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Monoclonal Antibodies Specific for Human Tumor-Associated Antigen 90K/Mac-2 Binding Protein: Tools to Examine Protein Conformation and Function

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Abstract As part of our effort to identify glycoproteins that contribute to colon cancer progression, we have previously described a family of structurally related glycoproteins expressing β1–6 branched asparagine(Asn)-linked oligosaccharides defined by monoclonal antibody (MAb 1H9), which are differentially expressed, processed, and glycosylated by human colon carcinoma cell lines (Laferté and Loh [1992]; Biochem J; 283:193–201). MAb 1H9 immunoprecipitates three glycoproteins having apparent sizes of 92–100, 66–70, and 25 kDa, the size heterogeneity attributable to cell-type specific glycosylation differences. We report on the basis of partial protein and cDNA sequence information, that the 100-kDa glycoprotein detected by MAb 1H9 is identical to the 90-kDa glycoprotein variably known as tumor-associated antigen 90K (TAA90K), Mac-2 binding protein, and cyclophilin C-associated protein. Using a PCR-based cloning strategy, the complete cDNA encoding TAA90K was cloned into the eukaryotic expression vector pCDNA-3 (pCD-TAA90K^{wt}) and the protein expressed in COS-1 cells. A [^{35}S]methionine-labeled 60-kDa polypeptide, processed to an endoglycosidase H-sensitive 74-kDa glycoprotein in the presence of dog pancreas microsomes, was detected in a coupled transcription/translation in vitro reaction. The in vitro-translated 60-kDa polypeptide and N-glycanase-treated TAA90K (60-kDa species) immunoprecipitated from HT29 cells were shown to be structurally identical by limited proteolytic peptide mapping. Using a new panel of 11 TAA90K-specific monoclonal antibodies, including five specific for human TAA90K and six cross-reactive with a 90-kDa species expressed by COS-1 cells, we have detected conformational differences between recombinant wild-type TAA90K, in vitro-synthesized TAA90K, and mutant forms of TAA90K containing point mutations at residues 189, 223, and 259. Furthermore, we have shown that these mutant forms of TAA90K, as well as a truncated form of TAA90K containing amino acid residues 1–383, are defective in secretion. These studies demonstrate the potential usefulness of TAA90K-specific monoclonal antibodies for examining the structure and function of TAA90K, and highlight the contribution of specific amino acid residues to its normal processing and secretion. J. Cell. Biochem. 77:540–559, 2000.

Key words: TAA90K; Mac-2 binding protein; monoclonal antibodies; protein conformation

Abbreviations used: TAA90K, tumor-associated 90K glycoprotein; Mac-2 BP, Mac-2 binding protein; Asn-linked, asparagine-linked; MAb, monoclonal antibody; CyCAP, cyclophilin C-associated protein; MAMA, murine adherent macrophage-associated protein; SRCR, scavenger receptor cysteine-rich domain; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; NMS, normal mouse serum; TBT, 50 mM Tris-HCl pH 8.0, 0.1% bovine serum albumin, 0.025% Tween-20, 0.02% sodium azide; TBS, Tris-buffered saline (50 mM Tris-HCl pH 8.0, 0.02% sodium azide); BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; IP, immunoprecipitation; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; CMV, cytomegalovirus; D-PBS, Dulbecco's phosphate-buffered saline (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 5.4 mM KCl, 137 mM NaCl, pH 7.4); PBS, phosphate-buffered saline (2.5 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 144 mM NaCl, pH 7.2).

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The tumor-associated antigen 90K (TAA90K) is a secretory glycoprotein present in normal human fluids, including plasma, breast milk, saliva, tears, and urine [Koths et al., 1993; D'Ostilio et al., 1996], a wide range of epithelial tissues [Linsley et al., 1986; Iacobelli et al., 1986, 1993; Koths et al., 1993; Ullrich et al., 1994; Jallal et al., 1995] and elevated in the sera of patients suffering from cancer [Linsley et al., 1986; Iacobelli et al., 1986, 1993; Natoli et al., 1993; Ullrich et al., 1994; Jallal et al., 1995] and acquired immunodeficiency syndrome [Iacobelli et al., 1995]. In addition, the level of TAA90K in the blood of breast cancer patients and individuals infected with the human immunodeficiency virus appears to be prognostic of disease progression [Iacobelli et al., 1994, 1995]. Recent studies have implicated TAA90K as an immunomodulator, playing a role in host-defense mechanisms against various pathogens and cancer cells, and mediating its immunomodulatory effects by interacting with accessory cells of the immune system (e.g., monocytes and macrophages) [Ullrich et al., 1994; Jallal et al., 1995; Powell et al., 1995; Trahey and Weissman, 1999]. Although the mechanism responsible for its putative immunomodulatory activity is not completely understood, it has been proposed that TAA90K may mediate its biological effects by modulating expression of cell adhesion molecules such as ICAM-1. More recently, Sasaki et al. [1998] have demonstrated that TAA90K can mediate tumor cell adhesion in a β1-integrin-dependent manner, suggesting that TAA90K may represent a multi-functional adhesive protein.

Surprisingly, results from an initially unrelated area of research indicated that TAA90K was identical to the newly characterized Mac-2 binding glycoprotein (Mac-2 BP), which interacts with the β-galactoside-specific mammalian lectin galectin-3 (also known as Mac-2) found on the surface of inflammatory but not resting macrophages [Rosenberg et al., 1991; Koths et al., 1993; Barondes et al., 1994]. This finding suggested that TAA90K may also play a role in carbohydrate-dependent cell adhesive interactions. Concurrently, two virtually identical murine glycoproteins with apparent molecular mass of 77 kDa, the cyclophilin C-associated protein (CyCAP) [Friedman et al., 1993] and the murine adherent macrophage-associated protein (MAMA) [Chicheportiche and Vassalli, 1994], were found to be murine homologues of TAA90K, sharing up to 80% homology in amino acid sequences in the most conserved region. Although the presence of a signal sequence on both cyclophilin C (CyC) and CyCAP indicates that a CyC-CyCAP complex could form in vivo within the endoplasmic reticulum, Golgi apparatus, or extracellularly, the existence of such a complex or its function has not yet been documented. The fact that *cis-trans* peptidylprolyl isomerases have been implicated in protein folding [Kay, 1996] suggests that CyC, a member of this family of isomerases, may play a role as a chaperone, facilitating TAA90K protein folding. Collectively, these studies point to the potential importance of TAA90K in health and disease and highlight the need for detailed structural analyses of TAA90K.

The human TAA90K glycoprotein isolated from human serum or conditioned medium migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a species having an apparent molecular mass of 90–100 kDa [Linsley et al., 1986; Iacobelli et al., 1986; Rosenberg et al., 1991]. However, TAA90K migrates in gel filtration as a very large homopolymer with an apparent molecular mass of 1,000–1,500 kDa [Linsley et al., 1986; Iacobelli et al., 1986; Koths et al., 1993; Sasaki et al., 1998] and forms ring-like structures of 30–40 nm [Sasaki et al., 1998]. TAA90K is synthesized initially as a 74-kDa precursor containing high-mannose N-linked chains, which are processed to complex-type chains found on the mature 90-kDa species [Linsley et al., 1986]. Glycoprotein species of 67–70 and 25–27 kDa have also been detected in immunoprecipitation reactions using TAA90K-specific monoclonal antibodies, but these are believed to arise by proteolytic cleavage of the mature 90 kDa form [Linsley et al., 1986; Rosenberg et al., 1991; Koths et al., 1993; Ullrich et al., 1994]. The isolation of complete cDNA clones of TAA90K by two independent laboratories [Koths et al., 1993; Ullrich et al., 1994] revealed an open reading frame (ORF) that encodes a polypeptide of 585 amino acids rich in cysteine, proline, and tryptophan residues. In addition to the presence of a signal sequence and 7 putative N-linked glycosylation sites, TAA90K contains a 120-amino acid N-terminal domain highly homologous to the scavenger receptor cysteine-rich domain (SRCR) found in members of the

macrophage scavenger receptor superfamily [Kodama et al., 1990; Krieger, 1994; Resnick et al., 1994; Pearson, 1996], a central mucin-like domain and a 27-kDa C-terminal domain [Sasaki et al., 1998]. The availability of domain-specific monoclonal antibodies that specifically detect each of these three domains [Linsley et al., 1986; Iacobelli et al., 1986; Tinari et al., 1997] will facilitate structural and functional studies of TAA90K.

As part of our ongoing studies aimed at identifying glycoproteins bearing β1–6 branched asparagine-linked oligosaccharides that play a role in colon cancer progression, we have previously reported the initial characterization of a family of structurally related glycoproteins detected by monoclonal antibody 1H9, which are differentially expressed, processed and glycosylated in human colon carcinoma cell lines [Laferté and Loh, 1992]. MAb 1H9 detects a conformational determinant and immunoprecipitates three size classes of glycoproteins with apparent molecular weights of 92–100, 66–70, and 25 kDa. The size heterogeneity within each class appears to be due to cell-type specific glycosylation differences. As described in the present report, partial protein sequence analysis of the 100- and 70-kDa glycoproteins and subsequent isolation of a partial cDNA clone encoding the N-terminal half of the 100-kDa glycoprotein revealed it to be identical to TAA90K/Mac-2 binding protein [Koths et al., 1993; Ullrich et al., 1994]. In this article, we describe a new panel of 11 TAA90K-specific monoclonal antibodies, including five antibodies specific for human TAA90K and six antibodies that detect a species of 90 kDa in COS-1 cells that may correspond to the simian homologue of TAA90K. We also demonstrate that these antibodies detect conformational differences between recombinant wild-type TAA90K, TAA90K synthesized in vitro, and a mutant form of TAA90K. In addition, we show that mutant forms of TAA90K that contain point mutations at residues 189, 223, and 259, as well as a truncated form of TAA90K containing the first 383 amino acids, are defective in secretion. Thus, we show for the first time the importance of specific regions within TAA90K on protein conformation and function, and demonstrate the potential usefulness of TAA90K-specific monoclonal antibodies for structure/function studies of native and recombinant TAA90K.

MATERIALS AND METHODS

Cell Lines

The human colon carcinoma cell lines HT29 (HTB 38), COS-1 (CRL 1650), Rat-2 (CRL 1764), and Balb/3T3 clone A31 (CCL 163) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's minimum essential medium (DMEM, high glucose) containing 10% FBS (Gibco-BRL, Burlington, Ontario, Canada). In routine testing, all the cell lines used were found to be free of *Mycoplasma* contamination.

Preparation of Detergent Lysates of HT29 Cells

HT29 cells (5-ml cell pellet) were homogenized using a Polytron (Brinkmann Instruments, Edmonton, Alberta, Canada) in 4 vol 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, 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 supernatant was assayed for protein content, using the Bio-Rad DC protein assay kit.

