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Micro-sequencing strategies for the human A33 antigen, a novel surface glycoprotein of human gastrointestinal epithelium

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Abstract

Monoclonal antibody (mAb) A33, which recognizes a M_r~43 000 differentiation antigen (A33) expressed in normal human colonic and small bowel epithelium as well as in 95% of colon cancers, shows specific targeting of colon cancer in humans and is currently being evaluated for clinical use. Here, we describe strategies for the purification and structural analysis of the A33 antigen from the human colorectal carcinoma cell lines LIM1215 and SW1222. Edman degradation of the intact protein and nine peptides, derived by proteolytic digestion of the A33 antigen with Asp-N endoproteinase, thermolysin, trypsin and pepsin followed by micropreparative reversed-phase high-performance liquid chromatography, allowed the unambiguous sequence assignment of 153 amino acid residues; these data reveal one N-glycosylation sequeon in Asp-N endoproteinase peptide D4, and a disulfide linkage between peptides D1 and D4. This amino acid sequence information has facilitated the cloning and subsequent sequencing of a cDNA for the A33 antigen which demonstrates that it is a novel human cell surface molecule of the immunoglobulin superfamily. © 1998 Elsevier Science B.V.

Keywords: Antigens; Glycoproteins; Proteins

1. Introduction

The monoclonal antibody A33 (mAbA33) recognizes a cell surface differentiation antigen of normal human gastrointestinal epithelium that is expressed in 95% of primary or metastatic colon cancer cells, but which is absent in most other normal tissues and tumor types [1,2]. Some colon cancer cell lines express large amounts of the A33 antigen, binding up to 800 000 Ab molecules per cell [3]. The A33 antigen is not secreted or shed into the blood stream. Upon binding to the A33 antigen, the A33 antibody is internalized into an incompletely characterized vesicular compartment, and a significant fraction of the internalized antibody appears to be recycled back to the extracellular environment [3]. The A33 antigenic system is the focus of several clinical studies in patients with colon cancer. Phase I/II clinical trials have shown that the mouse mAbA33 localizes with high specificity to colon cancer and is retained for prolonged periods (up to 6 weeks) by tumour cells but is cleared rapidly from the normal colon (5–6 days). As a carrier of ^{125}I or ^{131}I, the mAbA33 has been shown to have anti-tumor activity [1,4,5]. The mAbA33 has now been humanized [6] and the humanized antibody is currently being evaluated in phase-I clinical trials.

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At the protein level the A33 antigen has been only partially characterized. We have recently described a M_r~43 000 protein in Triton X-114 detergent extracts from LIM1215 [7] and SW1222 human colon cancer cells [8] that is recognized by mAbA33 by biosensor analysis and Western blot analysis under nonreducing conditions [9,10]. In the present report, we have used both a multidimensional micropreparative HPLC protocol and an efficient two-step chromatographic procedure [immunoaffinity chromatography followed by micropreparative reversed-phase high-performance liquid chromatography (RP-HPLC)] to purify sufficient (μg) quantities of protein for further characterization of the A33 antigen.

2. Experimental

2.1. Antibodies, lectins, enzymes and cells

Mouse mAbA33 and mAb100.310 (IgG2a) and humanized mAbA33 (IgG1) have been described elsewhere [1,6]. Rabbit polyclonal IgG antiserum was raised against the N-terminus of the A33 antigen (residues 2–20) [9]. All three mAbs recognize nonreduced A33 antigen only, while the polyclonal antiserum recognizes both reduced and nonreduced A33 antigen. Pepsin was obtained from Sigma (St. Louis, MO, USA). Thermolysin, trypsin, and Asp-N-endoproteinase were from Boehringer Mannheim (Germany).

2.2. Radioactive labeling of cultured cells

Tumor cell lines were obtained from the tumor cell banks at the New York and Melbourne Branches of the Ludwig Institute for Cancer Research. Cells were metabolically labeled with 50 μCi/ml Tran [^{35}S]-label (mixture of [^{35}S]-methionine and -cysteine) (ICN Biomedicals, Cosa Mesa, CA, USA) for 18 h at 37°C. Labeled cells were extracted with lysis buffer [phosphate buffered saline (PBS) containing 0.3% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml antipain, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) and 1 m*M* EDTA].

2.3. Radioimmunoprecipitations, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography and immunoblotting

Immunoprecipitations of lysates from radioactive labeled cells were performed as described [10]. SDS-PAGE was performed using polyacrylamide Tris–glycine pre-cast gels (Bio-Rad, Hercules, CA, USA, Novex, San Diego, CA, USA and Pharmacia Biotech, Uppsala, Sweden). Radiolabeled proteins were visualized by autoradiography of dried gels with Kodak X-Omat AR-5 films. SDS-PAGE separated proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Australia). The A33 epitope was detected by Western blotting and bound antibody was visualized by chemiluminescence (ECL, Amersham, UK).

2.4. Purification of the A33 antigen

In this study, the A33 antigen was purified using two different chromatographic protocols, essentially as described elsewhere [9,11,12].

