



cis-Determinants in the cytoplasmic domain of CEACAM1 responsible for its tumor inhibitory function

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CEACAM1, also known 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 Immuno-receptor 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.

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

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 (Kinzler and Vogelstein, 1996; Shibata *et al.*, 1997), and proteins involved in TGF- β signaling such as Dpc4

(Smad4) (Thiagalingam *et al.*, 1996; Moskaluk and Kern, 1996). Compound mutations, such as those observed in Apc and Smad4 proteins, also have an additive effect on colorectal tumor development (Takaku *et al.*, 1998).

We and others have shown that another cell adhesion molecule identified as biliary glycoprotein 1 (BGP in mouse, Bgp1 in human), is implicated in colorectal tumor development (Neumaier *et al.*, 1993; Rosenberg *et al.*, 1993; Kunath *et al.*, 1995). BGP is a member of the carcinoembryonic antigen (CEA) family (Hinoda *et al.*, 1988). The nomenclature of this gene family has recently been redefined and the C-CAM or BGP proteins are now referred to as CEACAM1 proteins (Beauchemin *et al.*, 1999). 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 (Frängsmyr *et al.*, 1995). In addition, the CEACAM1 protein is also found at the lateral borders of enterocytes (Hansson *et al.*, 1989). However, upon cellular transformation, the CEACAM1 expression is down-regulated in colonic tissue (Rosenberg *et al.*, 1993; Neumaier *et al.*, 1993). This occurs at an early period in tumor development as intestinal adenomas (Nollau *et al.*, 1997; Ilantzis *et al.*, 1997) or stage A tumors are devoid of CEACAM1 expression (Rosenberg *et al.*, 1993). This is not unique to colonic tissue, since CEACAM1 expression is also decreased in hyperplastic prostatic and mammary glands (Kleinerman *et al.*, 1995a; Riethdorf *et al.*, 1997), in transformed hepatic tissue (Hixson *et al.*, 1985; Tanaka *et al.*, 1997) and in bladder and endometrial tumors (Kleinerman *et al.*, 1996; Bamberger *et al.*, 1998). Interestingly, this protein has also been identified by the SAGE technique as a down-regulated participant in colorectal tumor development (Zhang *et al.*, 1997). However, primary lung tumors (squamous cell carcinoma, adenocarcinoma and small cell carcinoma) and stomach tumors overexpress the CEACAM1 protein (Ohwada *et al.*, 1994; Kinugasa *et al.*, 1998). Interestingly, in metastatic lesions of these lung cancers, CEACAM1 mRNA expression is decreased compared to the primary site (Ohwada *et al.*, 1994).

The CEACAM1 gene lies on human chromosome 19q13.1-2 (Thompson *et al.*, 1991) or mouse chromosome 7 (Robbins *et al.*, 1991). There is only one human and rat CEACAM1 gene (Barnett *et al.*, 1989; Najjar *et al.*, 1993), but two very similar genes have been identified in the mouse (*Ceacam1* and *Ceacam2*) (Nédellec *et al.*, 1994, 1995). The CEACAM1 genes in all three species generate a significant number of alternative splicing variants. The extracellular domains

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Received 9 February 1999; revised 27 April 1999; accepted 27 April 1999

form typical Ig folds with one variable Ig-like domain and one to three C2-set Ig-like domains (Barnett *et al.*, 1989). 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 ten residues (identified as S). Both forms are tandemly expressed in most *CEACAM1*-positive tissues in a characteristic ratio ranging from 1-3L:7-9S (Lin and Guidotti, 1989; Turbide *et al.*, 1997).

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 (Kunath *et al.*, 1995). A major difference was noticed however, between the *CEACAM1* isoforms: only the *CEACAM1* splice variant expressing the long cytodomain possessed tumor inhibitory activity. Similar results were obtained by studying a rat *CEACAM1* variant in a prostatic PC-3 carcinoma model (Hsieh *et al.*, 1995). In addition, down-regulation 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* (Hsieh *et al.*, 1995). 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 (Turbide *et al.*, 1997).