Monoclonal Antibodies

The isolation and characterization of MAb 1H9 have been described previously [Laferté and Loh, 1992]. The isolation of additional monoclonal antibodies specific for TAA90K (Table I) was carried out as described previously [Prokopishyn et al., 1999] after immunization of mice with a partially purified glycoprotein fraction isolated from detergent-solubilized HT29 cell lysates by lectin affinity chromatography on *Helix pomatia* (HPA)-agarose. Hybridoma supernatants were screened by indirect immunofluorescence, using HT29 cells, as described [Prokopishyn et al., 1999], and positive clones subcloned twice by limiting dilution using standard procedures [Hammarstrom et al., 1977]. Hybridomas were injected into Balb/c mice for the production of ascites, as described [Laferté and Loh, 1992]. Isotyping of the antibodies was carried out using the Bio-Rad Isotyping kit (Bio-Rad, Mississauga, Ontario, Canada), according to manufacturer's intructions.

Immunoprecipitation

[^{35}S]methionine-labeled conditioned media and cell lysates isolated from various cell lines

TABLE I. Characteristics of TAA90K-Specific Monoclonal Antibodies

Clone	Isotype	Western blotting[a,b]	Recombinant TAA90K (1-383)[c]	Recombinant TAA90K[d] (in vitro)	COS-1[e]
1G3	IgG_1	−	+	+++	−
1H9	IgG_1	−	+++	−	+++
2A11	IgG_1	−	+++	+	+++
2F9	IgG_1	−	+++	−	+++
4D1	IgG_1	−	+++	+	+++
5B11	IgG_1	−	+	+++	−
5H4	IgG_1	−	+	+++	−
9D11	IgG_1	−	+++	−	−
10A10	IgG_1	−	+++	+	+++
10E10	IgG_{2a}	−	+++	+++	+++
10G12	IgG_1	−	+	−	−
12D4	IgG_{2b}	++	−	+++	+++

[a]Plus signs indicate the relative amount of TAA90K detected: +++, strong; ++, moderate; +, weak; −, undetectable.
[b]A concentrated fraction containing TAA90K was prepared from HT29 conditioned medium after precipitation with 90% ammonium sulfate and then dialyzed and analyzed by Western blotting using TAA90K-specific monoclonal antibodies.
[c]Monoclonal antibodies were tested for their ability to immunoprecipitate detergent-solubilized cell lysates prepared from COS-1 cells transfected with the pSV-TAA90K-dC383 plasmid encoding the first 383 amino acids of TAA90K.
[d]Monoclonal antibodies were tested for their ability to immunoprecipitate wild-type recombinant TAA90K encoded by the pCD-TAA90K^{wt} plasmid expressed in the TNT-coupled transcription/translation system.
[e]Conditioned medium recovered from [^{35}S]-methionine-labeled COS-1 cells was immunoprecipitated with each of the monoclonal antibodies.

or [^{35}S]-labeled in vitro translation products (see below) were incubated overnight at 4°C with 3 μl of normal mouse serum or TAA90K-specific monoclonal antibodies; 100 μl of a 1:1 suspension of protein A-Sepharose-4B beads (Pharmacia, Montreal, Canada) was then added and the suspension gently rocked at 4°C for 1 h. After washing the beads once with immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 mM benzamidine, 1% deoxycholate, 1% Triton X-100, 0.02% sodium azide), twice with IP buffer containing 0.5 M NaCl, twice with IP buffer containing 0.1% SDS, and once with IP buffer, the immunoprecipitated proteins were eluted by boiling for 5 min in SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol). Samples to be digested with glycosidases were immunoprecipitated with protein A-Sepharose and washed as described above. After the last wash, beads were resuspended in glycosidase buffer, as specified by the manufacturer, boiled for 1–2 min, cooled, and incubated with glycosidase for the indicated period of time at 37°C. The glycosidases used in individual samples were as follows: (1) endoglycosidase H (endo H), 1 U (Boehringer-Mannheim, Laval, Quebec, Canada), (2) neuraminidase type X from *Clostridium perfringens*, 0.1 U (Sigma, Oakville, Ontario, Canada), and (3) N-glycanase, 0.3 U (Glyko, Novato, CA). Samples were adjusted to 1× SDS-sample buffer, boiled for 5 min, and analyzed by SDS-PAGE and fluorography.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli et al. [1970]. [^{35}S]-methionine-labeled proteins separated by 6% or 7.5% SDS-PAGE were visualized by fluorography [Bonner and Laskey, 1974], after enhancement with Enlightning (NEN, DuPont, Mississauga, Ontario, Canada).

Western Blotting Analysis

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose (Schleicher and Schull, Mandel, Guelph, Ontario, Canada) for 1 h at 100 V. Nitrocellulose blots were blocked 1 h at room temperature or overnight at 4°C in TBT/4% BSA. After three 5-min washes in TBT buffer, blots were incubated 2 h with monoclonal antibody diluted 1:500 in TBT, washed three times with TBT, and incubated 1 h with alkaline phosphatase-coupled affinity purified goat anti-mouse Ig diluted 1:3,000 in TBT (Bio-Rad). After three 5-min washes in TBT, one

wash in TBS containing 0.05% Tween-20, and one wash in TBS, blots were developed colorimetrically by incubation with the substrates BCIP and NBT (Bio-Rad).

Partial Protein Sequence Analysis of the 100- and 70-kDa Glycoproteins

A detergent-solubilized HT29 cell lysate prepared as described above from 10 ml of pelleted cells was divided into 1-ml aliquots, and each aliquot was immunoprecipitated with 3 μl of MAb 1H9. The immunoprecipitated proteins were separated by preparative SDS-PAGE on a 7.5% gel and transferred electrophoretically to Immobilon membrane (Millipore, Etobicoke, Ontario, Canada). After the membrane was stained for 5 min with 1% Ponceau Red and destained for 1 min in a solution of 1% acetic acid, visible bands were excised from the membrane, washed three times in distilled water and stored at −20°C until analysis [Aebersold et al., 1987]. Membrane strips containing the immunobilized 100- and 70-kDa glycoproteins were sent to Harvard Microchem (Boston, MA) for amino acid sequence analysis. The amino acid sequence of the N-terminal peptide obtained for the 100- and 70-kDa glycoproteins was VNDGDMRLADGGATNQGRVEIFYRG, while the amino acid sequences of two tryptic peptides derived from the 70-kDa glycoprotein were QWGTVSDNL-DLTDA-VV (peptide 1) and AVDTWSWDER (peptide 2). These sequences were used to design degenerate oligonucleotide primers, which were used in polymerase chain reaction (PCR)-based methods to isolate cDNA clones.

Isolation of cDNA Clones

The N-terminal peptide sequence was used to generate the upstream degenerate oligonucleotide primer N, GAATTCAGTIAA(T/C)GA-(T/C)GGIGA(T/C)ATG, while the sequence of peptide 2 was used to generate the downstream degenerate oligonucleotide primer P2, GAA-TTC(G/T)(T/C)TCICCCCAI(C/G)(A/T)CCAIGT-(A/G)TC (University Core DNA Synthesis, University of Calgary, Calgary, Alberta, Canada). Poly(A^+) RNA isolated from 10^6 HT29 cells, using the Microfast-track RNA isolation kit (Invitrogen, Carslbad, CA), was transcribed into cDNA using reverse transcriptase (RT) in the presence of random hexamers using the Perkin-Elmer RT-PCR kit (PE Applied Biosystems, Mississauga, Ontario, Canada). The resulting cDNAs were amplified by PCR using the upstream primer N and the downstream primer P2. Initial amplification conditions included 1 cycle of denaturation for 2 min at 95°C, followed by 5 cycles of denaturation at 95°C for 60 s, annealing at 47°C for 30 s, and extension at 72°C for 90 s. This was followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. Analysis of the reaction products by agarose gel electrophoresis revealed a band of 900 bp. Sequencing of the 900-bp cDNA clone indicated that it was identical to the N-terminal half of the 90-kDa tumor-associated glycoprotein (TAA90K) [Koths et al., 1993] and Mac-2 binding protein (Mac-2 BP) [Ullrich et al., 1994].

The complete cDNA clone encoding TAA90K was subsequently isolated using published sequence information and a PCR-based cloning strategy. The entire coding sequence was amplified from phage DNA isolated from a human colon carcinoma cDNA library (Stratagene, La Jolla, CA) in three overlapping segments using either Taq polymerase or Pfu DNA polymerase (Stratagene) and the following primer pairs: U1 (GGTACCATGACCCCTCCGAGGCTCTTC) and L1 (GTCCCTGACCATGGGCACACACT); U2 (TGCCCATGGTCAGGGACCTTCTCAG) and L2 (TGGAATTCCAGGGCCTGCAGAGTCTT); U3 (TGCAGGCCCTGGAATTCCACACTGT) and L3 (GGGGATCCTGGGGTTCTCCGGTTCTCAC) (University Core DNA Synthesis, University of Calgary, Calgary, Alberta, Canada). Each primer was designed to incorporate a unique restriction site (underlined in the primer sequences) such that the amplified sequence could be removed as a transportable cassette. In particular, *Kpn*I and *Bam*H1 sites were created at both ends of the ORF, and the unique *Nco*I and *Eco*R1 sites within the coding sequence (Fig. 1) were used in the design of the primers. The cDNAs obtained using the U1/L1, U2/L2, and U3/L3 primer pairs were 627 bp (fragment KN flanked by *Kpn*I and *Nco*I sites), 547 bp (fragment NE flanked by *Nco*I and *Eco*RI sites), and 673 bp in length (fragment EB flanked by *Eco*RI and *Bam*HI sites), respectively. The amplified cDNA fragments were separated by agarose gels electrophoresis, purified using Geneclean (Biocan, Mississauga, Ontario, Canada), and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems). Nucleotide sequences de-

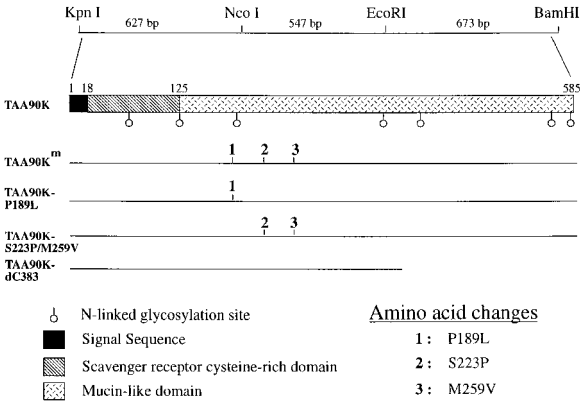


Fig. 1. Schematics for TAA90K and TAA90K mutants. The top line represents the cDNA encoding TAA90K with the locations of the unique restriction sites described under Materials and Methods. The open reading frame (ORF) of TAA90K and the locations of the putative N-linked glycosylation sites (at residues 69, 125, 192, 363, 398, 551, 580) are shown immediately underneath as shaded boxes. The amino acid residue number delineating the boundaries defined by Tinari et al. [1997] are shown above these boxes. The site-specific and deletion mutants of TAA90K described in this report are listed with the locations of the three mutations clearly marked. The relevant amino acid changes are: proline to leucine at residue 189 (P189L), serine to proline at residue 223 (S223L), and methionine to valine at residue 259 (M259V).

rived from cDNA fragments amplified with Pfu DNA polymerase were found to be identical to the published sequences [Koths et al., 1993; Ullrich et al., 1994].