2.4.1. Protocol A: multidimensional chromatography

Briefly, the A33 antigen was extracted from the human colonic carcinoma cell line LIM1215 by Triton X-114 phase partitioning and purified by sequential use of ligand dye (Green-Sepharose HE-4DB), anion-exchange (Mono Q HR 10/10), size-exclusion (Superose 12 HR 10/30) and micropreparative reversed-phase HPLC (Brownlee Aquapore RP 300, 30×2.1 and 100×1 mm I.D.). The chromatographic purification was monitored by biosensor analysis employing surface plasmon resonance detection [13] using a F(ab)′2 fragment of the humanised A33 mAb immobilised onto the sensor surface, and Western blot analysis using the A33 mAb under nonreducing conditions [9,10].

2.4.2. Extraction of A33 antigen from LIM1215 cells with Triton X-100 or Triton X-114

LIM1215 colonic cells (~$1 \cdot 10^9$ cells) were harvested, washed in PBS and solubilised (10^8 cells/ml) for 30 min at 4°C with 1% (v/v) Triton X-114 in 15 m*M* Tris–HCl (pH 7.4) containing 1 m*M* PMSF, 1 m*M* pepstatin, 0.1 m*M* leupeptin and 0.01 U/ml

aprotinin. The resulting extracts were centrifuged twice at 4°C for 20 min at 14 000 *g*. The Triton X-100 supernatant was taken directly for Green-Sepharose HE-4 BD chromatography. The Triton X-114 extracted supernatant was layered over 6% (w/v) sucrose in 15 m*M* Tris–HCl buffer (pH 7.4) with 0.06% (v/v) Triton X-114 and containing the protease inhibitors listed above. The tubes were incubated at 37°C for 30 min and then centrifuged at 25°C for 15 min at 5000 *g*. The detergent phase was collected for chromatographic purification.

2.4.3. Green-Sepharose chromatography

To remove actin, which had been found to bind to the Fc region of the A33 mAb [9], the Triton X-114 detergent phase was diluted to a final concentration of 0.1% (w/v) detergent and loaded at 4°C onto a Green-Sepharose HE-4BD column (100×10 mm I.D.) connected to a fast protein liquid chromatographic (FPLC) system (Pharmacia Biotech). The column was equilibrated with 10 m*M* Tris–HCl buffer (pH 7.4) containing 0.1% (w/v) 3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS). Bound proteins, including actin, were eluted with a step gradient to 1 *M* NaCl. The breakthrough, which contained the A33 antigen, was collected for anion-exchange HPLC.

2.4.4. Anion-exchange HPLC

The Green-Sepharose breakthrough was injected at 4°C onto a Mono Q HR 10/10 column previously equilibrated in 10 m*M* Tris–HCl buffer (pH 7.4) containing 0.1% (w/v) CHAPS. The proteins were eluted from the column using a linear 0 to 1 *M* NaCl gradient generated over 90 min at a flow-rate of 1 ml/min. Fractions (1 ml) were collected automatically (FRAC 100, Pharmacia Biotech). Proteins were detected by absorbance at 280 nm.

2.4.5. Size-exclusion HPLC

The A33-containing fractions eluted from the Mono Q column (10 ml) were concentrated ten-fold using a centrifugal concentrator (Speed Vac, Savant Instruments, NY, USA), dialysed against PBS containing 0.05% CHAPS (w/v) and loaded onto a Superose 12 HR 10/30 column connected to a FPLC system. The column temperature was 4°C. Proteins were eluted with PBS containing 0.05% (w/v) CHAPS at a flow-rate of 0.5 ml/min, and collected automatically in 0.5-ml fractions. Proteins were detected at 280 nm.

2.4.6. Micropreparative reversed-phase HPLC

A33 antigen-containing fractions from the Superose 12 column (2.5 ml) were loaded at a flow-rate of 1 ml/min, by multiple 1-ml injections, onto a Brownlee Aquapore RP 300 micropreparative RP-HPLC column (30×2.1 mm I.D.) equilibrated with the primary solvent, 0.15% (v/v) trifluoroacetic acid (TFA) in water. As we have described previously [14], the use of short columns permits high flow-rates to be used during loading without generating excessive back pressure. The flow-rate was then lowered to 100 μl/min and proteins eluted with a linear 60 min gradient to aqueous *n*-propanol–0.125% TFA (60:40, v/v). The column temperature was 45°C, and eluting proteins were detected by absorbance at 215 nm. The A33 antigen was specifically detected using both biosensor and Western blot analysis. Fractions containing the A33 antigen were repurified and further concentrated using a Brownlee Aquapore RP 300 micropreparative RP-HPLC column (100×1 mm I.D.) prior to N-terminal sequence analysis, using the gradient conditions described above at a flow-rate of 50 μl/min. Eluent fractions were recovered manually.

