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CEACAM1 as a tumor cell inhibitor

by

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A thesis submitted to the Falculty of Graduates Studies and Research of McGill University in partial fulfilment of the requirements for the degree of Master of Science.

Abstract

CEA-related cell adhesion molecule 1 (CEACAM1), a member of the carcinoembryonic antigen (CEA) family, is downregulated in a variety of malignancies including colon, prostate, liver, breast and endometrial cancers. Previous studies have shown that expression of the CEACAM1 isoform containing the long cytoplasmic domain (aa 448-521 in mouse; CEACAM1-L) inhibited tumor growth *in vivo*, while the expression of the CEACAM1 isoform bearing the short cytoplasmic tail (aa 448-458 in mouse; CEACAM1-S) had no effect. Therefore, the long cytoplasmic domain contains determinants which are crucial for the growth inhibitory phenotype. We have demonstrated that sequence elements within the C-terminal region of CEACAM1-L are responsible for the inhibition of colonic tumor cell growth. Mutation of Tyr488, Val518 and Lys519-521 averted CECAM1-L's tumor suppressive activity. Furthermore, the role of CEACAM1's adhesive properties in the tumor inhibition phenotype was also investigated. Truncation of the N-terminal domain of CEACAM1 abrogated CEACAM1's ability to sustain intracellular adhesion but had no effect on the tumor inhibitory function. Hence, while the adhesion domain is not implicated in CEACAM1's tumor inhibitory activity, the C-terminal region of the long cytoplasmic domain plays an important role.

Résumé

CEACAM1 (CEA-related cell adhesion molecule 1) est un membre de la famille des antigènes carcinoembryonnaires (carcinoembryonic antigen; CEA). L'expression de CEACAM1 est diminuée dans plusieurs cancers dont les carcinomes de côlon, prostate, foie, sein et endomètre. Des études ont demontré que l'expression de l'isoforme de CEACAM1 possédant un long domaine cytoplasmique (aa 448-521 chez la souris; CEACAM1-L) suprimait la croissance des tumeurs *in vivo,* tandis que l'expression de l'isoforme de CEACAM1 contenant un court domaine cytoplasmique (aa 448-458 chez la souris; CEACAM1-S) n'avait aucun effet. Il semble donc que le long domaine cytoplasmique contienne des motifs qui soient essentiels à la fonction d'inhibiteur de tumeur de CEACAM1. Nous avons démontré que l'inhibition de tumeur par CEACAM1 dépendait d'éléments présents dans la région C-terminale du long domaine cytoplasmique. La mutation de Tyr488, Val518 et des Lys519-521 révertait l'activité anti-tumorale de CEACAM1-L. De plus, le rôle des propriétés adhésives de CEACAM1 dans l'inhibition de tumeur a aussi été examiné. La délétion du domaine N-terminal de CEACAM1 abolissait son activité adhésive, mais n'altérait pas son activité anti-tumorigénique. Ainsi, alors que le domaine d'adhésion de CEACAM1 ne participe pas à la fonction d'inhibiteur de tumeur, la portion C-terminale du long domaine cytoplasmique y joue un rôle important.

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List of Abbreviations

aa	amino acids
Ab	antibody
Ala	alanine
APC	adenomatours polyposis coli
Asn	asparagine
Asp	aspartic acid
BCR	B-cell receptor
BGP	human biliary glycoprotein
Bgp	mouse biliary glycoprotein
C-CAM	cell-cell adhesion molecule
CAM	cell adhesion molecule
CaM	calmodulin
CD22	cluster of differentiation 22
CD66a	cluster of differentiation 66a
cDNA	complimentary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CEACAM1	CEA-related cell adhesion molecule 1
dpc	days post coitus
Dpc4	Deleted in pancreatic cancer 4
ECM	extracellular matrix
EGFr	Epidermal Growth Factor receptor
ER	endoplasmic reticulum
FcγRIIB	Fc gamma receptor II B isoform
GTP	guanosine triphosphate
Ig	immunoglobulin

Ig C2-set	immunoglubulin constant C2-set domain
IgCAM	immunoglobulin-like cell adhesion molecule
IgSF	immunoglobulin supergene family
Ig V	immunoglobulin variable domain
IR	insulin receptor
IRS-1	insulin receptor substrate 1
ITIM	immunoreceptor tyrosine-based inhibition motif
JNK	Jun kinase
kb	kilobase
kDa	kiloDalton
KIR	killer cell inhibitory receptor
LIR	leucocyte immunoglobulin-like receptor
Lys	lysine
M.W.	molecular weight
Mb	megabase
MHV	mouse hepatitis virus
*mm*CGM1	*Mus musculus CEA* gene family member 1
*mm*CGM2	*Mus musculus CEA* gene family member 2
mRNA	messenger ribonucleic acid
NK	natural killer
Opa	opacity
PAK	p21-activated protein kinase
PDGFR	Platelet-Derived Growth Factor Receptor
Phe	phenylalanine
PIR-B	paired immunoglobulin-like receptor B
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate

Ser	serine
SH2	Src homology 2
SHP-1	SH2-containing protein tyrosine phosphatase 1
SHP-2	SH2-containing protein tyrosine phosphatase 2
SIRP	signal regulatory proteins
Smad	human homolog of drosophila MAD (mothers against decapentaplegic) protein
TCR	T-cell receptor
Thr	threonine
TGF-β	Transforming Growth Factor beta
Tyr	tyrosine
UTR	untranslated region
Val	valine

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Preface

In accordance with Thesis Specification #1, section C, of the "Guidelines for Thesis Preparation" for the faculty of Graduate Studies and Research, McGill University, the following has been reproduced in full for the benefit of the external examiner (the reference to a doctoral oral defence is not applicable, since this thesis is submitted for a Master of Science degree):

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> As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers that have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:
>
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>
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>
> 4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in

sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis.** The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

8.. **In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis.**

As permitted by Thesis Specification #1 of the "Guidelines for the Thesis Preparation" for the Faculty of Graduate Studies and Research, McGill University, Chapter 2 of this thesis is presented in the form of a manuscript. **This manuscript has recently been accepted for publication in *Oncogene*.**

Contributions of Authors

The following describes the contribution of each co-author for the manuscript: Izzi, L., Turbide, C., Houde, C., Kunath, T., and Beauchemin, N. (1999) *cis*-Determinants in the Cytoplasmic Domain of CEACAM1 Responsible for its Tumor Inhibitory Function. Accepted for publication in *Oncogene* (Chapter 2).

Luisa Izzi derived CT51 populations expressing CEACAM1/Y488F, CEACAM1/Y515F, CEACAM1/Y488,515F, CEACAM1/V518A, CEACAM1/3K→3R, CEACAM1/Δ4-122/L, CEACAM1/Δ4-122/S. This was accomplished by retroviral-mediated infection and immunoselection with polyclonal anti-CEACAM1 antibodies (231 and 655). Expression of CEACAM1 in the derived populations was assessed by Western blotting (Figure 2-2A) and by FACS analysis (Figure 2-2B).

Luisa Izzi and Caroline Houde contributed equally to the generation of the following mutants: CEACAM1/V518A and CEACAM1/3K→3R. These mutants were generated by PCR site-directed mutagenesis.

Tilo Kunath derived CT51 populations expressing CEACAM1/Δ518, CEACAM1/Δ483, CEACAM1-4S and CEACAM1-4L. This was achieved by retroviral-mediated infection and immunoselection using polyclonal anti-CEACAM1 antibody (231). He also derived the control CT51-neo population. He originally evaluated the expression of CEACAM1 in all these derived populations. This was done by FACS analysis and immunoblotting. Luisa Izzi re-evaluated the expression of CEACAM1 in

the derived CT51 populations prior to injection in BALB/c mice. Again, this was achieved by cytofluorometric and immunoblotting analyses (Figure 2-2).

Luisa Izzi amplified the CT51 populations expressing the CEACAM1 proteins and harvested the populations for injection in BALB/c mice. Claire Turbide injected the derived CT51 populations sub-cutaneously in syngeneic BALB/c mice. She also sacrificed the mice and resected the tumors induced by the derived CT51 populations. Furthermore, she weighed and measured each tumor. Measurements were done with calipers.

Ms Izzi observed that CT51 populations expressing CEACAM1/Y488F, CEACAM1/V518A, CEACAM1/Δ518 and CEACAM1/3R→3R formed tumors as readily as CT51 neo control cells (Table 2-1). Furthermore, she observed that CEACAM1-L was able to inhibit tumor growth *in vivo,* while no inhibition was noted for parental CT51 cells and CEACAM1-S expressing cells (Table 2-1). These observations were in accordance with previously published data by Kunath *et al,* 1995 (1). Ms Izzi also noted that CEACAM1/Y515F inhibited tumor growth at the same extent as wild-type CEACAM1-L; whereas CEACAM1/Y488,515F led to a partial tumor inhibition (Table 2-1).

Luisa Izzi derived NIH 3T3 populations expressing either CEACAM1-L, CEACAM1-S, CEACAM1/Δ4-122L, or CEACAM1/Δ4-122S. This was done by retroviral-mediated infections and immunoselection using polyclonal anti-CEACAM1 antibodies (231 or 655). Expression of CEACAM1 proteins was evaluated by FACS

analysis and immunoblotting (data not shown). Ms Izzi performed aggregation assays with the derived NIH 3T3 populations. She observed that NIH 3T3 cells expressing CEACAM1/Δ4-122L, or CEACAM1/Δ4-122S were unable to aggregate, while NIH 3T3 populations expressing either CEACAM1-S or CEACAM1-L were able to form aggregates (Figure 2-3). Ms Izzi observed no cell aggregation with parental NIH 3T3 cells.

Ms Turbide performed all the statistical analysis of data collected from the tumorigenicity assays (Table 2-1). Ms Izzi performed the statistical analysis of data obtained from the aggregation assays (Figure 2-3).

CHAPTER 1

GENERAL INTRODUCTION

Discovery and Nomenclature of CEACAM1

Svenberg first described biliary glycoprotein (BGP) from human bile as a carcinoembryonic antigen (CEA) cross-reacting antigen (2). Since the discovery of human BGP, a bewildering number of BGP splice isoforms and allelic variants have been characterized in human (3, 4), rat (5) and mouse (6, 7). The genes and their products have been assigned various names by different research groups, leading to a complex and confusing nomenclature. Recently, a new nomenclature has been redefined and adopted by a majority of researchers in the field (8). Appellations for biliary glycoprotein in human, mouse and rat include BGP, CD66a, Bgp, *mm*CGM1, *mm*CGM2, cell-CAM105, C-CAM, rat ecto-ATPase, pp120, HA4, and gp 110. According to the new nomenclature, biliary glycoprotein will now be referred to as CEACAM1 (CEA-related Cell Adhesion Molecule 1) in all species. Table 1.1 summarizes the nomenclature of CEACAM1 allelic and splice variants.

Structure of CEACAM1 proteins

CEACAM1 proteins are type I transmembrane proteins which are highly conserved between human, rat and murine species (Figure 1-1) (4, 5, 7). The molecular weights (M.W.) of the various proteins range from 83 to 160 kDa (4, 5, 7, 9). All precursor CEACAM1 proteins bear a 34 amino acid (aa) leader peptide required for the protein's transport in the endoplasmic reticulum (ER) and its expression at the cell surface. CEACAM1 proteins comprise 1 to 4 immunoglobulin (Ig) domains which include a N-terminal Ig variable (V) fold, and up to 3 Ig C2-set domains.

TABLE 1-1: New Nomenclature for CEACAM splice variants in different species

Species	Gene	New Gene Name	Old Splice Variant Protein Name	New Splice Variant Protein Name	Structure	Other Names
Human	*BGP*	*CEACAM1*	BGPa	CEACAM1-4L	N, A1, B, A2, TM, L	BGP1, CD66a, TM-CEA
			BGPb	CEACAM1-3L	N, A1, B, TM, L	
			BGPc	CEACAM1-4S	N, A1, B, A2, TM, S	
			BGPd	CEACAM1-3S	N, A1, B, TM, S	
			BGPh	CEACAM1-3	N, A1, B	W233
			BGPx	CEACAM1-1L	N, TM, L	
			BGPx'	CEACAM1-1S	N, TM, S	
Mouse	*Bgp1*^{a}	*Ceacam1*^{a}	BgpA	CEACAM1^a-4S	N^a, A1^a, B^a, A2^a, TM, S	mmCGM1, mmCGM1a, MHVR1,
			BgpC	CEACAM1^a-2S	N^a, A2^a, TM, S	mCEA1, mmCGM2 B6
			BgpD	CEACAM1^a-4L	N^a, A1^a, B^a, A2^a, TM, L	MHVR1(2d)$_s$, MHVR1(4d)L
			BgpG	CEACAM1^a-2L	N^a, A2^a ,TM, L	MHVR1(2d)L
	Bgp1^b	*Ceacam1*^{b}	BgpB	CEACAM1^b-2S	N^b, A2^b, TM, S	mmCGM2, MHVR2, mmCGM2(2d)
			BgpE	CEACAM1^b-4S	N^b, A1^b, B^b, A2^b, TM, S	mmCGM1 SJL, mmCGM2(4d)
			BgpF	CEACAM1^b-4L	N^b, A1^b, B^b, A2^b, TM, L	mmCGM2(4d)L, bb-1
			BgpH	CEACAM1^b-2L	N^b, A2^b, TM, L	mmCGM2(2d)L
	Bgp2	*Ceacam2*	Bgp2C	CEACAM2-2S	N, A2, TM, S	
Rat	*C-CAM1*^{a}	*Ceacam1*^{a}	C-CAM1^a	CEACAM1^a-4L	N^a, A1^a, B^a, A2^a, TM, L	Cell-CAM105, pp120, HA4, gp110, ecto-ATPase, CBATP
			C-CAM2^a	CEACAM1^a-4S	N^a, A1^a, B^a, A2^a, TM, S	C-CAM2a
			C-CAM3	CEACAM1^a-4C3	N^a, A1^a, B^a, A2^a, TM, C3	
	C-CAM1^b	*Ceacam1*^{b}	C-CAM1^b	CEACAM1^b-4S	N^b, A1^b, B^b, A2^b, TM, S	C-CAM/n, cell-CAM105

a,b; a,b	Allelic variants	Cn	Different termini generated by splicing
N	V-like Ig domain	L	Longer cytoplasmic domain (71-73 aa)
A1, B, A2	C2-like Ig domains	S	Shorter cytoplasmic domain (10-11 aa)
TM	Transmembrane		

Figure 1-1 Structure of CEACAM1 isoforms

The structure of CEACAM1 variants in human, mouse and rat are illustrated. The splice variants are grouped by species. Each splice variant is identified by an extension (e.g. 4L) which represents the number of Ig domains (1-4) and the length of the cytoplasmic domain (S, short; L, long; C3, C-termini 3). The Ig domains are illustrated as ovals. The type of domain (N, A, B) is indicated inside each oval. The transmembrane and cytoplasmic domains are drawn as solid lines.