The 1.174-kb *Kpn*I-*Eco*RI cDNA fragment, which comprised the KN and NE DNA fragments initially amplified by PCR, was inserted into *Kpn*I and *Eco*RI sites of the plasmid vector pTZ18R to create the plasmid pTZ-KE. Similarly, the EB fragment was cloned into *Eco*RI and *Bam*HI sites of the plasmid vector pTZ18R to create plasmid pTZ-EB, so that unique *Xba*I and *Hin*dIII sites were placed directly downstream of the TAA90 ORF behind the *Bam*HI site. Finally, the entire TAA90K ORF was reconstructed by inserting the 1.174 kb *Kpn*I-*Eco*RI fragment from pTZ-KE and the 0.67 kb *Eco*RI-*Hin*dIII fragment from pTZ-EB into *Kpn*I and *Hin*III sites of the plasmid vector RsetB (Invitrogen) to create RsetB-TAA90K. Consequently, an additional *Bam*HI site was placed directly upstream of the TAA90K translation start site. In this manner, the entire TAA90K ORF could be excised as either a 1.85-kb *Bam*HI fragment, or a similarly sized *Kpn*I-*Xba*I fragment.

Expression of TAA90K in eukaryotic systems was facilitated by inserting the 1.85 kb *Kpn*I-*Xba*I fragment from RsetB-TAA90K into compatible restriction sites of the expression vector pCDNA3 to create pCD-TAA90K, thereby placing a strong CMV immediate-early promoter in front of the TAA90K ORF. In addition, a phage T7 promoter was present on the vector, allowing the expression of TAA90K in an in vitro transcription/translation system. Alternatively, the *GAL4* coding sequences located between *Bgl*II-*Bam*HI sites of the plasmid pM2 [Sadowski et al., 1992] were replaced by the 1.85 kb *Bam*HI fragment from RsetB-TAA90K to create plasmid pSV-TAA90K. In this instance, the expression of TAA90K is driven by an SV40 promoter, allowing high level expression during transfection into COS-1 cells. Finally, the *GAL4* sequences in pM2 were also replaced with a 1.17 kb *Bam*HI-*Eco*RI fragment from RsetB-TAA90K. The resulting plasmid, pSV-TAA90K-dC383, encoded a C-terminal truncated version of TAA90K containing the first 383 amino acids of the protein.

Construction of Mutant Recombinant TAA90K

Sequencing of the pCD-TAA90K cDNA clone obtained by PCR using Taq polymerase revealed three point mutations (henceforth referred to as pCD-TAA90K^{m}) not present in the comparable wild-type clone isolated using Pfu DNA polymerase (i.e., pCD-TAA90K^{wt}). One of the mutations (C $\rightarrow$ T) present within the KN cDNA fragment resulted in a change in amino acid sequence from proline to leucine at residue 189 (denoted P189L). The other two mutations (T $\rightarrow$ C and A $\rightarrow$ G, respectively) found within the NE cDNA fragment resulted in two changes in the amino acid sequence; the serine at residue 223 was changed to a proline (S223P), while the methionine at residue 259 was changed to a valine (M259V). To examine the effect of these mutations on the structure and function of TAA90K, we constructed mutant TAA90K open reading frames that contain either the mutant KN fragment (pCD-TAA90K-P189L) or the mutant NE fragment (pCD-TAA90K-S223P/M259V) by exchanging the wild-type KN or NE fragments within pCD-TAA90K^{wt} with the respective mutant fragments. A plasmid encoding TAA90K containing the EB cDNA fragment obtained from pCD-TAA90K^{m} was also constructed (pCD-TAA90K-EB).

Transfection of Plasmid DNA Into COS-1 Cells

Transfection of plasmids pCD-TAA90K^{wt}, pCD-TAA90K^{m}, pSV-TAA90K, or pSV-TAA90K-dC383 into COS-1 cells (15 μg DNA/2 × 10^6 cells) was carried out by electroporation with the Gene Pulser apparatus (Bio-Rad) as previously described [Chu et al., 1987]. In some experiments, COS-1 cells were co-transfected with a plasmid encoding the β-galactosidase gene downstream from the SV40 early promoter (pSV-βgal), together with plasmids encoding wild-type or mutant TAA90K (i.e. pCD-TAA90K^{wt}, pCD-TAA90K^{m}, pCD-TAA90K-P189L, pCD-TAA90K-S223P/M259V, or pCD-TAA90K-EB). Typically, electroporation was carried out using a 0.4-cm cuvette at voltage and capacitance settings of 250 V and 500 μF, respectively.

Metabolic Labeling of Cells

HT29 and COS-1 cell monolayers grown to 70–80% confluence were washed twice with D-PBS and radiolabeled for 24 h in methionine-free DMEM supplemented with one-tenth the normal content of methionine, 10% FBS, and 50 μCi of [^{35}S]-methionine/ml (800 Ci/mmol; NEN/Mandel, Guelph, Ontario, Canada). For pulse-chase labeling experiments, cell monolayers grown in 60-mm dishes were washed in D-PBS and incubated for 1 h in methionine-free medium before the addition of [^{35}S]-methionine (100 μCi/ml). Cells were pulse-labeled for 30 min and chased for 5 h in DMEM containing 10% FBS and 50× the normal concentration of unlabeled methionine. At the indicated times, the medium was removed, centrifuged to remove cells, and saved for analysis of secreted antigens. The plates were then washed three times with ice-cold D-PBS, and the cells were harvested with a rubber policeman and pelleted. Cell pellets were solubilized on ice for 1 h in IP buffer. COS-1 cells transfected with plasmids encoding wild-type or mutant TAA90K, or cells co-transfected with plasmids encoding TAA90K and β-galactosidase were allowed to recover for 24 h before labeling with [^{35}S]methionine. In the case of co-transfection experiments, the cell lysate was also assayed for protein content, using the DC protein assay (Bio-Rad) and β-galactosidase activity [Sambrook et al., 1989]. To ensure that the expression of wild-type and mutant TAA90K in COS-1 transfected cells was corrected for transfection efficiency of various plasmid constructs, the volume of radiolabeled culture medium used in immunoprecipitation analyses was adjusted based on the specific activity of β-galactosidase in each of the co-transfected COS-1 cell lysates.

In Vitro Translation

[^{35}S]-methionine-labeled TAA90K was synthesized in a coupled transcription-translation reaction using the TNT T7-coupled reticulocyte lysate system (Promega, Nepean, Ontario, Canada); 1 μg of plasmid DNA purified by density-gradient centrifugation in cesium chloride or by precipitation with polyethylene glycol [Sambrook et al., 1989] was used in each reaction according to the manufacturer's instructions.

Peptide Mapping

Limited proteolytic peptide mapping was carried out by a protocol originally developed by Cleveland et al. [1977] and modified as previously described [Loh, 1991].

RESULTS

MAb 1H9 Detects TAA90K

In a previous study, we described the isolation of a hybridoma cell line producing MAb 1H9, which detects structurally related glycoproteins of 100 and 70 kDa with β1–6 branched Asn-linked oligosaccharides [Laferté and Loh, 1992]. Our interest in elucidating the biological function of these glycoproteins stemmed from the documented importance of β1–6 branched Asn-linked oligosaccharides in cancer metastasis [Dennis et al., 1987; Dennis, 1992], as well as the differential expression and processing of these glycoproteins in human colon carcinoma cell lines [Laferté and Loh, 1992]. As a first step toward identifying the glycoproteins detected by MAb 1H9 and elucidating their possible contribution to colon cancer progression, we obtained partial protein sequence information from the 100- and 70-kDa species. As described under Materials and Methods, both glycoproteins shared an identical 25-amino acid residue N-terminal sequence, suggesting that the smaller glycoprotein may be derived from the larger one by proteolytic cleavage or that the two glycopro-

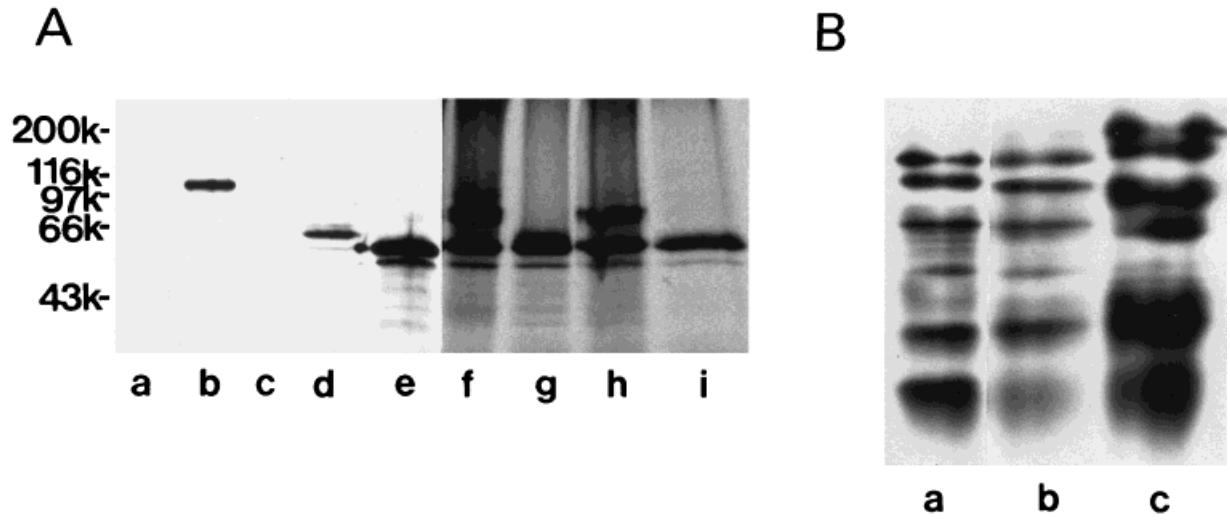


Fig. 2. Analysis of recombinant TAA90K synthesized in vivo and in vitro. **A:** [^{35}S]methionine-labeled conditioned medium recovered from HT29 cells was immunoprecipitated with NMS (**lanes a,c**) or MAb 1H9 (**lanes b,d**) and incubated for 18 h at 37°C with buffer alone (**lanes a,b**) or with buffer containing N-glycanase (**lanes c,d**). The products obtained after N-glycanase treatment were analyzed by 7.5% SDS-PAGE after fluorography and autoradiography and compared with recombinant TAA90K synthesized in vitro with the T7-coupled transcription-translation system (**lane e**). TAA90K was also synthesized in vitro in the presence of 2.5 μg of dog pancreas microsomes (**lanes f–i**). Aliquots of this reaction mixture were left untreated (**lane f**) or digested for 18 h at 37°C with endoglycosidase H (**lane g**), neuraminidase (**lane h**), or N-glycanase (**lane i**). **B:** [^{35}S]methionine-labeled TAA90K synthesized in vitro with the T7-coupled transcription-translation system (**lane a**) or N-glycanase-treated TAA90K immunoprecipitated from [^{35}S]methionine-labeled HT29 cell lysates (**lanes b,c**) were separated by 7.5% SDS-PAGE. Gel slices corresponding to the 60-kDa species (**lanes a,b**) or 64-kDa species (**lane c**) detected on the autoradiogram were excised from the unfixed dried gel and subjected to limited V8 proteinase peptide mapping, as described under Materials and Methods.

teins are members of a gene family. The more abundant 70-kDa species was subjected to tryptic proteolytic cleavage and the resulting peptide sequence information used to design degenerate oligonucleotide probes for use in RT-PCR (see Materials and Methods). A 900-bp partial cDNA clone encoding the N-terminal half of the 100 kDa was found to be identical to sequences encoding the N-terminal half of the 90 kDa glycoprotein variously known as tumor-associated 90K glycoprotein (TAA90K) [Ullrich et al., 1994], Mac-2 binding protein [Koths et al., 1993], and cyclophilin C-associated protein [Friedman et al., 1993] (data not shown).