2.4.7. Protocol B: affinity chromatography

In these studies, the A33 protein was purified from SW1222 cells using a modification of protocol A which included an affinity chromatography step. The A33 affinity column was prepared according to the protocol of Schneider et al. [15]. Briefly, murine mAbA33 was diluted to 1 mg/ml in 0.1 *M* borate buffer (pH 8.2) and incubated for 2 h at room temperature, or overnight at 4°C, with 1.5 ml protein A-Sepharose CL4 B (Pharmacia Biotech). After washing with 0.1 *M* borate buffer (pH 9.2), the protein-A–monoclonal antibody complex was incubated for 1 h at room temperature with 20 m*M* dimethyl pimelimidate dihydrochloride (ICN) in 0.1 *M* borate buffer (pH 9.2). Noncovalently bound antibody was removed with 50 m*M* glycine buffer (pH 2.5). The remaining active dimethyl pimelimidate groups were deactivated by washing with 0.1 *M* ethanolamine buffer (pH 8.0). The affinity support

was then equilibrated with PBS (pH 7.4) containing 0.05% (w/v) CHAPS.

The detergent phase from Triton X-100 SW1222 cell membrane extracts ($6 \cdot 10^8$ cells), containing the A33 antigen, was diluted five-fold with PBS, 0.05% CHAPS buffer (pH 7.4) and incubated overnight at 4°C with the A33 IgG–protein-A. The packing was then poured into an empty HR 10/10 column (Pharmacia Biotech) and attached to a FPLC system. After extensive washing with 10 m*M* Tris–HCl (pH 7.5) containing 0.3% Triton X-100, the column was eluted with 0.1 *M* sodium citrate buffer (pH 6.0, 4.0 and 3.0) and glycine (50 m*M*) buffered saline (pH 2.8). The elution profile was monitored by absorbance at 280 nm. Eluted fractions were concentrated by membrane filtration and aliquots monitored for the presence of A33 antigen by Western Blot analysis under nonreducing conditions (10% SDS-PAGE, chemiluminesence visualization) using humanized mAb A33 to reduce cross-reactivity with murine mAbA33, which was bleeding from the column matrix. The affinity purified material was then purified either using micropreparative RP-HPLC, as described above, or using micropreparative anion-exchange and size-exclusion chromatography (SEC). The latter procedure avoided partial denaturation cause by RP-HPLC and allowed the purification of the A33 protein to homogeneity under nondenaturing conditions. In this case, RP-HPLC was only used analytically to monitor sample purity. Material purified in this manner was used for kinetic [11] and lectin binding studies [12].

2.4.8. Micropreparative anion-exchange chromatography

The A33 antigen- containing fraction eluted from the affinity column was diluted two-fold with 20 m*M* Tris–HCl buffer (pH 7.5) 0.005% (v/v) Tween 20, adjusted to pH 7.5 with 1 *M* Tris–HCl buffer (pH 9.0) and injected onto a Mono Q PC 1.6/5 anion-exchange column connected to a SMART system (Pharmacia Biotech). Proteins were eluted with a linear 60 min gradient (0 to 1 *M* NaCl) at a flow-rate of 100 μl/min. Detection was performed at 215 nm, and 100-μl fractions were collected automatically.

2.4.9. Micropreparative size-exclusion chromatography

A33-containing fractions eluted from the Mono Q column (800 μl) were pooled and individual 100-μl aliquots were loaded onto a Superose 12 HR 3.2/30 size-exclusion column connected to the SMART system and equilibrated with PBS, 0.005% Tween-20. The column was eluted at a flow-rate of 100 μl/min. Detection was performed at 215 nm and 100-μl fractions were collected.

2.5. Biosensor analysis

Cell extracts and chromatographic fractions were monitored using an instrumental optical biosensor (BIAcore, Pharmacia Biosensor), with a F(ab)′2 fragment of humanized mAbA33 immobilized onto the biosensor surface using N-hydroxysuccinimide and ethyl-N′-dimethylaminopropyl carbodiimide at a flow-rate of 4 μl/min, as described previously [13]. Antigen binding to the F(ab)′2 fragment was detected by surface plasmon resonance which measures small changes in refractive index at, or near to, the gold sensor surface [16,17]. Prior to biosensor assay, cell extracts or aliquots of chromatographic fractions were diluted to a final volume of 100 μl in BIAcore buffer: 10 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 3.4 m*M* EDTA, 0.15 m*M* NaCl and 0.005% Tween 20. Samples (30 μl) were injected over the sensor surface at a flow-rate of 5 μl/min. Following completion of the injection phase, dissociation was monitored in BIAcore buffer at the same flow-rate for 360 s. Residual bound antigen was eluted and the surface regenerated between injections using 40 μl of 10 m*M* NaOH. This treatment did not denature the protein immobilised onto the sensor surface, as shown by equivalent signals upon re-injection of a sample containing the A33 antigen.

2.6. Western blot analysis

Electrophoresis and Western blot analysis were performed on precast Phastgels using a Phastsystem separation and control unit (Pharmacia Biotech). Cell extracts and chromatographic fractions were electrophoresed under nonreducing conditions on 8–25% SDS-PAGE Phastgels or 8–25% native Phastgels, transferred onto PVDF membranes and incubated with humanised mAbA33. IgG binding was probed

with horseradish peroxidase-labeled goat anti-human IgG and detected by ECL.