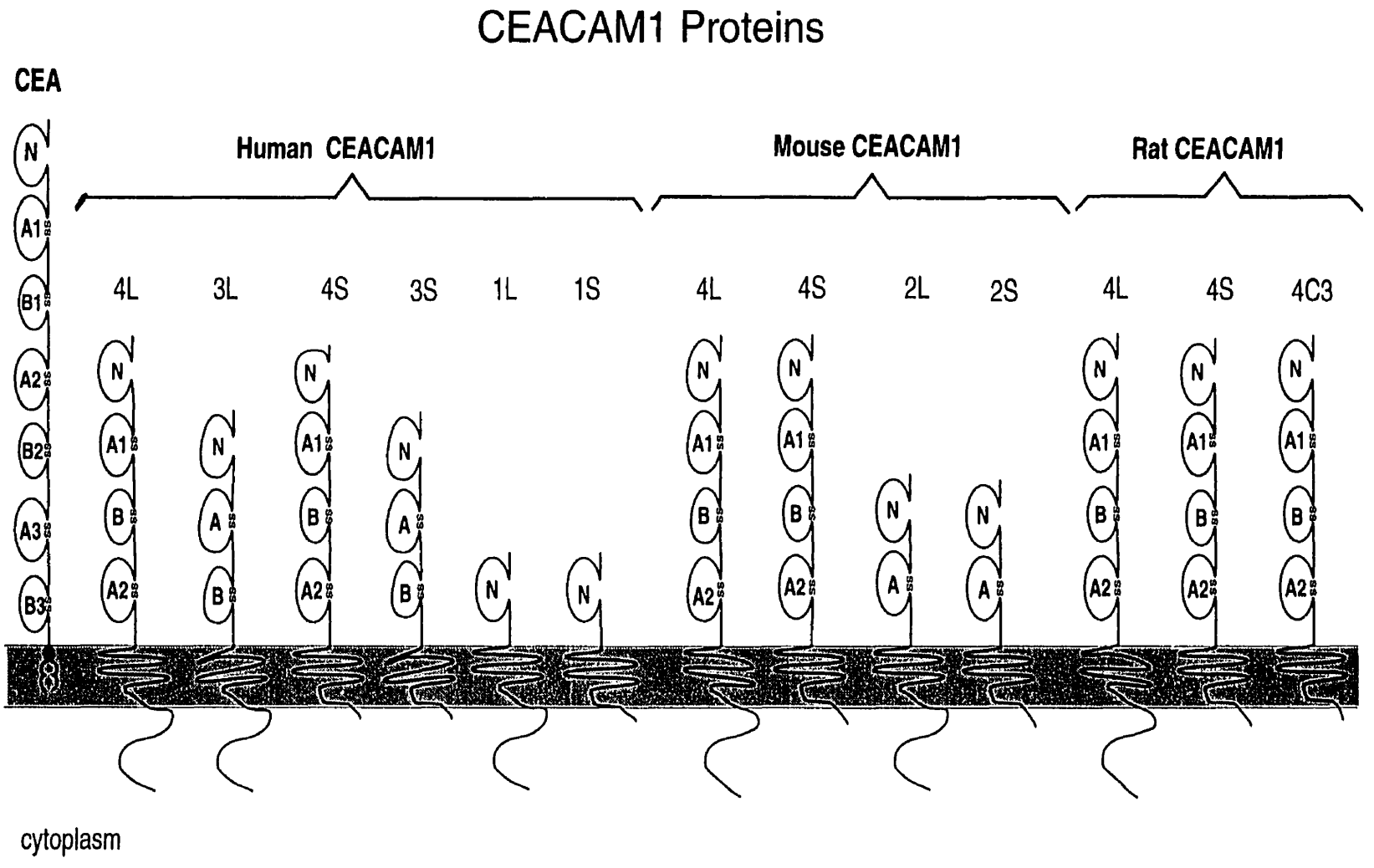

CEACAM1 Proteins
CEA
N
A1
B1
A2
B2
A3
B3
Human CEACAM1
Mouse CEACAM1
Rat CEACAM1
4L
3L
4S
3S
1L
1S
4L
4S
2L
2S
4L
4S
4C3
cytoplasm

Figure 1-1

The N-terminal domain consists of 108 aa. A salt bridge between highly conserved Asn and Asp residues maintains the characteristic Ig structure of the N-terminal domain. The C2-like Ig domains are of 2 types: **A** and **B**. **A** domains contain 92-93 aa, whereas **B** domains comprise 85-86 aa. As most Ig folds, the Ig C2-set domains are stabilized by a disulfide bridge formed between paired cysteine residues (4, 5, 7). Human and mouse CEACAM1 isoforms demonstrate variations in their extracytoplasmic regions. Such variations arise from the alternative splicing of the Ig C2-set domains (4, 5, 7). The primary amino acid sequence of CEACAM1 proteins predicts 16 potential Asn-linked glycosylation sites in rodents and 20 putative Asn-linked glycosylation sites in human (4, 5, 7). The alternative splicing of a 53 bp exon results in the expression of two CEACAM1 cytoplasmic isoforms which contain either a short or long cytoplasmic domain (4, 5, 7). The short tail is 10 aa-long in man and mouse and 11 aa-long in rat. Human and rat long cytoplasmic domains comprise 71 aa, while the murine long tail consists of 73 aa. CEACAM1-S and CEACAM1-L refer respectively to the short and long cytoplasmic variants (4, 5, 7). The implication of the splice variants in CEACAM1's biological functions is highlighted by the conservation of this splicing event from rodents to humans.

Chromosomal Localization, Allelic Variants and Genomic Organization

CEACAM1 is a member of the *CEA* gene family which is itself a subgroup of the immunoglobulin gene superfamily (IgSF) (10). To date, 29 human, 14 mouse and 7 rat *CEA*-related genes have been characterized. The human *CEA* gene family is located on

chromosome 19q13.2 (11) and occupies approximately 1.8 Mb. The mouse *CEA* gene cluster maps to chromosome 7 A1-A2 (12), which is syntenic to human 19q13 (13). The human *CEA* gene family is organized in two clusters which are 250 and 850 kilobase-long (Figure 2-1). A 700 kilobase region, containing genes unrelated to *CEA*-like genes, separates the two gene clusters. A similar organization is also observed in rodents (14).

Two allelic variants of the *Ceacam1* gene, denoted $Ceacam1^a$ and $Ceacam1^b$, have been characterized in mouse (15) and rat (16). The allelic divergence results in 16 aa substitutions between rat *Ceacam1* gene products (16) and 29 aa substitutions between mouse *Ceacam1* gene allelic variants (15). Only one *Ceacam1* gene has been characterized in human and rat; while two *Ceacam1* genes have been described in mouse (15). The second gene, referred to as *Ceacam2,* is highly homologous to the *Ceacam1* gene. The *Ceacam2* gene product is also a transmembrane protein which exhibits two Ig folds (1V- and 1 C2-set domain) and a short or long cytoplasmic region (17).

The exon-intron organization of the *CEACAM1* gene is well conserved across the species. *CEACAM1* genes consist of nine exons and 8 introns (Figure 1-3). The first exon encodes the 5' untranslated region (UTR) and 2/3 of the leader signal, while the second exon contains the last third of the leader sequence and the Ig V domain. Exon 3, 4 and 5 each encode one Ig C2-set domain. They can be alternatively spliced to produce isoforms with different extracytoplasmic regions. The transmembrane region is encoded by the sixth exon which also includes a portion of the cytolasmic domain. Exons 7, 8 and 9 encompass the intracytoplasmic domain and the 3' UTR. The alternative splicing of exon 7 determines the expression of either CEACAM1-S or -L (3-5, 15, 18).

Figure 1-2 Chromosomal arrangement of the human *CEA* gene family

The organization of the human *CEA* gene family is depicted. *CEA* genes are illustrated as gray boxes. The CEA gene family is subdivided in two clusters: *CEACAM* genes and *PSG* genes. A number identifies each gene (*ps* 1 and *ps* 2 are CEACAM pseudogenes). Black arrows show the direction of transcription. The centromere is represented as a black circle. The size of the gene family and its sub-clusters are also indicated.

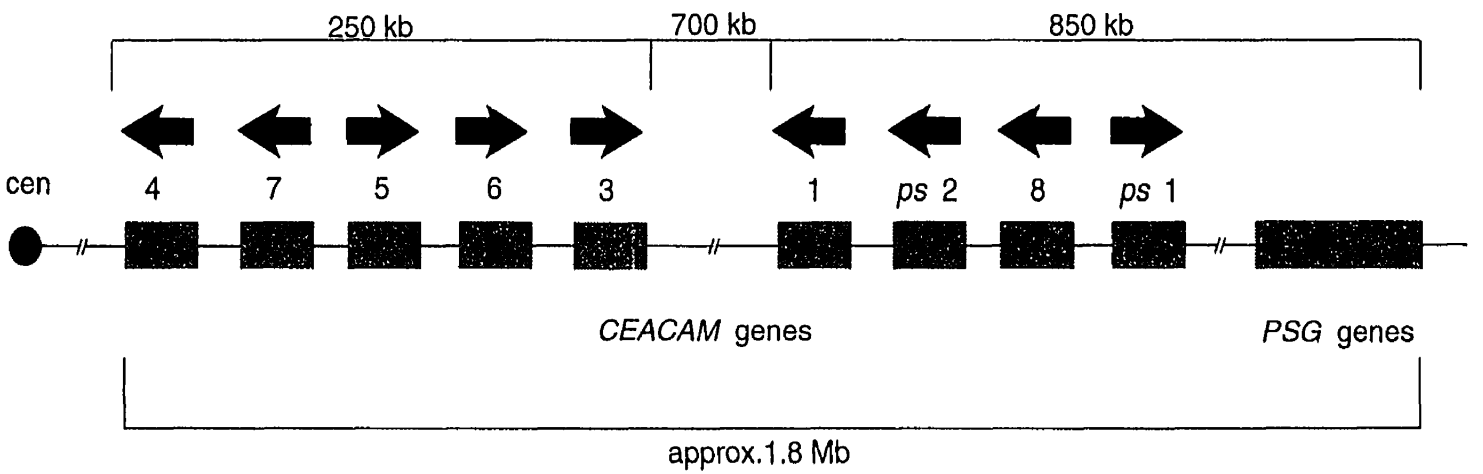
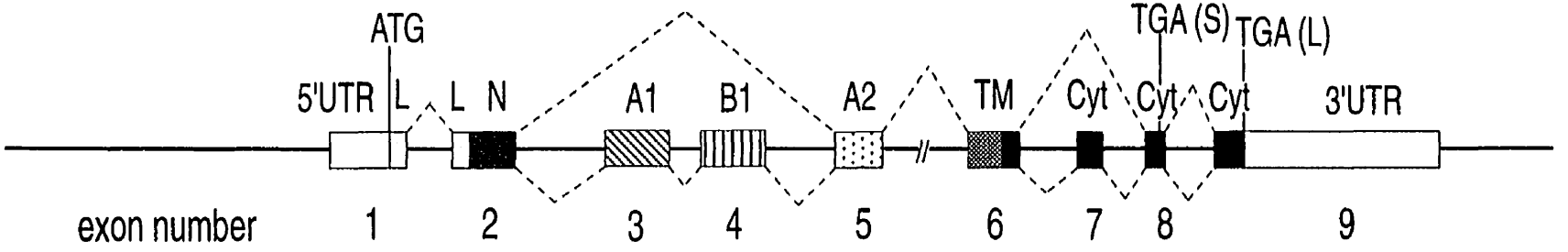

250 kb
700 kb
850 kb
cen
4
7
5
6
3
1
ps 2
8
ps 1
CEACAM genes
PSG genes
approx.1.8 Mb

Figure 1-2

Figure 1-3 Genomic exon-intron structure of mouse *Ceacam1* gene (A) *Ceacam1* gene transcripts (B).

(A) Exons are indicated by boxes. The exon number is also shown. Splice events are denoted by dashed lines. Exon 3 and 4 can be alternatively spliced as a single event to give rise to two different extracytoplasmic regions. Exon 7 can also be alternatively spliced to give rise to a short or long cytoplasmic domain. L, leader; N, N-terminal Ig V-like domain; A1, B1, A2, Ig C2-set domains; TM, transmembrane domain; Cyt, cytoplasmic domain; 5'UTR, 3'UTR, 5' and 3'untranslated regions; ATG, start codon; TGA(S), stop codon used to produce the short cytoplasmic domain; TGA(L), stop codon used to generate to long cytoplasmic domain. **(B)** CEACAM1-4L, CEACAM1-4S, CEACAM1-2L and CEACAM1-2S represent the transcripts generated by the alternative splicing of exons 3, 4 and 7.

A. *Ceacam1* gene



B. *Ceacam1* transcripts

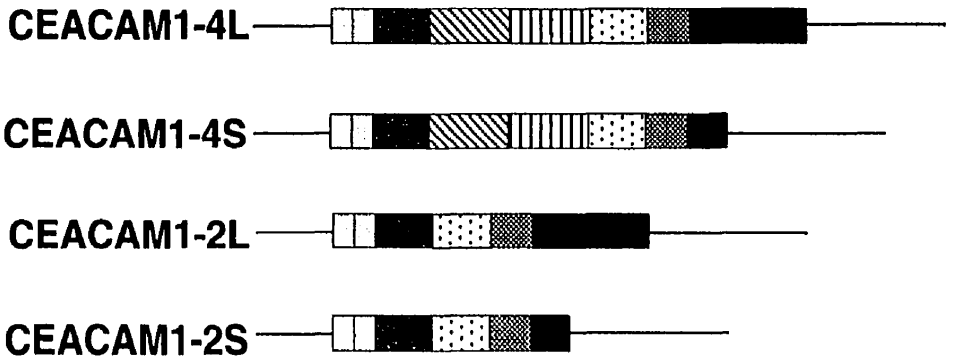


Figure 1-3

Normal Tissue Expression

Tissue expression of CEACAM1 has been studied at the mRNA and protein levels. Extensive expression studies in normal human, rat and mouse tissues demonstrated the presence of CEACAM1 in the epithelia of various tissues and organs and in the endothelial layer of several small vessels. Furthermore, CEACAM1 expression is also apparent in hematopoietic cells of myeloid origin (19-21).

CEACAM1 is abundant in human, rat and mouse liver. Intense apical staining is observed at the lateral membranes and bile canaliculi of hepatocytes (19-21). In human colon, CEACAM1 expression has been found at the apical surface of columnar absorptive cells in the upper third and luminal portion of the crypt. Ultrastructurally, the protein has been localized at the microvillar surface of the colonocytes lining the mouth of the crypt.(22) CEACAM1 has also been detected in BALB/c and CD-1 mice normal colonic tissue (20, 23, 24). Mouse CEACAM1^{a}-4L was detected by an antibody specific to the long tail in the proliferative region of the crypt (24) Mouse CEACAM1^{a}-4S is uniformly expressed along the crypt (24).

CEACAM1 is also apically expressed in epithelia of various organs of the digestive, urinary and reproductive system. CEACAM1 expression is detected in the esophagus, gallbladder, pancreas, renal proximal tubules, and urinary bladder (19-21). CEACAM1 is present in the extralobular and terminal ducts, and myoepithelial layer of the normal mature breast (25). The glandular and luminal epithelia of the endometrium also score positive for CEACAM1 staining (26, 27). In prostate, CEACAM1 was

detected at the apex of basal cells in the human organ and at the luminal surface of the rodent gland (28). This discrepancy is due to different antibody specifities (28).

CEACAM1 is detected in the endothelia of small vessels of all adult organs (19-21). Almost all endothelial cells of the renal glomeruli capillaries, vasa recta, adrenal sinusoids, and placental vessels present intense CEACAM1 staining. Endometrial, prostatic, and pancreatic vessels also exhibit CEACAM1 expression (21). Interestingly, blood vessels of the adult central nervous lack CEACAM1, while the protein is transiently expressed in microvessels of the fetal CNS (29).

The presence of CEACAM1 has been demonstrated at the surface of granulocytes, megakaryocytes, platelets, macrophages and B-lymphocytes (19, 30). Freshly isolated human T-lymphocytes and NK cells are CEACAM1-negative; however, upon IL-2 stimulation, CEACAM1 expression is upregulated in T cells and a subset NK cells ($CD16^-$, $CD56^+$) (31).

The spatiotemporal expression of CEACAM1 has also been well documented during rat and mouse embryogenesis. CEACAM1 is first detected in the trophoectoderm of the blastocyst during the pre-implantation period of the rat embryo (32). Loss of CEACAM1 expression in trophoblasts prior to the implantation of the rat embryo coincides with downregulation of CEACAM1 in the uterine wall (27). In mouse, CEACAM1 is absent from the early embryo; however, extraembryonic tissues were positive all throughout gestation. The earliest expression of CEACAM1 occurs within the embryo 8.5 days post-coitus (dpc) in the developing epidermis. CEACAM1 appears in the developing gut epithelium 10.5 dpc. By 15.5 dpc, CEACAM1 is detected in the

meninges, dermis, salivary glands, lung, pancreas, kidney and at active sites of myogenesis. These results indicate that CEACAM1 may participate in mesenchymal-epithelial interactions during fetal development (33, 34). Expression of CEACAM1 in the liver is a late event during embryogenesis, as hepatocytes become CEACAM1-positive only by 15.5 dpc. CEACAM1 is present in small intestine and kidney tubules at 17.5 dpc (35, 36). While it is not expressed in the adult central nervous system, CEACAM1 is detected in microvessels of the developing rat CNS by day 13.5 dpc. CEACAM1 levels are highest at about 18.5 dpc. Subsequently, expression slowly decreases and disappears by day 14 post partum (29).

