured form of the C215 antigen was eluted as a 600-kDa species by chromatography on Sepharose-6B (Fig. 4b) and yielded a 12-fold purification as it separated from very high M, proteins (e.g. mucins) as well as “low” M, proteins (e.g. intracellular proteins). SDS-hydroxylapatite chromatography (Fig. 4c) of denatured sample only

![Fig. 2. a, gel exclusion chromatography on Superose 6 of crude, non-denatured COLO 205 extract. Two-hundred μl sample was injected and eluted in 0.5-ml fractions at 0.5 ml/min with 50 mM Tris-HCl, 1 mM EDTA, 0.15 M NaCl, 0.1% Nonidet P-40, pH 7.4. Arrows indicate positions of excluded volume (V₀), M, standards (1, thyroglobulin, 670,000; 2, human IgG, 150,000; 3, BSA, 67,000; 4, carbonic anhydrase, 30,000; 5, ribonuclease A, 13,500), and C215 reactivity (bar); b, immunoblotting of crude tumor extract after isoelectric focusing under non-denaturing conditions. Four μl of 1:1 diluted (lanes 1 and 4) and undiluted (lanes 2 and 3) sample was applied in each well. Membrane strips were incubated in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of the C215 antibody. Development was made with the goat anti-mouse IgG horseradish peroxidase/ECL system. Positions of pI markers and bands at pH 6.1 and 7.1 are indicated.](https://example.com/fig2)

![Fig. 3. Western blot analysis of COLO 205 tumor extract after SDS-PAGE in 12.5% homogeneous gels. Non-reduced (lanes 1–4) and reduced (lanes 5–8) tumor extract incubated in the absence of primary antibody (lanes 4 and 8) or in the presence of mAb C215 (lanes 1 and 5), CO17–1A (lanes 2 and 6) and KS 1/4 (lanes 3 and 7) at 1, 5, and 1 μg/ml, respectively. Development was performed with the goat anti-mouse IgG AP system. Positions of M, standards are indicated.](https://example.com/fig3)
Glycoprotein Associated with Colon Adenocarcinoma

FIG. 4. Purification procedure for isolation of the C215 antigen from COLO 205 tumor extract. a, immunoaffinity chromatography on protein G-Sepharose: binding buffer, 10 mM phosphate buffered saline, pH 7.4, containing 0.1% Nonidet P-40; elution buffer, 50 mM glycine-HCl, pH 2.3, which was neutralized with 0.2 ml of 1 M Tris-HCl, pH 9.0, per ml (arrow indicates start of elution); b, Sepharose-6B chromatography under native conditions: conditions from Step I were applied on the column and eluted with 50 mM Tris-HCl, 1 mM EDTA, 0.15 M NaCl, 0.1% Nonidet P-40, pH 7.4. Arrows indicate positions of excluded volume (V), thyroglobulin (ThG, 670 kDa), egg albumin (OuA, 43.5 kDa), and myoglobin (Myo, 16.9 kDa); c, SDS-hydroxylapatite chromatography on Bio-Gel HTP: elution conditions, 0.01-0.40 M sodium phosphate, pH 6.8, containing 0.1% SDS and 0.1% Nonidet P-40. Arrows, straight line; gradient profile, dotted line; d, Lectin-affinity chromatography on SelectiSpher-10 (ConA): arrow indicates elution start with buffer containing methyl mannoside. Flow rate, 1 ml/min; e, SDS-hydroxylapatite chromatography (Bio-Gel HPHT) of reduced antigen: elution was carried out with a linear 0.01-0.40 M sodium phosphate gradient, pH 6.8, in 1 mM diethiothreitol and 0.1% SDS. Flow rate, 0.5 ml/min. Fractions displaying C215 reactivity in the dot-blot assay and that were pooled for further purification from each step are indicated.

