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# Tumor-Associated Antigen 90K/Mac-2-Binding Protein: Possible Role in Colon Cancer

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**Abstract** The tumor-associated antigen 90K (TAA90K)/Mac-2-binding protein implicated in cancer progression and metastasis is modified by β1-6 branched N-linked oligosaccharides in colon cancer cells, glycans shown to contribute to cancer metastasis. To elucidate the role of TAA90K in colon cancer, we examined its expression and function in human colon tumors and colon carcinoma cell lines. Immunohistochemical analyses of colon tumors revealed elevated expression of TAA90K in all samples analyzed compared to normal colon. To examine the function of TAA90K in colon cancer, we carried out protein and cell binding assays using TAA90K-His purified from HT-29 cells colon carcinoma cells infected with recombinant vaccinia virus expressing TAA90K containing a C-terminal poly-histidine tag. TAA90K-His bound to fibronectin, collagen IV, laminins-1, -5, and -10 and galectin-3 (Mac-2) but poorly to collagen I and galectin-1. As expected, binding of TAA90K to galectin-3 was dependent on carbohydrate since it was inhibitable by lactose and asiolofetuin, and a TAA90K-His glycoform purified from HT-29 cells treated with the glycosylation inhibitor 1-deoxymannojirimycin bound poorly to galectin-3. Unlike TAA90K isolated from other cell types, TAA90K-His isolated from colon cancer cells failed to mediate adhesion of colon cancer and normal cell lines, possibly due to cell-type specific glycosylation of TAA90K-His and/or its putative cellular receptor. However, at low concentrations, TAA90K-His enhanced galectin-3-mediated HT-29 cell adhesion while at high concentrations, it inhibited cell adhesion. Thus, a possible mechanism by which TAA90K may contribute to colon cancer progression is by modulating tumor cell adhesion to extracellular proteins, including galectin-3. J. Cell. Biochem. 98: 1351–1366, 2006. 

**Key words:** colon cancer; glycoproteins; cell adhesion; tumor-associated antigen 90K; galectins; immunohistochemistry

Colorectal cancer remains a significant cause of mortality affecting both men and women, thus posing the challenge to develop improved methods of early detection and define genes involved in disease progression [Weir et al., 2003; Kondo and Issa, 2004; Vogelstein and

Abbreviations used: GlcNAc, N-acetylglucosamine; CyCAP, cyclophilin C-associated protein; BSA, bovine serum albumin; DMJ, deoxymannojirimycin; DMEM, Dulbecco's minimum essential medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunoassay; ER, endoplasmic reticulum; FBS, fetal bovine serum; Gal, galactose; IgG, immunoglobulin G; IPTG, isopropylthiogalactoside; L-PHA, leucophytohemagglutinin; MAb, monoclonal antibody; NMS, normal mouse serum; ORF, open reading frame; PMSF, phenylmethylsulfonylfluoride; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 0.04% Tween-20; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TAA90K, tumor-associated antigen 90K; TK, thymidine kinase; TBS, Tris-buffered saline, 50 mM Tris-HCl, 0.15 M NaCl, 0.02% sodium azide; WGA, wheat-germ agglutinin.



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Kinzler, 2004]. Herein, we have focused on the tumor-associated antigen 90K (TAA90K)/ Mac-2-binding protein and its potential role in colon cancer.

TAA90K is a secreted glycoprotein of 90–100 kDa expressed in normal human fluids [Koths et al., 1993], epithelial cells [Jallal et al., 1995] and elevated in the serum of cancer patients [Iacobelli et al., 1993, 1994; Ozaki et al., 2002; Greco et al., 2004] and patients afflicted with viral infections [Iacobelli et al., 1995]. Northern blotting and immunohistochemical analyses have revealed elevated expression of TAA90K in pancreatic, breast, and lung cancers [Fusco et al., 1998; Künzli et al., 2002; Marchetta et al., 2002; Ozaki et al., 2002] and, in the cases of lung cancer and malignant mesothelioma, protein levels correlated with metastasis and predicted survival [Marchetta et al., 2002; Ozaki et al., 2002] or disease manifestation [Singhal et al., 2003], respectively. Similarly, serum levels of TAA90K in breast cancer patients were associated with poor prognosis [Iacobelli et al., 1994]. However, in this case, increased expression of TAA90K was derived from both tumor and peripheral blood mononuclear cells, suggesting that it plays a role in host anti-tumor immune responses [Fusco et al., 1998]. In support of this hypothesis, TAA90K was shown to stimulate cytokine production by T-cells and accessory cells which may lead to further increases in expression of TAA90K by cancer cells [Powell et al., 1995]. On the other hand, cyclophilin C-associated protein (CyCAP), the murine ortholog of TAA90K, down-modulated host pro-inflammatory responses [Trahey and Weissman, 1999], an arm of the immune system implicated in the development of some cancers, including stomach and colon [Coussens and Werb, 2002]. Thus, TAA90K may have multiple effects on immune function. Although the mechanism(s) leading to the effects of TAA90K is not well understood, it may mediate some of its biological effects by modulating cell adhesion. Sasaki et al. [1998] have demonstrated that TAA90K binds to extracellular matrix proteins including fibronectin, laminin-1, and various collagens and mediates tumor cell adhesion in a β1-integrin-dependent manner. In addition, the ability of TAA90K to be a ligand for galectins-3 (Mac-2 antigen), -1 and -7 implies that it can also participate in carbohydrate-dependent cell adhesive interactions [Koths et al., 1993; Müller et al., 1999; André et al., 2001; Tinari et al., 2001]. More recently, Bair et al. [2005] have demonstrated that TAA90K can induce expression of promatrilysin in LNCaP prostate cancer cells suggesting that it plays a role in extracellular matrix remodeling during tumor invasion and metastasis.

In previous studies aimed at identifying glycoproteins, which contribute to colon cancer progression [Laferté and Loh, 1992; Laferté et al., 2000], we identified a 100 kDa glycoprotein secreted by colon carcinoma cell lines which is modified with β1-6 branched N-linked oligosaccharides, glycan chains shown to play a role in cancer metastasis [Dennis et al., 1987; Fernandes et al., 1991]. We subsequently isolated the complete cDNA encoding this glycoprotein, established its identity as TAA90K/ Mac-2-binding protein and demonstrated that TAA90K expressed by colon cancer cells is further modified with poly-N-acetyllactosamine structures (repeating Galβ1-4GlcNAc units) [Laferté and Loh, 1992; Laferté et al., 2000], oligosaccharide chains which are suitable docking sites for galectins [André et al., 1999a]. Consistent with these data, binding of TAA90K via domains 3 and 4 to cell surface-associated galectin-3 was shown to cause tumor cell aggregation [Inohara et al., 1996], suggesting that extracellular co-localization of TAA90K and galectin-3 may contribute to malignancy by enhancing tumor emboli formation, an important step during the metastatic process.

In this study, we have examined the expression and potential function of TAA90K in human colon cancer. All colon tumors examined expressed elevated TAA90K levels compared to normal colon. Functional studies carried out using TAA90K-His purified from HT-29 colon carcinoma cells infected with recombinant vaccinia virus expressing TAA90K containing a C-terminal poly-histidine tag revealed that it binds to various extracellular matrix proteins including laminin-5 and laminin-10 as well as galectin-3. In contrast, TAA90K-His failed to mediate adhesion of HT-29 cells and other cell types, possibly due to its glycan structure. However, at low concentration, TAA90K-His enhanced galectin-3-mediated HT-29 cell binding while at higher concentrations, it inhibited cell binding. Collectively, these data highlighted a potential role for TAA90K in colon cancer progression.