Dysregulated expression of CEACAM in various cancers

The widespread CEACAM1 expression pattern prompted the comparison of CEACAM1 protein levels in tumor tissues versus their normal counterparts. Many groups reported a clear underexpression of CEACAM1 in several human cancers; however, there are also examples of upregulation.

CEACAM1 mRNA is decreased in 80% of colorectal cancers (37). More specifically, it is reduced by half in 80-90% of colorectal adenomas (38) and completely lost or severely downregulated in 55% of colorectal carcinomas (39). A marked reduction of *CEACAM1* mRNA is also observed in mouse primary colon tumors (23). An overall decrease of CEACAM1 expression occurs in hyperplastic prostatic epithelium, prostate intraepithelial neoplasia and primary prostate tumors (28). CEACAM1 is absent from hepatic tumors (40) and rat and murine hepatocellular carcinoma cell lines (23, 41).

Various human endometrial tumors ranging from low- to high-grade malignancies have been examined. Like normal endometrial tissues, low-grade carcinomas maintain apical CEACAM1 expression. However, as malignancy increases, CEACAM1 staining decreases in intensity and is often completely lost (26).

There have been many conflicting reports regarding the expression of CEACAM1 in primary breast tumors and mammary carcinoma cell lines (12, 23, 25). Robbins *et al.* showed that *CEACAM1* mRNA appeared to be overexpressed in murine mammary tumors induced by mouse mammary tumor virus or chemical carcinogens (12); however, Rosenberg *et al.* demonstrated that *CEACAM1* mRNA was undetectable in mouse breast tumor cell lines (23). In humans, although apical CEACAM1 staining is retained in benign and hyperplastic mammary lesions, CEACAM1 is underexpressed or lost in carcinoma *in situ* and invasive lesions (25).

Although it is apparent that CEACAM1 is downregulated in several types of epithelial cancers, there are also cases where expression of CEACAM1 is enhanced. CEACAM1 is overexpressed in primary lung tumors such as squamous cell carcinomas, small cell carcinomas and adenocarcinomas, while normal lung tissue is CEACAM1-negative. Interestingly, there is a net reduction in expression of CEACAM1 in resulting metastatic lesions (42). Human gastric adenocarcinomas also present higher levels of CEACAM1 than the normal adjacent tissues (43).

In summary, CEACAM1 expression is dysregulated in many types of epithelial cancers whether it is reduced or augmented. Furthermore, the localization of this protein changed upon transformation. In normal tissues or benign lesions, CEACAM1 was found

on the apical cell surface (21, 25). Neoplastic cells which have reduced expression of show staining all around the cell surface. (26, 28). Dysregulation of CEACAM1 expression is thought to be an early event in the development of certain malignancies as colonic polyps (39), Dukes Stage A adenomas (23), hyperplastic prostate (28) and benign breast lesions (25) show altered expression patterns of CEACAM1.

Functions of CEACAM1

Adhesion Molecule

CEACAM1 was first identified as a molecule involved in the aggregation of rat hepatocytes (44). Subsequent adhesion studies found that human (45), rat (18) and mouse CEACAM1 (6, 46) were all able to mediate homophilic intercellular adhesion. Human CEACAM1-4L, CEACAM1-3L and CEACAM1-4S cause reversible cell aggregation in a temperature-dependent manner (45, 47, 48). Similarly, murine CEACAM1^{a} and CEACAM1^{b} are both involved in homophilic intercellular aggregation (6, 46). Interestingly, CEACAM2 is unable to mediate cell aggregation (49). Rat CEACAM1^{a}-4L and -4S also sustain homotypic, reversible cell aggregation (18, 50, 51). CEACAM1^{a}-4C3 a truncated form of CEACAM1^{a}-4S, however, does not behave as a cell adhesion molecule (CAM). The cytoplasmic domains of CEACAM1^{a}-4C3 and CEACAM1^{a}-4S consist respectively of 6 aa and 10 aa; therefore, the tetrapeptide appears to have an important role in CEACAM1-mediated cell adhesion (18). These four residues may be binding cytoplasmic proteins which are crucial for cell aggregation. They

may also confer a certain structure to the protein which permits adhesion functions. Rat CEACAM1^b, an allelic variant of CEACAM1^a, does not exhibit cell aggregation (52).

The N-terminal Ig V-like fold has been identified as the adhesion domain in human (48), rat (53) and mouse (54) CEACAM1. Interestingly, the inability of murine CEACAM2 and rat CEACAM1^b to support cell adhesion may be due to amino acid differences in their N-terminal domain (49, 51). The Ig V-related folds of murine CEACAM1 and CEACAM2 diverge at 37 positions (49), whereas 16 substitutions have been recognized between the N-terminal domains of rat CEACAM1^a and CEACAM1^b (51).

Adhesion studies have been performed in a variety of cell lines which may explain the incongruities noted with respect to temperature requirements of CEACAM1-mediated adhesion. Amino acid divergence in the adhesion domains may likely be a factor. Regardless, human and murine CEACAM1 molecules may require physiological temperatures to achieve a conformation favorable for adhesion. Rat CEACAM1 may inherently possess this propitious structure. The possible involvement of certain enzymatic reactions such as phosphorylation in the adhesion process argues for the necessity of physiological temperature (55).

The cellular sublocalization of CEACAM1 corroborates its role as a CAM. The presence of CEACAM1 at the lateral cell borders of fetal and mature hepatocytes, enterocytes, basal and suprabasal layers of stratified epithelia, and endothelial cells supports its adhesive function (19, 56). Furthermore, the luminal expression of CEACAM1 on endothelial cells and its upregulation at the surface of platelets and

granulocytes during activation vouches for the protein's involvement in intercellular interactions, as activated platelets and granulocytes can bind to endothelia (19, 57) and possibly participate in rolling adhesion (58).

CEACAM1's adhesive properties may likely be required to maintain the cellular architecture of brush-border cells (59). Öbrink suggested that CEACAM1 proteins located on adjacent microvilli may participate in *trans*-interactions to assure proper brush-border structure (Figure 1-4A) (59). The increased expression of CEACAM1 on mature microvillar structures of colonic absorptive cells (22) concurs with this hypothesis. CEACAM1 is also highly expressed on the luminal aspect of bile canaliculi (19). In this case, CEACAM1 *trans*-interactions may be required for the maintenance of the canalicular opening (Figure 1-4B).

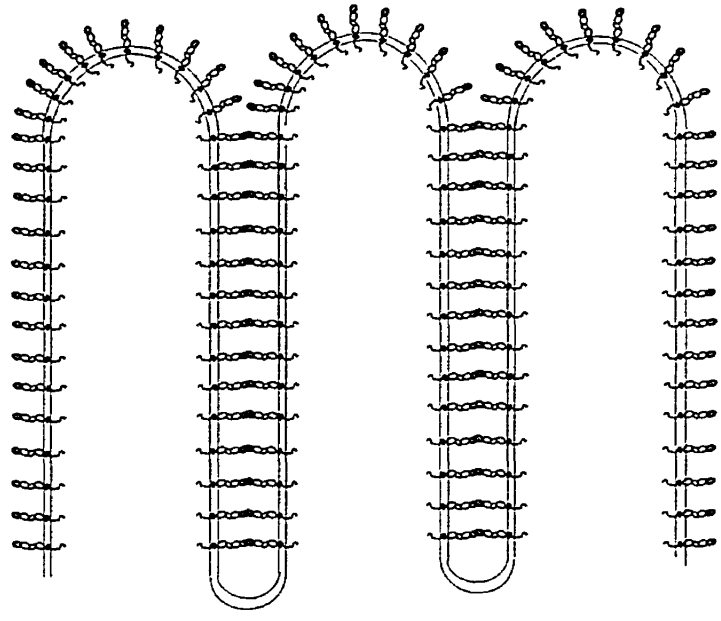
Viral and Bacterial Receptor

Using a variety of technical approaches, CEACAM1^{a} isoforms were all shown to be functional mouse hepatitis virus (MHV) receptors (60, 61). Mouse strains such as BALB/c which express these isoforms are susceptible to MHV-A59 infection, whereas SJL/J mice which produce the CEACAM1^{b} allelic variant are MHV-A59 resistant (62). CEACAM2 can also mediate MHV-A59 virion binding (17); however, it does so with less efficiency than CEACAM1^{a} (63). The soluble extracytoplasmic domain of CEACAM1^{a} has at least 1000-fold more MHV-A59 neutralizing activity than the soluble extracytoplasmic domain of CEACAM2 (63). MHV-binding, similarly to homophilic adhesion, is sustained by the N-terminal Ig V-like domain (64).

Figure 1-4 CEACAM1 binding in the maintenance of microvillar structures (A) and bile canalicular opening (B).

(A) Three microvilli are illustrated. At the surface of each structure, CEACAM1 proteins are depicted as 4 ovals and a solid black line. CEACAM1 *trans*-interactions may allow the proper structure of microvillar processes. **(B)** A bile canaliculus is pictured. CEACAM1 proteins are illustrated at the surface of irregular microvillar structures extending from hepatocytes. In this situation, CEACAM1 *trans*-interactions may be required to maintain the canalicular opening

A. Microvilli



B. Bile canaliculus

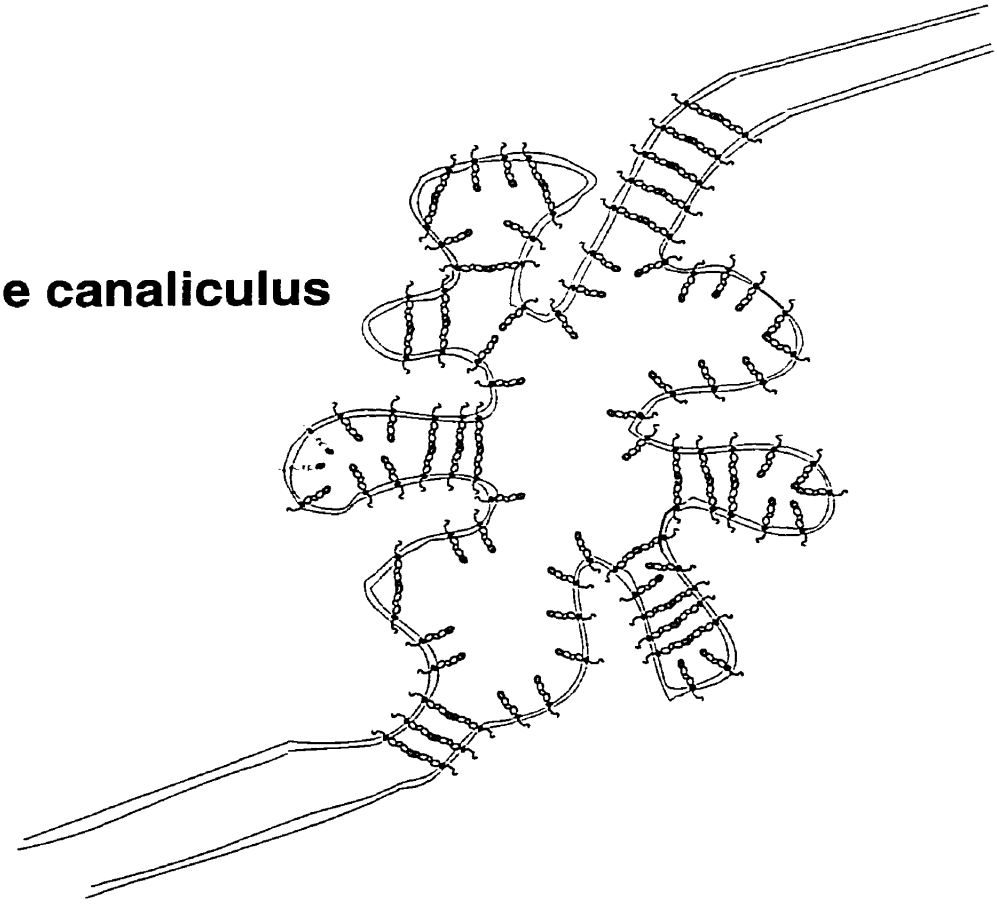


Figure 1-4

Interestingly, the N-terminal divergence between CEACAM1[a], CEACAM1[b], and CEACAM2 is suspected to be responsible for the different binding properties (17, 61, 64). Recently, Wessner *et al.* found that CEACAM1 residues 34 to 52 are critical for virion binding (65). Furthermore, the Ig constant folds enhance virion-receptor interactions mediated by the N-terminal domain (66).

CEA family members also bind to bacteria. *Escherichia coli* strains of human origins, three *Salmonella* species, and *Neisseria gonorrhea* and *meningitidis* (67-69) have all been shown to adhere to CEA-like proteins. *E. coli* and *Salmonella* strains bind CEA family member through high mannosyl-structures present on type I fimbrae (67, 70), Opacity (Opa) proteins are responsible for the adherence of *N. gonorrhea* to CEACAM1 (71). Monoclonal antibodies and site-directed mutagenesis of the N-terminal region demonstrated that the Ig V-related domain is critical for bacterial adherence (69, 70).

The expression of CEA-related proteins in the intestinal fuzzy coat (22) and their adherence to several bacteria suggests their involvement in normal bacterial colonization (67). By trapping pathogens in the glycocalyx and preventing them from invading intestinal cells, CEA family members may also participate in the innate immune response directed against bacteria. Furthermore, the induction of an oxidative response by polymorphonuclear neutrophils upon Opa proteins engagement with CEACAM1[a] further implies an immunoregulatory function for CEA-related proteins (72).

Tumor Suppressor

Various reports have indicated that CEACAM1 acts as a tumor cell inhibitor in several epithelial systems (1, 73-75). Expression of rat CEACAM1^a-4L in PC-3 human prostate carcinoma cells clearly affects their proliferation and tumorigenicity. PC-3 cells producing CEACAM1^a-4L form fewer colonies in soft agar than parental or vector control cells. Furthermore, mice injected with PC-3 cells expressing CEACAM1^a-4L had a lower tumor incidence than animals injected with parental or vector control cells (73). Downregulation of CEACAM1 expression in non-tumorigenic rat ventral prostate NbE cells by antisense technology induced the development of anaplastic or poorly differentiated tumors upon injection of these transformed cells in athymic mice (73). These experimental results concur with the observed CEACAM1 underexpression in primary prostate tumors (28). Rat CEACAM1^a-4L also arrested the progression of cancer in an orthotopic bladder model (75).

Expression of either murine CEACAM1^a-4L or -4S also prevented the proliferation of CT51 cells, which are highly tumorigenic and metastatic murine colon carcinoma cells. CEACAM1^a-transfected CT51 cells formed fewer colonies in soft agar and had a lower saturation density in culture than parental cells. Interestingly, when injected in syngeneic mice, CT51 cells expressing CEACAM1^a-4S produced tumors as readily as parental and vector control cells. However, CEACAM1^a-4L was able to reduce tumor incidence *in vivo* (1). The incongruity between the *in vitro* and *in vivo* models is not easily explained. BALB/c mice are deemed reliable for the assay, because CT51 cells originate from tumors chemically-induced in this strain of mice. Furthermore, this strain

of mice has an intact immune system. The clonogenic assay most likely does not mimic accurately the environment provided by an animal. For example, CEACAM1 ligands, which have yet to be identified, may be modulating the protein's anti-proliferative function (1). Regardless, the results of our laboratory were confirmed by another group who reported that the rat ortholog of CEACAM1^a-4S was unable to prevent the formation of breast cancer cells-borne tumors in nude mice (74).

Since CEACAM1^a-4L and -4S are expressed concomitantly in normal colonic epithelia in a 1-3L:7-9S ratio, the tumorigenic potential of CT51 cells producing both isoforms was evaluated. CT51 failed to form tumors as long as they expressed CEACAM1^a-4L and -4S isoforms in a ratio corresponding to that observed in normal cells. Any slight modification reversed the tumor inhibition phenotype (24). Taken together, these results suggests that CEACAM1^a-4L has a tumor inhibitory function and is dominant over CEACAM1^a-4S. Furthermore, residues or motifs among the 63 amino acids which differentiate the cytoplasmic isoforms are likely to control the tumor-suppressive activity exhibited by CEACAM1^a-4L.

The mechanisms by which CEACAM1^a-4L regulates tumor growth still remain to be elucidated. The long cytoplasmic domain contains 2 calmodulin binding sites (76), several protein kinase C (PKC) consensus sequences (10, 77, 78) and two immunoreceptor-tyrosine based motifs (ITIM; I/VXYXXL/V) (Figure 1-5) (79); thus, calmodulin binding and serine or tyrosine phosphorylation of the cytoplasmic domain are likely to be implicated in the regulation of tumor proliferation. Moreover, CEACAM1's adhesive function may also be associated with its tumor-suppressive properties.