The cDNA encoding the wild-type complete open reading frame of TAA90K (encoded by plasmid pCD-TAA90K^{wt}) was assembled from three overlapping cDNA clones, each encoding approximately one-third of the protein (see Materials and Methods and Fig. 1). As discussed in (Effect of Specific Mutations in TAA90K on Protein Biosynthesis, Conformation, and Secretion), a cDNA clone encoding a mutant form of TAA90K (pCD-TAA90K^{m}) containing three amino acid substitutions was also isolated (see Materials and Methods and Fig. 1), which provided an opportunity to test the effect of specific mutations on TAA90K structure and function. Subsequent cloning of the wild-type TAA90K cDNA into the eukaryotic expression vector pCDNA-3, and expression in the TNT T7-coupled transcription/translation system yielded a major translation product with an apparent molecular mass of 60 kDa (Fig. 2A, lane e). This is consistent with the predicted polypeptide size of 63 kDa for TAA90K [Koths et al., 1993]. The addition of dog pancreas microsomes to the transcription/translation system, which results in posttranslational modification of nascent polypeptides with high-mannose type Asn-linked oligosaccharides, resulted in the conversion of some of the 60-kDa species to a species having an apparent size of 74 kDa (Fig. 2A, lane f). This result agrees with our previous pulse-chase labeling experiments in HT29 cells [Laferté and Loh, 1992] as well as earlier studies by Linsley et al. [1986], which showed that TAA90K is derived from a 74-kDa glycoprotein with high-mannose

type oligosaccharides, processed to a glycoprotein with complex-type oligosaccharides of 90–100 kDa. This latter species of TAA90K is secreted into the culture medium (Fig. 2A, lane b). The presence of high-mannose type oligosaccharides on the in vitro translated 74-kDa species was confirmed by its susceptibility to endoglycosidase H (Fig. 2A, lane g), while its resistance to neuraminidase indicated a lack of complex-type oligosaccharides (Fig. 2A, lane h). The fact that the 74-kDa species was cleaved to a species of 60 kDa in the presence of N-glycanase (Fig. 2A, lane i) indicated that the glycan component on the in vitro synthesized glycoprotein consists primarily of Asn-linked oligosaccharides. In addition, the 60-kDa species synthesized in vitro and the lower band of the 64/60-kDa doublet obtained after N-glycanase treatment of TAA90K immunoprecipitated from [^{35}S]-labeled HT29 cell lysates produced identical peptide maps when subjected to limited protease V8 digestion (Fig. 2B, lanes a and b). Although some of the protease V8 peptides generated from the 64-kDa species co-migrated with those derived from the 60-kDa species (Fig. 2B, lane c, lower bands), other bands appeared to represent higher-molecular-weight forms of bands evident in the 60-kDa map. This is probably attributable to incomplete deglycosylation of the 64-kDa, which would be expected to generate some larger peptides. Furthermore, it would appear that TAA90K expressed by HT29 cells is somewhat resistant to complete deglycosylation, as evidenced by the low amount of the 60-kDa species obtained after N-glycanase treatment.

A New Panel of Monoclonal Antibodies Detects TAA90K

Having demonstrated that the 100-kDa glycoprotein previously identified by MAb 1H9 corresponds to TAA90K, we set out to isolate additional hybridoma lines secreting monoclonal antibodies specific for TAA90K that could prove useful in structure/function studies of wild-type and mutant TAA90K. As shown in Figure 3A and summarized in Table I, an additional 11 hydriboma lines producing monoclonal antibodies were isolated. These MAbs detected a 100-kDa species in the conditioned medium of HT29 cells, which co-migrates with TAA90K immunoprecipitated by MAb 1H9. To confirm that the 100-kDa species detected by the new antibodies corresponds to TAA90K, we repeated the immunoprecipitation experiment using [^{35}S]-labeled HT29 conditioned medium depleted of TAA90K by prior immunoprecipitation with MAb 1H9. As can be seen from Figure 3B, depletion of the HT29 conditioned medium of TAA90K, as evidenced by the lack of any detectable 100-kDa species in 1H9 immunoprecipitates (Fig. 3B, lane a), also removed the immunoreactive 100-kDa species detected by all other monoclonal antibodies. Thus, we had isolated a new panel of antibodies specific for TAA90K.

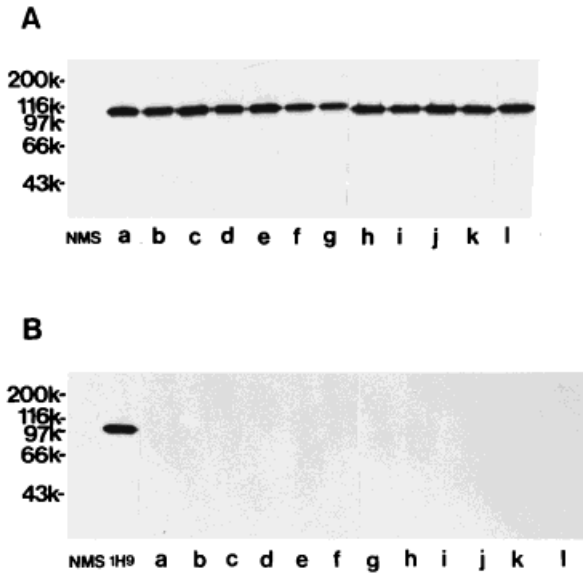


Fig. 3. Reactivity of new panel of monoclonal antibodies with human TAA90K. **A:** [^{35}S]methionine-labeled HT29 conditioned medium was immunoprecipitated with normal mouse serum (NMS) or monoclonal antibodies listed in Table I as follows: **lane a**, 1H9; **lane b**, 1G3; **lane c**, 2A11; **lane d**, 2F9; **lane e**, 4D1; **lane f**, 5B11; **lane g**, 5H4; **lane h**, 9D11; **lane i**, 10A10; **lane j**, 10E10; **lane k**, 10G12; **lane l**, 12D4. The products were analyzed by 7.5% SDS-PAGE, followed by fluorography and autoradiography. **B:** [^{35}S]methionine-labeled HT29 conditioned depleted of TAA90K by exhaustive immunoprecipitation with MAb 1H9 was immunoprecipitated with TAA90-specific monoclonal antibodies, as follows: **lane a**, 1H9; **lane b**, 1G3; **lane c**, 2A11; **lane d**, 2F9; **lane e**, 4D1; **lane f**, 5B11; **lane g**, 5H4; **lane h**, 9D11; **lane i**, 10A10; **lane j**, 10E10; **lane k**, 10G12; **lane l**, 12D4. As control, untreated HT29 conditioned medium was immunoprecipitated with NMS or MAb 1H9 (shown in the first two lanes). The products were analyzed by 7.5% SDS-PAGE, followed by fluorography and autoradiography.

In light of the extensive sequence homology existing between human TAA90K and cyclophilin C/MAMA [Friedman et al., 1993; Chicheportiche and Vassalli, 1994], two mouse homologues of human TAA90K, it was possible

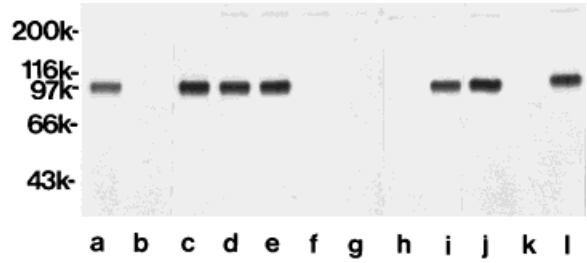


Fig. 4. Reactivity of TAA90K-specific monoclonal antibodies with a glycoprotein expressed by COS-1 cells. Conditioned medium isolated from [^{35}S]methionine-labeled COS-1 cells was immunoprecipitated with TAA90-specific monoclonal antibodies as follows: **lane a**, 1H9; **lane b**, 1G3; **lane c**, 2A11; **lane d**, 2F9; **lane e**, 4D1; **lane f**, 5B11; **lane g**, 5H4; **lane h**, 9D11; **lane i**, 10A10; **lane j**, 10E10; **lane k**, 10G12; **lane l**, 12D4. The products were analyzed by 7.5% SDS-PAGE followed by fluorography and autoradiography.