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* favoring 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 (Rosenberg *et al.*, 1993; Ocklind and Öbrink, 1982). However, *CEACAM1* expression is lost or greatly reduced in colonic, prostatic, breast, hepatic and endometrial carcinomas (Kunath *et al.*, 1995; Hsieh *et al.*, 1995; Luo *et al.*, 1997; Tanaka *et al.*, 1997; Bamberger *et al.*, 1998). In addition, the tumor inhibitory phenotype is dependent on the long cytoplasmic domain of *CEACAM1*-L and not on the shorter variant, *CEACAM1*-S (Kunath *et al.*, 1995; Turbide *et al.*, 1997).

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 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, Δ 483 (Figure 1). *CEACAM1*/ Δ 518, in which the last three Lys residues were eliminated, was generated to determine the involvement of these residues in tumor cell growth inhibition (Figure 1). A *CEACAM1* triple point (3K \rightarrow 3R) was produced to verify if the charge of the terminal Lys residues was important in this phenotype (Figure 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 (Huber *et al.*, 1999); 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 tumor inhibition. *CEACAM1* has also been identified as an intercellular adhesion molecule (McCuaig *et al.*, 1992). 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 (Δ 4-122/S and Δ 4-122/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-*CEACAM1* polyclonal antibodies (Ab231 or 655) and Dynabeads[®]. Total *CEACAM1* protein expression was evaluated by immunoblotting analyses (Figure 2a). The *CEACAM1* proteins migrated as large heterogeneous bands due to their high content of glycosylated residues (16 Asn-linked carbohydrate moieties in the wild-type *CEACAM1* isoform). Within this large band, there appears to be two major entities (Figure 2a, first panel, Y488F or Y515F), most possibly corresponding to predominant glycoforms synthesized in this cell line. *CEACAM1*-S appeared as a band at \approx 120 kDa, whereas *CEACAM1*-L had a relative molecular weight of approximately 128 kDa. The *CEACAM1*/ Δ 4-122L and - Δ 4-122S mutants migrated as proteins of approximately 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 (Daniels *et al.*, 1996). All other mutants exhibited a relative molecular weight between 120 and 128 kDa. Radioactive quantitations of several representative experiments revealed that the *CEACAM1* proteins were overexpressed by factors ranging from 1.2–9.1 relative to the band of lowest intensity on each immunoblot