Figure 1-5 Consensus sequences present on the CEACAM1 cytoplasmic domain.

The amino acid sequence of the short and long CEACAM1 cytoplasmic domain are indicated in one letter code. The calmodulin (CaM) binding sites are delineated by a dash- lined rectangle. The PKC consensus sequence is boxed in a solid line rectangle. ITIM motifs (I/VXYXXL/V) are underlined by a thick black line.

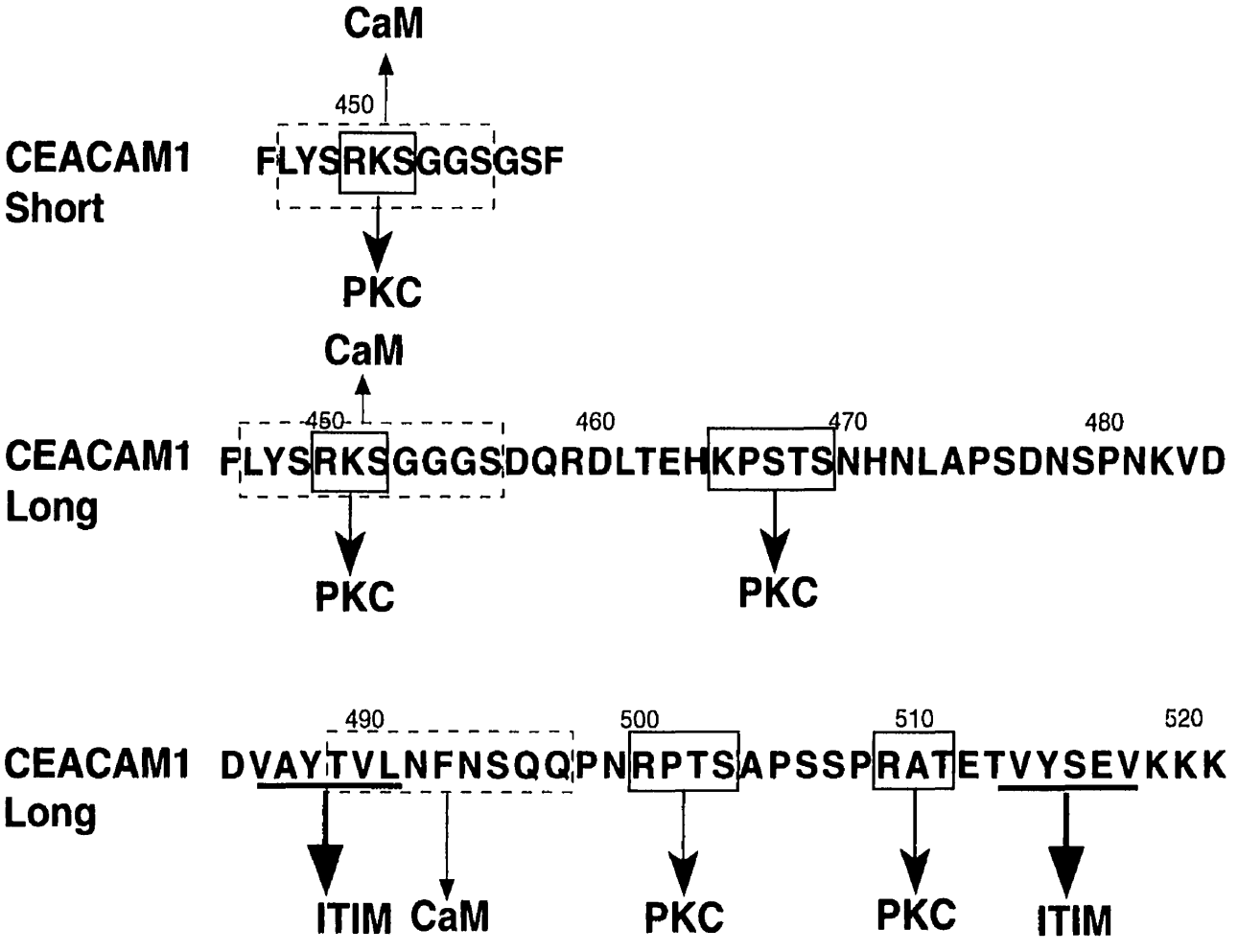

CaM
450
CEACAM1
Short
FLYSRKSGGSGSF
PKC
CaM
450
460
470
480
CEACAM1
Long
FLYSRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVD
PKC
PKC
490
500
510
520
CEACAM1
Long
DVAYTVLNFNSQQPNRPTSAPSSPRATETVYSEVKKK
ITIM
CaM
PKC
PKC
ITIM

Figure 1-5

The development of many cancers have been tied to the loss of E-cadherin, another CAM. (80). A similar situation may be occurring with CEACAM1.

Signal Transducer

CAMs are important participants in the activation of signaling pathways which regulate cellular processes such as adhesion, migration, proliferation, differentiation, and apoptosis. Recent reports indicate that CEACAM1, like other CAMs, may be implicated in signal transduction.

The long and short cytoplasmic domains of CEACAM1 isoforms contain a number of Ser and Thr residues which are located in putative protein kinase A (PKA) and PKC-dependent phosphorylation sites (10, 77, 78). Situated in a perfect PKC consensus site, KXXXS, Ser503 has been reported as a serine phosphorylation site in CEACAM1^{a}-4L (Figure 1-5) (81). Site-directed mutagenesis of Ser503 reduces insulin receptor internalization mediated by rat CEACAM1-4L (81).

CEACAM1-4L also contains two Tyr residues which are conserved in man (Tyr488, Tyr513), rat (Tyr488, Tyr513) and mouse (Tyr488, Tyr515) (Figure 1-5) (7, 82, 83). Phosphorylation of these elements by tyrosine kinases elicits signaling cascades which induce CEACAM1-4L's numerous functions. Several reports indicate that Tyr488 is the major tyrosine phosphorylation site of CEACAM1-4L in resting, insulin-induced and pervanadate-treated cells (79, 82, 84). Whereas phosphotransfer on Tyr513 in rat CEACAM1^{a}-4L is undetectable (78, 82), the phosphorylation of mouse CEACAM1^{a}-4L on Tyr515 has been detected in pervanadate-stimulated CT51 cells (85). Protein tyrosine

kinases phosphorylate human CEACAM1-4L on Tyr488 (82, 83, 86, 87). CEACAM1-4L is phosphorylated by Lyn and Hck in activated neutrophils (83), $pp60^{c\text{-}src}$ in HT29 human colon carcinoma cells (87) and the insulin receptor tyrosine kinase in hepatocytes (82). Recently, Hauck and co-workers showed that binding of Opa_{52} *Neisseria gonorrhea* proteins to CEA-related proteins, including CEACAM1, resulted in the stimulation of Hck and Fgr. Activated Hck and Fgr induced the activation of Rac-1, which in turn stimulated PAK and JNK. This newly described pathway involving CEACAM1 may have a critical role in the regulation of cell growth, inasmuch as JNK is involved in the induction of AP-1 transcriptional complexes and stress-induced apoptosis (88).

$CEACAM1^a$-4L also appears to be implicated in the transmission of inhibitory signals. $CEACAM1^a$-4L possesses two ITIM motifs centered around Tyr488 and Tyr515 (Figure 1-5). Recently, our group has demonstrated that the cytosolic tyrosine phosphatases SHP-1 and SHP-2 associate with $CEACAM1^a$-4L's ITIM motifs (79, 85). This binding necessitated the presence of both Tyr488 and Tyr515 and the phosphorylation of at least one Tyr residue (85). SHP-1 and SHP-2 each contain two SH2 domains. The interaction between $CEACAM1^a$-4L and SHP-1 occurs through one or both SH2 domains (79), while SHP-2 tethering to $CEACAM1^a$ -4L requires both SH2 domains (85). Furthermore, residues outside the ITIM encompassing Tyr515 regulate the association between $CEACAM1^a$-4L and SHP-1 or SHP-2 (85). Deletion of the C-terminal Lys, located at positions +4, +5, +6 relative to Tyr515, reduced tyrosine phosphorylation and SHP-1 and SHP-2 association. Although Tyr phosphorylation was

unaffected, site-directed mutagenesis of Val518 also abolished SHP-1 and SHP-2 binding (85).

Human CEACAM1-4L has been shown to contains a functional ITIM motif. Chen *et al.* demonstrated that a chimeric protein consisting of the extracellular domain of FcγRIIB and the cytoplasmic domain of CEACAM1-4L inhibited Ca^{2+} influx to the same extent as wild-type FcγRIIB. Mutation of Tyr488 in FcγRIIB-CEACAM1-4L abrogated the inhibition of the calcium mobilization, indicating that the ITIM motif was mediating the effect. Moreover, SHP-1 and SHP-2 appeared to play a critical role in the FcγRIIB-CEACAM1-4L inhibitory effect. The inhibiton of Ca^{2+} influx was diminished in SHP-$1^{-/-}$ and SHP-$2^{-/-}$ cells in comparison to wild-type cells. This data indicates that CEACAM1-4L functions as a negative modulator of B cell functions by associating with SHP-1 and SHP-2 (89).

CEACAM1 Binding proteins

In addition to Src-like kinases and tyrosine phosphatases SHP-1 and SHP-2, CEACAM1 interacts with calcium-binding proteins, actin, and an unidentifed 80 kDa protein. Annexin VI was the first calcium-binding protein reported to associate with CEACAM1(90). The interaction was first identified in the bile canaliculi of rat hepatocytes by immunofluorescence staining. Direct association between CEACAM1 and annexin VI was confirmed by chemical cross-linking techniques and by affinity chromatography using anti-CEACAM1 or anti-annexin antibodies (90). Calmodulin is an intercellular Ca^{2+}-binding protein expressed ubiquitously. It associates with the

cytoplasmic domains of both CEACAM1-4L and -4S in rat hepatocytes (91) in a calcium-dependent manner (92). Human, mouse and rat CEACAM1 variants contain respectively 1 or 2 calmodulin binding sites (76). Calmodulin binding possibly participates in the regulation of CEACAM1-mediated adhesion, as it reduces CEACAM1 self-association in dot blot assays (76).

Bifunctional cross-linking of rat liver membrane extracts identified an interaction between CEACAM1-4L and an unidentified protein with an apparent M.W. of 80 kDa (CAP-80). CAP-80 was also detected in liver membrane extracts immunoprecipitated with anti-CEACAM1 serum. Deletion of the terminal 37 a.a. of CEACAM1-4L abrogates the association with CAP-80. Furthermore, the interaction is independent of CEACAM1-4L Tyr488 phosphorylation. CAP-80 binding appears to be important for tumor cell inhibition, as the ability of CEACAM1-4L cytoplasmic mutants to associate with CAP-80 correlates with their anti-proliferative activity (93).

A yeast two-hybrid screen of the mouse CT51 colon carcinoma cDNA library identified actin as a CEACAM1 interacting partner (94). Only CEACAM1-4L appears to interact mouse β-actin. Immunofluorescence analyses co-localized CEACAM1-4L and actin at CT51 cell-cell contacts. Furthermore, CEACAM1-4L intercellular localization required polymerized actin and is regulated by Rho-like small GTPases (94).

Clinical Significance

Several studies have clearly demonstrated the downregulation of CEACAM1 in various human cancers of epithelial origin (26). Re-introduction of CEACAM1-4L

cDNA in murine colon carcinoma cells (1), rat prostate carcinoma cells (73) or human breast cancer cells (74) decreased tumor incidence *in vivo*.

A recent pre-clinical study examined CEACAM1 as a potential agent in prostate cancer gene therapy (95). A recombinant adenovirus containing the rat CEACAM1^a-4L cDNA sequence integrated in the viral genome was used as the delivering vector. The study reported that injection of CEACAM1-expressing adenoviruses to small PC3-induced tumors in nude mice arrested tumor proliferation for at least 3 weeks. A second inoculation extended the suppressive effect. CEACAM1-adenoviral therapy efficiently inhibited the growth of tumors ranging from 40 to 70 mm^3; however, no suppressive effect was observed for tumors larger than 100 mm^3 (95).

These preliminary findings indicate that CEACAM1 is a good candidate for cancer therapy in animal models. However, further studies are needed to establish the optimal therapeutic conditions and assess its efficiency in human cancers.

Objectives

CEACAM1 has been shown to be underexpressed in a number of malignancies of epithelial origin. Our laboratory and others investigated whether the re-introduction of CEACAM1 in tumorigenic cells inhibited the development of tumors *in vivo*. We observed that expression of CEACAM1-L (isoform with long cytoplasmic domain) inhibited the growth of CT51 colon carcinoma cells *in vivo*, while the expression CEACAM1-S (short cytoplasmic domain) did not alter their tumorigenicity. The first objective of this thesis was to define the sequence elements on the long cytoplasmic

domain of CEACAM1 responsible for the tumor inhibitory activity. Since CEACAM1 has been shown to behave as a intercellular adhesion molecule, the second goal of this thesis was to determine whether the adhesive activity was responsible for the tumor inhibitory effects exhibited by CEACAM1-4L.

CHAPTER 2

Cis-Determinants in the Cytoplasmic Domain of CEACAM1 Responsible for its Tumor Inhibitory Function

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cis-Determinants in the Cytoplasmic Domain of CEACAM1 Responsible for its Tumor Inhibitory Function

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Keywords: CEA, BGP, CD66a, C-CAM, tumor suppressor, colon cancer, SHP-1, SHP-2, Tyr phosphorylation

Abstract

CEACAM1, also know as C-CAM, BGP and CD66a, is a member of the carcinoembryonic antigen (CEA) family which is itself part of the immunoglobulin supergene family. CEACAM1 is involved in intercellular adhesion, signal transduction and tumor cell growth regulation. CEACAM1 is down-regulated in colon and prostate carcinomas, as well as in endometrial, bladder and hepatic tumors, and 30% of breast cancers. We have shown in a mouse colon tumor model that CEACAM1 with a long cytoplasmic domain inhibited the development of tumors whereas a splice variant lacking the cytoplasmic domain did not. In this study, we define the subregions of the long cytoplasmic domain participating in the tumor inhibition phenotype of CEACAM1. We show that a single point mutation of Tyr488, conforming to an Immunoreceptor Tyrosine Inhibition Motif (ITIM), was sufficient to reverse the *in vivo* tumor cell growth inhibition. Substitution or deletion of residues in the C-terminal region of the CEACAM1 cytoplasmic domain also led to reversal of tumor cell growth inhibition. This result is in agreement with our previous studies demonstrating the C-terminal region of the cytoplasmic domain influences the levels of CEACAM1 Tyr phosphorylation and its association with the protein Tyr phosphatases SHP-1 and SHP-2. Furthermore, removal of the N-terminal domain of CEACAM1, essential for intercellular adhesion, did not impair the tumor inhibitory effect. These results suggest that Tyr phosphorylation or dephosphorylation of the CEACAM1 cytoplasmic domain represents a crucial step in the control of epithelial cell proliferation.

Introduction

Colorectal tumor development is a consequence of the dysregulation or alteration of a number of key cellular proteins. Amongst them are oncogenes such as ras, tumor suppressors such as the APC and p53 proteins (96, 97), and proteins involved in TGF-ß signaling such as Dpc4 (Smad4) (98, 99). Compound mutations, such as those observed in Apc and Smad4 proteins, also have an additive effect on colorectal tumor development (100).

We and others have shown that another cell adhesion molecule identified as biliary glycoprotein 1 (BGP in human, Bgp1 in mouse), is implicated in colorectal tumor development (1, 23, 37). BGP is a member of the carcinoembryonic antigen (CEA) family (9). The nomenclature of this gene family has recently been redefined and the C-CAM or BGP proteins are now referred to as CEACAM1 proteins (8). The CEACAM1 protein is abundantly expressed in normal colonic tissue and is generally concentrated in the luminal glycocalyx and fuzzy coat of the intestinal epithelial cells (22). In addition, the CEACAM1 protein is also found at the lateral borders of enterocytes (56). However, upon cellular transformation, the CEACAM1 expression is down-regulated in colonic tissue (23, 37). This occurs at an early period in tumor development as intestinal adenomas (38, 101) or stage A tumors are devoid of CEACAM1 expression (23). This is not unique to colonic tissue, since CEACAM1 expression is also decreased in hyperplastic prostatic and mammary glands (25, 28), in transformed hepatic tissue (40, 102) and in bladder and endometrial tumors (26, 75). Interestingly, this protein has also

been identified by the SAGE technique as a down-regulated participant in colorectal tumor development (103). However, primary lung tumors (squamous cell carcinoma, adenocarcinoma and small cell carcinoma) and stomach tumors overexpress the CEACAM1 protein (42, 43). Interestingly, in mestastatic lesions of these lung cancers, *CEACAM1* mRNA expression is decreased compared to the primary site (42).