## MATERIALS AND METHODS

### Cell Lines

With the exception of the human colon carcinoma cell line KM20C and the human breast epithelial cell line HBL-100 (HTB 124, ATCC), which were obtained from Drs. I.J. Fidler (MD Anderson Cancer Centre, Houston, TX) and S. Carlsen (Saskatoon Cancer Research Unit, Saskatoon, SK), respectively, all cell lines were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD). Cells were cultured either in complete medium consisting of Dulbecco's minimum essential medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, and 2% MEM vitamin solution or F12/DMEM serum-free medium (Invitrogen, Burlington, ON) supplemented with 2 μg/ml insulin, 20 ng/ml epidermal growth factor, 13 ng/ml triiodothyronine, and 2 μg/ml transferrin (Sigma, Oakville, ON).

### Purification of Recombinant His-TAA90K From Bacteria

The entire open reading frame (ORF) of TAA90K [Laferté et al., 2000] was inserted in-frame behind the poly-histidine tag into the expression vector pRSETB (Invitrogen) for expression of His-TAA90K. Recombinant BL21 cells expressing His-TAA90K were grown at 30°C in Luria broth to an optical density of 0.6–0.8 at 600 nm and induced for 4 h with 1 mM isopropylthiogalactoside (IPTG) (Invitrogen, Burlington, ON). Bacteria were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 0.25 mM phenylmethylsulfonylfluoride (PMSF), 0.03 mg/ml lysozyme), sonicated and clarified by centrifugation. The pellet containing the inclusion bodies was solubilized in binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole) containing 1 mM PMSF and 6 M guanidine-HCl. Following centrifugation, the solubilized sample was applied to 0.5 ml of charged His-bind resin (Novagen, Madison, WI) pre-equilibrated in binding buffer containing 6 M guanidine-HCl. After washing, His-TAA90K was eluted from the beads with elution buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 M imidazole) containing 6 M urea and dialyzed against TBS buffer (50 mM Tris-HCl pH 7,4, 0.15 M NaCl, 0.02% sodium azide) containing decreasing concentrations of urea, down to a final concentration of 2 M urea.

### Preparation of Recombinant Vaccinia Virus Expressing TAA90K-His

For expression of TAA90K in eukaryotic cells, recombinant vaccinia virus encoding TAA90K containing a C-terminal poly-histidine tail was constructed. To this end, a histidine-tag consisting of six consecutive histidine residues was attached to the 3′ end of the TAA90K open reading frame [Laferté et al., 2000], as follows. First, using the Quikchange mutagenesis kit and the mutagenesis primers Mac2.SalU (CTCCTCAGGTGT**C**GAC*TAG*ACGGCGTGG) and Mac2.SalL (CCACGCCGTCTAGTC**G**AC-ACCTGAGGAG), a unique *Sal*I site (underlined) was created at the 3′ end of the TAA90K ORF just before the stop codon TAG (in italics) without altering its predicted amino acid sequence. The mutated nucleotide in the mutagenesis primer is shown in bold. Then, the 0.62 kb *Eco*RI-*Sal*I fragment representing the mutated 3′ end of the TAA90K ORF was inserted between the *Eco*RI and *Xho*I sites of the plasmid vector pCITE-2a to create pCITE-EB90His, thereby placing the coding sequence for the 6-histidine residues in frame with the 3′ end of the TAA90K ORF. However, the destruction of the *Sal*I site in the cloning process also resulted in a D to E conversion in the C-terminal amino acid residue of TAA90K.

The TAA90K ORF containing a C-terminal histidine tag (TAA90K-His) was reconstituted by replacing the 0.62 kb *Eco*RI-*Xba*I fragment of pCD90 [Laferté et al., 2000] with the 0.62 kb *Eco*RI-*Xba*I fragment from pCITE-EB90His to create pCD TAA90His. Similarly, the TAA90K-His ORF was introduced into the pSLAVE vector by replacing the *Eco*RI-*Bgl*II fragment of pSL-TAA90K with the 0.63 kb *EcoR*I-*Bgl*II fragment from pCITE-EB90His to create pSL-TAA90K-His. In this way, the TAA90K-His ORF was inserted behind a synthetic vaccinia late promoter and a site in the vector flanked by coding regions of the wild-type vaccinia virus thymidine kinase (TK) gene.

Recombinant vaccinia virus encoding TAA90K-His was constructed by disrupting the TK gene in wild-type vaccinia virus with the TAA90K-His coding sequence and propagated as previously described [Loh et al., 1994].

### Purification of TAA90K-His From Vaccinia-Virus-Infected HT-29 Cells

HT-29 cells cultured in complete medium containing 2% FBS were infected with recombinant vaccinia virus encoding TAA90K-His at a multiplicity of infection of 0.01. At 24 h post-infection, cells were washed in PBS and cultured in serum-free medium. At 48 and 72 h post-infection, conditioned medium was collected, centrifuged to remove cells, and the supernatant centrifuged for 4 h at 16,300*g* to pellet virus. The resulting supernatant was filtered through a 0.22 micron Millipore membrane (Fisher Scientific, Nepean, ON) and dialyzed against three changes of TBS, pH 7.4. The sample was adjusted to 0.5 M NaCl and applied to a 5 ml column of wheat germ agglutinin (WGA) agarose (E-Y Labs, San Mateo, CA) pre-equilibrated in 50 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, and 0.02% $NaN_3$ (WGA buffer). After washing with WGA buffer, TAA90K-His was eluted with TBS (pH 7.4) containing 5% N-acetylglucosamine (Sigma, Oakville, ON). Fractions containing TAA90K-His were pooled, dialyzed against 50 mM Tris-HCl pH 7.0, and applied to a 1 ml column of cation-exchange resin (HiTrap SP HP, Amersham Biosciences, Baie D'Urfé, Qc) equilibrated in the same buffer. The flow-through fractions containing TAA90K-His were pooled, adjusted to 0.2 M NaCl and purified further on a 1 ml column of anion-exchange resin (HiTrap Q HP, Amersham Biosciences, Baie D'Urfé, Qc) equilibrated in 50 mM Tris-HCl pH 7.0, 0.2 M NaCl. After washing, the anion-exchange column was connected to a fast performance liquid chromatography system (FPLC, Pharmacia), and TAA90K-His was eluted with a linear gradient of 0.2 to 1 M NaCl. The absorbance profile at 280 nm revealed a single peak eluting at 0.5 M NaCl which consisted of TAA90K-His, as detected by SDS–PAGE, and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (Proteomics Laboratory, Plant Biotechnology Institute, Saskatoon, SK). Fractions containing TAA90K-His were dialyzed against TBS (pH 7.4) and concentrated by re-application of the sample to the anion-exchange column and elution in a minimum volume of column buffer containing 0.5 M NaCl. The fraction containing TAA90K was desalted on a NAP-5 column (Amersham Biosciences, Baie D'Urfé, Qc). Typical yields of TAA90K ranged from 300 to 500 μg/L of conditioned medium.

For production of a TAA90K-His glycoform enriched with high-mannose-type N-linked oligosaccharides, HT-29 cells were grown for 24 h in medium containing 0.7 mM 1-deoxymannojirimycin (Sigma, Oakville, ON) prior to infection with recombinant vaccinia virus expressing TAA90K. Fresh 1-deoxymannojirimycin was added every 24 h until the medium was collected and processed, as described above. The dialyzed conditioned medium was applied to the WGA-agarose column. The flow-through fraction containing most of the TAA90K-His was applied to a HiTrap SP HP cation-exchange resin and eluted with a linear gradient of 0–1 M NaCl. Fractions containing TAA90K were dialyzed against 50 mM Tris-HCl, pH 7.0, 0.25 M NaCl, applied to the HiTrap Q HP anion-exchange resin and eluted with a linear gradient of 0.25–1 M NaCl. Fractions containing TAA90K-His were dialyzed and concentrated, as described above.