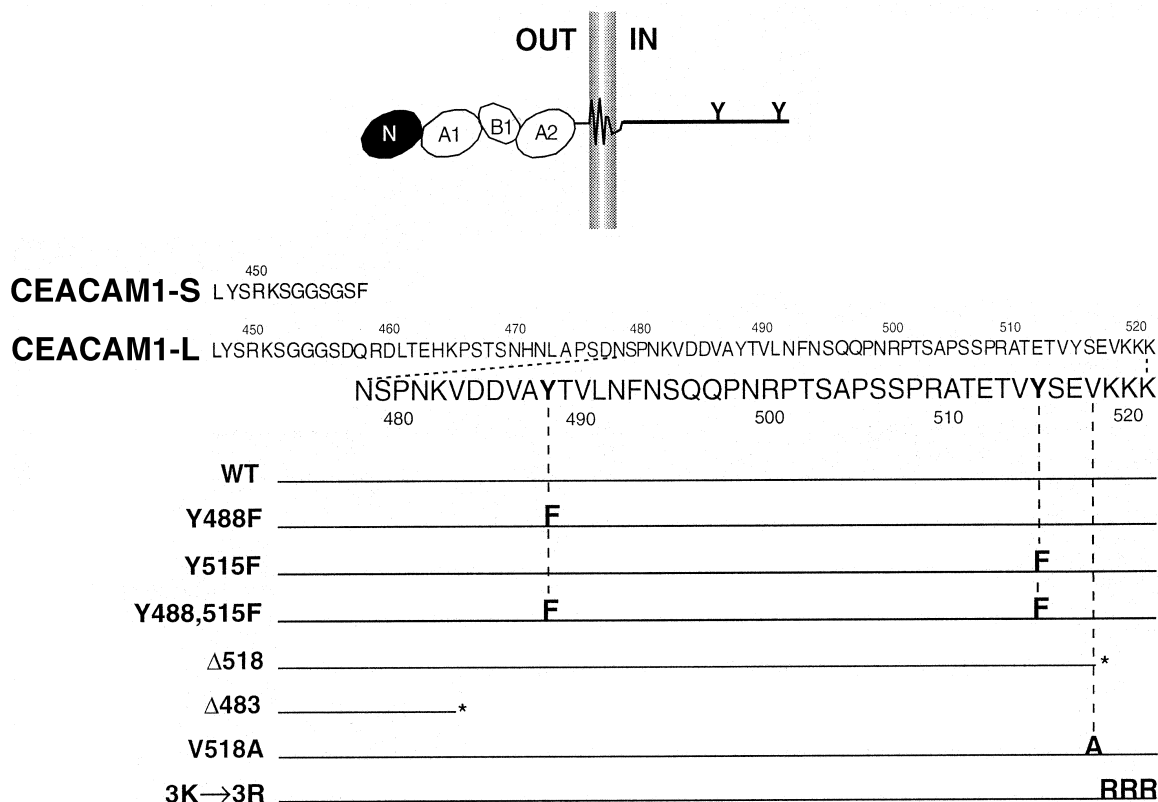


Figure 1 Schematic representation of CEACAM1 mutants used in this study. The four Ig extracellular domains of CEACAM1 are represented by ovals. The CEACAM1/ Δ 4-122 mutants eliminating the first Ig domain lack the domain indicated in black. The amino acid sequence in the one letter code representing sequences of the short (CEACAM1-S) and long (EACAM1-L) cytodomains 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

(Figure 2a). The tumor inhibitory properties of the CEACAM1 proteins may be related to their localization at the cell surface; therefore, cell surface expression of CEACAM1 proteins was estimated through cytofluorometric analyses. FACS profiles showed that the cell populations or clones used in our assays overexpressed the CEACAM1 proteins by factors ranging between 1.9 and 6.7 relative to the background fluorescence (Figure 2b). The expression levels of these mutants thus corresponded to the relative levels of expression of CEACAM1 found in normal colon (Turbide *et al.*, 1997).

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 (Turbide *et al.*, 1997). However, some discrepancies between the two different estimates were noticed with the CEACAM1/ Δ 518 and the CEACAM1/ Δ 4-122L and the / Δ 4-122S mutant proteins. It is possible that a non-negligible amount of the CEACAM1/ Δ 518 protein expressed may not reach the cell surface. As for the variation observed with the CEACAM1/ Δ 4-122L and -S mutant proteins, the specificity of antibody used (Ab655) may be responsible for the observed differences.

Tyr488 of CEACAM1 plays a major role in tumor growth inhibition

According to recent reports, CEACAM1-L participates in signal transduction events (Beauchemin *et al.*, 1997; Hauck *et al.*, 1998; Huber *et al.*, 1999). CEACAM1-L contains two consensus Immunorecep-Tyrosine-based Inhibition Motifs (ITIM) (Burshtyn *et al.*, 1996) where either Tyr residue is phosphorylated by protein Tyr kinases, such as Src in human colon carcinoma cells (Brümmer *et al.*, 1995), Lyn and Hck in activated neutrophils (Skubitz *et al.*, 1995) and the insulin receptor in hepatocytes (Rees-Jones and Taylor, 1985). CEACAM1-L Tyr phosphorylation is physiologically relevant during neutrophil respiratory bursts (Skubitz *et al.*, 1995), activation of neutrophils after binding of *N. gonorrhoea opa* proteins to CEACAM1-L (Hauck *et al.*, 1998) and in internalization of the insulin receptor (Formisano *et al.*, 1995). 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 (Beauchemin *et al.*, 1997; Huber *et al.*, 1999). 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 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). For subsequent comparisons, values obtained with the

