Purification and Characterization of a Novel Restricted Antigen
Expressed by Normal and Transformed Human Colonic Epithelium*

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B. Catimel‡, G. Ritter§, S. Welt§, L. J. Old§, L. Cohen§, M. A. Nerrie‡, S. J. White‡, J. K. Heath‡, B. Demediuk‡, T. Domagala‡, F. T. Lee§, A. M. Scott‡, G. F. Tu‡, H. Ji‡, R. L. Moritz‡, R. J. Simpson‡, A. W. Burgess‡, and E. C. Nice‡**

From the Ludwig Institute for Cancer Research, Melbourne Tumour Biology Branch, and the Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research, and The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia, the Joint Oncology Unit, Ludwig Institute for Cancer Research and Austin Hospital, Heidelberg, Victoria 3084, Australia, and the Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

A cell surface antigen that is expressed by normal and 95% of transformed colonic epithelium and is recognized by the monoclonal antibody A33 (Welt, S., Divgi, C. R., Real, F. X., Yeh, S. D., Garin-Chesa, P., Finstred, C. L., Sakamoto, J., Cohen, A., Sigurdson, E. R., Kemenyi, N., Carswell, E. A., Oettgen, H. F., and Old, L. J. (1990) J. Clin. Oncol. 8, 1894–1906) has been purified to homogeneity from the human colonic carcinoma cell line LIM1215. The A33 protein was purified from Triton X-114 extracts of LIM1215 cells under nondenaturing conditions. These extracts were applied sequentially to Green-Sepharose HE-4BD, Mono-Q HR 10/10, Superose 12 HR 10/30, and micropreparative Brownlee Aquapore RP 300. The purification was monitored by biosensor analysis using surface plasmon resonance detection with a F(ab′)2 fragment of the humanized A33 monoclonal antibody immobilized on the sensor surface and Western blot analysis following SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions using humanized A33 monoclonal antibody. The purified A33 antigen has a Mr of approximately 180,000 under native conditions on both size exclusion chromatography and native PAGE, possibly due to the formation of a homotetramer. N-terminal amino acid sequence analysis of the purified protein identified 34 amino acid residues of a unique sequence: IYETPQDVLRA5QKSGS-VTLPXHTH5XXRGLIQWQ. A polyclonal antibody was raised against a synthetic peptide corresponding to residues 2–20 of this sequence. The antipeptide serum recognized the purified protein using Western blot analysis under both nonreducing (Mr, 43,000) and reducing (Mr, 49,000) conditions.

Colorectal cancer is among the most common malignancies of the Western world and is a leading cause of cancer deaths (1). Advances have been made in the treatment of patients with advanced disease, but they have only had a modest impact on overall survival. Consequently, a need still exists to develop methods for effective treatment of metastatic colorectal cancer. Passive cancer immunotherapy using monoclonal or genetically engineered antibodies to deliver radioisotopes to tumor cells, can complement the effect of conventional treatments (surgery, chemotherapy, and radiotherapy), and a large number of monoclonal antibodies have already been administered to cancer patients following this approach.

The monoclonal antibody (mAb) A33 detects a cell surface antigen expressed by 95% of primary or metastatic colon cancer cells and normal colonic epithelium but not by most other normal tissues and tumor types (2, 3). Because of its restricted pattern of expression, the A33 antigen can be classified as a tissue-specific antigen for both normal and transformed colon, rectal, and small intestinal epithelium. Some human colon cancer cell lines express high levels of the A33 antigen, binding up to 800,000 molecules/cell. The A33 antigen is not secreted or shed, and cell-bound radiolabeled A33 mAb is rapidly internalized into antigen-positive cells. Phase 1 quantitative dosimetry and human biodistribution studies demonstrated the tumor targeting capabilities of A33 mAb (2). Phase I and II radioimmunotherapy studies with 131I- and 125I-labeled A33 mAb further demonstrated the localization capabilities of this antibody, and, in addition, some antitumor effects were observed in these heavily pretreated patients (3, 4). These studies have also shown that, although the isotope is rapidly cleared from the normal colon (5–6 days), it is retained for long periods (up to 6 weeks) in the tumor lesions, providing a rationale for radioimmunotherapy studies. The antibody has now been humanized (5), and studies are commencing using this reagent in multiple phase I trials.