### Monoclonal and Polyclonal Antibodies

TAA90K-specific monoclonal antibodies were prepared, as described previously [Laferté and Loh, 1992; Laferté et al., 2000]. IgG fractions were purified from mouse ascites by protein G-agarose chromatography according to manufacturer's instructions (Amersham Biosciences, Baie D'Urfé, Qc). Polyclonal antiserum specific for His-TAA90K was prepared in rabbits following an injection of 50 μg of bacterially expressed His-TAA90K followed by two antigen boosts at 4-week intervals, in accordance with protocols approved by the Canadian Council on Animal Care. Similarly, polyclonal antiserum against human galectin-3 was produced, the IgG-fraction was purified by protein A-Sepharose 4B chromatography and checked for reactivity to galectin-3 and absence of cross-reactivity against related proto- and tandem-repeat-type galectins by ELISA, Western blot and cell binding assays [André et al., 1999b; Wollina et al., 2002; Legendre et al., 2003].

### Immunohistochemistry

Sections of human colon tumors from 47 archived paraffin specimens collected at Royal University Hospital (Saskatoon, SK) as well as normal colon from five individuals who died of causes unrelated to cancer (Asterand, Inc.,

Detroit, MI) were stained with TAA90K-specific MAb 4D1 [Laferté et al., 2000]. Sections were deparaffinized in xylene and prepared for immunohistochemical staining with the Envision™ plus system mouse staining kit (Dako Cytomation, Mississauga, ON), according to manufacturer's instructions with the following modifications. Sections immersed in PBS were heated in a microwave oven for 20 min (4 × 5 min), cooled and washed in EDTA buffer at pH 9.0 prior to blocking of endogenous peroxidase. After washing in PBS, sections were incubated for 2 h with solutions containing either normal mouse IgG (Sigma, Oakville, ON) or 4D1 IgG (5 μg/ml in 1% bovine serum albumin). After chromogen treatment and washing, sections were dehydrated and mounted in Entellan (VWR, Edmonton, AB). A section of human colon tumor previously shown to stain strongly with MAb 4D1 was included in each experiment as an internal control. To confirm the specificity of MAb 4D1, blocking studies were carried out by pre-incubating the antibody solution overnight with 70 μg/ml purified TAA90K-His prior to staining of sections. Stained sections were examined independently by two pathologists using a Nikon Eclipse 80i microscope equipped with a digital imaging system and scored as positive if at least 10% of cells within the tumor stained with MAb 4D1 but not with normal mouse IgG.

### Preparation of Tissue Lysates

Samples of colon tumor and adjacent normal colon (400–500 mg; Asterand, Inc., Detroit Michigan) were homogenized using a Polytron homogenizer (Brinkmann Instruments, Edmonton, AB) in 8 volumes of 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM PMSF, 1 mM benzamidine, 10 μg/ml aprotinin, 0.5 μM leupeptin, and 0.7 μM pepstatin. The sample was adjusted to 1% Triton X-100, incubated on ice for 1 h and centrifuged at 20,000*g* for 30 min. The resulting supernatant was assayed for protein using the Bio-Rad DC protein assay kit (Mississauga, ON).

### Immunoprecipitation

Cell lysates containing 0.5 mg protein were incubated overnight at 4°C with 5 μl NMS or TAA90K-specific MAb 1H9, immunoprecipitated and analyzed by SDS–PAGE as described previously [Laferté and Loh, 1992; Laferté et al., 2000].

### Western Blotting Analysis

Proteins separated by SDS–PAGE were transferred electrophoretically onto nitrocellulose membranes (Schleicher and Schüll, Mandel Scientific, Guelph, ON) for 2 h at 100 V. Nitrocellulose membranes were processed for Western blotting as described previously [Laferté and Loh, 1992] except that membranes were incubated for 2 h with TAA90K-specific rabbit polyclonal antiserum (1:5,000 dilution) or anti-His monoclonal antibody (1:1,000 dilution) (Qiagen, Mississauga, ON).

### Protein Binding Assay

Binding of TAA90K-His to galectin-3 and extracellular matrix proteins was tested using a solid-phase assay. Prior to use, galectin-3 (VWR, Edmonton, AB) was subjected to chromatography on a NAP-5 column (Amersham Biosciences) to remove lactose. Solutions of galectin-3, collagens I and IV, fibronectin, laminin-1 (Sigma, Oakville, ON), laminins-5 and -10 (Chemicon) or BSA (Sigma, Oakville, ON) were added, in triplicate, to PolySorb 96-well plates and allowed to adsorb to the plastic surface overnight at 4°C. After blocking with PBS/5% BSA (blocking buffer) for 2 h at room temperature and washing with PBS/0.04% Tween (PBST), wells were incubated overnight at 4°C with 2 μg TAA90K-His or BSA diluted in blocking buffer. After washing with PBST, wells were incubated for 2 h with MAb 1H9 (1:1,000 dilution in blocking buffer). After washing, wells were incubated with alkaline phosphatase-labeled, affinity-purified goat anti-mouse Ig (1:3,000 dilution in PBST) (Bio-Rad Laboratories, Mississauga, ON). Bound TAA90K-His was detected colorimetrically at 405 nm following addition of the substrate *p*-nitrophenyl phosphate (BioRad EIA kit). To examine the effect of inhibitors of carbohydrate-dependent binding on the TAA90K-His/galectin-3 interaction, similar experiments were carried out except that wells coated with galectin-3 were incubated for 1 h with 500 mM lactose or 150 mM lactose containing 2 mg/ml asialofetuin (2× concentration) before addition of an equal volume of buffer containing TAA90K-His [Kopitz et al., 2001]. Solid-phase assays were also used to examine binding of human galectins-1, -2, -3, -4, -7, and -9 and the galactoside-binding *Viscum album* agglutinin to TAA90K as well as of galectin-3 and the

mannoside-specific Concanavalin A to the TAA90K glycoform produced in the presence of the glycosylation inhibitor 1-deoxymannojirimycin comparatively [André et al., 1999a, 2001, 2003; Dam et al., 2005]. The influence of galectin-3 oligomerization on binding via its N-terminal collagenase-sensitive stalk was also assessed by testing collagenase-treated galectin-3 [Agrwal et al., 1993; Kopitz et al., 2001]. Quantitation was performed spectrophotometrically at 490 nm after using the streptavidin-peroxidase conjugate and o-phenylenediamine/$H_2O_2$ as chromogenic substrates.

### Cell Adhesion Assay

Cell adhesion was measured using a colorimetric assay described previously [Loh et al., 2000] with the following modifications. After coating with protein solutions, wells were blocked with PBS/4% BSA for 1 h at room temperature, washed and incubated for 4 h at 37°C in a 5% $CO_2$ incubator with 0.2 ml DMEM supplemented with 32 μg/ml gentamycin containing $10^5$ cells. In some experiments, HT-29 cells were pre-treated for 48 h with the glycosylation inhibitor 1-deoxymannojirimicin (0.3 mM) (Sigma, Oakville, ON). In order to examine the ability of TAA90K-His to inhibit galectin-3-mediated cell binding, TAA90K-His diluted in DMEM supplemented with gentamycin was added to wells whose surface had previously been coated with galectin-3 and incubated for 1 h at 37°C prior to addition of cells.