2.7. Peptide mapping and amino acid sequence analysis

The A33 antigen-containing RP-HPLC fractions (~3 μg) were adjusted to 0.02% Tween-20 and concentrated a further four-fold (~50 μl) by centrifugal lyophilization. Digest conditions were as follows: (1) Asp-N; the sample was diluted to 500 μl with 0.05 *M* sodium phosphate buffer (pH 8.0) and digested at an enzyme–substrate ratio of 1:20 at 37°C for 16 h. (2) Trypsin; the sample was diluted to 500 μl with 1% (w/v) ammonium bicarbonate and digested at an enzyme–substrate ratio of 1:20 at 37°C for 16 h. (3) Thermolysin; the sample was diluted to 500 μl with 1% (w/v) ammonium bicarbonate and digested at an enzyme–substrate ratio of 1:20 at 50°C for 1 h. Following the initial Asp-N digestion, a small aliquot of the resultant digest (10%) was fractionated on a Brownlee RP-300 column (50×1 mm I.D.) using a trifluoroacetic acid–acetonitrile solvent system [18] and peptide fractions were collected manually and subjected to Edman degradation using a biphasic column protein sequencer operating with Routine 3.0 chemistry (Hewlett-Packard model G1005A) [19]. The remainder of the digest (90%) was chromatographed using identical conditions. To detect the presence of disulphide-bonded peptides in RP-HPLC fractions containing a mixture of peptides, as indicated by sequence analysis of the corresponding peptide fractions in the small scale peptide map, samples were reduced (in 5.3 *M* guanidinium hydrochloride (GdnHCl), 0.13 *M* dithiothreitol (DTT), 0.27 *M* Tris–HCl buffer (pH 8.5) for 30 min at 40°C) and re-chromatographed under identical conditions. Novel peaks were recovered manually and reduced again (as above) and S-pyridylethylated by the addition of 5 μl of 4-vinylpyridine (Aldrich, NSW, Australia) and incubation at 25°C, in the dark, for 30 min. Alkylation reactions were quenched by the addition of 10 μl of ß-mercaptoethanol and peptides were subjected to Edman degradation by direct loading onto the sequencer. Selected Asp-N peptide mixtures (e.g., peptide D5) were diluted ten-fold with 5% formic acid, sub-digested with pepsin (enzyme–substrate, 1:20) at 37°C for 1 h and the resultant peptides were fractionated by RP-HPLC, as described above.

2.8. Hemadsorption assay

The protein A hemadsorption assay which detects surface bound IgG antibody by adherence of protein A coated human RBC (blood group O) to target cells was performed as previously described [20].

3. Results and discussion

3.1. Expression of A33 antigen in cultured colon cancer cells

To identify an appropriate cell line for antigen purification, cell surface expression of A33 antigen was analyzed in 22 human colon cancer cell lines and in ASPC-1 (the human pancreatic cancer cell line originally used as immunogen for the generation of mAbA33) using mouse mAb A33 in a protein A hemadsorption assay. Mouse mAbA33 reacted with 10 of the 22 colon cancer cell lines and with ASPC-1 (Table 1). Expression of A33 was confirmed in most instances by radioimmunoprecipitation of A33 antigen and Western blot analysis. Murine mAbA33 specifically precipitated a protein with M_r~43 000 in SDS-PAGE under nonreducing conditions from colon cancer cell lines testing positive for A33 antigen expression by protein A hemadsorption ($A33^+$), but not from A33 negative cell lines ($A33^-$). A protein with the same SDS-PAGE characteristics was precipitated by mAb100.310 (a second generation mouse mAb with an almost identical serological reactivity pattern to mAbA33 but which, by biosensor analysis, appears to recognize a different epitope) but not by isotype matched control antibodies. SDS-PAGE analysis of the proteins immunoprecipitated from SW1222 cells by mAbA33 or mAb100.310 using reducing conditions followed by autoradiofluorography revealed a protein band with M_r~50 000 (Fig. 1, Inset A). Western blot reactivity of mouse mAbA33 and mAb100.310 with the 43 000 protein was completely abolished under reducing SDS-PAGE conditions. These characteristics of the A33 antigen isolated from SW1222 cells are identical to our findings of A33 isolated from LIM1215 colon cancer cells [9,10]. A33 antigen expression

Table 1
Reactivity of mAbA33 with human gastrointestinal cancer cell lines

Cell line	Hemadsorption titer[a] (ng IgG/ml)	Western blot[b]	Immune precipitation
LIM1215	2.5	+	+
LOVO	5	+	[c]
LS174T	5	ND	ND
LS180	10	+	ND
NCI-H508	5	+	+
SK-CO-17	40	+	ND
SK-CO-19	2.5	ND	ND
SNC-2B	5	ND	ND
SW403	2.5	+	ND
SW1222	5	+	+
COLO205[c]	ND	+	+
ASPC-1[d]	2.5	+	+
DLD1	−	ND	ND
HCT15	−	−	ND
HT29	−	−	ND
SK-CO-10	−	−	ND
SK-CO-11	−	ND	ND
SK-CO-15	−	ND	ND
SW480	−	ND	ND
SW620	−	−	−
SW837	−	ND	ND
SW1116	−	−	ND
SW1417	−	ND	ND