TABLE I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>Purification *-fold</th>
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<tbody>
<tr>
<td>1. COLO 205 tumor extract</td>
<td>1,290</td>
<td>1</td>
</tr>
<tr>
<td>2. Protein G-Sepharose</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3. Sepharose-6B (native conditions)</td>
<td>110</td>
<td>12</td>
</tr>
<tr>
<td>4. SDS-Bio-Gel-HP hydroxylapatite chromatography (denatured conditions)</td>
<td>68.6</td>
<td>19</td>
</tr>
<tr>
<td>5. ConA/HPLC lectin-affinity chromatography</td>
<td>2.3</td>
<td>570</td>
</tr>
<tr>
<td>6. SDS-HPHT hydroxylapatite chromatography (reducing conditions)</td>
<td>0.3</td>
<td>4,300</td>
</tr>
</tbody>
</table>

* Based on total protein in tumor extract.
† ND, not determined.

marginally improved the purification degree, whereas the lectin-affinity step in particular (Fig. 4d) and Bio-Gel HPHT chromatography under reducing conditions (Fig. 4e) markedly increased the purity of the resultant preparation. The results are summarized in Table I with a final yield of 0.3 mg at a 4,300-fold degree of purification. The homogeneity was tested by capillary electrophoresis and was found to be more than 95% (Fig. 5).

Amino-terminal Sequencing of C215 Antigen—The solubilized and purified C215 antigen was subject to 20 cycles of amino-terminal sequencing without prior reduction and alkylation. This revealed the following sequence: XxX-Ala-Lys-Pro-Glu-Gly-Ala-Leu-Gln-Asn-Asn-Asp-Glu-Leu-Tyr-Asp-Pro-Asp-XXX-Asp. The amino acid residues from cycles 1 and 19 could not be identified and may represent cysteins or post-translationally modified amino acids. The search for homologous sequences in the SWISSPROT (release 21.0) and NBRF-PIR (release 31.0) protein sequence databases using the program FASTA (32) revealed the close homology of the above sequence with the recently cloned carcinoma-associated
antigen GA733–2 (11). This antigen, which is expressed in both normal and cancerous colon epithelial cells, has been independently cloned by several groups, and referred to as KSA by Strnad et al. (7), the KS 1/4 epithelial carcinoma marker by Perez and Walker (8), and EGP by Simon et al. (12). The 17 identified contiguous amino terminal residues of the C215 antigen coincide exactly with amino acids 82–98 of the GA733–2 protein, as is illustrated in Fig. 6.

Epitope Characterization—The influence of N-linked carbohydrate on the recognition of the C215 epitope was studied after deglycosylation of the purified antigen by treatment with PNGaseF. The migration behavior of the deglycosylated C215 protein was examined in 12.5% homogeneous gels under reducing conditions (Fig. 7). Western blot analysis revealed a retained immunoreactivity, although at a reduced level, with a concomitant reduction in the molecular mass from 37 to 32 kDa (14%). In contrast, Pronase treatment completely abolished the C215 reactivity (data not shown), which further supports the non-carbohydrate nature of the C215 epitope.

The localization of the C215 epitope was investigated in a peptide ELISA using synthetic peptides derived from the predicted amino acid sequence of the GA733–2 protein (11). Twenty-six peptides, 22 residues long with an overlap of 12 residues, were prepared and extended over the entire extracellular domain of GA733–2. Of the consecutively numbered peptides, C215 reactivity was only demonstrated for peptides 4, 5, and 11, which contain residues 31–52, 41–62, and 101–122, respectively (Fig. 8). The strongest reactivity was obtained with peptides 4 and 5 whereas that of peptide 11 was relatively weak. The first two reactive peptides suggest an epitope localized close to the putative signal sequence ending at residue 23 (11). This localization corresponds to a hydrophilic peak at residues 41–46 in a Kyte-Doolittle hydropathy plot of the GA733–2 protein (11). In an additional experiment, the peptides were tested for their ability to inhibit binding of 125I-labeled mAb C215 to immobilized C215 antigen in an inhibition ELISA. Inhibition could only be demonstrated for peptide 4, which was found to be a ≥7,000-fold weaker competitor than the purified C215 antigen (data not shown). These results further support that this part of the C215 protein is a possible localization of the C215 epitope. The weak reactivity with peptide 11, and the fact that this peptide could not compete for binding to C215 with the purified antigen, may either be due to an irrelevant, weak-affinity epitope or that this peptide forms part of a conformational epitope of which the stretch in the overlap between peptides 4 and 5 is the main contributor to the antibody affinity.

