Structure and function of C-CAM1: effects of the cytoplasmic domain on cell aggregation

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C-CAMs are epithelial cell-adhesion molecules of the immunoglobulin supergene family with sequences highly homologous to carcinoembryonic antigen (CEA). C-CAMs and their human homologues, biliary glycoproteins, are unique among the CEA-family proteins in that they have cytoplasmic domains. Furthermore, alternative splicing generates C-CAM isoforms with different cytoplasmic domains, suggesting that the cytoplasmic domains of C-CAM may play important roles in regulating the function or functions of C-CAM. By using both sense and antisense approaches, we have shown that C-CAM1 is a tumour suppressor in prostate carcinogenesis. This observation raises the possibility that the cytoplasmic domain of C-CAM1 may be involved in signal transduction or interaction with cytoskeletal elements to elicit the tumour suppressor function. The cytoplasmic domain of C-CAM1 contains several potential phosphorylation sites, including putative consensus sequences for cyclic AMP-dependent kinase and tyrosine kinase. One of the potential tyrosine phosphorylation sites is located within the antigen-receptor homology (ARH) domain. The ARH domain of the membrane-bound IgM molecule is necessary for signal transduction in B-cells. These structural features suggest that the cytoplasmic domain of C-CAM1 may be important for signal

transduction. To test this possibility, we generated several site-directed C-CAM1 mutants and tested their ability to support adhesion and their abilities to be phosphorylated *in vivo*. Results from these studies revealed that Tyr-488 is phosphorylated *in vivo*. However, replacing this tyrosine with phenylalanine did not significantly compromise its adhesion function. Similarly, Ser and Thr residues are phosphorylated *in vivo*, but deletion of the potential cyclic AMP-dependent kinase site did not significantly reduce the adhesion function. These results suggest that the kinase phosphorylation sites in the cytoplasmic domain of C-CAM1 are not required for the adhesion function. However, these phosphorylation sites are probably involved in the regulation of C-CAM-mediated signal transduction. Thus, there are probably distinct structural requirements for the adhesion and the signal transduction functions of C-CAM. Incidentally, a C-CAM1 deletion mutant containing a 10-amino-acid cytoplasmic domain was able to support adhesion activity. This is in contrast to our previous finding that a C-CAM isoform, C-CAM3, with a 6-amino-acid cytoplasmic domain could not support cell adhesion. This result indicates that the extra four amino acids, which are absent in C-CAM3 and contain a potential Ser/Thr phosphorylation site, are important for the adhesion function.

INTRODUCTION

Cell-adhesion molecules (CAM) involved in complex intercellular interactions during organ development and differentiation are proposed to mediate these effects by providing signalling between the adjacent cells [1,2]. There is increasing evidence that adhesion mediated by the extracellular domains of CAM is the initial step in a cascade of events leading to membrane polarization and cellular differentiation [3,4]. In the case of the integrins, these post-adhesion events have been shown to involve the cytoplasmic domains of integrins [5]. Jaffe et al. [6] have also shown that specific interactions between the cytoplasmic domain of liver CAM (L-CAM) and certain cytoskeletal components are required for cell sorting and cell patterning. The cytoplasmic domain of N-cadherin was also found to be essential for embryogenesis [7].

C-CAMs are epithelial adhesion molecules of the immunoglobulin supergene family. The C-CAMs are highly homologous to carcinoembryonic antigen (CEA)-subfamily members [8]. C-CAMs and their human homologues, biliary glycoproteins (BGPs), are unique among the CEA-family proteins in that they contain cytoplasmic domains, whereas other CEA-like molecules are linked to cell membranes via phosphatidylinositol-glycan or are secreted proteins. The cytoplasmic domain of C-CAM1 contains several potential phosphorylation sites, including puta-

tive consensus sites for cyclic AMP-dependent kinase, C-kinase and tyrosine kinase phosphorylation. Furthermore, C-CAM1 also contains a typical antigen-receptor homology (ARH) domain (Figure 1). The ARH domain of the membrane-bound IgM (mIgM) is necessary for signal transduction in B-cells [9].