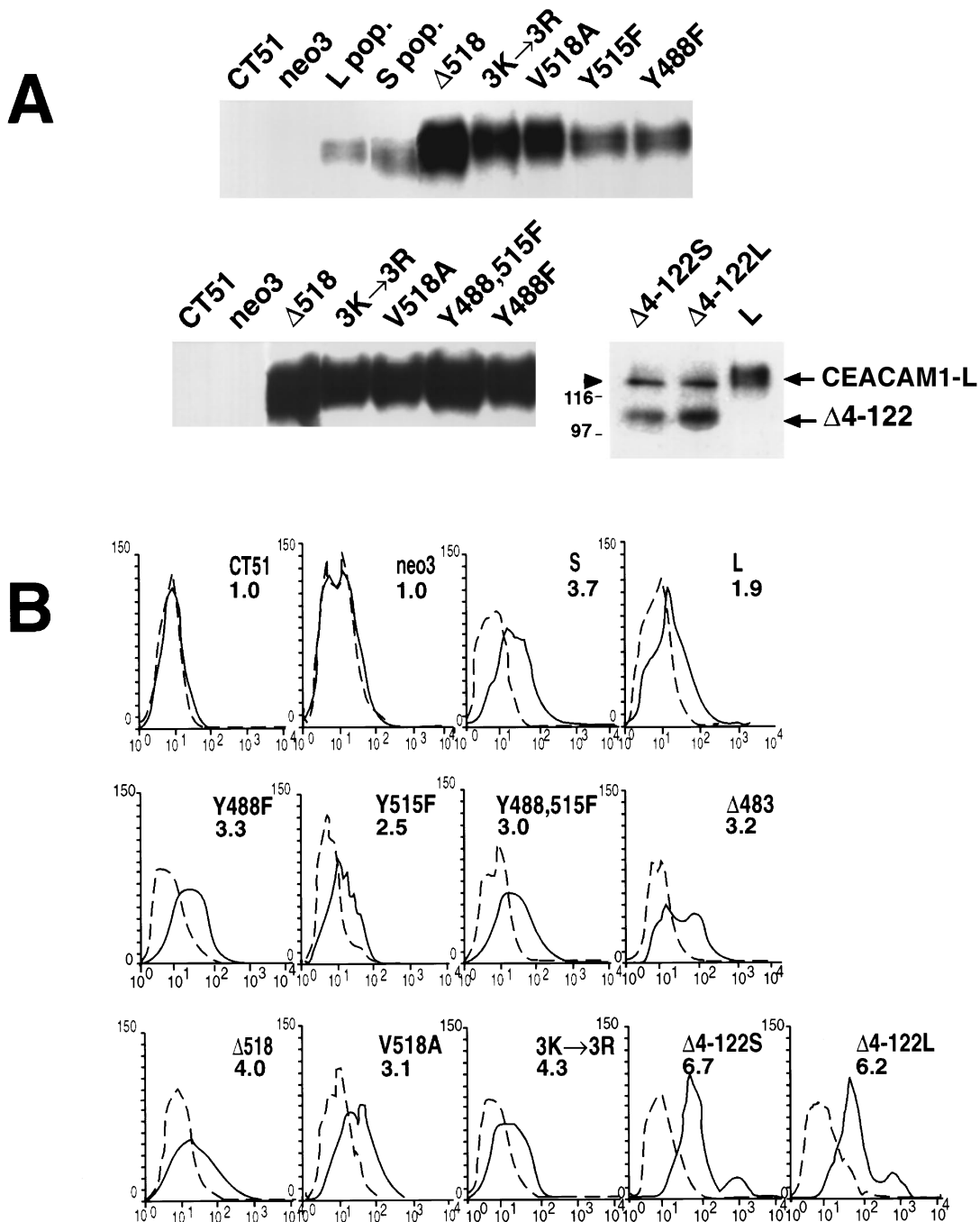


Figure 2 Expression of CEACAM1 proteins in CT51 colonic tumor cells. CT51 and neo3 are respectively wild-type and control transfected cells. CEACAM1-L represents the CT51 cell population expressing the long-tailed-CEACAM1 isoform. CEACAM1-S is a population expressing the short tailed-CEACAM1 isoform. 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 lysed 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 ¹²⁵I-labeled 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-labeled antibody only, whereas solid lines illustrate cells incubated with both primary (AgB10) and secondary antibodies

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 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 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 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/Y488,515Y) or a deletion of the region containing the Tyr residues (CEACAM1-L/ Δ 483) 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) (Lin and Guidotti, 1989; Sippel *et al.*, 1994) and a calmodulin binding site (Edlund *et al.*, 1996).