However, despite extensive immunochemical, immunohistochemical, and clinical studies, the antigen for the A33 mAb has not previously been identified. Our initial attempts to purify the A33 mAb antigen by immunochromatography were hindered by low yields and leakage of the antibody from the affinity matrix. Furthermore, nonspecific interaction between the Fc domain of the murine A33 mAb and actin was observed. Therefore, a multidimensional chromatographic protocol was developed for the purification of the protein from the human colonic carcinoma cell line LIM1215 (6), which expresses significant levels of A33 antigen, as demonstrated by both immu-

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**To whom correspondence should be addressed: Ludwig Institute for Cancer Research, P. O. Royal Melbourne Hospital, Parkville, Victoria, 3050, Australia. Tel.: 61-3-9347-3155; Fax: 61-3-9347-1938; E-mail: nice@licre.ludwig.edu.au.

1 The abbreviations used are: mAb, monoclonal antibody; RP, reversed-phase; HPLC, high performance liquid chromatography; ID, inside diameter; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; RU, resonance unit.

2 S. Welt, G. Ritter, and L. J. Old, unpublished results.
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chromatographic purification.

Green-Sepharose Chromatography—Triton X-100 extract or the Triton X-114 detergent phase was diluted to a final concentration of 0.1% detergent and loaded at 4°C onto a Green-Sepharose HE-4BD column (100 × 10 mm ID) connected to a fast protein liquid chromatography system (Pharmacia Biotech). The column was equilibrated with 10 mM Tris-HCl, pH 7.4, containing 0.1% (v/v) CHAPS. Bound proteins, including actin, were eluted stepwise with 1 M NaCl. The breakthrough contained the A33 antigen and was collected for anion exchange HPLC.

Anion Exchange HPLC—The Green-Sepharose breakthrough was injected at 4°C onto a Mono-Q HR 10/10 column previously equilibrated in 10 mM Tris-HCl, pH 7.4, containing 0.1% (v/v) CHAPS. The proteins were eluted from the column using a linear 0–1 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. The A33 antigen in eluant fractions was detected using both biotin-streptavidin blot analysis under nonreducing conditions, as described below.

Size Exclusion HPLC—The active fractions eluted from the Mono-Q column (10 ml) were concentrated 10-fold using a SpeedVac concentrator (Savant Instruments Inc., Farmingdale, NY), dialyzed against PBS containing 0.05% (v/v) CHAPS, and loaded at 4°C onto a Superose 12 HR 10/30 column. Proteins containing 0.05% (v/v) CHAPS were eluted using a linear 60-min gradient to 60% aqueous 0.15% (v/v) trifluoroacetic acid in water. The proteins were eluted with a linear 60-min gradient to 60% aqueous 0.15% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. Fractons (0.5 ml) were collected. Proteins were detected at 280 nm. The A33 antigen was monitored by both Western blot and biosensor analysis.

Reversed-Phase HPLC Chromatography—Superose 12 active fractions (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 ID) equilibrated with the primary solvent, 0.15% (v/v) trifluoroacetic acid in water. The proteins were eluted with a linear 60-min gradient to 60% aqueous 0.15% (v/v) trifluoroacetic acid at a flow rate of 100 μl/min. The column temperature was 45°C. Protein detection was performed at 215 nm. The A33 antigen was 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 ID) prior to N-terminal sequence analysis (12), using the gradient conditions described above at a flow rate of 50 μl/min. Eluant fractions were recovered manually.

Bioassay Analysis

Cell extracts and chromatographic fractions were monitored using an instrumental optical biosensor (BIAcore™, Pharmacia Biosensor), with an F(ab)₂ fragment of humanized A33 mAb immobilized onto the biosensor surface using N-hydroxysuccinimide and ethyl-N,N-dimethylamino propyl-carbodiimide at a flow rate of 4 μl/min as described previously (13). Antigen binding to the F(ab)₂ fragment is detected by surface plasmon resonance, which measures small changes in refractive index at, or near, the gold sensor surface (8, 9). Prior to biosensor assay, cell extracts or aliquots of chromatographic fractions were diluted to 100 μl final volume in BIAcore buffer (10 mM HEPES, pH 7.4, containing 3.4 mM EDTA, 0.15 mM 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 mM NaOH. This treatment did not denature the protein immobilized onto the sensor surface, as shown by equivalent signals on reionization of a sample containing the A33 antigen.