ARH1 motif

C-CAM1	D D S P N K V D D V S Y S V L N F N A
m MB-1	D M P D D Y E D E N L Y E G L N L D D
h MB-1	D A G D E Y E D E N L Y E G L N L D D
m B29	D G K A G M E E D H T Y E G L N I D Q
h B29	D S K A G M E E D H T Y E G L D I D Q
m TCR-ζc	E R R R G K G H D G L Y Q G L S T A T
m CD3-γ	D K Q T L L Q N E Q L Y Q P L K D R E
m CD3-δ	E V Q A L L K N E Q L Y Q P L R D R E
c T11.15	D R Q N L I A N D Q L Y Q P L G E R N

Figure 1 Comparison of the cytoplasmic sequence of C-CAM1 with the antigen receptor homology 1 motif (ARH1 motif) in several immunological receptor complexes [9]

Abbreviations used: ARH, antigen-receptor homology; BGP, biliary glycoprotein; CAM, cell adhesion molecule; C-CAM, cell-CAM105; CEA, carcinoembryonic antigen; L-CAM, liver CAM; mIgM, membrane-bound IgM; N-CAM, neural CAM.

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Blikstad et al. [10] and Edlund and Obrink [11] reported that calmodulin could bind to peptides corresponding to the sequences of the cytoplasmic domain of C-CAMs. We recently showed that C-CAM1 may function as a tumour suppressor in the progression of prostate cancer [12]. Although the mechanism by which C-CAM1 exerts its tumour-suppressive function is not clear, it is highly likely that C-CAM1 is involved in the signal transduction leading to the altered cell growth. Taken together, these observations lend support to the notion that the cytoplasmic domain of C-CAM1 may play an important role in the normal functioning of C-CAM.

We recently isolated a C-CAM isoform (C-CAM3) that has a 6-amino-acid cytoplasmic domain in contrast to the 71-amino-acid cytoplasmic domain of C-CAM1, and does not mediate adhesion [13]. This suggests that the 65-amino-acid fragment that is absent in C-CAM3 and which contains all the potential phosphorylation sites and the ARH domain might be important for C-CAM1-mediated adhesion. In this study, we examine whether kinase modification of the cytoplasmic domain is involved in C-CAM1 function. C-CAM1 mutants were constructed by site-directed mutagenesis and deletion, and their abilities to induce cell adhesion were assayed by using the insect cell system [14]. The results of this study indicated that a cytoplasmic domain as short as 10 amino acids was sufficient to produce cell adhesion and that the kinase regulatory elements of the C-CAM1 cytoplasmic domain were not required for this function.

EXPERIMENTAL

DNA constructs

C-CAM1 mutants with site-directed mutation or deletions in their cytoplasmic domains were constructed by PCR: 40 cycles of 1 min at 94 °C to denature the template DNA, 1 min at 50 °C to anneal the primers, and 3 min at 72 °C to extend the DNA. The sequences and positions of the oligonucleotides used to construct the C-terminal deletion mutants are shown in Table 1 and Figure 2(a), respectively. The nucleotides are numbered starting from the first nucleotide of the initiation methionine [15]. We made a mutation and deletions in the 3' coding region of C-CAM1 by replacing the wild-type C-CAM1 3'-end restriction fragment (0.5 kb) with mutant 3'-end fragments. These 3'-end mutant fragments were generated by PCR in three steps: (1) the 3'-end C-CAM1 DNA fragments containing the specific mutation or deletions were generated, (2) these fragments were subcloned into TA-cloning vector pCRII (Invitrogen, CA, U.S.A.) and sequenced to confirm their identity, and (3) the insert was excised from pCRII and used to replace the wild-type 3'-end through

Figure 2 Structures of C-terminal deletion mutants

(a) Generation of C-terminal deletion mutants. Arrows indicate 5' to 3' direction of oligonucleotides used for PCR. (b) Structure of mutant proteins. The Ig-like domains are labelled D1 to D4. Abbreviations: sig, signal sequence; TM, transmembrane domain; cyto, cytoplasmic domain.

Table 1 Oligonucleotides used to construct C-CAM1 mutants

The nucleotides are numbered according to Lin and Guidotti [15].

Oligo number	Position	Nucleotide sequence (5' to 3')
3	1037–1054 (sense)	CAAAGGACACTGGAGTCT
22	1452–1474 (sense)	TGACGTCTCATTCTGTCTGCTGA
23	1542–1560 (antisense)	TCACCTCTTTTGTACTAC
24	1479–1497 (antisense)	TTATTTGGATTGCTGGGCATT
25	1428–1446 (antisense)	CTACTTGTTAGGAGATCGTC
26	1399–1416 (antisense)	TCAATTGTGGCTGGAGGTGA
28	1372–1395 (antisense)	TTATTTGTGCTCTGTGAGATCTCGATG
29	1351–1374 (antisense)	TTAATGGTCACTCCCGGCCAGCTTT

restriction sites. The specific procedures for constructing each mutant are as follows.