[a] Lowest concentration of mAbA33 giving 50% rosetting.
[b] Utilizing mAbA33; SDS-PAGE was performed using nonreducing conditions.
[c] Not suitable for hemadsorption assay as cell line only grows in suspension.
[d] Pancreatic carcinoma.
ND=not determined.

detected by protein A hemadsorption assay, radioimmunoprecipitation and Western blot analysis correlated qualitatively and quantitatively. The cell lines SW1222 and LIM1215 were selected for further A33 antigen purification since they expressed relatively high levels of A33 antigen and grew rapidly in tissue culture.

3.2. Purification of the A33 antigen

A33 antigen was purified from Triton X-100 extracts of the human colon cancer cell lines SW1222 and LIM1215 by multidimensional chromatography (protocol A) in our initial studies [9], and an immunoaffinity chromatography based method (protocol B) in later studies. Preliminary attempts at purifying the A33 antigen by immunoaffinity chromatography using mAbA33-conjugated Affigel did not yield sufficient quantities of pure A33 antigen for sequence analysis due to low yields and considerable leakage of antibody from the affinity matrix [9]. To improve yields, mouse mAbA33 was bound by the Fc portion in a defined orientation to protein A of the gel matrix before covalent conjugation using dimethyl pimelimidate, allowing exposure of a higher number of free antibody binding sites for antigen binding. The affinity column was eluted with 0.1 *M* sodium citrate buffer with decreasing pH followed by glycine (50 m*M*) buffered saline (pH 2.8). The A33 antigen eluted in 0.1 *M* citrate buffer at pH 3.0 and in glycine (50 m*M*) buffered saline at pH 2.8, as monitored by Western blot analysis under nonreducing conditions using humanized mAbA33 (Fig. 1, inset B). A major protein band with a M_r~90 000 was also reactive with the humanized mAbA33 (Fig. 1, inset B, lane 4). The presence of oligomeric species of A33 has been noted previously [9]. The major contaminant in A33 antigen-containing frac-