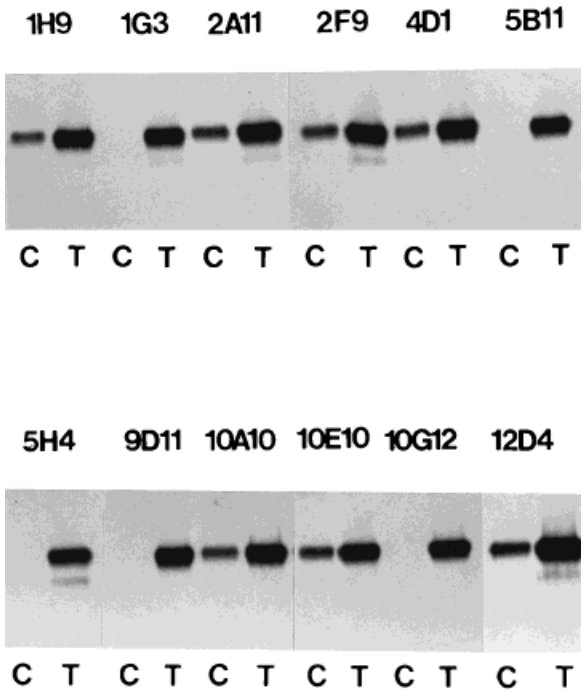


Fig. 5. Expression of recombinant human TAA90K in COS-1 cells. COS-1 cells were transfected with plasmid DNA encoding wild-type TAA90K (pCD-TAA90^{wt}) by electroporation and allowed to recover overnight before radiolabeling for 18 h with [^{35}S]methionine. Aliquots (0.5 ml) of radiolabeled conditioned medium from control (C) or transfected (T) cells were immunoprecipitated with TAA90K-specific monoclonal antibodies as indicated above each panel. The products were analyzed by 7.5% SDS-PAGE, followed by fluorography and autoradiography.

that some of the new monoclonal antibodies cross-reacted with homologous glycoproteins expressed by cells of different species. It was predicted that simian cells would be most likely to express glycoproteins immunologically cross-reactive with human TAA90K. Whereas TAA90K-specific monoclonal antibodies failed to detect cross-reactive glycoproteins in Balb/3T3 and rat-2 fibroblasts, 7 of 12 antibodies (1H9, 2A11, 2F9, 4D1, 10A10, 10E10, 12D4) immunoprecipitated a 90-kDa species from the conditioned medium of [^{35}S]methionine-labeled COS-1 cells (Fig. 4). Although smaller in size than TAA90K expressed in the human colon carcinoma cell line HT29 [Laferté and Loh, 1992], the 90-kDa species detected in COS-1 cells may be a simian homologue of TAA90K. The remaining five monoclonal antibodies, namely 1G3 (Fig. 4, lane b), 5B11 (lane f), 5H4 (lane g), 9D11 (lane h), and 10G12 (lane k), were unable to immunoprecipitate the 90-kDa species from COS-1 cells, indicating that they are human-specific. This was confirmed by demonstrating that these five monoclonal antibodies immunoprecipitate the recombinant human TAA90K in transfected COS-1 cells but not the simian 90-kDa glycoprotein (Fig. 5). By contrast, the seven cross-reactive monoclonal antibodies detected the recombinant human TAA90K as well as the simian 90-kDa glycoprotein, although the latter was expressed at a much lower level.

It was noted that the recombinant TAA90K expressed in transfected COS-1 cells migrated on SDS-PAGE with an apparent size of 90 kDa, which is smaller than the 100-kDa TAA90K species detected in HT29 cells (Fig. 6, lanes b and c). As shown in Figure 6, this size difference appeared to be due to the content of N-linked chains since treatment of TAA90K immunoprecipitated from COS-1 and HT29 cells with N-glycanase produced co-migrating doublets of 64 and 60 kDa (lanes e and f, respectively). Thus, TAA90K expressed in different cell types appears to be glycosylated differently. Furthermore, the difference in size between TAA90K expressed in HT29 cells and the 90-kDa species from COS-1 cells appears to be the result of differences in the number of N-linked chains. Limited N-glycanase treatment of TAA90K immunoprecipitated from the conditioned medium of HT29 cells, which can be used to estimate the number of N-linked chains in a glycoprotein [Carlsson et al., 1988], revealed seven partially deglycosylated species consistent with the seven potential sites of N-linked glycosylation in human TAA90K (Fig.

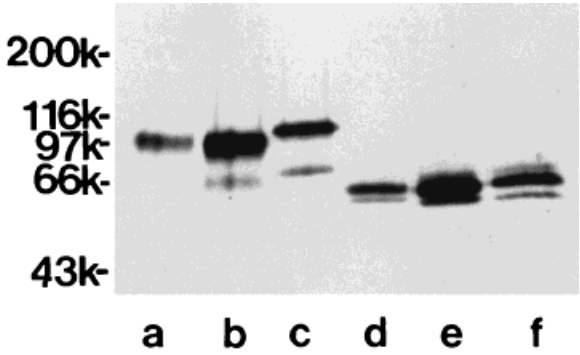


Fig. 6. Analysis of native and recombinant TAA90K in HT29 and COS-1 cells. [^{35}S]methionine-labeled conditioned medium recovered from COS-1 cells (**lanes a,d**), COS-1 cells transfected with pCD-TAA90K^{wt} plasmid DNA encoding wild-type TAA90K (**lanes b,e**) or HT29 cells (**lanes c,f**) was immunoprecipitated using either the cross-reactive monoclonal antibody 1H9 (**lanes a,d**) or the human-specific monoclonal antibody 1G3 (**lanes b,c,e,f**). Immunoprecipitates were incubated for 18 h at 37°C with buffer alone (**lanes a–c**) or with buffer containing N-glycanase (**lanes d–f**), and analyzed by 7.5% SDS-PAGE, followed by fluorography and autoradiography.

7A). Similar analysis of recombinant TAA90 expressed in COS-1 cells revealed at most five partially deglycosylated species (Fig. 7C). This was not limited to recombinant TAA90K, as the simian 90-kDa glycoprotein expressed by COS-1 cells produced a similar pattern of partially deglycosylated species (Fig. 7B). These results suggested that glycosylation of TAA90K in COS-1 cells differs significantly from that of HT29 cells, possibly due to differences in glycosyltransferase levels within each cell line.

Effect of C-Terminal Deletion on Antibody Reactivity

As seen previously with MAb 1H9, most of the new monoclonal antibodies detected conformational epitopes, since they were unable to detect TAA90K in Western blotting analysis (Table I). However, MAb 12D4 detected TAA90K in Western blotting analysis of HT29 conditioned medium (Fig. 8), suggesting that this antibody detects a linear epitope. In an attempt to localize the epitope detected by MAb 12D4 and to obtain information about the location of epitopes recognized by the other monoclonal antibodies, we transfected COS-1 cells with plasmid DNA encoding wild-type TAA90K (pSV-TAA90K) or a truncated form of TAA90K encompassing the first 383 amino acids (pSV-TAA90K-dC383), and immunoprecipitated the [^{35}S]-methionine-labeled cell lysates with

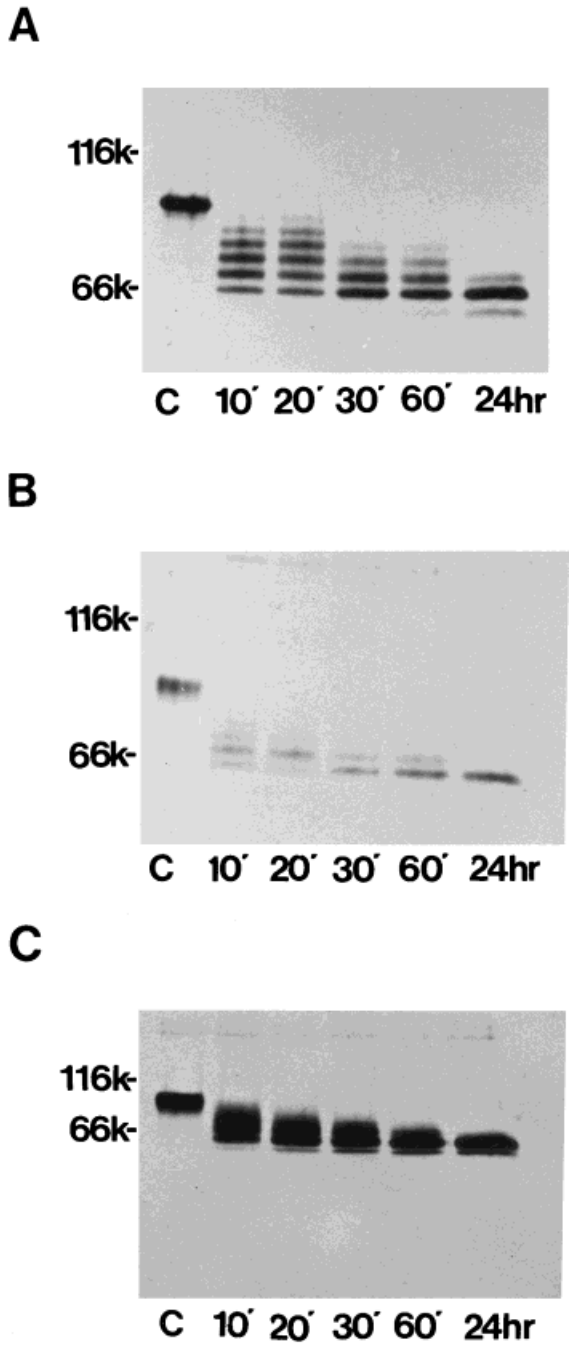


Fig. 7. Partial N-glycanase treatment of native and recombinant TAA90K expressed by HT29 and COS-1 cells. [^{35}S]methionine-labeled conditioned medium recovered from HT29 (**A**), COS-1 (**B**) or COS-1 cells transfected with pCD-TAA90K^{wt} plasmid DNA encoding wild-type TAA90K (**C**) was immunoprecipitated with MAb 12D4 (**A,B**) or 1G3 (**C**). Samples immobilized on protein A-Sepharose were incubated at 37°C with buffer alone (**C**) or with buffer containing N-glycanase for 10 min, 20 min, 30 min, 60 min, or 24 h. The products were separated by 6% (**A,B**) or 7.5% SDS-PAGE (**C**) and bands visualized after fluorography and autoradiography.

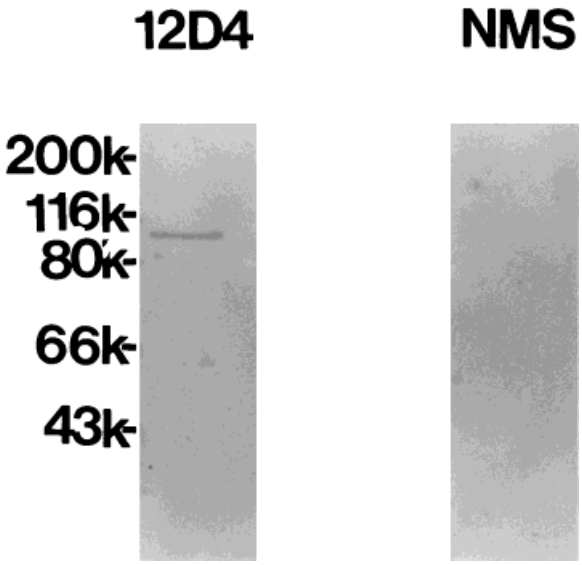


Fig. 8. Western blotting analysis of human TAA90K with MAb 12D4. HT29 conditioned medium containing TAA90K was precipitated with 90% ammonium sulfate, dialyzed against PBS, and concentrated 1,000-fold. An aliquot containing 20 μg total protein was analyzed by Western blotting using NMS (1:500 dilution) or MAb 12D4 (1:500 dilution), as described under Materials and Methods. The sizes of prestained molecular-weight markers are shown at the left of the figure.