## RESULTS

### Expression of TAA90K in Colon Cancer

In order to examine the biological role of TAA90K in human colon cancer, we carried out immunohistochemical analyses of paraffin-embedded colon tumors using MAb 4D1, a TAA90K-specific monoclonal antibodies previously developed in our laboratory [Laferté and Loh, 1992; Laferté et al., 2000]. We examined 47 colon tumors differing in histopathological grade [Sugarbaker et al., 1985] including 10 Duke's A, 5 Duke's B, 31 Duke's C and 1 Duke's D tumor. In contrast to normal colon, which lacked detectable TAA90K expression (Fig. 1, panel A), elevated expression of TAA90K was detected in all colon tumors examined (Fig. 1, panels C–H). Four types of staining patterns were detected among the tumor specimens. These included diffuse intracellular staining (panel C), punctate staining concentrated at the apical face of colon cancer cells consistent with proteins associated with secretory vesicles (panels D and E), luminal staining (panel F) and basolateral staining, more frequently associated with early-stage Duke's A tumors (panel G). In certain tumors, weak staining was also detected within transitional mucosa at the tumor margin (panel H). Blocking studies carried out with purified TAA90K-His revealed complete antigen dependence for staining by MAb 4D1, confirming that the complex pattern of signal generation observed is due to TAA90K expression (panels B and D). In addition, immunoprecipitation of TAA90K from detergent lysates of three colon tumors (Duke's B, C and D) and adjacent normal colon using MAb 1H9 followed by Western blotting analysis using TAA90K-specific polyclonal antiserum (Fig. 2) confirmed the quantitative increase in expression of TAA90K in colon tumors relative to adjacent histologically normal colon. The apparent mobility of TAA90K in the Duke's B, C and D samples was 106, 101, and 108 kDa, respectively, (Fig. 2) consistent with variable glycosylation of TAA90K previously detected in human colon carcinoma cells lines [Laferté and Loh, 1992]. The lower molecular mass species of 76–86 kDa species detected in each tumor sample likely results from proteolytic cleavage of TAA90K previously detected in various cell lines [Laferté and Loh, 1992; Koths et al., 1993; Sasaki et al., 1998]. Thus, TAA90K may represent a novel tumor-associated marker of colonic mucosa.

### Functional Studies of TAA90K

The elevated expression of TAA90K within colon tumors compared to normal colon as well as its cellular and tissue localization suggested that increased expression, secretion, and extracellular accumulation of TAA90K contribute to enhanced malignancy by altering tumor cell phenotype and/or tumor-host cell interactions. In order to examine the possible mechanism(s) of TAA90K function in colon cancer, we purified TAA90K from HT-29 human colon carcinoma cells in sufficient quantity to carry out functional studies. To this end, we constructed a recombinant vaccinia virus for expression of TAA90K-His which contains a C-terminal polyhistidine tag. The wide host-range specificity of vaccinia virus [Moss, 1996] made this an ideal system for increasing expression and secretion

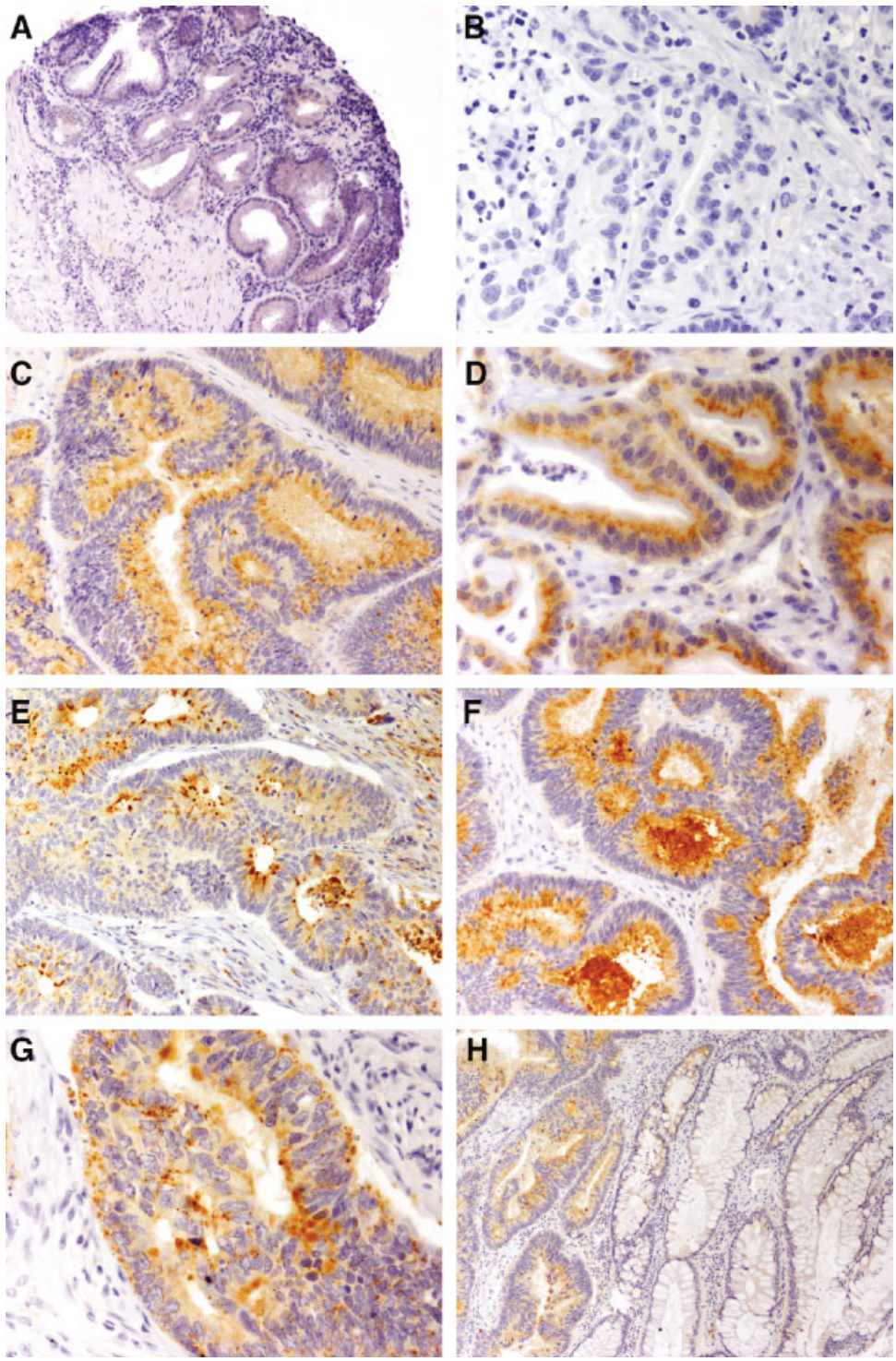


**Fig. 1.** Immunohistochemical staining of human colon tumors. Sections of human colon tumors were examined by immunohistochemical staining with MAb 4D1 (5 μg/ml) or normal mouse IgG. Staining patterns obtained with normal IgG are not shown since they yielded negative results. **A**: Normal colon, 400×, (**B**) Duke's B2 tumor (also shown in **panel D**) incubated with MAb 4D1 pre-absorbed with TAA90K-His (70 μg/ml), (**C**) Duke's C2 tumor showing diffuse intracellular staining, 200×, (D) Duke's B2 tumor showing granular staining at the apical border of colon cancer cells, 400×, (E) Duke's C1 tumor showing punctate staining consistent with secretory vesicles, 400×, (F) Duke's C2 tumor showing luminal staining, 400×, (G) Duke's A tumor showing apical and basolateral staining, 400×, and (H) Duke's C2 tumor showing staining of transitional mucosa at the tumor border, 200×.