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.* (1999) have shown that the C-terminal region influences the extent of CEACAM1-L Tyr phosphorylation. The three Lys residues were removed to generate CEACAM1/ Δ 518. Deletion of the Lys residues reversed the tumor inhibition phenotype exhibited by CEACAM1-L (Table 1c). Seventeen out of 20 mice injected with cells expressing this mutant developed tumors (Table 1c, $P > 0.05$ relative to neo3). To determine if the

Table 1 Tumorigenicity assays with CEACAM1 mutants

Cell line	Experiment	Latency (days)	Incidence	Total number of tumors	Inhibition (%)	P value relative to neo3	P value relative to CEACAM1-L
<i>(a) Controls</i>							
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
<i>(b) CEACAM1 mutants related to Tyr phosphorylation</i>							
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- Δ 483	1	42	6/10	6/10	40	0.056	0.48
<i>(c) Ceacam1 mutants in the C-terminal</i>							
CEACAM1- Δ 518	1	42	8/10				
	2	48	9/10	17/20	15	0.598	0.006
CEACAM1-3K \rightarrow 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
<i>(d) CEACAM1 mutants of the N-terminal domain</i>							
CEACAM1- Δ 4-122/S	1	53	8/10	8/10	20	0.518	0.08
CEACAM1- Δ 4-122/L	1	53	5/10	5/10	50	0.015	0.87

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 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.* (1999) 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. 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 1c, $P > 0.05$ relative to neo3). This result 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 (OcklinD and Öbrink, 1982; Rojas *et al.*, 1990; Oikawa *et al.*, 1992; McCuaig *et al.*, 1992). In the rat and human CEACAM1 protein, the first Ig domain is responsible for CEACAM1-dependent cell-cell aggregation (Cheung *et al.*, 1993; Watt *et al.*, 1994). We questioned whether the mouse CEACAM1 also used

this same domain for intercellular binding. To this end, deletion mutants lacking the first Ig domain ($\Delta 4$ -122/S or $\Delta 4$ -122/L) (Dveksler *et al.*, 1993) were transfected into NIH3T3 fibroblasts and tested in aggregation assays (Figure 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 NIH3T3 cells or cells transfected with an empty vector were used as negative controls, whereas CEACAM1-S- and CEACAM1-L-transfected NIH3T3 cells represented the positive controls. After 2 h incubations at 37°C, 86% of wild-type NIH3T3 cells and approximately 90% of CEACAM1/ $\Delta 4$ -122/L- or -/S-expressing cells were non-aggregated (Figure 3). Comparatively, 65% of cells expressing CEACAM1-L or 50% of those transfected with CEACAM1-S remained single (Figure 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/ $\Delta 4$ -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 1d *versus* those in Table 1a). On the other hand, CEACAM1/ $\Delta 4$ -122/L prevented tumor proliferation by 50%, also concurring with the 60% tumor inhibitory action of the full-length CEACAM1-