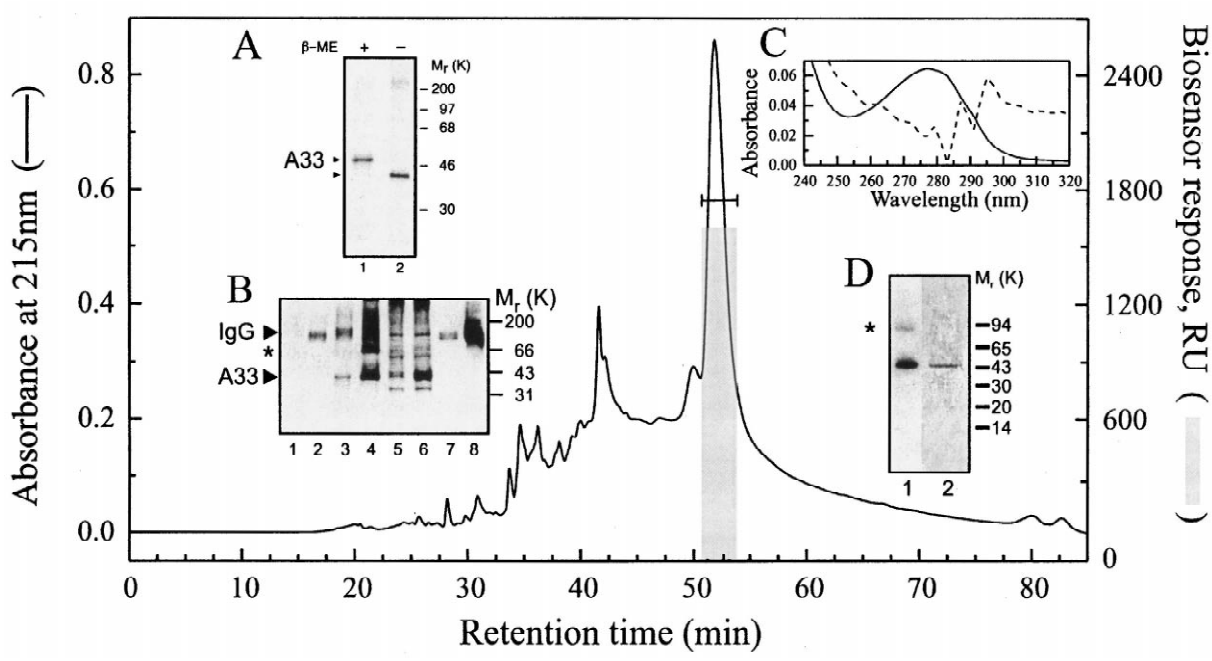

Fig. 1. Purification of A33 antigen by immunoaffinity chromatography and RP-HPLC. Main Panel: Micropreparative RP-HPLC of A33 antigen obtained by affinity chromatography. Samples (4×~100 μl) were loaded directly onto a Brownlee RP-300 column (50×1.0 mm I.D., Applied Biosystems, Foster City, CA), at a flow-rate of 500 μl/min employing a Hewlett-Packard 1090A liquid chromatograph fitted with a model 1040A diode-array detector, as described previously [18]. The column was previously equilibrated in buffer A (aqueous 0.1% TFA) and the chromatogram developed with a 60 min linear gradient with buffer B [aqueous 0.09% TFA–*n*-propanol (40:60, v/v)] at 40 μl/min at 40°C. Inset Panel A: Electrophoretic motility of A33 antigen under reducing and nonreducing conditions. [^{35}S]-labeled A33 antigen was obtained by immunoprecipitation with mouse mAb100.310 (anti-A33 antigen) from Triton X-100 (0.3% in PBS, pH 7.5) extracts of SW1222 colon cancer cells, resolved by SDS-PAGE on 10% gels under reducing (5% ME) or nonreducing conditions and visualized by autoradiofluorography. Under reducing conditions A33 antigen had an M_r~50 000 (lane 1), and under nonreducing conditions an M_r~43 000 (lane 2). Inset Panel B: Triton X-100 (0.3% in PBS) extracts of SW1222 colon cancer cells ($6{\cdot}10^8$) were incubated overnight at 4°C with mouse mAbA33 covalently immobilized on protein A Sepharose with dimethylpimelimidate (3 mg bound Ig/ml beads). After extensive washing with 10 m*M* Tris–HCl (pH 7.5) containing 0.3% Triton X-100, the column was eluted with 0.1 *M* sodium citrate buffer (pH 6.0) (lane 1), 4.0 (lane 2) and 3.0 (lane 3), and glycine (50 m*M*) buffered saline, pH 2.8 (lane 4). Eluted fractions were concentrated by membrane filtration and aliquots monitored for the presence of A33 antigen by Western Blot analysis under nonreducing conditions (SDS (10%)-PAGE, chemiluminesence visualization) using humanized mAb A33 to reduce cross-reactivity with murine mAbA33, which was bleeding from the column matrix (lanes 2, 3 and 4). A33 antigen eluted with 0.1 *M* citrate buffer, pH 3.0 (lane 3) and with glycine (50 m*M*) buffered saline, pH 2.8 (lane 4). Lanes 5 to 8: SW1222 Triton X-100 extract after (lane 5) and before (lane 6) incubation with affinity matrix; mouse (lane 7) and humanized (lane 8) mAb A33. *Indicates a dimeric form of A33 antigen. Inset Panel C: UV absorption spectrum of A33 antigen (retention time, 55 min) (−); with second derivative spectrum from 240–320 nm (− −). Inset Panel D: SDS-PAGE and Western blot analysis of purified A33 antigen. Purified A33 antigen was separated by SDS (8–25% Phastgel)-PAGE and analyzed by Western blot with murine mAbA33 (lane 1) or silver staining (lane 2). The asterix (*) indicates a dimeric form of A33 antigen.

tions (pH 3.0 and pH 2.8) was mouse mAb A33 which, even using the covalent coupling procedure described, still exhibited some leaking from the immunoaffinity column. No other major protein bands were detected in silver-stained gels (data not shown). A33 antigen-positive fractions were pooled, concentrated and further purified by microbore RP-HPLC (Fig. 1, main panel). A33 antigen chromatographed as the major peak (retention time 51–53 min) under these conditions. A33 antigen purified in this manner was homogenous as judged by SDS-PAGE (silver-staining; Fig. 1, inset D, lane 2) and yielded a single N-terminal sequence (Table 2); this material was immunoreactive with mAbA33 by biosensor and Western blot analysis (Fig. 1, inset D, lane 1). On-line spectral analysis of the A33 antigen

(Fig. 1 inset C) revealed the presence of tryptophan residues as evidenced by the extremum at 292 nm in the second-derivative spectrum [21]. The average yield of purified A33 antigen from $6 \cdot 10^8$ SW1222 cells using this two-step purification strategy was ~3 μg (~70 pmol).