To construct C-CAM1-F488, in which Tyr-488 was mutated to Phe-488, oligonucleotide-directed mutagenesis by PCR as described by Sarkar and Sommer [16] was performed. In the first PCR, the 3'-end 0.5 kb *PstI*/*NsiI* C-CAM1 cDNA fragment was used as the template and amplified with oligo 22, which contains the sequence to be mutated, and oligo 23, which is complementary to the last 18 nucleotides of the coding sequence. The 109 bp product was isolated and used with oligo 3 (nucleotides 1037–1054) as primers in the second PCR, in which the full-length C-CAM1 cDNA was used as the template. The 0.5 kb product (the 3'-end C-CAM1 DNA with a mutation at Tyr-488) was subcloned into pCRII, and its identity was confirmed by sequencing.

The 3'-ends of the C-CAM1 deletion mutants (C-CAM1-K499, -K482, -N472, -K465, and -H458) were generated with oligo 3 and oligos 24, 25, 26, 28, and 29 respectively, which contain the sequences complementary to the last six amino acids of each deletion mutant plus a termination codon. The full-

length C-CAM1 cDNA was used as the template. The PCR products containing the 3'-end C-CAM1 cDNA with deletions were subcloned into pCRII. After each deletion was confirmed by sequencing with a T7 Sequenase kit (United States Biochemical Corp., Cleveland, OH, U.S.A.) according to the method specified by the manufacturer, the inserts were excised from pCRII by digestion with *Pst*I and *Eco*RI.

The inserts were then used to replace the wild-type 3'-end C-CAM1 cDNA. The full-length C-CAM1 in pBS [15] was digested with *Xba*I and *Pst*I, and the 1.3 kb fragment containing the N-terminus of C-CAM1 was isolated and ligated with the mutated C-terminal *Pst*I/*Eco*RI fragments (see the previous section) into the *Xba*I and *Eco*RI sites of the plasmid pSK (Stratagene, La Jolla, CA, U.S.A.) to make pSK-F488, pSK-K499, pSK-K482, pSK-N472, pSK-K465 and pSK-H458. The entire mutant C-CAM1 cDNAs were then excised from pSK and subcloned into the *Not*I/*Eco*RI sites of the baculoviral transfer vector pVL1392 to generate plasmids pVL-F488, pVL-K499, pVL-K482, pVL-N472, pVL-K465 and pVL-H458.

Aggregation assays

The baculoviral transfer vectors containing mutant C-CAM cDNAs were introduced into the baculoviral genome by homologous recombination by co-transfecting the vector with baculoviral DNA (PharMingen, San Diego, CA, U.S.A.) according to procedures recommended by the manufacturer.

Aggregation of Sf9 cells can be assayed by infecting the cells with C-CAM1-containing vectors in either monolayer culture or suspension. In monolayer culture, C-CAM1-mediated adhesion causes Sf9 cells to form clusters of tightly attached cells [14]. In suspension culture, expression of C-CAM1 causes the formation of large multicellular aggregates that cannot be dissociated by mixing or repeated pipetting. To measure the degree of aggregation, the virus-infected Sf9 cells were grown in a test tube with constant mixing at 27 °C. At various times, aliquots of virus-infected Sf9 cells were removed, and the number of single cells in these aliquots was counted with a haemocytometer. Aggregation of Sf9 cells was characterized in terms of the decrease in the number of single cells.

Immunoblot analysis of C-CAM1 mutant proteins

Aliquots of cell lysate from infected and uninfected Sf9 cells were boiled in SDS sample buffer and analysed by SDS/PAGE [17]. Western immunoblotting with Ab669 and anti-C1, anti-C2 and anti-C3 antibodies was performed as previously described [18], except that the signal was detected with an Enhanced Chemiluminescence Kit (Amersham, Arlington Heights, IL, U.S.A.).