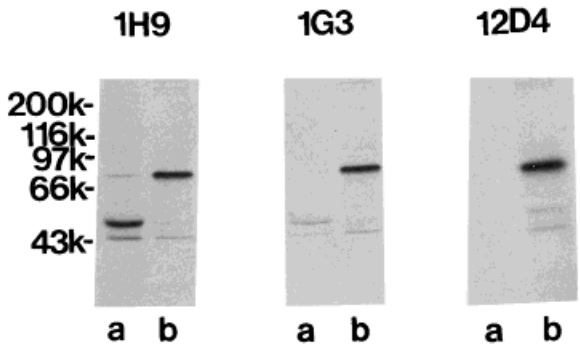


Fig. 9. Reactivity of MAbs 1H9, 1G3, and 12D4 with a truncated form of TAA90K. COS-1 cells were transfected with plasmid DNA encoding wild-type TAA90K (pSV-TAA90K^{wt}) or a truncated form of TAA90K consisting of amino acids 1-383 (pSV-TAA90K-dC383). At 24 h posttransfection, COS-1 cells were radiolabeled for 18 h with [^{35}S]-methionine. The detergent-solubilized cell lysate prepared from cells transfected with pSV-TAA90K-dC383 (**lane a**) or pSV-TAA90K^{wt} (**lane b**) was immunoprecipitated with MAbs 1H9, 1G3, or 12D4. The immunoprecipitated species were analyzed by 7.5% SDS-PAGE, followed by fluorography and autoradiography.

TAA90K-specific antibodies. All of the antibodies immunoprecipitated a 74-kDa species from COS-1 cells transfected with pSV-TAA90K plasmid DNA (data not shown). Representative immunoprecipitation results obtained with MAbs 1H9, 1G3, and 12D4 are shown in Figure 9. As discussed in (Effect of Specific Mutations in TAA90K on Protein Biosynthesis, Conformation, and Secretion), the 74-kDa species represents a processing intermediate found within COS-1 cells. Similar analysis of COS-1 cells transfected with the pSV-TAA90K-dC383 plasmid encoding the truncated TAA90K revealed that 7 of 12 antibodies reacted strongly with a 50-kDa species, suggesting that the epitope detected by these antibodies is located within the first 383 amino acids (Fig. 9 and Table I). The low amount of 74-kDa species detectable in COS-1 cells transfected with plasmid DNA encoding TAA90K-dC383 likely represents the cross-reactive protein expressed by COS-1 cells. By contrast, four of the antibodies, namely 1G3, 5B11, 5H4, and 10G12 immunoprecipitated low but detectable levels of the 50-kDa glycoprotein. This suggested that the epitope may be modified by improper folding, possibly due to the absence of residues within the C-terminal domain. In the case of MAb 12D4, which detects a linear epitope and failed to immunoprecipitate the 50-kDa species, our data indicated that the epitope detected by this antibody is destroyed by removal of the C-t erminal third of TAA90K (Fig. 9). It is noteworthy that the truncated form of TAA90K was not secreted into the culture medium (data not shown), possibly due to improper protein folding and subsequent retention within the endoplasmic reticulum [Plemper and Wolf, 1999].

Reactivity of Monoclonal Antibodies Against Recombinant TAA90K Synthesized In Vitro

The availability of the TNT T7 coupled transcription/translation system to produce recombinant proteins in pure form has provided unique opportunities for carrying out structure/function studies [Loh et al., 1994]. However, the successful application of this approach is dependent on the production of proteins in their native conformations. To examine whether recombinant TAA90K expressed in vitro assumes its native conformation, we tested the TAA90K-specific monoclonal antibodies listed in Table I for their ability to immunoprecipitate the unglycosylated and unprocessed 60-kDa species synthesized in vitro using the reticulocyte lysate system. As shown in Figure 10, four of the conformation-dependent antibodies, namely

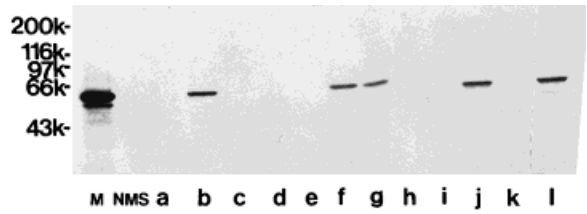
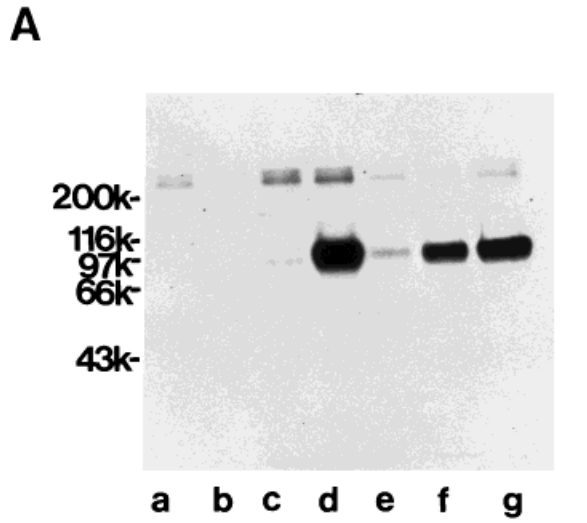


Fig. 10. Specificity of TAA90K-specific monoclonal antibodies for recombinant TAA90K synthesized in vitro. [^{35}S]methionine-labeled recombinant TAA90K synthesized in vitro, using the T7-coupled transcription-translation system was immunoprecipitated with NMS or TAA90K-specific monoclonal antibodies as follows: **lane a**, 1H9; **lane b**, 1G3; **lane c**, 2A11; **lane d**, 2F9; **lane e**, 4D1; **lane f**, 5B11; **lane g**, 5H4; **lane h**, 9D11; **lane i**, 10A10; **lane j**, 10E10; **lane k**, 10G12; **lane l**, 12D4. The products were analyzed by 7.5% SDS-PAGE, followed by fluorography and autoradiography. The first lane, labeled M, shows an aliquot of the in vitro translation reaction mixture.



1G3 (lane b), 5B11 (lane f), 5H4 (lane g) and 10E10 (lane j), as well as the Western blotting antibody 12D4 (lane l), reacted strongly with the 60 kDa translation product (Table I). Longer exposure of the autoradiogram revealed that MAbs 2A11 and 4D1 reacted weakly with the 60-kDa species (Table I). Thus, the epitope detected by each of these antibodies appears to be maintained in the in vitro-synthesized TAA90K. By contrast, the remaining six antibodies (1H9, 2F9, 4D1, 9D11, 10A10, 10G12) failed to immunoprecipitate in vitro-synthesized TAA90K (Table I). The addition of dog pancreas microsomes to the reticulocyte lysate used for the in vitro transcription/translation reaction, which yields a 74-kDa species containing high-mannose type Asn-linked oligosaccharides (Fig. 2), did not improve antibody reactivity (data not shown). This suggested that poor antibody reactivity could not be explained solely by the absence of glycosylation in the in vitro-synthesized TAA90K. Other posttranslational modifications may contribute to the native conformation of TAA90K, including proper protein folding facilitated by cyclophilins [Kay, 1996], calnexin [Bergeron et al., 1994], and calreticulin [Trombetta and Helenius, 1998], as well as correct formation of disulfide bonds.

Fig. 11. Reactivity of TAA90K-specific monoclonal antibodies with wild-type and mutant TAA90K. **A:** COS-1 cells were co-transfected with plasmids encoding β-galactosidase (pSV-βgal) and wild-type or mutant TAA90K by electroporation and allowed to recover 24 h before radiolabeling for 24 h with [^{35}S]methionine. The TAA90K-expressing plasmids used included pCD-TAA90K^{m} (**lane c**), pCD-TAA90K^{wt} (**lane d**), pCD-TAA90K-P189L (**lane e**), pCD-TAA90K-S223P/M259V (**lane f**), and pCD-TAA90K-EB (**lane g**). COS-1 cells subjected to the electroporation procedure without the inclusion of plasmid DNA and cells transfected with pSV-βgal plasmid DNA alone were used as controls (**lanes a,b**, respectively). Aliquots of radiolabeled conditioned medium normalized for β-galactosidase activity were immunoprecipitated with MAb 5B11. Samples were analyzed by 7.5% SDS-PAGE and bands visualized after fluorography and autoradiography. **B:** COS-1 cells were transfected with plasmid DNA encoding wild-type TAA90K (pCD-TAA90K^{wt}) (**lanes a–d**) or the triple mutant form of TAA90K (pCD-TAA90K^{m}) (**lanes e–h**). At 48 h posttransfection, cells were pulse-labeled with [^{35}S]-methionine for 30 min, followed by a 5-h chase labeling period. Radiolabeled conditioned medium (M) and detergent-solubilized cell lysates (C) from the pulse (**lanes a,b,e,f**) and 5-h chase period (**lanes c,d,g,h**) were immunoprecipitated with MAb 5B11. Samples were analyzed by 7.5% SDS-PAGE and bands visualized as described above.

Effect of Specific Mutations in TAA90K on Protein Biosynthesis, Conformation, and Secretion

During the screening of cDNA clones encoding TAA90K, we isolated a cDNA clone synthesized using Taq polymerase, which encodes a mutant form of TAA90K (referred to as pCD-TAA90K^m) containing three nucleotide substitutions resulting in specific changes in amino acid sequences (Fig. 1). One of the mutations was located within the first third of the protein, encoded by the KN cDNA fragment, and resulted in a change from proline to leucine at residue 189 (referred to as P189L). The other two mutations, found within the middle third of the protein encoded by the NE cDNA fragment, resulted in a change of serine to proline at residue 223 (S223P) and a change of methionine to valine at residue 259 (M259V). Availability of this mutant form of TAA90K provided an opportunity to examine the effect of specific mutations on the biosynthesis and processing of TAA90K as well as the conformation of epitopes detected by the five human-specific monoclonal antibodies. To this end, we also constructed mutant pCD-TAA90K plasmids that contain either the mutant KN fragment encoding the P189L mutation (pCD-TAA90K-P189L), or the mutant NE fragment encoding the S223P and M259V mutations (pCD-TAA90K-S223P/M259V). As a control, we constructed a plasmid encoding TAA90K containing the EB cDNA fragment synthesized by Taq polymerase, which encodes the wild-type sequence. In order to control for the transfection efficiency of various plasmid constructs, COS-1 cells were co-transfected with plasmids encoding β-galactosidase and wild-type or mutant TAA90K. The β-galactosidase activity was used to normalize the volume of [^{35}S]-methionine-labeled conditioned medium used in immunoprecipitation analyses.

As shown in Figure 11A (for MAb 5B11) and summarized in Table II, the human-specific monoclonal antibodies 1G3, 5B11, 5H4, 9D11, and 10G12 were very effective at immunoprecipitating wild-type TAA90K encoded by the pCD-TAA90K^{wt} plasmid (lane d). Similar results were obtained with wild-type TAA90K encoded by the pCD-TAA90K-EB plasmid (lane g). However, all five antibodies reacted poorly with the triple mutant TAA90K encoded by the pCD-TAA90K^m plasmid (Fig. 11A, lane c, and Table II). While monoclonal antibodies 1G3, 5B11, and 5H4 were still able to immunoprecipitate low amounts of the triple mutant TAA90K, antibodies 9D11 and 10G12 failed to do so (Table II).