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 (14) on 8–25% SDS-PAGE Phastgels or 8–25% native Phastgels and transferred onto polyvinylidene difluoride membranes and incubated with murine or humanized A33 mAb. RP-HPLC-purified A33 antigen was also analyzed by Western blot under nonreducing and reducing conditions using polyclonal anti-N-terminal peptide antibodies. IG binding was probed with horse radish peroxidase-labeled goat anti-mouse IgG, goat anti-human IgG, or goat anti-rabbit IgG and detected by enhanced chemiluminescence.

N-terminal Amino acid sequence analysis

N-terminal amino acid sequence analysis of purified A33 protein was performed on a Hewlett-Packard G1005A protein sequencer operated with the routine 3.0 sequencer program described previously (14).
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Fig. 1. Flow cytometry of LIM1215 and Hep-2 cells using A33 mAb. LIM1215 colonic carcinoma cells and Hep-2 epidermoid carcinoma cells were stained with murine A33 mAb followed by fluorescein-conjugated anti-murine IgG or with a nonrelated, isotypically matched IgG followed by fluorescein-conjugated anti-murine IgG and analyzed as described under “Materials and Methods.” The entire population of LIM1215 cells exhibited a strong homogeneous fluorescence (B) when incubated with the A33 mAb, compared with the fluorescence obtained with the control antibody (A). The profiles shown in the panels obtained with the HEp-2 cells (C and D) were overlapping, indicating no detectable A33 mAb binding to these cells. The x axis shows the fluorescence intensity (log scale), and the y axis shows the cell number.

A33 mAb and F(ab’)2 Fragment Purification
Murine (2) and humanized (5) A33 antibodies were purified from ascites or cell supernatants using protein A affinity chromatography. F(ab’)2 fragments were generated by pepsin (1% w/w) digestion (15) of 10 mg of humanized A33 mAb in 0.1 M sodium acetate, pH 3.5. F(ab’)2 fragments were purified by size exclusion chromatography on a Sephacryl S-200 (2.8 × 60 cm) column (Pharmacia Biotech) equilibrated with 50 mM sodium phosphate (pH 7.4) containing 0.15 mM NaCl. Elution was performed at a flow rate of 0.5 ml/min.

Polyclonal Antipeptide Antibody Production and Purification
Antibodies were generated in New Zealand White rabbits using as immunogen a chemically synthesized peptide corresponding to part of the N-terminal sequence of the A33 antigen described herein (amino acid residues 2–20) conjugated to keyhole limpet hemocyanin (Quality Controlled Biochemicals Inc., Hopkinton, MA) in complete Freund’s adjuvant/incomplete Freund’s adjuvant. IgG was purified from immune sera by protein A affinity chromatography. Purified IgG was analyzed for reactivity with LIM1215 cell lysates and purified A33 antigen by SDS-PAGE and Western blot analysis.

Protein Quantitation
Protein concentration was determined using the bicinchoninic acid protein assay (16) or from the ratio of A280/A260 (17).

RESULTS AND DISCUSSION
The colonic carcinoma cell line LIM12156 was shown by flow cytometry (Fig. 1) and immunocytochemistry and rosetting assays (data not shown) to strongly express the membrane antigen of the A33 mAb. Western blot analysis of a 0.3% Triton X-100 extract of LIM1215 cells under nonreducing conditions using both murine and humanized A33 mAb showed a major band with a Mr of 43,000 (Fig. 2A, lane 1). A Mr of 41,000 band was also recognized by Western blot analysis, as well as two other minor lower molecular weight bands (Fig. 2A, lane 1). Western blot analysis of a 0.3% Triton X-100 extract of Hep-2 cells, which were negative for A33 antigen expression by flow cytometry (Fig. 1), failed to demonstrate the Mr 43,000 band but detected both the Mr 41,000 and lower molecular weight bands (results not shown), suggesting that only the Mr 43,000 protein was specifically recognized by the A33 mAb. The A33 mAb recognized a conformationally sensitive epitope, as evidenced by nonreactivity in Western blot analysis under reducing conditions.