The synthetic peptides were also examined for KS 1/4 and C017–1A reactivity in the peptide ELISA (data not shown). At high concentrations, the C017–1A antibody showed a weak, but specific, reaction only with peptide 11. In contrast, KS 1/4 exhibited a more heterogeneous peptide-binding profile, where the strongest reactivity was demonstrated in the same region of the GA733–2 protein as was observed for C215 (i.e. including binding to peptides 4–6) and to a much lesser extent with peptides 11 and 15.

Molecular Cloning and Expression of GA733–2 cDNA—In order to test further whether the C215 antigenic epitope is encoded within the GA733–2 protein, a cell line expressing a recombinant form of this antigen was established. To obtain cDNA encoding GA733–2, cytoplasmic mRNA was purified from COLO 205 cells and used as a template for PCR after single strand cDNA synthesis. One pair of oligonucleotide primers were used so that the resulting PCR fragment would
cover the entire coding sequence of the GA733-2 cDNA. After cloning into an appropriate expression plasmid, the DNA sequence was determined and the region encoding the GA733-2 open reading frame was found to be identical to that reported by Szala et al. (11). The resulting expression vector, pKGE796, contains the SV40 early promoter/enhancer, immediately followed by GA733-2 cDNA, which in turn is followed by a polyadenylation sequence derived from the SV40 early region, as depicted in Fig. 9.

In order to derive mammalian cells constitutively expressing the cDNA encoded GA733-2 antigen, the above plasmid was co-transfected with the plasmid pSV2neo (31) into K36.16 mouse T cell leukemia cells. After selection with G418, the emerging clones were pooled, expanded, and checked for their expression of the C215 antigen with FACS.

Expression of C215 Antigenic Structures in the K36.16 Transfectant Cell Line—A surface expression of the C215 antigen was demonstrated in the K36.16 transfectant cells when subject to FACS analysis without any previous sorting and/or cloning of the cells (Fig. 10a). The KS 1/4 antibody was bound to antigenic structures on the transfected cells at a similar density, whereas it was not possible to demonstrate any reactivity for CO17-1A in these cells. As expected, the parental K36.16 cells showed no expression of either C215 or KS 1/4 antigens (data not shown). For comparison, FACS data for COLO 205 cells are also shown (Fig. 10a). C215 and KS 1/4 showed the same degree of binding. In contrast to the negative finding of the CO17-1A binding to the K36.16 transfectant cells, COLO 205 indicated the surface expression of this epitope, although at about a 3-fold lower rate than that observed for the corresponding epitopes for C215 and KS 1/4. These data show that the expression level of the C215 antigen was substantially lower in the K36.16 transfectant cell line than in the COLO 205 cells.

The relationship between the C215 epitope and the epitopes recognized by the other two antibodies in both the K36.16 transfectant cell line and in COLO 205 cells was also investigated (Fig. 10b). Only pre-incubation with C215 was able to block the subsequent binding of fluorochrome-labeled C215, whereas KS 1/4 and CO17-1A could not. Hence, this demonstrates that C215 identifies an epitope distinct from those identified by KS 1/4 and CO17-1A.

Structural Relationship of Antigen Expressed in COLO 205 and K36.16 Transfectant Cells—The structural identity between the C215 antigen in COLO 205 cells and the expression product in the GA733-2 cDNA transfected K36.16 cells was examined by Western blotting after SDS-PAGE in 12.5% gels (Fig. 11). C215 reactivity migrated exclusively as a 42 kDa band in the K36.16 transfectant cells under both denaturing and reducing conditions, thus contrasting with the decrease in the mass observed after the reduction of the C215 antigen.
in the COLO 205 tumors (Fig. 3). As is also shown in Fig. 11, the parental K36.16 cells did not exhibit any specific reactivity toward the C215 antibody.