The *CEACAM1* gene lies on human chromosome 19q13.1-2 (104) or mouse chromosome 7 (12). There is only one human and rat *CEACAM1* gene (3, 5), but two very similar genes have been identified in the mouse (*Ceacam1* and *Ceacam2*) (15, 17). The *CEACAM1* genes in all three species generate a significant number of alternative splicing variants. The extracellular domains form typical Ig folds with one variable Ig-like domain and one to three C2-set Ig-like domains (3). The most conserved feature between the *CEACAM1* gene products of various species is found in their cytoplasmic region. Insertion of exon 7, comprised of 53 bp, into the *CEACAM1* mRNA leads to the translation of a protein with a long cytoplasmic domain of 71-73 amino acids (denoted L), while its absence creates a cytoplasmic tail of 10 residues (identified as S). Both forms are tandemly expressed in most CEACAM1-positive tissues in a characteristic ratio ranging from 1-3L:7-9S (10, 24).

We have demonstrated that insertion of the CEACAM1 protein into highly tumorigenic and metastatic mouse CT51 colon carcinoma cells led to inhibition of tumor cell growth *in vitro* and *in vivo* in BALB/c syngeneic mice (1). A major difference was noticed however, between the CEACAM1 isoforms: only the CEACAM1 splice variant expressing the long cytoplasmic domain possessed tumor inhibitory activity. Similar

results were obtained by studying a rat CEACAM1 variant in a prostatic PC-3 carcinoma model (73). In addition, downregulation of CEACAM1 expression in non-tumorigenic ventral prostatic NbE cells by antisense technology induced the development of anaplastic and poorly-differentiated tumors when transfected cells were injected *in vivo* (73). As CEACAM1-L and -S are both expressed in normal cells, we questioned if changing the expression ratio of these isoforms would influence tumor development. We found that as long as the ratio of CEACAM1-S: CEACAM1-L was similar to that found in normal cells, tumor development was inhibited. However, reversing the ratio led to the formation of tumors with shortened latency (24).

Using deletion and point mutations within the CEACAM1 long cytoplasmic domain, we have now delineated some of the sequence elements within this region responsible for colonic tumor cell growth inhibition. Our results indicate that Tyr488 and a number of C-terminal residues of the CEACAM1 cytoplasmic domain play a major role in the tumor inhibition phenotype, whereas mutation of Tyr515 to Phe was inconsequential. In addition, we demonstrate that deletion of the N-terminal domain of the CEACAM1 protein, instrumental in mediating intercellular adhesion, did not influence its tumor inhibitory phenotype. We conclude that the CEACAM1 cytoplasmic domain may probably adopt a particular conformation *in vivo* favouring cooperation of Tyr488 and C-terminal Lys519-521 in mediating the tumor inhibition phenotype or that several cytoplasmic proteins, normally binding to these regions exert their phenotypic effects by altering the signal transduction cascades leading to increased cell proliferation.

Results

Expression of CEACAM1 proteins

We and others have already shown that the CEACAM1 proteins are expressed in normal tissues such as colon and liver (23, 44). However, CEACAM1 expression is lost or greatly reduced in colonic, prostatic, breast, hepatic and endometrial carcinomas (1, 26, 40, 73, 74). In addition, the tumor inhibitory phenotype is dependent on the long cytoplasmic domain of CEACAM1-L and not on the shorter variant, CEACAM1-S (1, 24).

We sought to define carboxy-terminal subregions or residues within the long cytoplasmic isoform responsible for tumor cell growth inhibition. To attain our objectives, several C-terminal and N-terminal deletion mutants were generated (Figure 2-1). The following mutants were devised to establish the impact of Tyr phosphorylation and/or dephosphorylation on CEACAM1-dependent tumor inhibition: CEACAM1/Y488F, Y515F and Y488,515F, Δ483 (Figure 2-1). CEACAM1/Δ518, in which the last three Lys residues were eliminated, was generated to determine the involvement of these residues in tumor cell growth inhibition (Figure 2-1). A CEACAM1 triple point (3K→3R) was produced to verify if the charge of the terminal Lys residues was important in this phenotype (Figure 2-1). We have recently shown that the Val at position 518 is important for the association of CEACAM1-L to the SHP-2 protein Tyr phosphatase (85); therefore, a CEACAM1 protein exhibiting a Val to Ala substitution at position 518 (CEACAM1/V518A) was included in our assays to evaluate its role in

Figure 2-1 Schematic representation of mutants used in the tumorigenicity assays

The four Ig extracellular domains of CEACAM1 are represented by ovals. The CEACAM1/Δ4-122 mutants eliminating the first Ig domain lack the domain indicated in black. The amino acid sequence in the one letter code representing sequences of the short (CEACAM1-S) and long (CEACAM1-L) cytodoplasmic domains are found below the drawing. A portion (amino acids 478-521) of the long cytoplasmic domain is highlighted as an inset. The two Tyr residues (Tyr488 and Tyr515) are shown in bold letters. The mutations in the cytoplasmic domain are indicated in bold letters over the lines. Asterisks correspond to the stop codon created in the deletion mutants.

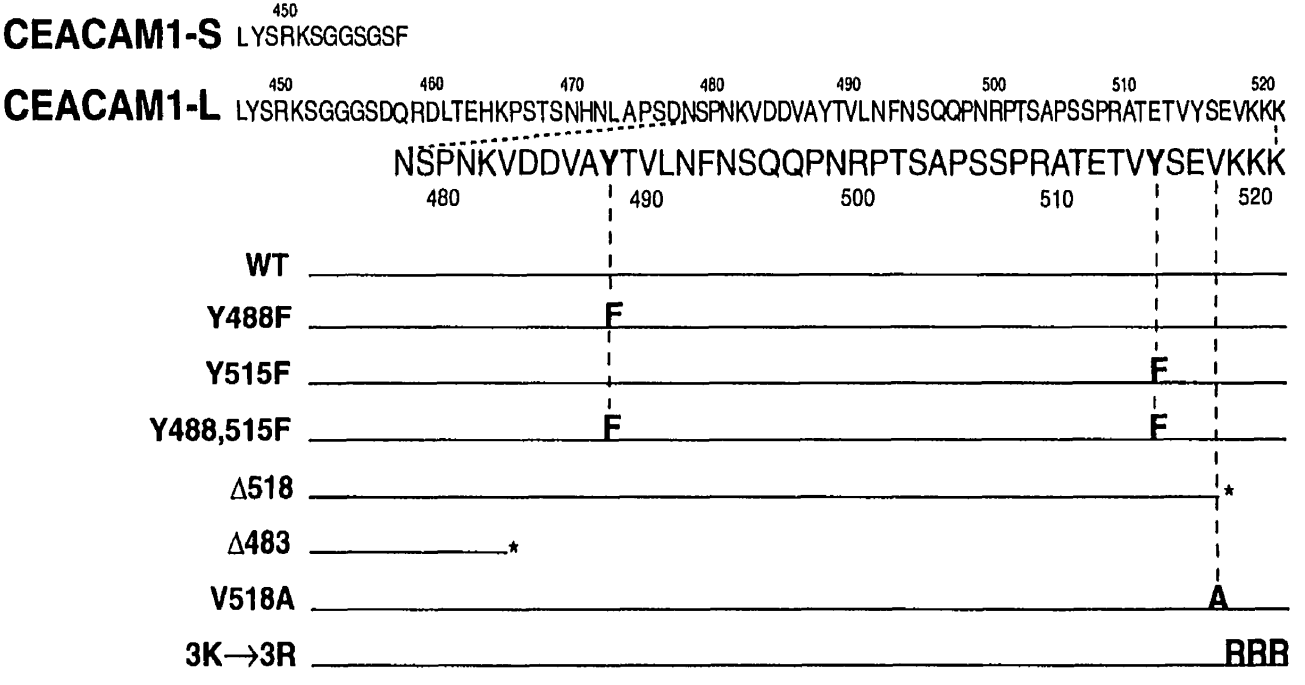

CEACAM1-S
450
LYSRKSGGSGSF
CEACAM1-L
450 460 470 480 490 500 510 520
LYSRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVDDVAYTVLNFNSQQPNRPTSAPSSPRATETVYSEVKKK
NSPNKVDDVAYTVLNFNSQQPNRPTSAPSSPRATETVYSEVKKK
480 490 500 510 520
WT
Y488F
Y515F
Y488,515F
Δ518
Δ483
V518A
3K→3R
F
F
F
F
*
*
A
RRR

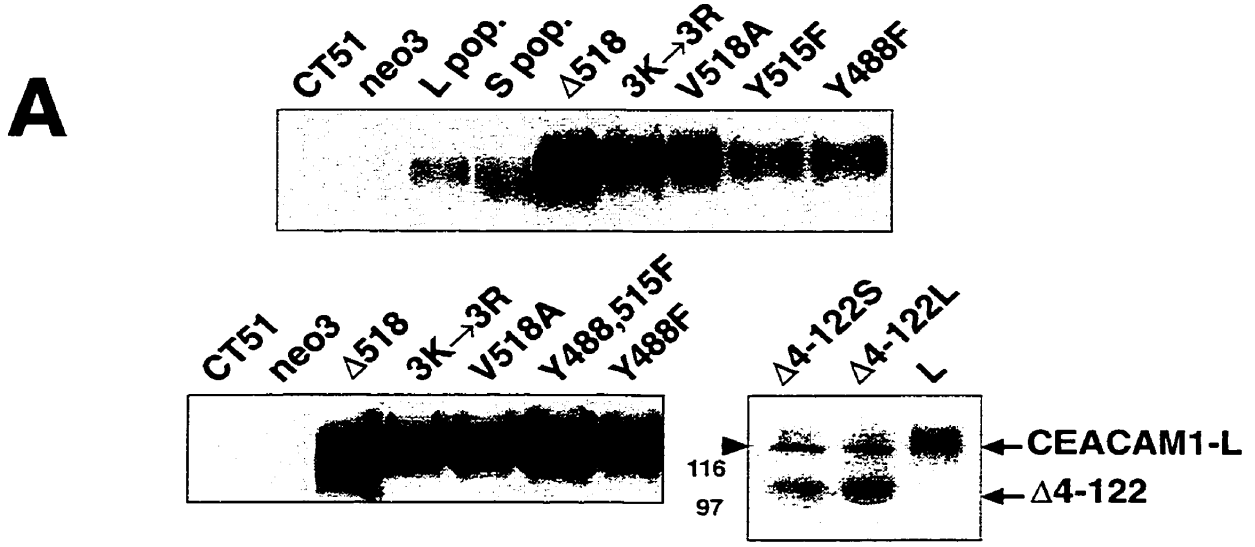
Figure 2-1

tumor inhibition. CEACAM1 has also been identified as an intercellular adhesion molecule (6). In this regard, we have questioned the role of the extracellular domain responsible for cell adhesion by studying the tumorigenicity of N-terminal deletion mutants (Δ4-122/S and Δ4-122/L).

The cDNAs corresponding to these CEACAM1 proteins were inserted in CT51 colon carcinoma cells by retroviral-mediated infections. Enrichment of cells expressing the CEACAM1 proteins was performed by immunoselection using anti-CEACAM-1 polyclonal antibodies (Ab231 or 655) and Dynabeads®. Total CEACAM1 protein expression was evaluated by immunoblotting analyses (Figure 2-1A). The CEACAM1 proteins migrated as large heterogeneous bands due to their high content of glycosylated residues (16 Asn-linked carbohydrate moeities in the wild-type CEACAM1 isoform). Within this large band, there appears to be two major entities (Figure 2-2A, first panel, Y488F or Y515F), most possibly corresponding to predominant glycoforms synthesized in this cell line. CEACAM1-S appeared as a band at ≈120 kDa, whereas CEACAM1-L had a relative molecular weight of approximately 128 kDa. The CEACAM1/Δ4-122L and Δ4-122S mutants migrated as proteins of aproximately 95 kDa. The use of Ab655 was necessary for detection in this case, as Ab231 only recognizes epitopes in the N-terminal domain of the CEACAM1 proteins (33). All other mutants exhibited a relative molecular weight between 120 and 128 kDa.

Figure 2-2 Expression of CEACAM1 proteins in CT51 colonic tumor cells

CT51 and neo3 are respectively wild-type and control transfected cells. CEACAM1-L represents the CT51 cell population expressing the long tailed-CEACAM1 isoform. CEACAM1-S is a population expressing the short tailed-CEACAM1 isoform. Δ518, 3K→3R, V518A, Y488F, Y515F, Y488,515F, Δ4-122-L and Δ4-122-S are CT51 populations expressing mutant CEACAM1 proteins. (**A**) CT51 parental, neo3 control or CT51 cells expressing CEACAM1 proteins were analysed by immunoblotting. Cells were lyzed and 100 µg of total cellular proteins were resolved on 8.0% SDS-PAGE gels. Proteins were transferred to membranes which were incubated with rabbit anti-CEACAM1 antibodies (231 or 655) and ^{125}I-labelled protein A. Bands were subjected to radioactive quantification. Molecular weight markers are illustrated on the left of the panel. The arrowhead on the left of the third panel indicates the presence of a non-specific band whereas the arrows on the right represent the full length CEACAM1-L or Δ4-122 mutants. (**B**) Surface expression of CEACAM1 proteins were evaluated by cytofluorometry using anti-CEACAM1-specific rat monoclonal antibody (AgB10). Fluorescence is depicted on a log scale. Dashed lines represent cells incubated with the secondary fluorescein-labelled antibody only, whereas solid lines illustrate cells incubated with both primary (AgB10) and secondary antibodies.