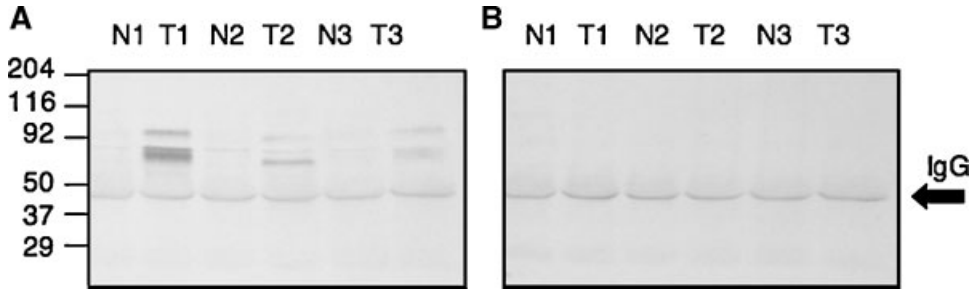


**Fig. 2.** Analysis of TAA90K expression in human colon tumors and adjacent normal colonic mucosa. TAA90K was immunoprecipitated from 0.5 mg of detergent-solubilized human colon tumors (T1 to T3) or adjacent normal colonic mucosa (N1 to N3) using 5 μl of either MAb 1H9 (**panel A**) or NMS (**panel B**). Immunoprecipitates were analyzed by Western blotting using rabbit anti-TAA90K antiserum (1:2,000 dilution). The samples designated T1 to T3 and N1 to N3 correspond to Duke's B, Duke's C and Duke's D tumors and their adjacent normal colonic mucosa, respectively. The size of the molecular weight markers in kilodaltons is shown at the left of the figure. The mobility of the immunoglobulin band (IgG) is shown at the right of the figure.

of TAA90K-His as well as insuring that the secreted glycoprotein contains the proper post-translational modifications. In addition, the presence of the poly-histidine tag would allow purification of TAA90K-His by metal-chelation affinity chromatography on His-bind resin. Although TAA90K-His could be isolated using this type of resin, the yields were low, apparently due to poor binding of the histidine tag in the native protein. Using a combination of lectin (WGA) affinity chromatography, which binds glycoproteins harboring N-acetylglucosamine or sialic acid [Merkle and Cummings, 1987], cation- and anion-exchange chromatography, TAA90K-His was purified to near homogeneity (~400 μg/L conditioned medium), as indicated by the single band migrating in SDS–PAGE with an apparent mobility of 105 kDa (Fig. 3A). Its identity as Mac-2-binding protein was confirmed by mass spectrometry. The minor species migrating at 82 kDa was shown by mass spectrometry to correspond to a proteolytic cleavage product of TAA90K-His. No other bands were detected in the gel. As further evidence for extensive glycosylation of TAA90K-His, we purified recombinant His-TAA90K from bacteria by metal-chelation affinity chromatography. His-TAA90K migrated on SDS–PAGE with an apparent molecular mass of 62 kDa (Fig. 3B), consistent with an unglycosylated polypeptide tagged with a poly-histidine sequence, as detected by Western blotting analysis using anti-His monoclonal antibody [Trahey and Weissman, 1999]. The glycan chains—in addition to the protein part—may be relevant to TAA90K function.

Since cancer progression and metastasis are characterized by altered cell–cell and cell-extracellular matrix interactions, we examined the ability of colon cancer-derived TAA90K-His to bind to extracellular matrix proteins and galectins, As is shown in Fig. 4, TAA90K-His binds to galectin-3 (panel A), collagen IV (panel A), and laminin 1 (panel B), as described previously [Sasaki et al., 1998; Hellstern et al., 2002], but poorly to collagen I (panel A) and homodimeric galectin-1 (panel A), the latter possibly due to cell-type-specific characteristics of its glycan structure. In addition, TAA90K-His bound to the extracellular matrix proteins laminin-5 and laminin-10 (Fig. 4B). To examine

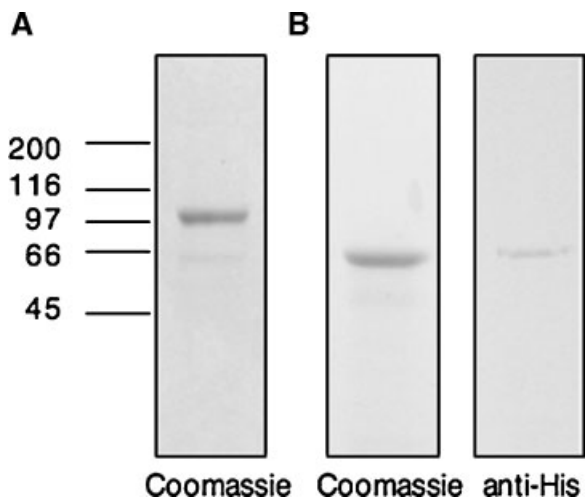


**Fig. 3.** Analysis of purified TAA90K by SDS–PAGE. **A**: Coomassie-stained gel showing 1 μg of TAA90K-His purified from conditioned media of HT-29 infected with recombinant vaccinia virus expressing TAA90K-His. **B**: Coomassie-stained gel and anti-His Western blot (1:1,000 dilution) of 1 μg of His-TAA90K purified from recombinant BL21 bacteria expressing His-TAA90K. The size of the molecular weight markers in kilodaltons is shown at the left side of the figure.

further the relative specificity of galectin binding to glycan chains of TAA90K-His from HT-29 cells, we also tested human galectins-2, -4, -7, and -9. Relative levels of carbohydrate-dependent binding were 0.6%, 27.4%, 63.0%, and 32.7% compared to galectin-3. To underscore that the mere presence of N-glycans will not automatically lead to binding, we tested the galactoside-specific lectin from *Viscum album*, which also bound poorly to this TAA90K preparation. In addition to this remarkable level of selectivity, binding of galectin-3 to TAA90K was found to be dependent on this galectin's ability to oligomerize since a truncated version of galectin-3 reached only about a fifth of the binding level of the full-length protein.

Although it is not known whether binding of TAA90K to the extracellular matrix proteins is mediated by protein–protein or protein-carbohydrate interactions, the interaction of TAA90K with galectin-3 was mediated at least in part by carbohydrate since addition of 250 mM lactose or 75 mM lactose combined with 1 mg/ml of the glycoprotein asialofetuin, which presents bi- and triantennary complex-type N-linked glycans containing terminal N-acetyllactosamine (Galβ1-4GlcNAc) or its β1-3-linked isomer with a 6,000-fold increased affinity in the first of nine binding steps relative to free disaccharide [Dam et al., 2005], significantly reduced their interaction (Fig. 4C). Furthermore, a TAA90K-His glycoform purified from HT-29 cells treated with 1-deoxymannojirimycin (TAA90K-DMJ), which inhibits conversion of high-mannose-type to complex-type N-linked glycans [Bischoff and Kornfeld, 1984], bound rather poorly to galectin-3 (Fig. 5), as expected from the carbohydrate specificity of galectin-3. Evidence in support of altered glycosylation of TAA90K-DMJ includes reduced size on SDS–PAGE