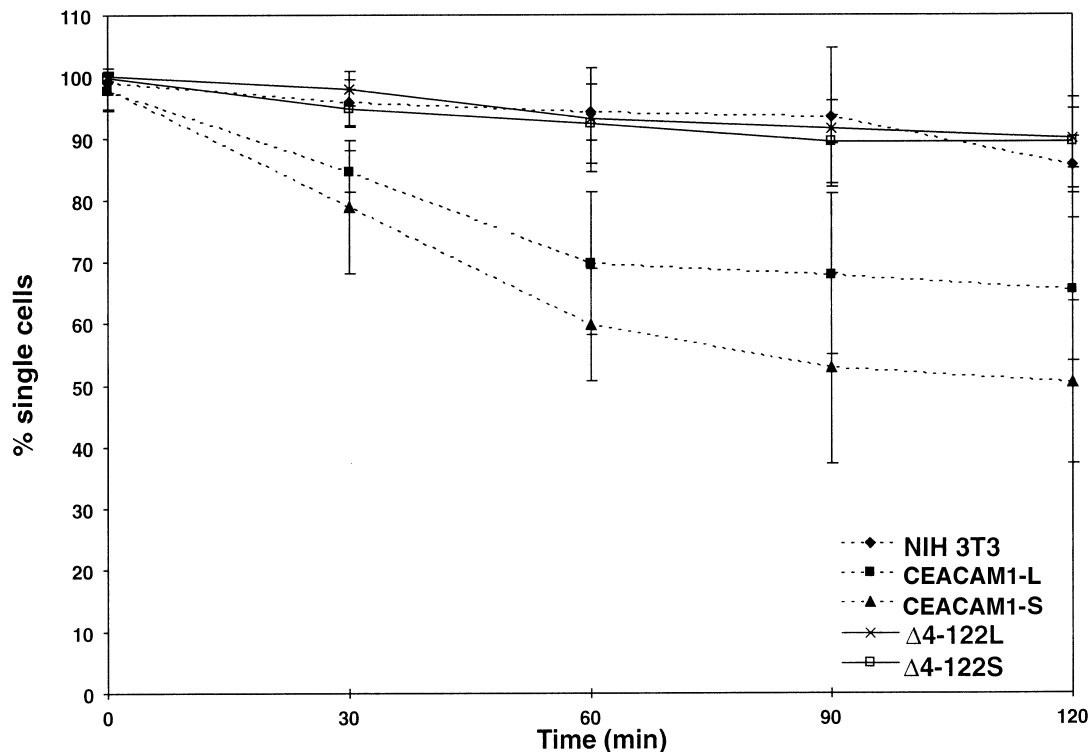


Figure 3 Aggregation assays. Wild-type NIH3T3, CEACAM1-L and -S-transfected cells as well as $\Delta 4$ -122-L or -S mutant CEACAM1 cells were subjected to *in vitro* aggregation assays at 37°C as described in Materials and methods. Individual cells or aggregates were counted at various time points and plotted as number of single cells *versus* time. Each assay was repeated in triplicate and standard deviations were computed. A representative experiment is presented

L (Table 1d, $P < 0.05$, relative to neo3). Tumors expressing the CEACAM1/ Δ 4-122/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.

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 (Kunath *et al.*, 1995; Hsieh *et al.*, 1995; Luo *et al.*, 1997; Tanaka *et al.*, 1997; Bamberger *et al.*, 1998). The evidence for its down-regulation is convincing in early stages of some cancers i.e. intestinal adenomas (Nollau *et al.*, 1997), hyperproliferative prostate tissue (Kleinerman *et al.*, 1995a) or hepatic or breast tumors (Tanaka *et al.*, 1997; Riethdorf *et al.*, 1997). 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 (Ohwada *et al.*, 1994; Kinugasa *et al.*, 1998). Moreover, most human colon tumor cell lines tested express CEACAM1 abundantly (Barnett *et al.*, 1989). 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. In addition, Tyr-phosphorylated CEACAM1 has been implicated in the activation of Rac1, PAK and Jun kinase in *N. gonorrhoea*-activated neutrophils (Hauck *et al.*, 1998) as well as in respiratory bursts in neutrophils (Skubitiz *et al.*, 1995). Association of CEACAM1 with protein tyrosine kinases of the Src family (Brümmer *et al.*, 1995; Skubitiz *et al.*, 1995) and with the protein tyrosine phosphatases SHP-1 and SHP-2 (Beauchemin *et al.*, 1997; Huber *et al.*, 1999) argues in favor 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 Fc γ 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 (Tie Chen *et al.*, submitted).