3.3. Amino acid sequence analysis of the A33 antigen

Edman degradation of the A33 antigen obtained from the final micropreparative RP-HPLC step established the N-terminal 40 residues of the molecule (Table 2). Additional sequence information was obtained by Edman degradation of peptides produced by proteolytic fragmentation of a subsequent preparation. A preliminary low level peptide map followed by microsequence analysis was performed using 10% of the sample (approximately 7.5 pmol). This allowed the homogeneity of the generated fractions to be determined, and facilitated the development of a suitable strategy to generate unique peptides. Asp-N endoproteinase mapping of the A33 antigen (Fig. 2A) revealed five major peptide fractions. Of these, peptide fractions D2 and D3 yielded unambiguous sequences (39 and 15 residues, respectively) (Table 2), while Edman degradation of peptide fraction D1–D4 revealed the presence of two peptides in approximately equimolar quantities. Sequence analysis of peptide fraction D5 was complex, indicative of several N-termini. Upon reduction, and subsequent rechromatography under identical chromatographic conditions, peptide fraction D1–D4 was resolved into two discrete peptides, D1 and D4, (Fig. 2B) which yielded unambiguous sequences (30 and 12 amino acids, respectively) (Table 2). These data are consistent with a disulfide bridge between peptides D1 and D4. Subdigestion of peptide fraction D5 with pepsin yielded peptides Pc1 and Pc2 (Fig. 2C) and a further unambiguous assignment of 16 and 21 amino acid residues (Table 2). The sequence of two additional peptide fragments (T-1, T-2) (Table 2) was obtained after tryptic digestion of A33 antigen purified from LIM1215 colon cancer cells using the multidimensional chromatographic approach described (protocol A). In total, the unambiguous assignment of 153 amino acid residues was possible. The amino acid sequence information obtained from this study enabled the isolation, and subsequent oligonucleotide sequence analysis, of an A33 antigen cDNA from a LIM1215 cDNA library [22]. All peptide sequences obtained by Edman degradation were in agreement with the peptide sequences predicted from the A33 antigen cDNA (see Fig. 3). The

Table 2
Summary of amino acid sequence data for the A33 antigen

	Analyzed sample sequence[a]
N-terminus	ISVETPQDVLRASQGKSVTLPCTYHTSTSSREGLIQWDKL (1–40)
Peptides[b,c]	
D-1	DVLRASQGKSVTLPCTYHTSTSSREGLIQW (8–37)
D-2	DKLLLTHTERVVIWPFSNKNYIHGELYKNRVSISNNAEQ (38–76)
D-3	ELYKNRVSISNNAEQ (62–76)
D-4[d]	DXGTYECSVSLM (90–101)
Pc-1	IQLTCQSKEGSPTPQY (137–152)
Pc-2	LVLVPPSKPECGIEGETIIGN (115–135)
P-1	ILNQEQPLAQPASGQPV (159–175)
T-1	EAYEEPPEQLR (258–268)
T-2	VVIWPFSNK (48–56)

[a] One-letter notation used for amino acids.
[b] Peptide nomenclature: D, Asp-N endoproteinase; P, pepsin; Pc, peptides recovered from 'core material' following thermolysin/pepsin/Asp-N treatment of A33 antigen (see Fig. 1), T, trypsin.
[c] Amino acid numbering was derived from the A33 antigen-cDNA derived sequence [22].
[d] No amino acid was detected at cycle 2 (X).

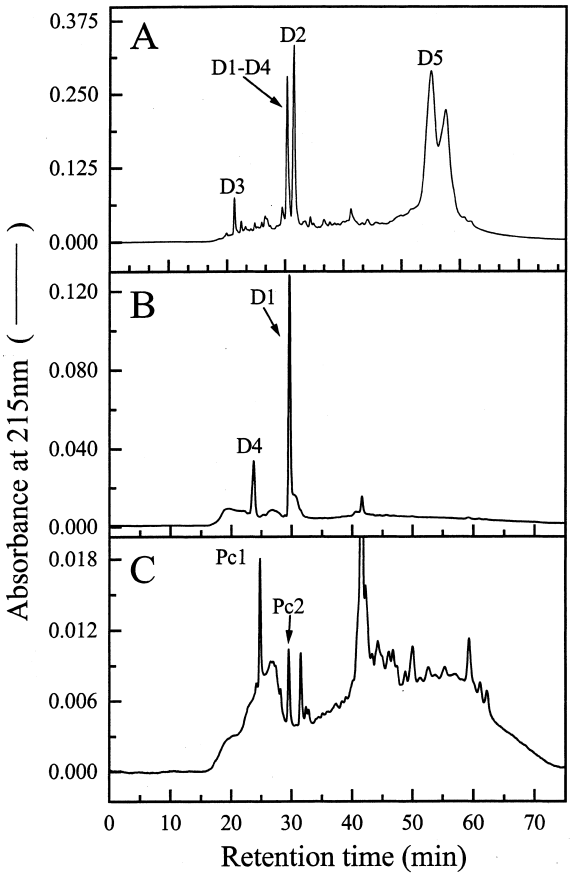

Fig. 2. RP-HPLC of A33 antigen following proteolytic digestion. Panel A: Separation of Asp-N endoproteinase peptides obtained from RP-HPLC-purified A33 antigen (Fig. 1) The peak eluting after D5 is undigested core material. Panel B: Rechromatography of reduced Asp-N endoproteinase peptide D1–D4 from A. Panel C: Chromatography of pepsin sub-digest of Asp-N endoproteinase of peptide D5. Chromatographic conditions as described in Fig. 1.

fact that LIM1215-derived peptide fragment T-2 and the 34 amino acids identified from the N-terminal residue of the A33 antigen purified from this source [9] were contained in SW1222-derived peptide fragment D-2 and in the 40 amino acids of the N-terminal sequence of the corresponding protein indicates that both A33 proteins were identical, although they were obtained from different colon cancer cell lines using alternative purification protocols.