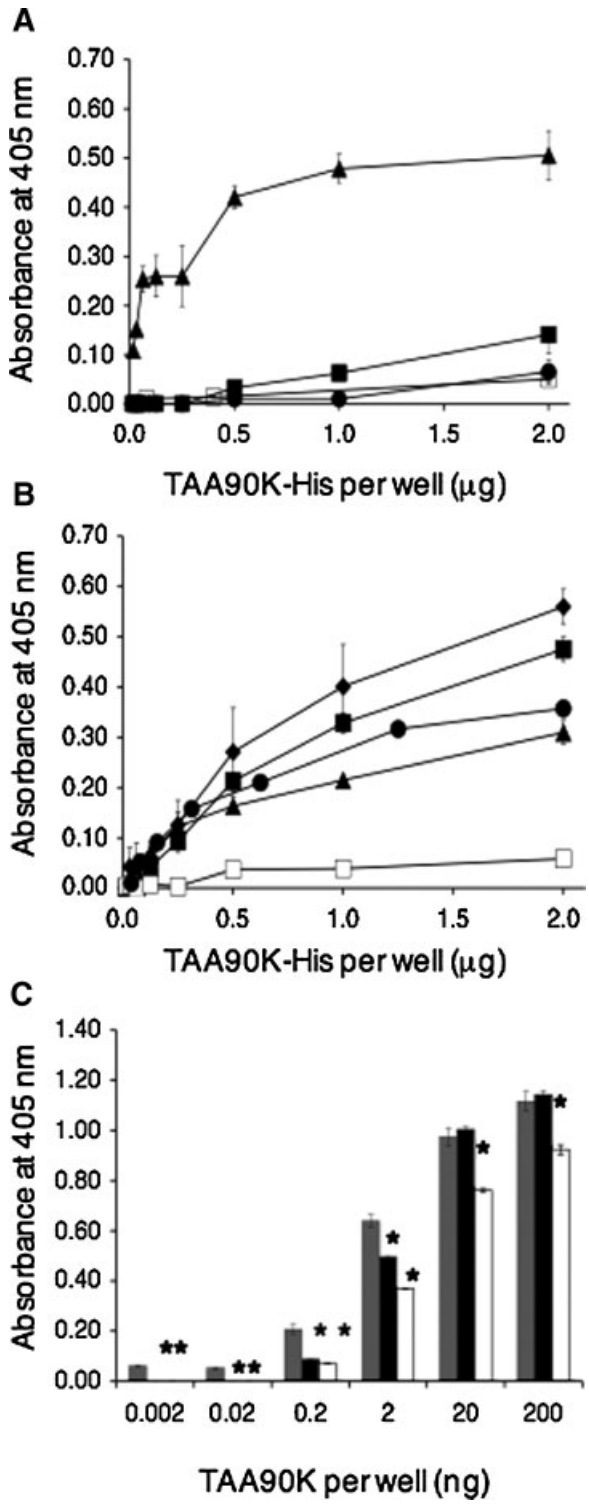


**Fig. 4.** Binding of TAA90K-His to immobilized galectin-3 and extracellular matrix proteins. For experiments shown in **panels A** and **B**, wells were coated with the following proteins at 0.5 μg/ml except for fibronectin, which was used at 1 μg/ml. Panel A: collagen I (closed circle), collagen IV (closed square), galectin-1 (open square), galectin-3 (closed triangle); Panel B: laminin-1 (closed square), laminin-5 (closed triangle), laminin-10 (closed diamond), fibronectin (closed circle), BSA (open square). For the experiment shown in **panel C**, wells were coated overnight with galectin-3 alone (0.2 μg/ml; gray bar) or galectin-3 followed by 250 mM lactose (black bar) or 75 mM lactose containing 1 mg/ml asialofetuin (white bar). After washing and blocking, all wells (panels A–C) were incubated with increasing amounts of TAA90K-His (with or without lactose and asialofetuin), followed by MAb 1H9 and alkaline phosphatase-labeled goat anti-mouse Ig. Specific binding of TAA90K was detected spectrophotometrically at 405 nm. The mean and standard deviation of triplicate samples are shown. The asterisk indicates statistical significance with a *P*-value less than 0.05.

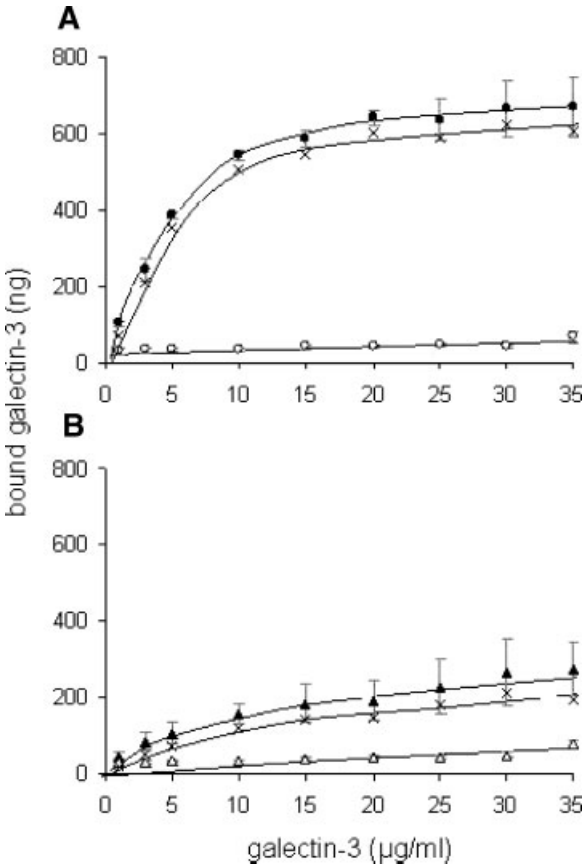


**Fig. 5.** Effect of type of N-linked glycosylation on TAA90K binding to galectin-3. The level of carbohydrate-inhibitable binding (×) of biotinylated human galectin-3 to surface-immobilized TAA90K (**A**) and its variant from 1-deoxymannojirimycin-treated HT-29 cells (**B**) was determined using a solid-phase assay. Binding was detected spectrophotometrically at 490 nm. Total binding (closed circle, closed triangle) to the matrix using 100 ng glycoprotein per well for adsorption was reduced by the extent of carbohydrate-independent binding (open circle, open triangle) determined in the presence of a mixture of 75 mM lactose and 1 mg asialofetuin/ml as inhibitor (crosses).

(90 kDa vs. 105 kDa; data not shown), as well as loss of binding to WGA-agarose and retention by a cation-exchange resin, possibly facilitated by loss of the negatively charged sialic acid residues present on TAA90K [Laferté and Loh, 1992]. In contrast to galectin-3, binding to the plant lectin concanavalin A, which accommodates the trimannoside core of N-glycans, was not significantly affected. Thus, N-glycosylation of TAA90K-DMJ was altered but not impaired.

We also examined the ability of TAA90K-His derived from HT-29 cells to mediate cell adhesion. TAA90K-His was unable to mediate adhesion of HT-29 and six other colon carcinoma cells lines (SW403, SW620, LS123, CaCo-2, LoVo, and KM20C) above the BSA control (data not shown). Similarly, the normal rat intestinal cell line IEC-6, rat-2 fibroblasts and HBL100 breast epithelial cells were unable to adhere to TAA90K-His. The absence of cell binding was specific to TAA90K-His since HT-29 cells adhered to fibronectin, collagen I, and galectin-3 used as positive controls (Fig. 6A). Thus, TAA90K-His obtained from HT-29 cells is a poor cell-adhesive substratum. The inability of TAA90K-His to mediate cell adhesion cannot be attributed to the presence of the C-terminal histidine tag since identical results were obtained with TAA90K purified from the conditioned medium of HT-29 cells infected with vaccinia virus expressing wild-type TAA90K (data not shown). Thus, we hypothesized that the lack of cell adhesion to TAA90K-His may result from cell-type-specific glycosylation of TAA90K and/or its cellular receptor(s) resulting in an anti-adhesive rather than adhesive interplay. To test this hypothesis, we first examined the adhesion of HT-29 cells to bacterially produced His-TAA90K devoid of glycan chains. Low cell binding was observed (Fig. 6B), as was seen with HT-29-derived TAA90K-His. However, treatment of HT-29 cells with 1-deoxymannojirimycin to alter cell-surface glycosylation resulted in enhanced cell binding to bacterially produced His-TAA90K (Fig. 6B). Since this version of His-TAA90K maintained conformational determinants, based on binding of 5 of 12 conformation-dependent anti-TAA90K monoclonal antibodies (data not shown) [Laferté et al., 2000], the adhesive interactions between TAA90K and its putative cellular receptor(s) may be modulated by substratum- and cell-type-dependent N-linked glycosylation.