A combined immunopurification and biosensor approach was initially designed in an attempt to purify the A33 ligand, using Triton X-100 extracts of LIM1215 cells as the starting material. The biosensor technology monitors protein-protein interactions in real time, using an optical detection principle based on surface plasmon resonance (9). In these initial experiments the murine A33 mAb was immobilized onto the surface of the sensor chip, whereas antigen-containing fractions were injected in a continuous flow over the surface. The surface plasmon resonance response reflects a change in refractive index, and hence a change in mass, at the detector surface as the antigen binds or dissociates from the immobilized antibody. For the immunochromatographic step, the murine A33 mAb was conjugated to Affi-Gel 10 (N-hydroxysuccinimide esters of a derivatized cross-linked agarose). By using this matrix, similar immobilization chemistry was used for both the biosensor analysis and affinity chromatography step, allowing the appropriate dissociation conditions for immunochromatography (10 mM NaOH) to be rapidly determined using biosensor analysis (18, 19).

A major protein of Mr 43,000 was immunopurified and found to be N-terminally blocked on amino acid microsequence analysis. Internal sequence analysis following “in gel” trypic digestion (20) identified this protein as actin. Further biosensor
studies confirmed this in vitro interaction between actin and immobilized A33 mAb. When a preparation of actin was injected over A33 mAb (Fig. 3) or nonrelated monoclonal antibodies of the same immunoglobulin subclass (IgG2a) immobilized onto the biosensor surface, a strong positive binding was observed. By contrast, when A33 F(ab')2 fragments were immobilized, actin binding was no longer observed, suggesting that actin was binding to the Fc domain of the antibody (Fig. 3).

We therefore developed a multidimensional chromatographic protocol for the purification of the A33 mAb ligand. To avoid actin interaction during the screening of the chromatographic fractions, A33 F(ab')2 fragments were immobilized onto the biosensor surface. Active fractions detected by biosensor analysis were confirmed positive by Western analysis following SDS-PAGE under nonreducing conditions.

The focus of the initial purification steps was to remove actin, which had been shown to be present in the cell extracts in significant quantities. Studies with Triton X-100 LIM1215 cell lysates showed that actin could be selectively separated from the A33 antigen by Green-Sepharose HE-4BD ligand dye chromatography. The A33 ligand did not bind to the Green-Sepharose matrix (Fig. 2A, lane 2), whereas actin was retained and could be eluted with 1 M NaCl (Fig. 2A, lane 3). Actin, purified from rabbit muscle, was weakly recognized on Western analysis with A33 mAb under nonreducing (a minor band of M, 41,000; Fig. 2A, lane 4) and reducing conditions (data not shown), confirming the cross-reactivity observed using the biosensor (Fig. 3).

Although the Green-Sepharose chromatography was effective for removal of actin, Triton X-114 extraction and phase separation was found to be preferable to Triton X-100 to obtain a detergent phase enriched in hydrophobic membrane proteins (Fig. 2B). The A33 antigen was detected in the Triton X-114 extract (Fig. 2B, lane 1) and in the detergent-rich phase following phase extraction (Fig. 2B, lane 3) but not in the aqueous phase (Fig. 2B, lane 2). The presence of the A33 antigen in the detergent-rich phase suggested that either the A33 antigen is an integral membrane protein or a glycosylphosphatidylinositol-anchored protein (21). The actin band of M, 41,000 and the other nonspecific low molecular weight components observed with Triton X-100 (Fig. 2A, lane 1) were no longer detected using Triton X-114 extraction. The combination of Triton X-114 phase separation followed by Green Sepharose ligand dye chromatography to remove all traces of actin was therefore used as the primary purification step for the A33 antigen.

Injection of Triton X-114 extracts onto humanized A33 mAb immobilized to various chromatographic matrices (Affi-Gel 10, CNBr-activated Sepharose, and N-hydroxysuccinimide-activated Sepharose) resulted in the recovery of only very low amounts of a Mr 43,000 protein. Furthermore, leakage of significant quantities of antibody from the supports (22) hampered the practical use of these methods to purify and identify the antigen. This, coupled with the fact that specific detection (biosensor or Western blot) was also based solely on affinity methods, led us to exclude an affinity step in the purification protocol.