**DISCUSSION**

In the present study, we have characterized and isolated the antigen defined by the anti-colon adenocarcinoma mAb C215. It seems to be a monomeric glycoprotein with two non-reduced forms, 40 kDa (major form) and 45 kDa (minor form), respectively, and with a molecular mass of 36 kDa following reduction of intrachain disulfide bridges. Hence, the M', observed after gel filtration under non-denaturing conditions (Figs. 3a and 4b) may be regarded as a detergent artifact. At first, we attempted to use immunoadsorbents for the isolation of the C215 antigen from COLO 205 tumors grown in athymic mice. Despite the fact that the C215 mAb binds to its antigen with a relatively high affinity (K \( \approx 2 \times 10^{-9} \) M)\(^4\) and different functional groups were tested for covalent coupling to the matrix, none of the immunoaffinity columns worked satisfactorily due to the impaired immunoreactivity of the immobilized antibody and/or a significant antibody leakage from the column. Therefore, a multi-step procedure was utilized, far more time consuming and probably resulting in a poorer yield but with the benefit of giving more information as regards the chemical nature of the antigen than a single-step procedure could.

Our first step in determining the partial NH\(_2\)-terminal amino acid sequence of the C215 antigen was to sequence the partially purified antigen after the ConA/HPLC step by electropherophoresis to a siliconized glass fiber support, which had been designed for direct protein chemical analysis according to Eckerskorn et al. (33), after SDS-PAGE in 12.5% gels under reducing conditions. No phophoethanolamidomethylated amino acids could be identified with this approach, suggesting a blocked amino terminus. However, after the additional DSHS-hydroxylapatite step under reducing conditions, we succeeded in determining 17 contiguous amino acids after 20 cycles of NH\(_2\)-terminal sequencing (Fig. 6). The homology with residues 82-98 of the recently cloned GA733-2 antigen (11) motivated the use of GA733-2 cDNA for cloning of the C215 protein from COLO 205 cells and GA733-2 derived synthetic peptides for identification of the C215 epitope. A likely C215 epitope was localized in the extracellular domain of the GA733-2 protein and C215 reactivity was demonstrated by FACS analysis for the expressed GA733-2 cDNA in the K36.16 expression system, thus, providing data which substantiate the notion that the C215 and GA733-2 antigens might be one and the same molecular entity. However, the establishment of a possible identity between these antigens must await the presentation of the complete sequence of the C215 antigen.

Szlak et al. (11) reported two forms of the GA733-2 antigen in the human SW948 colorectal adenocarcinoma cell line: a blocked 40-kDa form consisting of 314 amino acids encoded by the full-length GA733-2 cDNA, which we have used for transfection into the K36.16 cell line, and a 30-kDa form which is probably a product of cleavage at a dibasic site at the Arg\(^{269}-\)Arg\(^{270}\) position. Furthermore, the 40-kDa form contains a potential signal sequence with the signal peptidease site located at Ala\(^{264}\)Glu\(^{24}\). Therefore, they suggested that the 30-kDa breakdown product might represent either the native membrane form, resulting from post-translational processing of the immature, intracellular 40-kDa species, or simply a nonspecific cleavage product obtained by the action of lysosomal proteases released during purification. The fact that only the 42-kDa species was observed in the K36.16 transfectant cells may support the first alternative and suggests that the specific pathway for post-translational processing of this protein (i.e. only one out of two potential dibasic cleavage sites seems to be hydrolyzed, namely Arg\(^{269}\)-Arg\(^{270}\) but not Arg\(^{215}\)-Arg\(^{216}\)) is absent in these cells; this reflects the fact that the K36.16 cell line, in contrast to COLO 205 cells, is of non-epithelial origin. We have also calculated the theoretical pI values from the primary amino acid sequence of GA733-2 using the Isoelectric Protein Analysis Program of the Genetics Computer Group of the University of Wisconsin (34). A pI value of 7.38 and 6.31 was obtained for the "intracellular" form, without the potential signal sequence, and the "mature," cleaved form, respectively. These values are similar to the bands visualized at pH 6.1 (major form) and 7.1 (minor form) on immunoblots after isoelectric focusing under non-denaturing conditions, but do not explain the diffuse and extended staining at more acidic pH values (Fig. 2b). On the other hand, the likely localization of the C215 epitope (i.e. in the part of the C215 molecule that is supposed to be cleaved off) is more consistent with the second alternative as we have demonstrated a strong binding of C215 to the surface of COLO 205 cells in FACS analysis.