A
CT51
neo3
L pop.
S pop.
Δ518
3K→3R
V518A
Y515F
Y488F
CT51
neo3
Δ518
3K→3R
V518A
Y488,515F
Y488F
Δ4-122S
Δ4-122L
L
116
97
←CEACAM1-L
←Δ4-122

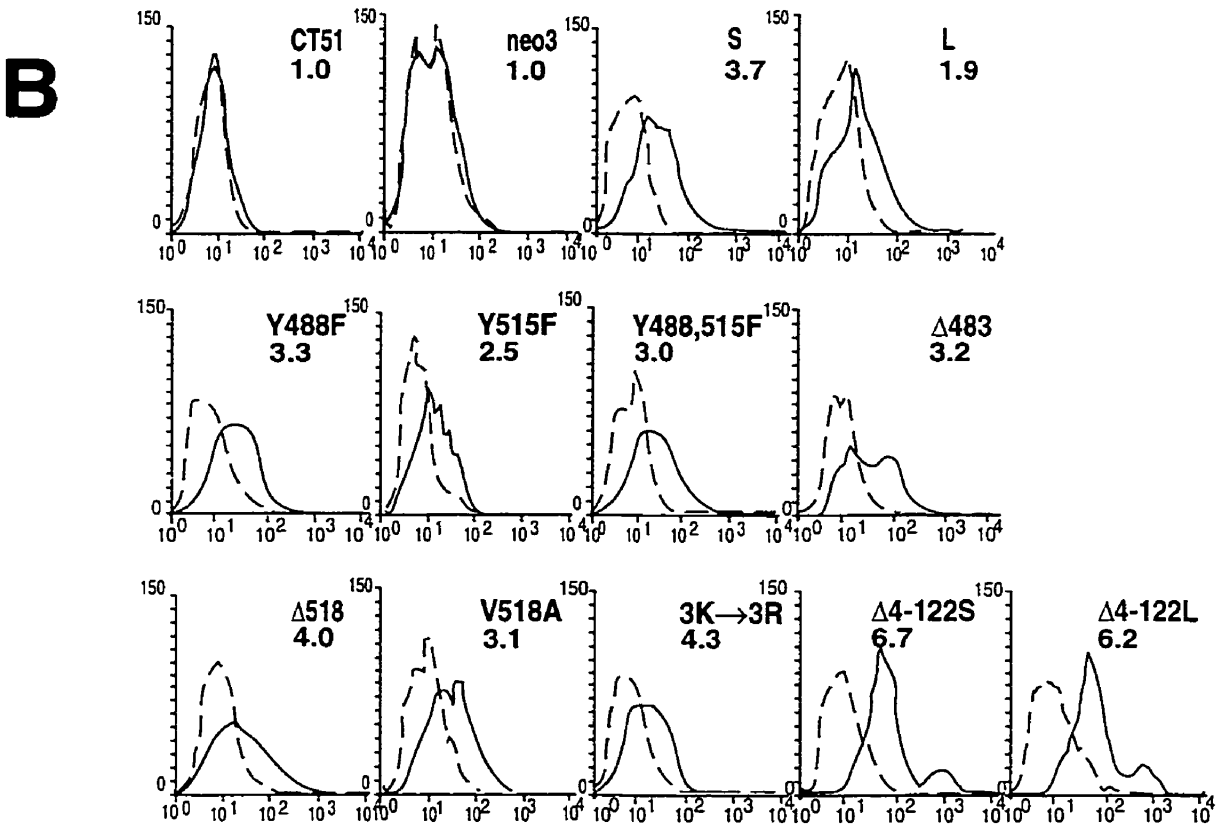

B
CT51 1.0
neo3 1.0
S 3.7
L 1.9
Y488F 3.3
Y515F 2.5
Y488,515F 3.0
Δ483 3.2
Δ518 4.0
V518A 3.1
3K→3R 4.3
Δ4-122S 6.7
Δ4-122L 6.2

Figure 2-2

Radioactive quantitations of several representative experiments revealed that the CEACAM1 proteins were overexpressed by factors ranging from 1.2 to 9.1 relative to the band of lowest intensity on each immunoblot (Figure 2-2A). The tumor inhibitory properties of the CEACAM1 proteins may be related to their localizaton at the cell surface; therefore, cell surface expression of CEACAM1 proteins was estimated through cytofluorometric analyses. FACS profiles showed that the cell populations used in our assays overexpressed the CEACAM1 proteins by factors ranging between 1.9 and 6.7 relative to the background fluorescence (Figure 2-2B). The expression levels of these mutants thus corresponded to the relative levels of expression of CEACAM1 found in normal colon (24).

Although the data calculated from fluorescence profiles and radioactive quantitations cannot be directly compared, it is apparent that most cell populations expressed the CEACAM1 proteins in the same relative range. Surface expression of the CEACAM1 proteins was comparable to the expression of CEACAM1 found in normal colonic cells (24). However, some discrepancies between the two different estimates were noticed with the CEACAM1/Δ518 and the CEACAM1/Δ4-122L and the /Δ4-122S mutant proteins. It is possible that a non-negligeable amount of the CEACAM1/Δ518 protein expressed may not reach the cell surface. As for the variation observed with the CEACAM1/Δ4-122L and -S mutant proteins, the specificity of antibody used (Ab655) may be responsible for the observed differences.

Tyr488 of CEACAM1 plays a major role tumor growth inhibition

According to recent reports, CEACAM1-L participates in signal transduction events (79, 85, 88). CEACAM1-L contains two consensus Immunoreceptor Tyrosine-based Inhibition Motifs (ITIM) (105) where either Tyr residue is phosphorylated by protein Tyr kinases, such Src in human colon carcinoma cells (87), Lyn and Hck in activated neutrophils (83) and the insulin receptor in hepatocytes (106). CEACAM1-L Tyr phosphorylation is physiologically relevant during neutrophil respiratory bursts (83), activation of neutrophils after binding of *N. gonorrhea opa* proteins to CEACAM1-L (88) and in internalization of the insulin receptor (81). We have demonstrated that, in mouse CT51 colon carcinoma cells, the presence of both CEACAM1-L Tyr residues and the phosphorylation of at least one of them are required for association with the protein Tyr phosphatases SHP-1 and SHP-2 (79, 85). Thus, we assessed the role of CEACAM1-L Tyr residues in tumor growth inhibition by testing single and double point mutants of Tyr488 and Tyr515 in *in vivo* tumor assays.

Two completely independent experiments were performed, i.e. expression of CEACAM1-L mutant proteins in CT51 cells, isolation of CT51-transfected mutant sub-populations, and injection of cells in BALB/c syngeneic mice. As expected, the CT51 parental cells, the CT51 neo-transfected control cells and the CEACAM1-S-expressing CT51 cells readily formed tumors when injected in mice (Table 2-1A). The differences in tumor incidence noted between the wild-type and the control transfected CT51 cells (15/20 *versus* 19/20) fell within experimental variation as defined by statistical evaluation ($P > 0.05$, relative to neo3, or < 0.05 when compared to CEACAM1-L).

TABLE 2-1. TUMORIGENICITY ASSAYS WITH CEACAM1 MUTANTS

Cell Line	Experiment	Latency (days)	Incidence	Total # tumor	inhibition (%)	*P*value relative to neo3	*P*value relative to Ceacam1-L
A. CONTROLS							
CT51	1	42	7/10				
	2	48	8/10	15/20	25	0.18	0.04
neo3	1	48	9/10				
	2	56	10/10	19/20	5	0.47	0.00
CEACAM1-S	1	42	12/15				
	2	56	9/10	21/25	16	0.49	0.004
CEACAM1-L	1	42	7/15				
	2	56	3/10	10/25	60	0.0004	0.77
B. CEACAM1 MUTANTS RELATED TO TYR PHOSPHORYLATION							
CEACAM1/Y488F	1	53	10/10				
	2	56	14/14	24/24	0	0.926	0.0001
CEACAM1/Y515F	1	42	8/15				
	2	69	4/14	12/29	59	0.0004	0.86
CEACAM1/ Y488,515F	2	53	14/20	14/20	30	0.096	0.09
CEACAM1/Δ483	1	42	6/10	6/10	40	0.056	0.48
C. CEACAM1 MUTANTS IN THE C-TERMINAL REGION							
CEACAM1/Δ518	1	42	8/10				
	2	48	9/10	17/20	15	0.598	0.006
CEACAM1/3K→3R	1	56	7/10				
	2	53	9/10	16/20	20	0.338	0.02
CEACAM1/V518A	1	56	5/10				
	2	53	9/10	14/20	30	0.096	0.09
D. CEACAM1 MUTANTS OF THE N-TERMINAL DOMAIN							
CEACAM1/Δ4-122/S	1	53	8/10 8/10		20	0.518	0.08
CEACAM1/Δ4-122/L	1	53	5/10	5/10	50	0.015	0.87

For subsequent comparisons, values obtained with the control transfected cells were mostly used. As described previously, the expression of CEACAM1-L in CT51 cells reduced the development of tumors; 10 out of 25 mice injected with these cells exhibited tumor growths in these experiments (corresponding to an inhibition of 60%), whereas the majority of mice injected with control cells developed tumor growths (Table 2-1A). The CEACAM1-L-dependent inhibition was statistically significant when compared to results obtained with the control cells or the CEACAM1-S-expressing cells ($P<0.05$, relative to neo3). Furthermore, tumors that developed with cells expressing CEACAM1-L were generally smaller than those seen with control cells within the same experiment (data not shown). Mutation of Tyr488 to a Phe had a dramatic effect, reversing CEACAM1-L's tumor growth inhibition effect. In this case, 24 out of 24 mice injected with the CEACAM1-L/Y488F-expressing cells formed tumors (Table 2-1B, $P>0.05$ relative to neo3). Mutation of Tyr515 within the CEACAM1 tail, however, did not influence the inhibitory effect, as 12 out of 29 mice injected produced tumors. This was comparable to the tumor incidence of CEACAM1-L ($P > 0.05$, relative to CEACAM1-L) which represented a 59% inhibition (Table 2-1B, $P<0.05$ relative to neo3). In the second assay with this mutant, the tumors were allowed to develop for 69 days instead of 53-56 days to verify whether later onset would occur. The number of tumors remained lower than with the Tyr488 mutant and the size of the tumors was also comparatively smaller (data not shown). Mutations of both Tyr residues to Phe (CEACAM1/Y488,515F) or a deletion of the region containing the Tyr residues (CEACAM1-L/Δ483) resulted in a

partial inhibition, preventing tumor development by 30% or 40%, respectively. These values appear to be significant as the *P* values are equal to 0.05 or slightly higher than 0.05 (0.096). These results indicate that phosphorylation of Tyr488 is crucial for CEACAM1-L's tumor growth inhibition effect. Phosphorylation of Tyr515, however, seems dispensable for this phenotype ($P > 0.05$, relative to CEACAM1-L). On the other hand, the results obtained with the doubly-mutated Tyr residues and the deletion mutant suggest that there may be some inter-dependence of these two Tyr residues.

Effects of the C-terminal region of CEACAM1-L in tumor development

In addition to the Tyr phosphorylation sites, other motifs are present in the C-terminal region of the long cytoplasmic tail such as a protein kinase C consensus site (Ser503) (10, 78) and a calmodulin binding site (76).

In order to define motifs, other than the ITIMs, involved in colonic tumor development, several mutations were introduced in the C-terminus of CEACAM1-L. One of the conserved features between the rodent and human cytoplasmic domains is the presence of three terminal Lys residues at the C-terminus of the protein. Huber *et al.* have shown that the C-terminal region influences the extent of CEACAM1-L Tyr phosphorylation (85). The three Lys residues were removed to generate CEACAM1/Δ518. Deletion of the Lys residues reversed the tumor inhibition phenotype exhibited by CEACAM1-L (Table 2-1C). Seventeen out of 20 mice injected with cells expressing this mutant developed tumors (Table 2-1C, $P>0.05$ relative to neo3). To determine if the tumor inhibitory action of wild-type CEACAM1-L was due to the

identity or the charge of the residues, an additional mutant was tested in which the three Lys residues were replaced by three Arg. When injected in BALB/c mice, CT51 cells expressing CEACAM1/3K→3R easily formed tumors. In fact, 16 out of 20 mice developed abnormal growths, (Table 2-1C, $P>0.05$ relative to neo3). Hence, this result suggested that the presence of the three terminal Lys residues is important for CEACAM1-L's anti-proliferative effect.

Huber *et al.* also showed that replacing Val518 by an Ala prevented SHP-2 association with CEACAM1-L, although this mutant had no significant effect on the Tyr phosphorylation status of the protein (85). CT51 cells expressing CEACAM1/V518A were included in our tumorigenicity assays to determine the importance of this residue in tumor inhibition. Fourteen out of 20 mice injected with this cell population presented tumors (Table 2-1C, $P>0.05$ relative to neo3). This results hints that Val518 influences the CEACAM1-L-dependent tumor inhibition.

The mouse CEACAM1 intercellular adhesion domain is not involved in its tumor inhibitory effects

CEACAM1 has been shown by several groups to function as an intercellular adhesion molecule (6, 44, 45, 107). In the rat and human CEACAM1 protein, the first Ig domain is responsible for CEACAM1-dependent cell-cell aggregation (53, 108). We questioned whether the mouse CEACAM1 also used this same domain for intercellular binding. To this end, deletion mutants lacking the first Ig domain (Δ4-122/S or Δ4-122/L) (64) were transfected into NIH 3T3 fibroblasts and tested in aggregation assays (Figure 2-

3). The fibroblast cellular background was chosen for these experiments as these cells do not form aggregates, whereas the CT51 cells express the E-cadherin cell adhesion molecule (data not shown) and tend to form clumps. Parental NIH 3T3 cells or cells transfected with an empty vector were used as negative controls, whereas CEACAM1-S- and CEACAM1-L-transfected NIH 3T3 cells represented the positive controls. After 2 h incubations at 37°C, 86% of wild-type NIH 3T3 cells and approximately 90% of CEACAM1/Δ4-122/L or -/S-expressing cells were non-aggregated (Figure 2-3). Comparatively, 65% of cells expressing CEACAM1-L or 50% of those transfected with CEACAM1-S remained single (Figure 2-3). This confirms that the first Ig domain is responsible for the adhesive properties of the mouse CEACAM1.

To clarify the role of the adhesion domain in tumor growth suppression, CT51 cells expressing the N-terminal deletion mutants were introduced into BALB/c syngeneic mice. Tumor development was not inhibited by cells expressing the CEACAM1/Δ4-122/S mutant. This is consistent with the lack of significant tumor inhibition seen with the CEACAM1-S-producing cells (16%, $P > 0.05$, relative to neo3) (compare results in Table 2-1D *versus* those in Table 2-1A). On the other hand, CEACAM1/Δ4-122/L prevented tumor proliferation by 50%, also concurring with the 60% tumor inhibitory action of the full-length CEACAM1-L (Table 2-1D, $P < 0.05$, relative to neo3). Tumors expressing the CEACAM1/Δ4-122/L mutant remained small (data not shown); hence, this mutant clearly reduced tumor growth. We conclude that the N-terminal adhesion domain does not participate in the tumor inhibitory effects exhibited by CEACAM1-L.

Figure 2-3 Aggregation assay

Wild-type NIH 3T3, CEACAM1-L and -S-transfected cells as well as Δ4-122-L or -S mutant CEACAM1 cells were subjected to *in vitro* aggregation assays at 37°C as described in Materials and Methods. Individual cells or aggregates were counted at various time points and plotted as number of single cells *versus* time. Each assay was repeated in triplicate and standard deviations were computed. A representative experiment is presented.

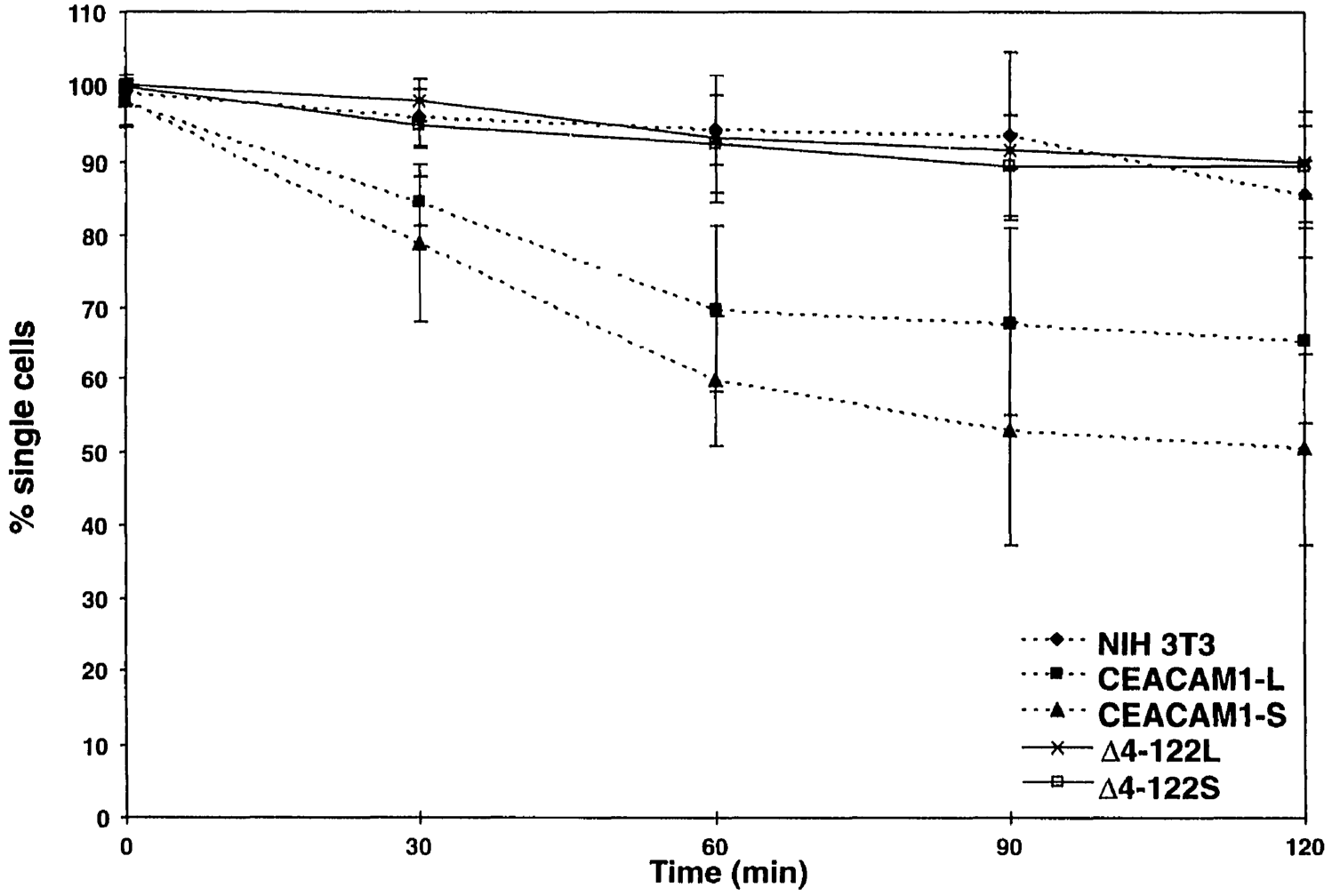

110
100
90
80
70
60
50
40
30
20
10
0
% single cells
0
30
60
90
120
Time (min)
NIH 3T3
CEACAM1-L
CEACAM1-S
Δ4-122L
Δ4-122S

Figure 2-3

Discussion

The results presented in this report focus on the motifs or domains of the CEACAM1 glycoprotein involved in inhibition of *in vivo* colonic tumor cell growth. The tumor growth inhibition role played by this glycoprotein in various diseased epithelial tissues is likely to be similar since CEACAM1 is down-regulated or absent in a number of malignancies such as intestinal, hepatic, prostate, breast, endometrial and bladder cancers (1, 26, 40, 73, 74). The evidence for its down-regulation is convincing in early stages of some cancers i.e. intestinal adenomas (38), hyperproliferative prostate tissue (28) or hepatic or breast tumors (25, 40). However, CEACAM1 down-regulation does not appear to represent an all or nothing phenomena; it appears that there may be differences in the cellular behavior of this protein, as CEACAM1 is overexpressed in at least two types of cancers i.e. lung and gastric carcinomas (42, 43). Moreover, most human colon tumor cell lines tested express CEACAM1 abundantly (3). Cell surface expression of CEACAM1 seems therefore to be actively modulated; however, the responsible mechanisms have so far remained ill-defined. In this respect, CEACAM1 may not qualify as a "classical" tumor suppressor protein.