The detergent phase of the Triton X-114 LIM1215 cell extracts, after passage through Green-Sepharose HE-4BD, was loaded onto a Mono-Q anion exchange HPLC column. Triton X-114 was exchanged with the zwitterionic detergent CHAPS after absorption of the detergent-membrane complex to the chromatographic support. CHAPS was found to greatly improve resolution on both the anion exchange and the subsequent size exclusion chromatography. Application of a 0–1 M

**Fig. 3. Biosensor analysis of the interaction between actin and either A33 IgG or the A33 F(ab')2 fragment.** A preparation of rabbit muscle actin (0.5 μg) was injected at a flow rate of 5 μl/min over a sensor surface onto which either murine A33 IgG (upper trace) or A33 F(ab')2 (lower trace) had been immobilized. Protein-protein interactions were monitored by surface plasmon resonance. At the end of the injection pulse, a signal of 247 RU was observed due to actin binding to A33 IgG, whereas the signal corresponding to actin binding to A33 F(ab')2 was only 4 RU (arrows).

**Fig. 4. Anion exchange HPLC of the A33 antigen.** Proteins contained in the Green-Sepharose breakthrough fraction were loaded onto a Mono-Q HR 10/10 anion exchange column and eluted at a flow rate of 1 ml/min with a linear NaCl gradient as indicated (— — —). One-ml fractions were collected, and aliquots (20 μl) of each fraction were taken for biosensor assay. The M, 43,000 antigen was detected by Western blot analysis under nonreducing conditions (inset) in the labeled fractions.
Protein aliquots (2 ml) of each fraction were taken for biosensor analysis. The M₄, 43,000 antigen was also detected by Western blot analysis under nonreducing conditions (inset A) in the fractions indicated. Western analysis of a pool of the Superose 12 activity (fractions 2–5) using an 8–25% native gel revealed that the A33 antigen migrated under native conditions (no SDS) with a M₄ of 180,000 (inset B).

NaCl gradient resulted in the elution of at least 15 major protein peaks from the anion exchange column (Fig. 4). A strong biosensor signal (>200 RU) was registered when aliquots of fractions 2–11 were injected over the biosensor surface (Fig. 4). The presence of the M₄, 43,000 A33 antigen was confirmed by Western blot analysis (Fig. 4, inset), in fractions 2–9, eluting between 0.45 and 0.55 M NaCl. The subsequent fractions, eluting between 55 and 68 min, gave a small residual biosensor signal (approximately 100 RU) but were negative by Western blot analysis. The most active Mono-Q fractions (fractions 3–7) were pooled, concentrated 10-fold using a Savant SpeedVac concentrator, and further purified by size exclusion chromatography on Superose 12 HR 10/30 (Fig. 5). Interestingly, the M₄, 43,000 A33 mAb antigen was detected in both Western blot (Fig. 5, inset A) and biosensor analysis (Fig. 5) in early eluting fractions corresponding, by correlation with the retention time of protein standards of known molecular weight, to an apparent M₄ of 160,000–200,000. Western analysis of the pooled Superose 12 active fractions (fractions 2–5), using a 8–25% native Phastgel confirmed this result by revealing a protein migrating under native conditions (in the absence of SDS) with an apparent M₄, 23 of 180,000 (Fig. 5, inset B).

RP-HPLC of the Superose 12 active fractions (Fig. 5, fractions 1–5) on a Brownlee RP 300 micropreparative column (100 × 2.1 mm ID) using n-propyl alcohol as the organic modifier allowed further purification of the A33 antigen (Fig. 6A). The M₄, 43,000 A33 antigen was found by biosensor analysis (Fig. 6, A and B) to be associated with one symmetrical peak eluting from the RP-HPLC column between 45 and 48 min, demonstrating the highly hydrophobic character of this protein. Aliquots (2 µl) of the active fractions were analyzed by SDS-PAGE with silver staining (Fig. 6A, inset A). Whereas fraction 3 contained traces of a higher molecular weight component, fractions 4 and 5 were essentially homogeneous and revealed a M₄, 43,000 protein, corresponding to the band recognized by A33 mAb using Western blot analysis (Fig. 6A, inset B). The overall yield, based on comparison of the UV absorption at 215 nm with that of an ovalbumin standard, was approximately 1 µg.