Several studies have indicated that the mAbs GA733 (4, 10, 11), KS 1/4 (8), CO17-1A (4-6), and HEA-125 (12) as well as the mAb 323/A3, which reacts with a mammary cancer-associated glycoprotein (35), define different epitopes of the same antigen. All these studies have utilized an approach where surface-labeled antigenic structures have been immunoprecipitated, thus favoring the analysis of the mature membrane form. Furthermore, this approach has enabled the detection of the corresponding antigen in its reduced form although all these antibodies, in contrast to mAb C215, are reported to be unable to react with the reduced antigen. Simon et al. (12) reported a 40- and 34-kDa form of EGP after \(^{125}\)I-labeling of cell surface proteins on HT-29 cells and subsequent immunoprecipitation with mAb HEA-125. The 34-kDa species was the predominant form, which they claimed supported the notion that this form represents mature EGP, inserted in the plasma membrane, and the minor 40-kDa species the intracellular form. Similar results were reported by Perez and Walker (8) who demonstrated a major 35-kDa species and a minor 40-kDa protein in the UCLA-P3 human lung adenocarcinoma cell line by immunoprecipitation with mAb KS 1/4 following iodination of surface proteins. In addition, they could identify a minor 42-kDa form differing from the 40-kDa species only in the degree of N-linked glycosylation. Both the 40- and 34/-35-kDa species are glycosylated and the removal of carbohydrates by N-glycanase treatment decreased the mass by 8 and 14%, respectively. The latter figure corresponds well to the decrease in mass caused by the deglycosylation of the purified C215 antigen (i.e. from 37 to 32 kDa or by 14%). Accordingly, the two non-reduced forms of the C215 antigen in the crude COLO 205 extract may be explained by different degrees of glycosylation. Göttlinger et al. (6) reported a non-reduced 37- and a reduced 33-kDa species in three colon adenocarcinoma cell lines, HT-29, DLD-1 and WiDr, after the immunoprecipitation of \(^{125}\)I-labeled surface antigens with mAb CO17-1A. Despite the lower molecular weights observed for these forms, their findings are consistent with our data for the C215 antigen, where the reductive cleavage of intrachain disulfide bridges results in an altered shape of this protein and a faster migratory behavior in SDS gels and in the same diminution in mass (i.e. 11%). In our experiments with COLO 205 cells, it is evident that the predominant form of the C215

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\(^4\) P. Björk, unpublished results.

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antigen in the crude cell extract under non-reducing conditions is the 40-45-kDa form. If this form is identical to the “intracellular” 40-42-kDa form described by Perez and Walker (8) and Szala et al. (11), this is contradictory to the fact that both C215 and KS 1/4 seem to recognize an epitope that should be absent in the dibasic-site cleaved form. Pulse labeling studies must be performed to verify the nature of these two forms and whether it is likely that the dibasic cleavage of this antigen plays a role in its post-translational processing.

The GA733-2 protein (11) was found to differ from KSA in lung adenocarcinoma cells (7, 8) and EGP in colon epithelial carcinoma (12) in only one residue located in the transmembrane domain (Ile instead of Met at position 278), whereas it only displayed a 49% identity and, when accounting for conserved substitutions, 67% similarity with the other member of the GA733 gene family, the GA733-1 antigen (11). In this study, a close structural relationship between the C215 and KS 1/4 defined antigens was indicated since: (i) FACS data showed identical antigen densities on COLO 205 as well as K36.16 transfectant cells, and (ii) a similar staining pattern was obtained by the immunoblotting of crude COLO 205 tumor extract after SDS-PAGE under non-reducing conditions. Furthermore, KS 1/4 was successfully used as a coating antibody in an ELISA developed for analysis of the C215 antigen-antibody interaction. However, we were not able to fully establish a similar relationship with the CO17-1A antigen since a significantly lower degree of expression of this epitope was observed in COLO 205 cells and we failed to demonstrate any expression in the K36.16 transfectant cells (despite the fact that the three mAbs used for the FACS analysis were of the same IgG subclass and titration was performed to compensate for the lower affinity of CO17-1A, Ref. 4). Thus, C215 and KS 1/4 seem to define distinct epitopes on the same antigen, whereas it is still somewhat unclear whether CO17-1A reacts with the same antigen despite the fact that this antibody displayed a weak reactivity with peptide 11 in the peptide ELISA.