In order to determine which of the three mutations could account for this altered antibody reactivity, we examined the ability of these antibodies to immunoprecipitate the single and double mutant form of TAA90K encoded by pCD-TAA90K-P189L and pCD-TAA90K-S223P/M259V plasmids, respectively. As shown in Figure 11A (lane f) and summarized in Table II, the double mutant had a modest effect on the ability of the antibodies to immunoprecipitate TAA90K. However, mutation of the proline residue at residue 189 had a dramatic effect on the amount of TAA90K immunoprecipitated by each of the antibodies (Fig. 11A, lane e and Table II). In fact, this single point mutation appears to be sufficient to account for the low level of the triple mutant form of TAA90K in the culture medium (Fig. 11A, lane c). This would suggest that Pro 189 makes up part of the epitope recognized by these antibodies or alternatively, this proline residue affects the conformation of distant regions of the protein.

There are two possible interpretations of these results. The first is that mutations at residues 189, 223, and 259 of TAA90K alter the conformation of epitopes detected by the five human-specific monoclonal antibodies. The second is that these mutations affect the rate of secretion of TAA90K into the conditioned medium, effectively reducing the amount of TAA90K immunoprecipitated by these antibodies. To test the latter possibility, we compared the biosynthetic processing and secretion of wild-type and mutant TAA90K in transfected COS-1 cells. In order to detect the intermediate forms in the processing and secretion of TAA90K, COS-1 cells transfected with pCD-TAA90K^{wt} or pCD-TAA90K^m were radiolabeled for a short 30-min pulse (instead of 18 h) followed with a 5-h chase period, a time interval sufficiently long to permit secretion of TAA90K into the conditioned medium. As shown in Figure 11B, using 5B11 as the immunoprecipitating monoclonal antibody, and summarized in Table II for other human-specific antibodies, wild-type TAA90K was synthesized initially as a 74-kDa species (lane a). The pulse-labeling period was too short to allow secretion of TAA90K into the conditioned medium (lane b). Five hours later, the 74-kDa species was processed to a less abundant spe-

TABLE II. Relative Amount of Wild-Type, Mutant, or Truncated TAA90K Detected in Cell Lysates and Conditioned Medium of Transfected COS-1 Cells*

MAb	TAA90K^{wt}		TAA90K$^-$ dC383		TAA90K^m		TAA90K$^-$ P189L		TAA90K$^-$ S223P/ M259V	
	74 K/72 K	90 K	50 K	58 K[b]	74 K/72 K	90 K	74 K/72 K	90 K	74 K/72 K	90 K
1G3	+++[a]	+++	+	−	+++	+	+	+/−	+++	+
5B11	+++	+++	+	−	+++	+	+	+/−	+++	+
5H4	+++	+++	+	−	+++	+	+	+/−	+++	+
9D11	+++	+++	+++	−	−	−	+	+/−	++	+
10G12	+++	+++	+	−	−	−	+	+/−	++	+
12D4	+++	+++	−	−	+++	++	++	+	+++	++

*COS-1 cells transfected with plasmid DNA encoding wild-type TAA90K full-length (pCD-TAA90K^{wt} or pSV-TAA90K^{wt}) or trunctated TAA90K (pSV-TAA90K-dC383), the triple mutant form of TAA90K (pCD-TAA90K^m), as well as the single (pCD-TAA90K-P189L) and double (pCD-TAA90K-S223P/M259V) mutant forms of TAA90K were pulse-labeled with [^{35}S]-methionine for 30 min, followed by a 5-h chase period. Detergent-solubilized cell lysates and conditioned medium from pulse (74/72-kDa species) and 5-h chase (90 kDa species) samples were immunoprecipitated with the five human-specific monoclonal antibodies (1G3, 5B11, 5H4, 9D11, 10G12) or the cross-reactive monoclonal antibody 12D4. The products were analyzed by 7.5% SDS-PAGE and bands visualized after fluorography and autoradiography. Representative examples are shown in Figs. 11 and 13.

[a]Plus and minus signs designate the relative amount of TAA90K detectable in immunoprecipitates. +++, strong; ++, moderate; +, weak; +/−, negligible; −, undetectable.

[b]Expected approximate size of secreted truncated form of TAA90K.

cies of 72 kDa (lane c), accompanied by the secretion of a 90-kDa species into the culture medium (lane d). Similar analysis of COS-1 cells transfected with the triple mutant form of TAA90K encoded by pCD-TAA90K^m revealed a 74-kDa species synthesized during the pulse-labeling period (Fig. 11B, lane e), which persisted 5 h later (lane g). However, very little of the 90-kDa mature glycoprotein was detected in the culture medium in the 5-h chase sample (lane h), suggesting that the triple mutant form of TAA90K is defective in secretion. Susceptibility of the 74-kDa and 72-kDa of both wild-type and triple mutant TAA90K to endoglycosidase H (Fig. 12A,B, lanes b and e) indicated that each of these species contains high-mannose type oligosaccharides, characteristic of glycoproteins within the endoplasmic reticulum. By contrast, resistance of the wild-type 90-kDa species secreted by COS-1 cells to endoglycosidase H but susceptibility to neuraminidase (Fig. 12A, lanes h and i, respectively) was consistent with conversion of high-mannose type oligosaccharides to complex type structures on glycoproteins destined for the plasma membrane or secretion. The fact that low levels of neuraminidase-sensitive 90-kDa species were detected in the conditioned medium of COS-1 cells transfected with plasmid DNA encoding the triple mutant (Fig. 12B, lanes g–i) suggested that processing of Asn-linked oligosaccharides from high-mannose to complex-type structures occurred normally within these cells. Thus, as a result of mutations at residues 189, 223, and 259, TAA90K^m appears to accumulate in the endoplasmic reticulum, thereby affecting its rate of secretion into the extracellular milieu. This would explain the low amount of TAA90K^m detected in the conditioned of transfected COS-1 cells using MAbs 1G3, 5B11, and 5H4 (Fig. 11A and Table II). However, this is not the case for MAbs 9D11 and 10G12, as these antibodies were unable to immunoprecipitate the mutant 74- or 72-kDa species from transfected COS-1 cells (Table II). The lack of reactivity of MAbs 9D11 and 10G12 for TAA90K^m provided evidence that the triple mutant has an altered conformation that could account for its accumulation in the endoplasmic reticulum.

In order to determine which of the three mutations accounts for poor secretion of mutant TAA90K, we examined the ability of each of the five human-specific monoclonal antibodies to immunoprecipitate mutant TAA90K from COS-1 cells transfected with the plasmid encoding the single mutant (pCD-TAA90K-P189L) or the double mutant (pCD-TAA90K-S223P/M259V). We also included MAb 12D4 in this study as a control for the total amount of TAA90K present in cell extracts and conditioned medium. As shown in Figure 13 for

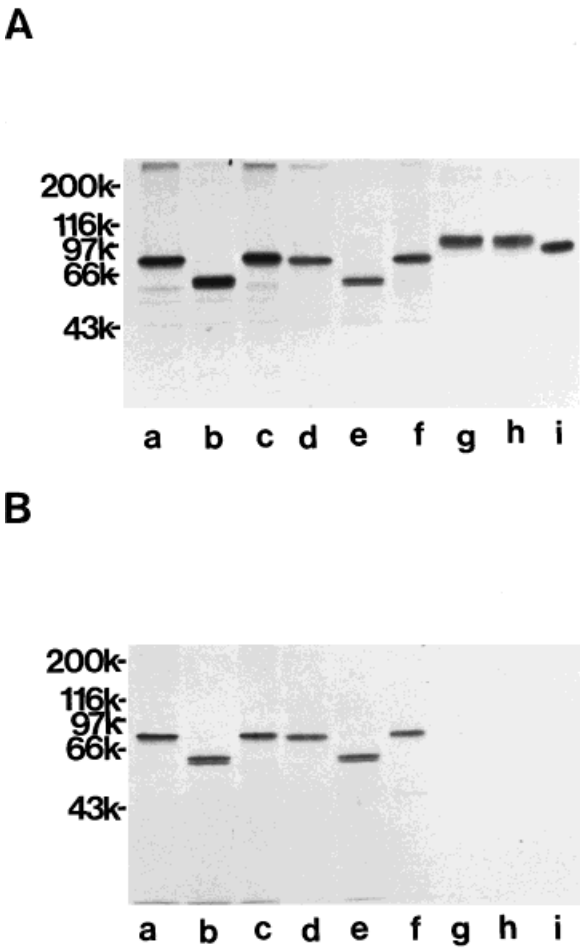


Fig. 12. Susceptibility of pulse-chase labeled recombinant wild-type and mutant TAA90K to endoglycosidase H and neuraminidase. COS-1 cells transfected with plasmid DNA encoding wild-type TAA90K (pCD-TAA90K^{wt}) (**A**) or the triple mutant form of TAA90K (pCD-TAA90K^{m}) (**B**) were pulse-labeled with [^{35}S]-methionine for 30 min, followed by a 5-h chase period. Detergent-solubilized cell lysates from pulse-labeled samples (**lanes a–c**) and 5-h chase samples (**lanes d–f**), as well as conditioned medium from the 5-h chase samples (**lanes g–i**) were immunoprecipitated with MAb 5B11. Samples immobilized to protein A-Sepharose were incubated for 18 h at 37°C with buffer alone (**lanes a,d,g**) or with buffer containing endoglycosidase H (**lanes b,e,h**) or neuraminidase (**lanes c,f,i**). Samples were analyzed by 7.5% SDS-PAGE and bands visualized after fluorography and autoradiography.

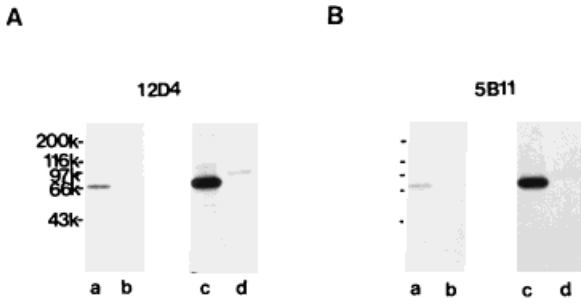


Fig. 13. Biosynthesis and secretion of single and double mutant forms of recombinant TAA90K in COS-1 cells. COS-1 cells transfected with plasmid DNA encoding the single mutant form of TAA90K (pCD-TAA90K-P189L) (**lanes a,b**) or the double mutant form of TAA90K (pCD-TAA90K-S223P/M259 V) (**lanes c,d**) were pulse-labeled with [^{35}S]methionine for 30 min, followed by a 5-h chase period. Detergent-solubilized cell lysates (**lane a**) and conditioned medium (**lane b**) from the 5-h chase period were immunoprecipitated with MAbs 12D4 (**A**) or 5B11 (**B**). Samples were analyzed by 7.5% SDS-PAGE and bands visualized after fluorography and autoradiography.