Lastly, we examined the effect of TAA90K-His on galectin-3-mediated HT-29 cell binding. As shown in Figure 6A,C, galectin-3 mediated adhesion of HT-29 cells. However, in the presence of a high concentration (5 µg/well) of TAA90K-His (Fig. 6C), galectin-3-mediated cell binding was significantly lowered ($P < 0.0001$). Surprisingly, as the concentration of TAA90K was decreased to an optimum value of approximately 8 ng/well, binding of HT-29 cells to galectin-3 was enhanced ($P < 0.0001$). Thus, at high concentration, TAA90K exhibits an anti-adhesive phenotype since it interferes with galectin-3-mediated HT-29 cell binding whereas, at low concentration, it exhibits an adhesive

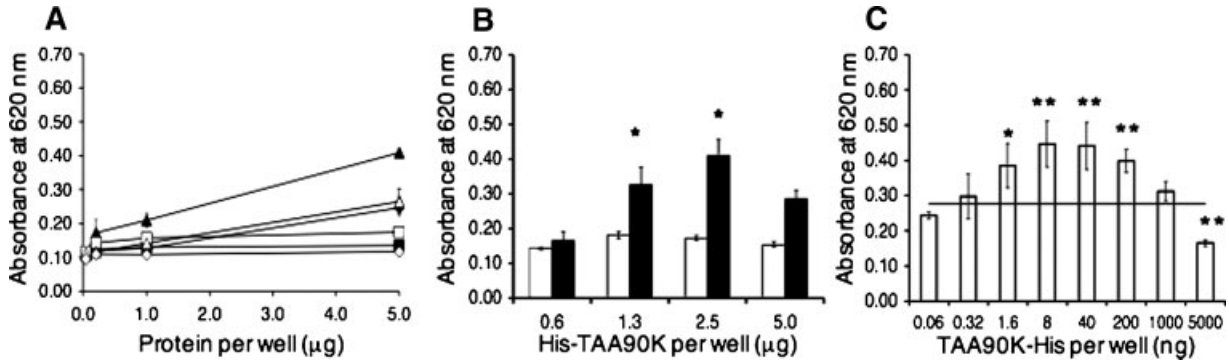


**Fig. 6.** Adhesion of HT-29 cells to various proteins. Wells were coated with extracellular matrix proteins, galectin-3 or BSA (**panel A**), recombinant His-TAA90K purified from BL21 bacteria (**panel B**) or galectin-3 (**panel C**). Adhesion of HT-29 cells (panels A–C) or HT-29 cells pre-treated with 1-deoxymannojirimycin (panel B) was detected spectrophotometrically at 620 nm. Panel A: fibronectin (closed diamond), laminin-1 (closed square), collagen I (closed triangle), collagen IV (open square), galectin-3 (open triangle), BSA (open diamond). Panel B: untreated HT-29 cells (white bar), 1-deoxymannojirimycin-treated HT-29 cells (black bar). The mean and standard deviation of triplicate samples are shown. The asterisk indicates statistical significance with a *P*-value less than 0.05. Panel C: Wells coated with 5 μg of galectin-3 were incubated with increasing amounts of purified TAA90K-His followed by addition of $10^5$ HT-29 cells. The horizontal line running through the chart corresponds to the amount of HT-29 cell binding observed with galectin-3 alone (panel A). The mean and standard deviation of triplicate samples from three separate experiments are shown. The asterisks indicate statistically significant differences in cell binding compared to that observed with 5 μg galectin-3 alone. ($*P < 0.005$; $**P < 0.0001$).

phenotype by enhancing galectin-3-mediated cell binding of HT-29 cells.

## DISCUSSION

In this report, we have examined the expression and possible function of TAA90K in human colon cancer. Our interest in studying TAA90K was two-fold. First, several studies have documented increased expression of TAA90K in cancerous tissues and correlated these cancer-associated changes with metastatic disease and/or prognosis [Iacobelli et al., 1993, 1995; Fusco et al., 1998; Künzli et al., 2002; Marchetta et al., 2002; Ozaki et al., 2002]. Secondly, in our previous studies of glycoproteins in colon cancer cells [Laferté and Loh, 1992; Prokopishyn et al., 1999; Laferté et al., 2000], we identified TAA90K as a major carrier of β1-6-branched N-linked oligosaccharides, glycan chains implicated in cancer metastasis [Dennis et al., 1987; Fernandes et al., 1991]. Thus, studies of TAA90K in colon cancer cells have the potential to elucidate its function in health and disease and the contribution of its glycan chains to this function.

Our immunohistochemical analyses of colon tumors revealed elevated intracellular and/or luminal expression of TAA90K in all tumor samples examined compared to normal colon, suggesting that TAA90K is a marker of cancer-associated changes within colonic mucosa. The complex immunohistochemical staining pattern of TAA90K expression in colon tumors provided clues about the possible function of this glycoprotein during colon cancer progression. The intense intracellular expression of TAA90K detected in most colon tumors likely resulted from increased transcription of the TAA90K gene as a result of genetic changes associated with the carcinogenic process [Vogelstein and Kinzler, 2004]. The presence of TAA90K on the basolateral face of colon cancer cells is consistent with its ability to bind to the extracellular matrix proteins laminin-5, laminin-10 and collagen IV. Since basolateral expression of TAA90K was more prevalent in early-stage Duke's A tumors than in more advanced tumors, this pattern of TAA90K expression may resemble closely its subcellular localization in normal colon. Although we were unable to detect TAA90K in normal colon, this may be due to the level of sensitivity associated with immunohistochemical staining. In this regard, Northern blot analyses have detected mRNA coding for TAA90K in numerous epithelial tissues, including colon [Ullrich et al., 1994], suggesting that TAA90K is expressed at low level in normal colon. The prevalent luminal expression of TAA90K in colon tumors suggested that this is due in part to increased secretion of TAA90K from colon cancer cells in

vivo, supported by the presence of stained granules consistent with glycoproteins associated with secretory vesicles. The presence of TAA90K in granules is consistent with studies by Fernandes et al. [1991] who showed punctate staining of colon tumors when using leucophytohemagglutinin (L-PHA), a plant lectin which binds β1-6-branched N-glycans. Significantly, they demonstrated a correlation between L-PHA binding and progression of colon and breast carcinoma [Fernandes et al., 1991]. Since we showed previously that TAA90K is the major L-PHA binding glycoprotein secreted by colon carcinoma cells [Laferté and Loh, 1992], we may have identified one of the L-PHA-binding glycoproteins likely contributing to colon cancer progression.