Formisano *et al.*, 1995 have reported that the phosphorylation of CEACAM1 correlates with internalization of the insulin receptor (81). In addition, Tyr-phosphorylated CEACAM1 has been implicated in the activation of Rac1, PAK and Jun kinase in *N.gonorrhea*-activated neutrophils (88) as well as in respiratory bursts in neutrophils (83). Association of CEACAM1 with protein tyrosine kinases of the Src

family (83, 87) and with the protein tyrosine phosphatases SHP-1 and SHP-2 (79, 85) argues in favour of its involvement in signaling. CEACAM1 may either respond to or be involved in activation of a number of signal transduction cascades.

In light of this, we surmised that CEACAM1-L Tyr-phosphorylation could impact upon the role of this glycoprotein in inhibition of tumor cell growth. In our analyses, a single point mutation of the Tyr488 residue was sufficient to convert the inhibitory effect of the wild-type protein to that of a dominant tumor developing phenotype. In this case, tumors developed in all mice tested. In contrast, mutation of Tyr515 did not significantly affect the inhibition of tumor cell growth. Each CEACAM1 Tyr residue may be involved separately in intermolecular cross-talk (i.e. specific protein-protein interactions) requiring some degree of coordination for an overall effect to be produced. This would affect different signaling cascades which may antagonize each other. CEACAM1 Tyr residues may also be involved in some intramolecular interactions within the cytoplasmic domain, possibly requiring phosphorylation of the Tyr or Ser residues for effective folding of the cytoplasmic tail. The dominant tumor growth inhibitory role of Tyr488 is in complete agreement with a recent report identifying the role of Tyr488 as an ITIM motif in DT40 B cells: a chimera composed of Fcγ receptor IIB fused to the cytoplasmic domain of human CEACAM1 inhibited calcium influx in these cells, as did the wild-type receptor. The effect was abrogated by a mutation at Tyr488 and reduced in SHP-1- or SHP-2-deficient DT40 B cells (89).

Our results with Tyr488 are in contrast with those published by Luo *et al.*, 1997 (74). In that report, Tyr488 of the rat C-CAM1 homolog was mutated to a Phe and expressed into the MDA-MB-468 human breast tumor cell line using adenoviral infections. Tumorigenicity assays were performed in nude mice. In these assays, CEACAM1/Y488F behaved like the wild-type protein. Whether the discrepancies in these analyses are due to differential modulation of the protein in the breast *versus* the colonic tumor cell lines or to the amount of protein expressed at the cell surface remains to be evaluated. We have shown that truncation of the CEACAM1 cytoplasmic domain at residue 518, eliminating the three C-terminal lysines, provoked a reduction in its Tyr phosphorylation and a decrease in its association with the protein Tyr phosphatases SHP-1 and SHP-2 (85) as well as reversing the inhibition of tumor cell growth. Hence, mutations within CEACAM1 leading to abrogation or diminution of its overall Tyr-phosphorylation levels appear critical for the proliferation of cellular growth *in vivo* and the development of tumors. These results also reinforce the notion that association of the SHP-1 and/or SHP-2 Tyr phosphatases to the CEACAM1 Tyr-phosphorylated protein and their subsequent dephosphorylation of this substrate may then provoke a switch of these epithelial cells from the growth inhibitory mode to that of active proliferation.

The last residues of C-terminal end of CEACAM1 are important in tumor cell growth inhibition. Most mutations introduced within these residues (Δ518, V518A or 3K→3R) led to the development of tumors. Interestingly, this region of the cytoplasmic domain, which contains the potential ITIM sequence (pYXXV) (85), is one of the most

conserved across species (109). This region may also be involved in protein-protein interactions. A calmodulin binding site overlaps with the C-terminal end of CEACAM1-L region (76). Truncation of the last 39 amino acids of the rat CEACAM1 homolog abrogates the binding of an unknown 80 kDa protein (93). Its binding appears important for tumor cell growth inhibition. Clearly, more studies are necessary to understand the mechanisms governing tumor cell growth inhibition mediated by the CEACAM1 C-terminal region.

Our results also indicate, as previously shown with the rat and the human CEACAM1 proteins (53, 108), that the first Ig domain of the mouse CEACAM1 glycoprotein encodes the intercellular adhesion domain. However, the N-terminal domain does not apparently play a role in inhibition of colonic tumor development as a CEACAM1 deletion mutant excluding this domain retained its growth inhibition potential providing that the construct expressed the long cytoplasmic tail. A similar result has been obtained in a breast cancer model (74). Although the adhesion domain appears dispensable for the tumor inhibition phenotype, the role of the other extracellular Ig domains or the membrane-anchorage of this glycoprotein remains to be defined relative to tumor inhibition. In this respect, a parallel between the cadherins, another class of cell adhesion molecules, and CEACAM1 could be invoked to explain some of our findings. We have recently shown using a variety of techniques that the CEACAM1 long cytoplasmic domain is connected with the actin cytoskeleton in the CT51 epithelial cells. In addition, microinjections of the CEACAM1-L cDNA into confluent Swiss 3T3 fibroblasts indicate that this glycoprotein is associated with the actin cytoskeleton.

Localization of CEACAM1-L to sites of cell-cell contacts depends upon the activation of the Rho-like GTPases (94). This family of GTP-binding proteins are actively involved in modulation of cell morphology, differentiation programs and cell motility (110). The cadherins are also tightly associated with the actin cytoskeleton via the catenins (80, 111). Loss of cadherin and ß-catenin expression has been well documented in intestinal cancer (96). In fact, disruption of the cadherin-mediated signaling pathways leads to overt intestinal malignancies; these effects are highly dependent upon the sequestration of ß-catenin away from the cadherins resulting in the loss of epithelial integrity (112). As with the cadherins, it appears that cytoskeletal associations of CEACAM1-L takes precedence over its function as a cell adhesion molecule relative to tumor inhibition. Many questions will, however, need further investigation. For instance, does the down-regulation of CEACAM1 in epithelially-derived cancers early in the transformation process lead to major cytoskeletal reorganizations ? Are these events conducive to greater mobility of the epithelial cells or altered differentiation programs ? Do CEACAM1$^{-/-}$-engineered mice develop more tumors than their normal littermates ? These represent some of the issues that we are currently studying. However, one important point has already deserved some attention: expression of CEACAM1 *via* adenoviral infections in an *in vivo* prostate tumor model is maintained long after the virus has disappeared, making this protein an interesting candidate for gene therapy trials (95). This suggests that the mechanisms underlying the role of CEACAM1 in cellular growth control are crucial.

Materials and Methods

Cell Culture

The mouse CT51 colon carcinoma cells used in these experiments were a kind gift of Dr. Michael G. Brattain, Medical College of Ohio, Toledo, Ohio (113). CT51 cells were established from transplantable chemically-induced tumors in BALB/c mice. When injected subcutaneously in BALB/c syngeneic mice, these cells form tumors readily. Parental and transfectant cells were maintained in a-modified Eagle's medium supplemented with 10 % fetal bovine serum (Gibco BRL, Hamilton, Ont.), 50 Units/ml of penicillin, 50 mg/ml streptomycin at 37°C in 5% CO_2-humidified air. Transfectant cells were selected and grown in the presence of 750 μg/ml of active Geneticin (G418) (Gibco BRL, Hamilton, Ont.).

Antibodies

Polyclonal rabbit anti-mouse CEACAM1 antibodies (either serum 231 or 655) were used to detect the wild-type and mutant forms of CEACAM1. The generation of serum 231 was reported in McCuaig *et al.*, 1992 (6). Serum 655 was a generous gift of Dr. K. V. Holmes (U. of Colorado) and has previously been described (114). Drs. Kuprina and Rudinskaya (Moscow, Russia) kindly provided us with the AgB10 rat anti-mouse CEACAM1-specific monoclonal antibody used in our cytofluorometric analyses.

Site-directed mutagenesis

Point and deletion mutations of the CEACAM1-L cytoplasmic region have previously been described (85). Briefly, the Δ518 deletion mutant was created by inserting a TGA stop codon at position 519. Point mutants Y515F, Y488F, Y488,515F, V518A, 3K→3A were generated by overlap PCR mutagenesis (115). The N-terminal deletion mutants for the long and short isoform of CEACAM1 (Δ4-122/S, Δ4-122/L) were provided by Dr. Gabriela Dveskler (64).

Transfections and establishement of stable cell lines

CEACAM1-L, CEACAM1-S and all mutant CEACAM1-L cDNAs were cloned in a pLXSN vector (1) and transfected in Ψ2 packaging cells by calcium phosphate co-precipitation (116). Introduction of the cDNAs in CT51 or NIH 3T3 cells was accomplished by retroviral-mediated infections (1). G418 selection was applied on cell populations 48 hrs post infection. Following G418 selection, CEACAM1-expressing cell populations were immunoselected using the anti-CEACAM1 rabbit polyclonal antibodies (serum 231 or 655) and Dynabeads® (Dynal, Great, Neck N.Y). FACS and Western analyses were carried out to confirm the expression of CEACAM1 in the immunoselected cells.

Cytofluorometric analyses

Surface expression of CEACAM1 proteins was assesed by cytofluorometry using rat anti-CEACAM1 monoclonal antibody AgB10 and fluorescein-labelled goat anti-rat

antibody (Cappel). Expression of the CEACAM1/Δ4-122/L and /Δ4-122/S mutants was detected with serum 655. Fluorometric detections were analysed with a FACScan Becton-Dickinson program. Fold expression was determined by calculating the ratio of the median expression of cells incubated with both the primary and secondary antibodies over the median expression of cells exposed only to the fluorochrome-labelled antibody.

Western analyses

Expression of CEACAM1 proteins in protein lysates was verified by immunoblotting. Cells were lifted, pelleted by centrifugation, and resuspended in a lysis buffer (50mM Tris-HCL pH 8.0, 5mM EDTA, 1% Nonidet-P40, 150mM NaCl and 10 μg/ml of each: leupeptin, aprotinin, pepstatin, N-phenylmethylsulfonylfluoride, N-a-p-tosyl-1-lysine chloromethyl ketone, N-tosyl-1-phenylalanine chloromethyl ketone) and incubated on ice for 10 min. Lysates were centrifuged at 15,000 g for 10 min at 4^0 C. Total protein concentration was evalutated using a BCA Protein Assay kit (Pierce Chemicals, Rockford, IL). Cell lysate proteins (100 μg) were resolved on 8% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). CEACAM1 proteins were detected with either 231 or 655 antibodies at a 1:500 dilution and ^{125}I-labelled protein A. A Fuji BioImager 2000 was used for protein quantification.

Intercellular adhesion assays

Cell aggregation assays were performed using either wild-type NIH 3T3 cells or populations stably transfected with either the CEACAM1-L or -S or the Δ4-122/L or -/S

mutant cDNA constructs or an empty control vector. Cells were removed from the tissue culture dishes with a solution of PBS-citrate containing 0.125% trypsin. Single cell suspensions were produced by 3 passages through 27-gauge needles. Viability of cells was determined by trypan blue dye exclusion and shown to be > 95% in every assay. Three million cells of each transfectant clone were incubated in 3 ml of a-MEM medium containing 0.8% fetal bovine serum and DNase I (10 μg/ml) for 2 h at 37°C with constant stirring at 100 rpm; samples were retrieved at 30 min intervals and were evaluated by hemocytometer for both single cells and number of cells in aggregate. Standard deviations on the average of one representative experiment were calculated.

Tumorigenicity assays

Four million viable CT51 wild-type or transfected cells resuspended in 200 μl of α-MEM were injected s.c. in the flank of 6-8 week-old female BALB/c mice, weighing 20-22g. A minimum of 10 mice per cell line were used for each assay. Mice were checked every 5-7 days for tumor growth. The duration of the assay was 42 to 69 days depending on the experiment. Mice were sacrificed, tumors were resected, weighed and measured with calipers. Statistical analysis of our data was done as described in Kunath *et al.*, 1995.

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CHAPTER 3

GENERAL DISCUSSION

Discussion

According to the American Cancer Society, cancer accounts for approximately 23% of deaths in the United States. In 1999, 563 100 Americans are expected to die of cancer. This grim statistic makes cancer the second leading cause of death after cardiovascular disease (117). Neoplasms arise from excessive cell growth. Mutation or dysregulated expression of proteins implicated in the regulation of cell proliferation are important factors in the development of cancer (118).

CEACAM1 expression is downregulated in a number of malignancies of epithelial origin such as colon (37), liver (40), prostate (28), breast (25) and endometrial (26) carcinomas. Loss of CEACAM1 expression during the development of cancer is suggestive of a tumor inhibitory role for CEACAM1. Functional studies have demonstrated that introduction of CEACAM1 in tumorigenic carcinoma cells reduced their ability to form tumors *in vivo* (1, 73, 74). However, not all CEACAM1 isoforms are able to inhibit tumor development (1). While CEACAM1^{a}-4S, an isoform with a short cytoplasmic (10aa) region, was unable to arrest tumor development *in vivo*, CEACAM1^{a}-4L, a variant possessing a long cytoplasmic (73aa) region, efficiently reduced tumor growth (1). As the CEACAM1^{a}-4S and CEACAM1^{a}-4L isoforms only differ in their cytoplasmic region (7), the long cytoplasmic domain is believed to contain determinants crucial to the growth inhibitory phenotype. The objective of this thesis was to define the residues or motifs within the CEACAM1 long cytoplasmic region responsible for its tumor inhibitory activity.

As the CEACAM1^a-4L cytoplasmic domain comprises a number of motifs (Figure 1-5), the work presented in this thesis focused on determinants located in the C-terminal half of the cytoplasmic region. Since this portion of the glycoprotein contains two Tyr residues which have been shown to be phosphorylated *in vivo* (81, 83, 85, 87), the influence of Tyr phosphorylation in the CEACAM1^a-4L tumor inhibitory effect was investigated. Site-directed mutagenesis of Tyr488 abolished the glycoprotein's ability to arrest tumor cell growth, whereas a single point mutation of Tyr515 did not interfere with the tumor inhibitory phenotype. Interestingly, only a 30% reduction in tumor incidence was observed when both tyrosine residues were replaced by Phe. This result was surprising as a complete reversion of the inhibitory phenotype was expected. According to these results, phosphorylation of Tyr488 is crucial to the tumor suppressive activity exhibited by CEACAM1^a-4L, while phosphorylation of Tyr515 seems to be irrelevant. Tyr488 may be involved in protein-protein interactions important for the transmission of signals which result in the termination of cellular proliferation. Although Tyr515 does not appear to be directly implicated in the tumor inhibitory phenotype, its presence and phosphorylation may be required for a full tumor inhibitory effect.