As we have noted previously (19), one of the advantages of using the biosensor to monitor chromatographic purification is that complimentary kinetic data describing the specific interaction can also be obtained simultaneously. Using nonlinear least squares regression analysis (24, 25) of the biosensor curves shown in Fig. 6B, the apparent Kₛ for the interaction between the purified A33 antigen and the immobilized A33 F(ab′)₆ fragment was found to be in the low nanomolar range.

The purified protein was subjected to N-terminal amino acid sequence analysis in which, in an initial experiment, a unique sequence of 33 amino acids was obtained: XVSETTPQGDVLRAIQGKSVTLPXHILSRKRVKVRGIAWQVQK. The initial yield was approximately 10 pmol. This is the anticipated yield (50%) for the quantity loaded onto the sequencer (0.8 µg after aliquots had been taken for Western blot and biosensor analysis (see Table I) and indicates that no N-terminally blocked proteins were present. In a subsequent experiment, in which purified protein from two preparations was loaded onto the sequencer, the initial yield was 20 pmol. In this experiment isoleucine was identified as the N-terminal residue. A sequence similarity search of the available protein and nucleotide data bases failed to reveal any significant sequence identity. In particular, we noted that the sequence obtained for the A33 antigen showed no significant similarity with the epithelial glycoprotein 40,
also known as the 17-1A antigen or epithelial cell adhesion molecule (26). This Mr 41,000 protein is expressed by many carcinomas, and the 17-1A antibody has also been used in adjuvant immunotherapy for colorectal carcinoma (27).

Rechromatography of the purified protein on size exclusion chromatography or native PAGE followed by Western blot analysis indicated, as seen previously prior to the final RP-HPLC stage (Fig. 5), an apparent Mr of approximately 180,000. A single protein peak was observed with the size exclusion chromatography. The recovery was essentially quantitative (based on peak area calculations), suggesting that no additional species had dissociated during the chromatography. In fact, even after treatment of the purified protein with 8 M urea, no change in elution position on size exclusion chromatography was observed. Furthermore, the A33 antigen did not appear to be complexed with another protein, since co-immunoprecipitation from [35S]methionine-radiolabeled LIM1215 cell lysates by the A33 mAb yielded a single species with the characteristic apparent Mr on SDS-PAGE of 43,000 under nonreducing conditions and 50,000 following reduction (data not shown). Taken together with the initial yields obtained during N-terminal sequence analysis, these observations suggest that the 180,000 form of the antigen observed under nondenaturing conditions could be a nonequivalently associated homotetramer.

To further confirm the biological specificity of the N-terminal sequence, a polyclonal antibody was raised in rabbits against a synthetic peptide corresponding to residues 2–20 of the generated sequence. Purified IgG from immunoreactive sera recognized the same Mr 43,000 RP-HPLC-purified protein as the A33 mAb under nonreducing conditions (Fig. 7, lane 1). The peptide antisera also recognized a single protein spot with an apparent Mr of 49,000 under reducing conditions (Fig. 7, lane 2), in agreement with the mobility observed for the immunoprecipitated material obtained from [35S]methionine-radiolabeled LIM1215 cell lysates under reducing conditions (see above). The antipeptide antibody reactivity appeared to be stronger against the reduced form of the protein.

The yields obtained for the individual purification steps, calculated from the biosensor responses (13), are summarized in Table I. For this purpose, a calibration curve of response (RU) again dilution was constructed retrospectively using the purified antigen. The responses observed for all of the purification steps, with the exception of the final reversed-phase chromatography (Fig. 6B), were found to fall in the linear range of the calibration curve (0–700 RU). For quantitation of the RP-HPLC fractions, in which higher responses were obtained, values were extrapolated to the linear fit.