Our findings suggest that the C215 antigen is closely related to a number of carcinoma antigens. Apparently, these proteins constitute a fairly immunodominant antigenic structure in mice, since immunization with human colon adenocarcinoma cells and several cell lines from other epithelial carcinomas has resulted in a number of mAbs with different epitopes on the same protein. A general structural pattern for these antigens is the very short, highly charged cytoplasmic domain, a highly conserved transmembrane domain, and the cystein-rich extracellular domain with three potential N-glycosylation sites in the uncleaved form and with the loss of one site in the dibasic-site cleaved form. The extracellular domain constitutes almost 80% of the cleaved protein and probably has both potential N-glycosylation sites in the glycosylated state. This has raised the question of the biological function of this antigen. The fact that the protein is expressed at high levels of density and adenocarcinomas and, to a varying extent, in normal epithelium implies an important role in the biology of normal and malignant epithelium. Linnenbach et al. (10) reported on the homology of the extracellular domain of the GA733-2 antigen with the type I repeat of human and bovine thyroglobulin and with the MHC class II-associated invariant chain. They also found a possible relationship to the alpha subunit of the interleukin-2 receptor. In particular, Perez and Walker (8) focused on the 12 cysteine residues in the first 135 amino acids of the uncleaved form with the potential of forming six intradsulfide bridges, which may be important both for function (e.g., receptor binding sites) and stability.

The inability of all mAbs, except for the C215 antibody, to react with the reduced antigen suggests that their immunoreactive properties require intact disulfide bonds. Consequently, the C215 antibody may be advantageous inasmuch as it seems to recognize a distinct epitope which is not susceptible to disulfide bridge cleavage. Finally, Simon et al. (12) demonstrated a significant degree of sequence homology between EGP and nidojen, a matrix adhesion molecule, and the placental protein 12, an insulin-like growth factor-I-binding protein secreted by the placenta that is suggested to play a role in fetal growth regulation. Hence, although the C215 antigen is a transmembrane protein, in contrast to the extracellular matrix component nidojen and the secretory placental protein 12, it may well interact with extracellular matrix components and/or belong to the growth factor antigen family by binding to a hitherto unknown ligand. The mammalian expression system devised for the C215 antigen and described in the present study may help in further elucidating the biological function of this protein.

The primary aim of the present investigation was to purify the C215 antigen for transfection into a suitable mammalian expression system in order to design adequate animal models for the in vivo testing of the tumoricidal effects of antibody-targeted drugs. Here, we have demonstrated that this protein shares structural homologies with a number of epithelial carcinoma antigens used as targets in antibody therapy. The favorable results reported in colon adenocarcinoma patients treated with the mAb CO17-1A have placed further emphasis on the potential use of the C215 antibody in the targeting of superantigens to human colon adenocarcinomas. By transfection into the murine K36.16 T cell leukemia cell line, we have proven the efficacy of the expression vector described in this paper which causes the expression of a structurally and immunochemically homologous membrane form of the C215 antigen in these cells. The same expression vector has been used to transfect the B16/F10 mouse melanoma cell line (36). When transplanted into syngenic mice, a much higher expression of the C215 antigen was demonstrated by FACS analysis3 than that observed in the K36.16 transfected cells. The B16/F10 melanomas form multiple metastases in the lung, which are easily visualized and can be quantified when assessing anti-tumoral effects. This model may turn out to be ideal for the assessment of both the anti-tumoral activity and pharmacokinetic properties of the intact C215 antibody as well as monovalent Fab or single chain Fv fragments.

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