The cDNA derived sequence contains three potential N-linked glycosylation sites [22]. The amino acid sequence analysis of peptide D-4 revealed a Xaa–Gly–Thr motif whereby residue X is consistent with an N-glycosylated asparagine (Table 2), residue X corresponding to Asn 91 in the deduced protein sequence [22]. Additionally, immobilized A33 antigen, purified under nondenaturing conditions using affinity chromatography followed by micropreparative anion-exchange and SEC, showed specific binding to concanavalin A and Lens culinaris using biosensor technology [12], suggesting that the A33 antigen is a glycoprotein containing branched mannose residues.

An initial search of available nucleotide and protein databases did not reveal any match between the A33 sequence and any known protein, although manual inspection revealed the presence of several amino acids which are conserved in members of the Ig superfamily [22]. However, we have recently noted (Fig. 3) significant (~30%) structural homology with CTX, a developmentally regulated type-I transmembrane protein belonging to the immunoglobulin superfamily which is specifically expressed on cortical thymocytes in *Xenopus* [23]. It is interesting to note that both the A33 antigen and CTX also appear related (Fig. 3) to the recently reported common receptor for Coxsackie B viruses and Adenoviruses 2 and 5 (CAR) [24]. Both mature proteins have a similar number of amino acid residues (299 and 351, respectively) to the A33 antigen (298 residues) with short intracellular domains and contain two putative immunoglobulin domains in the extracellular region. However, to date, the biological function of A33 and CTX remains unknown.

4. Conclusions

The methods presented herein have allowed the unambiguous identification of 153 amino acid residues of the A33 antigen using approximately 6 μg of protein (133 pmol) purified from the colon carcinoma cell lines LIM1215 and SW1222. The N-terminal sequence obtained facilitated the cloning of the corresponding cDNA [22]. The internal pep-

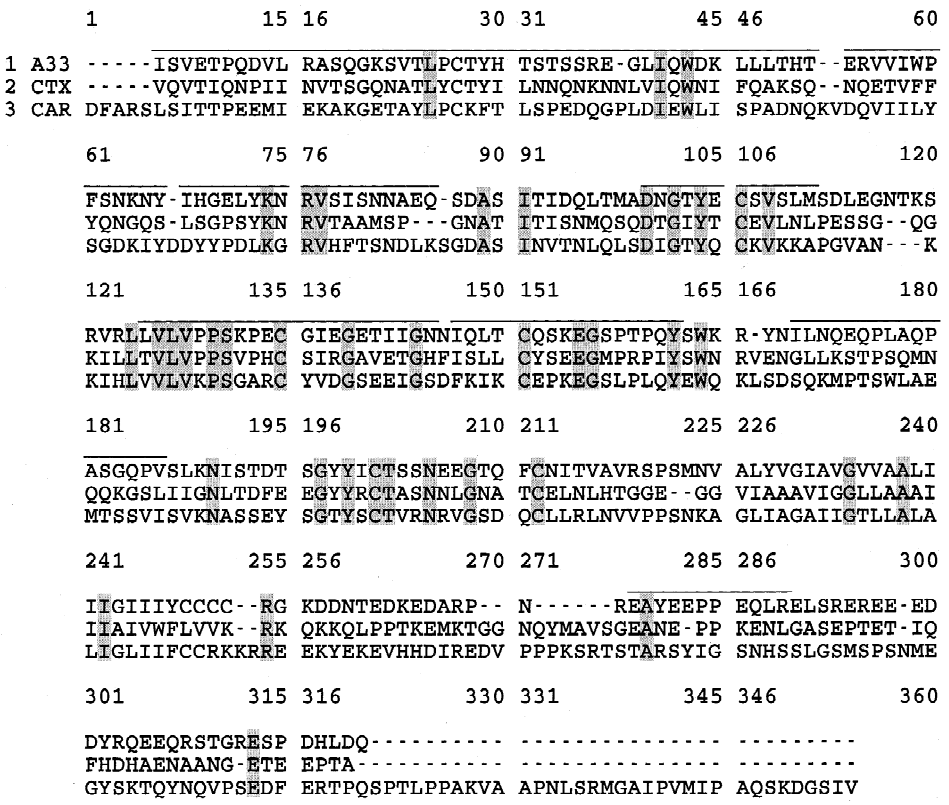

Fig. 3. Comparison of A33 related amino acid sequences. Amino acid sequences are shown in one-letter code. The A33 antigen sequence is from the cDNA-derived sequence [22]; CTX is a type-1 transmembrane protein expressed in the amphibian *Xenopus* [23]; CAR is a common receptor for coxsackie B viruses and adenoviruses 2 and 5 [24]. Overlined sequences were derived by Edman degradation of the mature A33 antigen and related peptides (see Table 2). Amino acids which are conserved in all three sequences are highlighted.

tide sequences generated in this study were invaluable in ensuring that no reading errors were introduced during the interpretation of the oligonucleotide sequence. The ability to readily purify microgram-quantities of protein will facilitate future structure–function studies to determine the biological role of the A33 antigen.

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