The Triton X-114 phase extraction was a useful initial purification and enrichment step, since almost 70% of the total extracted protein partitioned into the aqueous phase, whereas there was good recovery (83%) of the A33 ligand activity in the corresponding detergent phase. Recovery from the Green-Sepharose ligand dye column was 92%, with a 50% increase in the specific activity. The recovery from the Mono-Q column (fractions 2–10) was only 39%, possibly due to a number of factors, including the hydrophobic nature of the molecule, the detergent exchange from the Triton X-114 to CHAPS that was performed at this stage, and the use of a semipreparative Mono-Q HR 10/10 column (the use of the smaller HR 5/5 column resulted in unacceptably high back pressures). Additionally, it should be noted that, due to the large number of chromatographic fractions and the reduction in protein concentration, the fractions were monitored by UV absorption at 260 nm compared with an ovalbumin standard.

For the calculation of the specific activity (SA): (i) the protein concentration was measured as described; (ii) a 10-μl aliquot from the chromatographic fraction was diluted with 90 μl HBS buffer, and 30 μl of this sample was injected over the biosensor surface; and (iii) the RU values obtained were correlated with the amount of protein injected over the biosensor.

Determined by BCA reaction (16).

Monitored from UV absorption at A260/A280 (17).

Table I

| Purification step | Protein | RU | Total recovery | Step recovery | SA | Purification factor |
|------------------|--------|----|---------------|--------------|-----|-------------------|
| LIM1215 Triton X114 extract | 61.0 | 2,670 | 100 | 44 |
| Triton X114 aqueous phase | 44.5 | 384 | 83 | 8 |
| Triton X114 detergent phase | 12.5 | 2,228 | 83 | 180 | 4 |
| Green-Sepharose breakthrough | 8.0 | 2,054 | 77 | 260 | 6 |
| Mono-Q, fractions 2–10 | 0.560 | 745 | 30 | 1,330 | 30 |
| Superose, fractions 1–5 | 0.190 | 482 | 18 | 2,540 | 60 |
| Brownlee RP 300 | 0.001 | 185 | 6 | 165,000 | 3750 |

* Calculated as a percentage of RU in initial extract.

b Calculated as a percentage of RU in initial extract.

c Calculated as a percentage of RU in initial extract.

d Calculated as a percentage of RU in initial extract.

e Calculated as a percentage of RU in initial extract.

FIG. 7. Western blot analysis of the A33 antigen using an antipeptide IgG. HPLC-purified A33 antigen (0.1 μg) was electrophoresed on an 8–25% SDS-PAGE Phastgel under nonreducing (lane 1) and reducing (lane 2) conditions and analyzed by Western blot analysis using an antipeptide IgG, raised against residues 2–20 of the N-terminal sequence, as described under “Materials and Methods.”

1 2 Mr(K)
--- 143
97
50
35
30
22
of approximately 3750 and an overall recovery of 6%. By reinjection of an aliquot of the purified protein back onto the Brownlee RP 300 column and monitoring both the relative peak recovery from the chromatographic trace at 215 nm and binding response on the biosensor, it was established that the protein recovery from the RP-HPLC column was approximately 45%, whereas the recovery of biosensor activity was only 23%, suggesting that there were both on-column protein losses and partial denaturation of the ligand during this chromatographic procedure. If this loss of reactivity is taken into account, an overall purification of approximately 15,000-fold could be calculated. Additionally, it should be noted that these purification factors are based on the protein content of the initial LIM1215 detergent extract and not the total protein content of the cells, which would have been considerably higher.

In conclusion, a method for the purification and characterization of a novel tissue-specific cell surface marker for normal and transformed human colonic epithelium has been described in this article. The biosensor analysis of the interactions between the A33 mAb and its antigen, together with Western blot analysis performed under nonreducing conditions, provided a rapid, specific, and sensitive method for monitoring the purification of a novel cell protein. The availability of pure A33 antigen will now allow us to perform structural and functional studies on this molecule, as well as detailed kinetic studies on the interaction with the A33 mAb and related molecules. Kinetic characterization of intact IgG, F(ab’)2 and Fab’ fragments, and corresponding antibody conjugates will be studied using the biosensor in conjunction with their use in clinical imaging and therapeutic trials. An understanding of the structure and function of this interesting new tissue-specific antigen for normal and transformed colonic epithelium could lead to the development of new and improved reagents and methods for the diagnosis and treatment of colon cancer.