Our results with Tyr488 are in contrast with those published by Luo *et al.* (1997). In that report, Tyr488 of the rat C-CAM1 homologue 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 (Huber *et al.*, 1999) 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 (Δ 518, V518A or 3K \rightarrow 3R) led to the development of tumors. Interestingly, this region of the cytoplasmic domain, which contains the potential ITIM sequence (pYXXV) (Huber *et al.*, 1999), is one of the most conserved across species (Beauchemin and Lin, 1998). This region may also be involved in protein-protein interactions. A calmodulin binding site overlaps with the C-terminal end of CEACAM1-L region (Edlund *et al.*, 1996). Truncation of the last 39 amino acids of the rat CEACAM1 homologue abrogates the binding of an unknown 80 kDa protein (Luo *et al.*, 1998). 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 (Cheung *et al.*, 1993; Watt *et al.*, 1994), 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 prostate cancer model (Luo *et al.*, 1997). 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 CEACAM-1-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 (Sadkova, Lamarche, Li and Beauchemin, submitted). This family of GTP-binding proteins are actively involved in modulation of cell morphology, differentiation programs and cell motility (Hall, 1998). The cadherins are also tightly associated with the actin cytoskeleton via the catenins (Takeichi, 1988, 1991). Loss of cadherin and β -catenin expression has been well documented in intestinal cancer (Kinzler and Vogelstein, 1996). In fact, disruption of the cadherin-mediated signaling pathways leads to overt intestinal malignancies; these effects are highly dependent upon the sequestration of β -catenin away from the cadherins resulting in the loss of epithelial integrity (Pennisi, 1998). 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 (Kleinerman *et al.*, 1995b). 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) (Brattain *et al.*, 1980). 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 α -modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL, Hamilton, Ontario, Canada), 50 units/ml of penicillin, 50 mg/ml streptomycin at 37°C in 5% CO₂-humidified air. Transfectant cells were selected and grown in the presence of 750 μ g/ml of active Geneticin (G418) (Gibco-BRL, Hamilton, Ontario, Canada).

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. Serum 655 was a generous gift of Dr KV Holmes (University of Colorado, USA) and has previously been described (Pensiero *et al.*, 1992). 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 (Huber *et al.*, 1999). Briefly, the Δ 518 deletion mutant was created by inserting a TGA stop codon at position 519. Point mutants Y515F, Y488F, Y488,515F, V518A, 3K \rightarrow 3A were generated by overlap PCR mutagenesis (Vallejo *et al.*, 1994). The N-terminal deletion mutants for the long and short isoform of CEACAM1 (Δ 4-122/S, Δ 4-122/L) were provided by Dr Gabriela Dveksler (Dveksler *et al.*, 1993).

Transfections and establishment of stable cell lines

CEACAM1-L, CEACAM1-S and all mutant CEACAM1-L cDNAs were cloned in a pLXSN vector (Kunath *et al.*, 1995) and transfected in Ψ 2 packaging cells by calcium phosphate co-precipitation (Southern and Berg, 1982). Introduction of the cDNAs in CT51 or NIH3T3 cells was accomplished by retroviral-mediated infections (Kunath *et al.*, 1995). G418 selection was applied on cell populations 48 h 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, NY, USA). 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 assessed by cytofluorometry using rat anti-CEACAM1 monoclonal antibody AgB10 and fluorescein-labeled goat anti-rat antibody (Cappel). Expression of the CEACAM1/ Δ 4-122/L and / Δ 4-122/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-labeled 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 (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% Nonidet-P40, 150 mM NaCl and 10 μ g/ml of each: leupeptin, aprotinin, pepstatin, N-phenylmethylsulfonylfluoride, N-a-p-tosyl-l-lysine chloro-

methyl ketone, N-tosyl-L-phenylalanine chloromethyl ketone) and incubated on ice for 10 min. Lysates were centrifuged at 15 000 g for 10 min at 4°C. Total protein concentration was evaluated using a BCA Protein Assay kit (Pierce Chemicals, Rockford, IL, USA). 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 ¹²⁵I-labeled protein A. A Fuji BioImager 2000 was used for protein quantification.

Intercellular adhesion assays

Cell aggregation assays were performed using either wild-type NIH3T3 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 three 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 α-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 r.p.m.; 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.

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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 20–22 g. A minimum of ten 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 for 42–69 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).

Acknowledgments

The authors are greatly indebted to Dr Gabriela Dveksler (US University of the Health Sciences, Bethesda, MD, USA) for providing the CEACAM1/Δ4-122 mutants and Dr Kathryn V Holmes (University of Colorado, Denver, CO, USA) for the 655 serum and Dr Michael Brattain (University of Toledo, Toledo, OH, USA) for providing the mouse CT51 cells. These studies were funded by the Cancer Research Society Inc. L Izzi and T Kunath are supported by studentships from the Medical Research Council of Canada. C Houde and N Beauchemin are respectively funded by a studentship and a Senior Scholarship from the Fonds de la Recherche en Santé du Québec.

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