The three Lys residues present at the C-terminus of CEACAM1^a-4L also contribute to the tumor inhibitory phenotype. Truncation of the terminal Lys residues results in a loss of CEACAM1^a-4L tumor suppressive activity. The effect of their deletion on the tumor inhibitory function may be related to their ability to modulate the extent of CEACAM1^a-4LTyr phosphorylation (85).

The mechanism by which the CEACAM1^a-4L phosphotyrosines mediate tumor inhibition is still unknown. Not long ago, our group has demonstrated the association of protein Tyr phosphatases SHP-1 and SHP-2 with the tyrosyl-phosphorylated ITIM motifs contained within the C-terminal region of CEACAM1^a-4L (79, 85). It has also been demonstrated that human CEACAM1-4L contains a functional ITIM motifs surrounding Tyr488 (89). Furthermore, the CEACAM1-4L ITIM is required for SHP-1 or SHP-2 to mediate inhibitory signals in DT40 B cells (89). SHP-1 is predominantly expressed in hematopoeitic cells and some epithelial cells (119) including CT51 carcinoma cells (85). The phosphatase associates with the ITIM motifs of a variety of cell surface receptors including FcγRIIB (105, 120), BCR (121), CD22 (122), KIR (123), LIR (124), and PIR-B (125). SHP-1 negatively modulates signaling pathways to which it is targeted (126). SHP-2, on the other hand, is ubiquitously expressed (127). Upon stimulation of cells, it is recruited to the plasma membrane where it associates with specific proteins (127). SHP-2 binding partners include PDGFR (128), EGFr(128), erythropoietin receptor (129), TCR (130), IR (131), IRS-1 (132), SIRP (133), PIR-B (134), KIR (135), FcγRIIB (136). Depending on the substrate tethering to SHP-2, the effect of the phosphatase on the signaling cascade can be either downregulatory or upregulatory (127).

Since SHP-1 and SHP-2 are required for the inhibitory signals mediated by CEACAM1-4L in DT40 B cells (89), the binding of SHP-1 or SHP-2 to CEACAM1^a-4L was also thought to be involved in the tumor inhibition phenotype. Mutational evidence supports the implication of SHP-1 and SHP-2 in tumor suppression mediated by

CEACAM1[a]-4L in CT51 cells: site-directed mutagenesis of Tyr488 or Val518 abolished CEACAM1[a]-4L binding of SHP-1 and SHP-2 (85) and abrogated the glycoprotein's tumor inhibitory activity (54). Moreover, CEACAM1[a]-4L's anti-proliferative phenotype and its ability to associate with SHP-1 and SHP-2 were also lost when the terminal Lys were eliminated (54, 85). However, there is also tumor incidence data obtained with other cytoplasmic mutants which argues against the participation of SHP-1 and SHP-2 in the regulation of tumor cell proliferation by CEACAM1[a]-4L. Mutation of Tyr515, necessary for SHP-1 and SHP-2 binding, did not affect the tumor suppressive activity of CEACAM1[a]-4L. Similarly, mutagenesis of both Tyr residues, which has been documented to completely abrogate recruitment of SHP-1 and SHP-2 (85), led to a partial tumor inhibition. The CEACAM1[a]-4L cytoplasmic mutant in which the C-terminal Lys were substituted by Arg residues retained its ability to associate with SHP-1 and SHP-2 (85), but failed to arrest CT51 proliferation *in* vivo (54). Clearly, from this data, it cannot be concluded with certainty that SHP-1 and SHP-2 are involved in CEACAM1[a]-4L-mediated tumor inhibition.

In order to clarify the issue, CEACAM1[a]-4L's tumor suppressive activity needs to be examined in the context of CT51 cells in which the recruitment of SHP-1 and SHP-2 by CEACAM1[a]-4L is disrupted. Several strategies may be used to assess the importance of SHP-1 and SHP-2 in CEACAM1-4L's tumor inhibitory function. Firstly, endogenous expression of SHP-1 and SHP-2 could be downregulated by antisense technology. Secondly, the involvement of SHP-1 and SHP-2 in CEACAM1-4L-mediated tumor inhibition can be studied by expressing catalytically inactive variants of the phosphatases.

Such mutants contain Cys-to-Ser substitutions in the catalytic domain which inactivate the phosphatase activity without affecting substrate binding. However, downregulation of endogenous SHP-1 and SHP-2 or overexpression of dominant negative mutants may be difficult to obtain as CT51 cells produce substantial amounts of the phosphatases. Expression of short peptides encompassing the two SH2 domains present in SHP-1 and SHP-2 may be easier to achieve. Like dominant negative mutants, the shorter peptides would bind CEACAM1^{a}-4L and prevent its interaction with functional SHP-1 and SHP-2 (85). Modulation of CEACAM1^{a}-4L's anti-proliferative activity by SHP-1 and SHP-2 may thereby be disrupted. Whether or not CT51 cells expressing wild type CEACAM1^{a}-4L and SHP-1 and SHP-2 mutants were able to produce tumors *in vivo* would determine the participation of the phosphatases in CEACAM1^{a}-4L tumor inhibition. Impeding the binding of SHP-1 and SHP-2 to CEACAM1-4L using the latter approach is by no means a perfect system. The expression of the shorter peptides will most likely affect all the signaling pathways which recruit SHP-1 and SHP-2. Therefore, it would be wise to design an inducible system which could be specific to CEACAM1^{a}-4L. On the other hand, the role of SHP-1 and SHP-2 in CEACAM1^{a}-4L's anti-proliferative activity could also be investigated by transfecting normal colonocytes with dominant negative mutants of CEACAM1^{a}-4L which are unable to bind SHP-1 and SHP-2. Such mutants include CEACAM1/Y488F, CEACAM1/Y515F, and CEACAM1/Y488,515F. Tranformation of normal colonocytes upon expression of these CEACAM1^{a}-4L mutants could be suggestive of the involvement of SHP-1 and SHP-2 in CEACAM1^{a}-4L's anti-proliferative function.

Tumor inhibition by CEACAM1[a]-4L may be due to mechanisms other than those involving tyrosine phosphorylation and recruitment of SHP-1 and SHP-2. The cytoplasmic domain of CEACAM1-4L contains a number of Ser residues which are potential sites of phosphorylation (86). Several studies have demonstrated that Ser503 undergoes phosphorylation (78, 81). As Ser503 is located in a potential PKC consensus site, phosphorylation by PKC was investigated by a member of our group. Treatment of CEACAM1-transfected CT51 cells with phorbol-12-myristate-13-actetate (PMA), a PKC activator, enhanced Ser503 phosphorylation, while treatment with the broad spectrum protein kinase inhibitor, staurosporine, decreased phosphorylation of Ser503. Interestingly, treatment with calphostine, a specific PKC inhibitor did not affect phosphorylation of Ser503 (Sadekova and Beauchemin, unpublished results). These results indicate that phosphorylation of Ser503 depends on a protein kinase activated by PKC. The identity of the kinase responsible for the phosphorylation of Ser503 remains unknown. Although it is not clearly established how it occurs, phosphorylation of Ser503 influences the tumor inhibitory effect. Site-directed mutagenesis of Ser503 abrogates CEACAM1-4L's ability to arrest tumor growth (unpublished data).

CEACAM1-4L has been reported to associate with calmodulin (CaM) both *in vivo* and *in vitro*. CaM binding may influence CEACAM1 functions, as it has already been shown to downregulate CEACAM1 self-association *in vitro* (76). Recruitment of CaM may modulate the tumor inhibitory function by obstructing the association of CEACAM1-4L with other binding proteins. In addition, the presence of CaM may regulate the activity of kinases and phosphatases associating with CEACAM1-4L (109).

Emerging evidence indicates that CaM and PKC impede each other's action as PKC phosphorylation sites and CaM binding domains are often superimposed in a growing number of substrates in neurons and other cells (137). Since PKC consensus sequences also overlap or are co-terminal to CaM binding sites in CEACAM1-4L (Figure 1-5), the tumor inhibitory function may depend on a fine balance between the action of these two proteins. C-terminal truncations of CEACAM1^a-4L which eliminated the CaM binding domains also averted the tumor suppressive function (unpublished data). However, due to the presence of many motifs in the C-terminus, the role of CaM binding in the tumor inhibition effect could not be established with certainty by deletion analysis. Point mutants specific to the calmodulin binding sites would probably be better tools to assess the participation of CaM in CEACAM1^a-4L tumor suppression.

The C-terminal half of the rat CEACAM1^a-4L cytoplasmic domain also associates with the newly identified CAP-80 protein (93). Recruitment of CAP-80 correlates with CEACAM1-4L tumor growth inhibition. Cytoplasmic mutants able to bind CAP-80 were capable of arresting tumor growth, while mutants which failed to interact with CAP-80 lacked tumor inhibitory activity (93). As nothing is known about the structure and biochemistry of CAP-80, it is impossible to speculate on a possible mechanism of action. The characterization of CAP-80 will undoubtedly offer clues to elucidate the role of this protein in tumor inhibition sustained by CEACAM1-4L.

A recent study by Hunter *et al.* has demonstrated the dimerization of CEACAM1 within rat liver membranes (138). While both CEACAM1-4L and CEACAM1-4S are capable of forming homodimers, there is no evidence of heterodimerization between the

long and short cytoplasmic variants (138). It is not clear how dimerization occurs *in vivo*. It may depend on ligand binding; however, CEACAM1 ligands have yet to be isolated. Homodimerization may result from the action of an intracellular enzyme which cross links the cytoplasmic domains. The cytoplasmic domains of mouse and rat CEACAM1-4L have been shown to be substrates for transglutaminase (139). Therefore, it is thought that higher molecular weight forms of CEACAM1-4L are formed by transglutaminase modification (139). Dimerization of the CEACAM1 cytoplasmic domains may allow cellular proteins to interact with each other and induce signaling cascades which terminate cellular proliferation.

The engagement of CEACAM1 in intercellular adhesion may induce signaling cascades which regulate cellular proliferation; therefore, tumor inhibition by CEACAM1 may be related to the glycoproteins's adhesive function. A study investigating a potential correlation between the two functions was also pursued for this thesis. The first goal was to determine the murine CEACAM1^{a}-4L adhesion domain. Previous studies demonstrated that the Ig V-like fold was responsible for both human and rat CEACAM1-mediated cell aggregation (48, 53). Aggregation assays performed with fibroblasts expressing N-terminal deletion mutants of CEACAM1 revealed that murine CEACAM1-mediated adhesion also required the Ig V-like domain. However, truncation of the adhesion domain did not interfere with the tumor suppressive effect. Similar results were obtained by Luo and co-workers (74). These authors found that rat CEACAM1^{a}-4L lacking the N-terminal domain retained its ability to inhibit the proliferation of mammary carcinoma cells (74). Taken together these results indicate that CEACAM1-4L's tumor

suppressive activity is not dependent on the protein's ability to function as a CAM. Although it is not pertinent to early tumorigenesis, loss of CEACAM1-mediated adhesion may participate in later stages of cancer progression. This possibility will be discussed below.

CEACAM1-4L has clearly been demonstrated to function as a tumor growth inhibitor in several epithelial models (1, 73, 75, 95). Furthermore, it may be involved in other processes crucial to the development of cancer. As growing tumors require increasing blood supply, angiogenesis is a critical step in cancer progression (118). The transient expression of CEACAM1 in fetal brain microvessels (29) may be suggestive of an angiogenic function for CEACAM1. A number of tumors expressing a cytoplasmic mutant of CEACAM1^{a}-4L (not identified purposely) appeared to be gorged with blood, while tumors originating from parental CT51 cells contained little blood (unpublished data). Although interesting, these findings need to be further investigated as tumors were only checked macroscopically. Tumors need to be examined at the microscopic level to determine if the presence of blood is due to the formation of new blood vessels. It may well be that the presence of blood at the surface of certain tumors is simply due to an inflammation reaction. CEACAM1^{a}-4L's anti-proliferative activity contrasts with a potential function as an angiogenic factor. However, a study by Turbide *et al.* showed that a significant overexpression of CEACAM1^{a}-4L resulted in the reversal of the tumor inhibitory phenotype (24). CEACAM1^{a}-4L's ability to promote angiogenesis may depend on its levels and sites of expression.

Metastasis is a multi-step process in which cancerous cells detach from the primary tumor, penetrate the surrounding extracellular matrix (ECM), intravasate in the circulatory system, migrate to the capillary network of target organs, extravasate from the vasculature, and establish themselves in the target tissue (118). Normal cells are attached to each other and the ECM by a adhesion molecules such as cadherins, integrins and IgCAMs (118). CEACAM1 localizes to cell-cell contacts of hepatocytes, enterocytes and other epithelial cells (19-21, 56). Downregulation of CEACAM1 during cell transformation may contribute indirectly to metastasis, as intercellular connections are loosened and cancerous cells may detach from the tumor and invade their surroundings. E-cadherin functions as a metastasis suppressor through its association with the actin cytoskeleton (80). CEACAM1-4L also interacts with polymerized actin through the C-terminal half of its cytoplasmic domain (94). Disruption of the interaction between CEACAM1-4L and the actin cytoskeleton, as a consequence of the downregulation of CEACAM1-4L during the transformation process, may result in the loss of CEACAM1-mediated adhesion, which in turn may lead to greater cell motility. Parental CT51 cells have been reported to be highly metastatic (113); therefore, it would be interesting to investigate the effects of CEACAM1 expression on the invasive potential of CT51 cells. Various cellular invasion assays may be used to evaluate the invasive and metastatic potential of CEACAM1-transfected cells. These assays include modified Boyden chambers, Matrigel outgrowths, and monolayer wound models (140).

CEACAM1-4L has convincingly been shown to act as a tumor growth inhibitor in several models (1, 73, 75, 95). Moreover, the cytoplasmic domain appears to be crucial

for this function (1). Site-directed and deletion mutagenesis demonstrated that the C-terminal half of the cytoplasmic domain played a critical role in the tumor inhibitory phenotype (54). This region binds several cytosolic proteins (79, 85, 92, 94) and contains multiple phosphorylation sites (78 , 79, 85, 86). A number of these elements are most likely involved in a complex signaling cascade regulating the tumor suppressive effect mediated by CEACAM1^{a}-4L. Characterizing the interactions between CEACAM1^{a}-4L and its cytosolic partners will help define the mechanism eliciting the tumor inhibitory phenotype.

Future Directions

The finding that CEACAM1-4L acts as a tumor inhibitor offers new possibilities for the development of cancer treatment. A pre-clinical study by Kleinerman *et al.* has already demonstrated the effective use of rat CEACAM1^{a}-4L-dependent as a gene therapy agent in the treatment of prostatic tumors in nude mice (95). Before CEACAM1-4L gene therapy approaches can be used in the treatment of human cancers, the mechanisms underlying tumor inhibition by CEACAM1-4L need to be better defined. The work reported in this thesis has pinpointed several *cis*-determinants in the C-terminal half of the cytoplasmic domain which are crucial to the tumor inhibitory activity. Future investigations on the interactions between these elements and cytosolic proteins should provide insight on the signaling cascades regulating tumor growth.

In order to assess adverse side-effects resulting from CEACAM1-4L gene therapy, future studies should address the normal biological functions of CEACAM1.

The generation of a CEACAM1-null mouse in our laboratory should, hopefully, shed some light on the functions performed by CEACAM1 in normal tissues.

Future experiments should also be designed to determine how CEACAM1-4L inhibits tumor growth. CEACAM1-4L may arrest cell proliferation or induce cell death. TUNEL assays should determine if cells expressing CEACAM1-4L are more susceptible to cell death than non-expressing cells. On the other hand, analysis of CEACAM1-4L expression during the cell cycle should provide knowledge on the action of the glycoprotein.

The complex mechanism regulating CEACAM1-4L's function as a tumor suppressor probably involves cross-talk between a number of signaling cascades. The elucidation of the pathways implicated in the modulation of tumor growth by CEACAM1-4L will undoubtedly provide new and exciting information on the role of CAM in different cellular processes.

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