Although TAA90K has been implicated in cancer progression [Iacobelli et al., 1994; Marchetta et al., 2002; Ozaki et al., 2002], its mechanism of action is not known. The extracellular localization of TAA90K within colon tumors as well as its reported role as an adhesive protein mediating both cell-cell and cell-extracellular matrix interactions [Sasaki et al., 1998; André et al., 2001; Tinari et al., 2001; Bair et al., 2005] highlighted the potential for functions of TAA90K during colon cancer progression. Due to the emerging importance of cell-type-specific glycosylation for glycoproteins, we deliberately focused on colon cancer cells as a source for the glycoprotein. To elucidate its function in colon cancer cells, we isolated TAA90K from the conditioned medium of HT-29 colon carcinoma cells infected with recombinant vaccinia virus expressing TAA90K-His. This proved to be an excellent approach for high yield expression and proper post-translational modifications including cell-type-specific glycosylation. Our studies demonstrated that TAA90K-His (or TAA90K) expressed by colon cancer cells, like TAA90K isolated from fibroblasts [Sasaki et al., 1998; Hellstern et al., 2002], exhibits multi-adhesive properties since it bound fibronectin, collagen IV, laminin-1, and to a lesser extent collagen I. In addition, TAA90K-His bound to laminins-5 and -10, components of basement membranes shown to modulate adhesion and proliferation of the human colon carcinoma cell line CaCo-2/TC7 [Turck et al., 2005]. TAA90K-His also bound well to galectin-3 in a carbohydrate-dependent manner. In contrast to TAA90K derived from fibroblasts [Tinari et al., 2001], the HT-29-derived glycoprotein bound rather poorly to galectin-1, indicating cell-type-specific glycosylation detectable by different galectins. It is noteworthy that the HT-29 cell line was established from a patient of histo-blood group A status, a 30-fold preferred ligand for galectin-3 but not galectin-1 [Sparrow et al., 1987]. We have shown previously that the cell adhesion receptor α3β1 integrin expressed by HT-29 cells is also modified with blood group A epitopes [Prokopishyn et al., 1999]. Similarly, TAA90K secreted by HT-29 cells is decorated with blood group A determinants (data not shown). Thus, it appears reasonable that TAA90K binds better to galectin-3 than galectin-1. In addition, our binding assays using a panel of galectins from the three subfamilies clearly revealed specificity for galectin-3 as the major family member to bind TAA90K-His. Because the architectures of the binding sites of galectins for structures extended beyond the galactose core differ, inherent selectivity for distinct glycan profiles can arise among family members [Ahmad et al., 2002; Siebert et al., 2003; André et al., 2005]. Thus, the term galectin, which pertains to the galactoside specificity of this family of endogenous lectins, should not lead to an assumption of common reactivity.

We also examined the ability of TAA90K-His derived from colon cancer cells to mediate cell adhesion directly or in cooperation with other extracellular proteins. In contrast to previous reports demonstrating that TAA90K from other cell sources can mediate adhesion of A431 epidermoid carcinoma cells and HBL-100 breast epithelial cells [Sasaki et al., 1998; André et al., 2001; Hellstern et al., 2002], TAA90K isolated from colon carcinoma cells was unable to mediate adhesion of seven colon cancer cell lines and a sampling of normal cell lines including HBL-100 cells. Since HT-29 cells adhered to other extracellular proteins, these data suggested that TAA90K-His produced by HT-29 cells is a poor cell-adhesive substratum. Cell-type-specific glycosylation of TAA90K and/or its cellular receptor(s) may yield an anti-adhesive rather than adhesive glycoprotein, resulting in poor cell adhesion. In support of this hypothesis, we have shown that HT-29 cells treated with 1-deoxymannojirimycin, which inhibits the conversion of high-mannose-type to complex-type N-glycans, adhered better to bacterially expressed TAA90K devoid of oligosaccharides than properly glycosylated TAA90K-His. Thus, the

adhesive interactions between TAA90K and its putative cellular receptor(s) may be modulated by substratum- and cell-dependent N-linked glycosylation, underscoring the informational role of glycans by interfering with molecular recognition or serving as docking sites [Reuter and Gabius, 1999; Gabius et al., 2004]. Regulation of these functions by changes in activity of N-acetylglucosaminyltransferases-III and -V, including the responsiveness of galectins-1 and -3 [Dennis et al., 2002; André et al., 2004], have been documented. Importantly, the presence of the β-1,6-branch (*Mgat5*-modified N-glycan) has been shown to give rise to lattice-like galectin-3/T cell receptor complexes, an indication for a role of this branch in regulating receptor activity [Dennis et al., 2002]. Since this branching structure is also prominently present within N-glycans of colon-cancer-derived TAA90K [Laferté and Loh, 1992], it may modulate the function of TAA90K in this disease.

In light of our results demonstrating preferential binding of TAA90K-His to galectin-3, we examined the effect of TAA90K-His on galectin-3-mediated cell adhesion. Previous studies revealed that galectin-3 can mediate adhesion of a number of cell types, including macrophages and neutrophils [Woo et al., 1990; Kuwabara and Liu, 1996; André et al., 1999a]. In addition, Inohara et al. [1996] have shown that addition of TAA90K to melanoma cells which express galectin-3 on the cell surface resulted in tumor cell aggregation, suggesting that the interaction between TAA90K and galectin-3 may be important in the metastatic spread of these cancer cells. We demonstrated for the first time that TAA90K can modulate galectin-3-mediated HT-29 cell binding. At high concentration, TAA90K inhibited galectin-3-mediated HT-29 cell binding but at lower concentrations, it enhanced cell binding. Interestingly, an inverse relationship has been demonstrated for galectin-3. At sub-micromolar concentration, galectin-3 inhibits cell adhesion while at increased concentration, it promotes cell adhesion [Inohara et al., 1996]. Although this dual role of galectin-3 may result from its ability to oligomerize at high concentration [Ahmad et al., 2004], thereby cross-linking suitable cell surfaces and extracellular matrix proteins, increased secretion of TAA90K and subsequent binding to galectin-3 may also contribute to this phenomenon by reducing the availability of galectin-3 for cell binding [Inohara et al., 1996]. Of note in this respect, galectin-3 has been assigned prognostic value especially at early stages of colon cancer progression in the galectin network [Schoeppner et al., 1995; Legendre et al., 2003; Nagy et al., 2003]. In light of this new information, the relative ratio between TAA90K and galectin-3 secreted by colon cancer cells in vivo may play an important role during colon cancer progression and metastasis by modulating tumor cell adhesion. Because other family members, for example galectin-4, which has prognostic value in Duke's A and B stages and is expressed in T84 colon adenocarcinoma cells at sites of cell adhesion, or galectins-2, -8, and -9 are present in colon cancer [Huflejt et al., 1997; Lahm et al., 2001, 2004; Nagy et al., 2003], it is intriguing to see the inherent selectivity of the HT-29-derived TAA90K for galectin-3.

Since TAA90K and galectin-3 have distinct subcellular localizations (ER/Golgi vs. cytoplasm, respectively), any biological role mediated by association of these two proteins would likely occur extracellularly following classical and non-classical secretion of each protein, respectively [Inohara et al., 1996]. The presence of galectin-3 within the underlying stroma of colonic mucosa [Greco et al., 2004] and the basolateral expression of TAA90K in some colon tumors suggest that co-localization of TAA90K and galectin-3 within the basement membrane alters colon tumor cell adhesion to galectin-3 and/or extracellular matrix proteins and contributes to tumor progression, galectin-3 being a pro-metastatic factor in a tumor model [Bresalier et al., 1998]. Since galectin-3 is also present within luminal secretions of colonic polyps [Greco et al., 2004], a pattern which parallels TAA90K expression in colon tumors, it is also possible that association of these proteins leads to signaling events at the apical face of colon cancer cells which contribute to cancer progression. In rat cardiac fibroblasts, for instance, galectin-3 is the signal for enhanced collagen production resulting in a three-fold preference of collagen I over collagen III [Sharma et al., 2004]. Since both TAA90K and galectin-3 have been implicated in immunomodulation, including modulation of T-cell responses and stimulation of the pro-inflammatory responses [Powell et al., 1995; Trahey and Weissman, 1999; Rabinovich et al., 2002; Nakahara et al., 2005], their presence in the luminal spaces of a tumor

may modulate host immune responses in a manner which promotes tumor progression.

In conclusion, we have shown that colon carcinogenesis is associated with elevated expression of TAA90K and that extracellular interactions between TAA90K and galectin-3, which could be governed by cell-type-specific glycosylation, may contribute to enhanced malignancy.

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