MAbs 5B11 and 12D4, and summarized in Table II for other monoclonal antibodies, both single (lanes a and b) and double (lanes c and d) mutant forms of TAA90K were defective in secretion, suggesting that amino acid substitutions at proline 189 or serine 223 and methionine 259 alter the conformation of TAA90K in a way which affects its rate of secretion. Interestingly, MAbs 9D11 and 10G12 were now able to immunoprecipitate the intracellular 74/72-kDa species from cells transfected with the single and double mutants (Table II), emphasizing the potential contribution of residues 189, 223, and 259 to the epitope structure detected by these antibodies. Furthermore, these studies highlighted the cumulative effect of mutations on TAA90K conformation.

DISCUSSION

Using a combination of biochemical, molecular, and immunological approaches, we have demonstrated in this report that the 100-kDa glycoprotein expressing β1–6 branched Asn-linked oligosaccharides, previously identified in human colon carcinoma cell lines by MAb 1H9 [Laferté and Loh, 1992], corresponds to TAA90K/Mac-2 BP, a secretory glycoprotein implicated in cancer progression, viral pathogenesis, and immunoregulation [Linsley et al., 1986; Iacobelli et al., 1986, 1995; Natoli et al., 1993; Powell et al., 1995]. Subsequent cloning of the wild-type complete open reading frame into the eukaryotic expression vectors pCDNA3 and pM2 facilitated expression of recombinant TAA90K in COS-1 cells. However, the fact that COS-1 cells express a 90-kDa glycoprotein which is cross-reactive with MAb 1H9, as well as several of the new TAA90K-specific monoclonal antibodies described herein, indicated that the suitability of these cells for transfection studies was dependent on the availability of human-specific mono-

clonal antibodies. Fortunately, five of the new monoclonal antibodies isolated (1G3, 5H4, 5B11, 9D11 and 10G12) were shown to be specific for human TAA90K (Table I).

The immunological cross-reactivity between human TAA90K and the 90-kDa simian glycoprotein suggested that the latter may correspond to a simian homologue of human TAA90K. Although the mature glycoproteins expressed in HT29 and COS-1 cells differed in size, this appeared to be due to glycosylation differences. This is probably attributable to cell-type specific differences in glycosyltransferase levels. In this regard, colon carcinoma cells express highly branched Asn-linked oligosaccharides containing poly-N-acetyllactosamine structures (i.e. repeating Galβ1-4GlcNAcβ-R) [Fernandes et al., 1991; Saitoh et al., 1992], which would be expected to contribute to the larger size of TAA90K expressed in HT29 cells. In fact, we showed previously that TAA90K expressed by colon carcinoma cells expresses β1–6 branched Asn-linked oligosaccharides [Laferté and Loh, 1992]. Differential expression of glycosyltransferases involved in the biosynthesis of O-linked oligosaccharides, characteristic of mucin glycoproteins secreted by colon cells [Strous and Dekker, 1992], may also contribute to the apparent glycosylation differences between human TAA90K and the simian 90-kDa glycoprotein. The fact that recombinant human TAA90K expressed in COS-1 cells also migrated as a smaller species of 90 kDa, which appeared to have fewer N-linked chains, provided evidence for cell-type specific glycosylation differences. Thus, it should be possible to generate different glycoforms of recombinant human TAA90K glycoproteins by selecting an appropriate host cell for transfection studies.

The availability of a large panel of monoclonal antibodies specific for human TAA90K, including 11 antibodies which detect conformational epitopes and 1 antibody that detects a linear epitope (Table I), provided an excellent opportunity to examine and compare the conformation of TAA90K synthesized in vivo and in vitro, as well as determine the effect of specific mutations on its conformation, biosynthesis and secretion. In our initial experiments, we were able to show for the first time that TAA90K can be synthesized in vitro using the T7-coupled transcription/translation system. Although we demonstrated using limited proteolytic peptide mapping that the 60-kDa species synthesized in vitro is identical to the 60-kDa species obtained from TAA90K immunoprecipitated from HT29 cells after N-glycanase digestion, we observed that this species of TAA90K is recognized only by a subset of conformation-dependent monoclonal antibodies. This finding suggested that only certain regions within the unprocessed and unglycosylated protein assume the native conformation. Although the reason for this is unclear, proper folding of TAA90K and its assembly into oligomers may necessitate the involvement of accessory proteins which facilitate glycoprotein folding, such as cyclophilin C [Friedman, 1993; Kay, 1996], calnexin [Bergeron, 1994] and calreticulin [Trombetta and Helenius, 1998], which would likely be absent from the in vitro transcription/translation reticulocyte lysate system. In addition, proper disulfide bond formation may not occur during in vitro synthesis in the absence of peptide disulfide isomerase, a resident protein of the endoplasmic reticulum [Ferrari and Söling, 1999]. This may be an important factor since we demonstrated previously that the 100- and 70-kDa glycoproteins detected by MAb 1H9 migrate faster in SDS-PAGE carried out under nonreducing conditions [Laferté and Loh, 1992], consistent with the presence of disulfide bonds. This has been confirmed recently by Hohenester et al. [1999], who located three disulfide bonds between cysteine residues 31 and 95, 44 and 105, as well as 75 and 85 within the crystal structure the SRCR domain of human Mac-2 BP.

The second approach used to probe the conformation of TAA90K consisted of testing the ability of TAA90K-specific monoclonal antibodies to detect a trunctated from of TAA90K consisting of the first 383 residues (referred to as TAA90K-dC383). This 50 kDa form of TAA90K retains the SRCR domain within the first 125 amino acids of the mature protein, followed by the first 158 amino acids of the C-terminal mucin-like domain (Fig. 1). Seven of 12 monoclonal antibodies (1H9, 2A11, 2F9, 4D1, 9D11, 10A10, 10E10) reacted strongly with the truncated TAA90K protein, suggesting that the native conformation of the epitopes detected by these antibodies is preserved and localized within the N-terminal two-thirds of the protein. By contrast, MAb 12D4 failed to react with the trunctated protein, indicating that the structural epitope detected this antibody was destroyed by removal of the C-terminal portion of TAA90K. This result suggested that the epitope detected by MAb 12D4 is either located

within the deleted C-terminal portion of the protein or that this deletion has a detrimental effect on 12D4 antibody reactivity. Interestingly, four of the five human-specific monoclonal antibodies (1G3, 5B11, 5H4, 10G12) reacted weakly with the truncated TAA90K protein, suggesting that the epitopes detected by these antibodies are modified in the absence of the C-terminal portion of the TAA90K protein, possibly as a result of aberrant folding. Poor reactivity of these four antibodies also provided evidence that the epitope(s) detected by these antibodies are distinct from that detected by MAb 9D11, the other human-specific monoclonal antibody. Previous studies have shown that misfolded proteins destined for secretion or the plasma membrane are retained within the endoplasmic reticulum and subsequently degraded via retrograde transport to proteosomes located within the cytosol [Plemper and Wolf, 1999]. The fact that the 50-kDa species is not secreted into the conditioned medium of HT29 cells (data not shown) may provide some evidence that the truncated form of TAA90K is misfolded.

The third approach used to probe TAA90K conformation relied on the availability of mutant forms of TAA90K carrying point mutations within its coding sequence. The most dramatic effect on protein conformation was observed with TAA90K-P189L, with a leucine at residue 189 instead of proline. This single amino acid substitution had an effect on reactivity of most human-specific monoclonal antibodies (Table II) and resulted in a block in secretion of TAA90K into the conditioned medium. Whether these effects are mediated directly by the change of proline 189 is not known. On the basis of predicted structural motifs within TAA90K [Chou and Fasman, 1978], proline 189 is part of a β turn structure. It is possible that mutation of proline 189 to a hydrophobic residue such as leucine not only alters the loop structure in this region of TAA90K but also impacts on other secondary structure motifs within the protein. Additional mutations at residues 223 and 259 were shown to cause further conformational changes, as evidenced by lack of reactivity of MAbs 9D11 and 10G12 with the triple mutant TAA90K^{m}. The block in secretion observed with the single and triple mutant forms of TAA90K, and to a lesser extent with the double mutant, is consistent with the production of partially misfolded TAA90K. As a result, mutant TAA90K glycoproteins may associate transiently with calnexin/calreticulin, a lectin-like transmembrane protein of the endoplasmic reticulum involved in glycoprotein folding [Bergeron et al., 1994; Trombetta and Helenius, 1998], thereby retarding its rate of secretion.

In addition to demonstrating the potential usefulness of our panel of monoclonal antibodies for structure/function studies of TAA90K, we have also attempted to map some of the epitopes detected by these antibodies. On the basis of reactivity of these monoclonal antibodies with wild-type and trunctated TAA90K, in vitro-synthesized TAA90K or the 90-kDa simian glycoprotein, it is apparent that there are at least six different epitopes on human TAA90K. As shown in Table I, the human-specific monoclonal antibodies fall within three groups. MAbs 1G3, 5B11, and 5H4 are noted for their ability to detect in vitro-synthesized TAA90K. The other two antibodies, 9D11 and 10G12, can be distinguished on the basis of their reactivity with the trunctated form of TAA90K.

Among the group of monoclonal antibodies that cross-react with the 90 kDa glycoprotein from COS-1 cells, there appear to be at least three different specificities (Table I). MAb 12D4 detects a linear epitope possibly within the C-terminal portion of TAA90K, which is retained in the in vitro-synthesized protein. On the other hand, MAb 10E10 detects a conformational epitope within the first 383 amino acids of TAA90K, which is also retained in the in vitro-synthesized TAA90K. The third group of cross-reactive antibodies consisting of MAbs 1H9, 2A11, 2F9, 4D1, and 10A10 detect an epitope within the first 383 amino acids of TAA90K which is poorly preserved in the in vitro-synthesized TAA90K.

In conclusion, the studies described herein highlight the potential usefulness of a new panel of TAA90K-specific monoclonal antibodies as tools for examining the structure and function of TAA90K. In addition, characterization of mutant forms of TAA90K has identified for the first time specific amino acid residues which may contribute to its structure and function. Although little is known about the prevalence of natural mutations in TAA90K expressed by different individuals or within cancerous tissues and cells, their occurrence could have detrimental effects on secretion of TAA90K into the extracellular milieu. Since TAA90K appears to function extracellularly

[Ullrich et al., 1994; Powell et al., 1995; Sasaki et al., 1998], mutations affecting the rate of secretion of TAA90K would effectively block its function. Conversely, enhancing the rate of secretion of TAA90K may constitute effective therapy for the treatment of viral, bacterial, and neoplastic diseases.

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