Phosphorylation of C-CAM1 mutants expressed in Sf9 cells

Sf9 cells in 6-well dishes were infected with recombinant viruses. At 48 h post-infection, the cells were washed three times (15 min each) with phosphate-free insect cell medium and further incubated in phosphate-free insect cell medium containing 0.35 mCi of [³²P]phosphate (1000 Ci/mmol; Amersham) in a total volume of 0.5 ml per well for 16 h. The cells were washed with PBS and extracted with 200 µl of lysis buffer (0.5 % Triton X-100, 0.3 M NaCl, 0.02 M octyl glucoside, 0.025 M sodium phosphate, pH 7.4) at room temperature for 5 min. After removal of insoluble material by centrifugation, the supernatants were diluted with immunoprecipitation buffer (150 mM NaCl, 1 mM EDTA, 0.5 % deoxycholate, 1 % Nonidet P-40, 25 mM Tris/HCl, pH 7.5) to a final volume of 1 ml and incubated with 50 µl

of Affi-gel Ab669, which was prepared by coupling Ab669 IgG to Affi-gel 10 (Bio-Rad) for 16 h at 4 °C with constant mixing. After removal of the supernatant, the gels were washed once with 0.8 ml of immunoprecipitation buffer containing 0.1 ml of saturated NaCl, then with 0.8 ml of immunoprecipitation buffer containing 0.1 % SDS, and finally with 0.8 ml of immunoprecipitation buffer. The materials bound to Affi-gel Ab669 were eluted by boiling the gels in SDS sample buffer. The eluted samples were analysed by SDS/PAGE followed by transferring the proteins on to nitrocellulose filters. The ³²P-labelled proteins were detected by exposing an X-ray film to this filter. After autoradiography, the same nitrocellulose filter was further probed with anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology Incorporated) and horseradish peroxidase-conjugated anti-mouse antibody. The phosphotyrosine-containing proteins were detected with Enhanced Chemiluminescence using a kit from Amersham (Arlington Heights, IL, U.S.A.). Finally, anti-phosphotyrosine antibody was stripped off the nitrocellulose filter by treatment with stripping buffer (2 % SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris/HCl, pH 6.7) for 1 h at 60 °C. After washing with Tris-buffered saline, the filter was further probed with Ab669, followed with alkaline phosphatase-conjugated goat anti-rabbit antibody. Proteins reactive with Ab669 were then detected with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the substrates of alkaline phosphatase.

To test whether C-CAM1 expressed in insect cells could be phosphorylated at tyrosine residues, Sf9 cells were infected with recombinant viruses. At 48 h post-infection, sodium vanadate (final concentration 100 µM) was added to the medium to inhibit the phosphatase activity. The cells were harvested at 72 h post-infection and the proteins extracted and immunoprecipitated as described above. The samples were then separated by SDS/PAGE, transferred on to nitrocellulose membrane and sequentially immunoblotted with anti-phosphotyrosine antibody and Ab669 as described above.

RESULTS

Generation and expression of mutant C-CAM1 proteins in Sf9 cells

To study the significance of the cytoplasmic domain of C-CAM1 in C-CAM-mediated adhesion, mutant C-CAM1 cDNAs with deletions or mutation in the cytoplasmic domain, as shown in Figure 2, were generated. We had previously shown that expression of wild-type C-CAM1 in Sf9 cells resulted in cell aggregation and that this aggregation could be inhibited with an antibody against C-CAM (Ab669) [19]. These observations suggested that the adhesion activity was due to direct involvement of C-CAM1 and that the Sf9 cells are suitable for structural and functional studies of C-CAM1-mediated adhesion. Therefore, we constructed C-CAM1 cytoplasmic-domain mutant cDNAs in baculoviral vectors to express the mutant C-CAM1 molecules in Sf9 cells. Western immunoblotting with polyclonal antibody Ab669, which was generated against purified C-CAM [18] (Figure 3a), showed that the mutant proteins with deletions in the cytoplasmic domain migrated slightly faster on SDS/polyacrylamide gels than did the wild-type C-CAM1 (Figure 3a). The specificities of these mutant proteins were further confirmed by using the specific anti-C-terminal peptide antibodies anti-C1 (against amino acids 460–475), anti-C2 (against amino acids 478–496), and anti-C3 (against amino acids 497–519) [18]. As predicted, wild-type C-CAM1 and CAM1-F488 were detected with all three anti-C-terminal antibodies, CAM1-K499 with anti-C1 and anti-C2, and CAM1-K482 and CAM1-N472 with

Figure 3 Immunoblot analysis of C-terminal mutant proteins expressed from Sf9 cells

The control is cell lysate from Sf9 cells. Cell lysates from cells infected with recombinant virus are indicated at the top of each lane. The lysates were analysed by immunoblotting with (a) antibody Ab669 diluted 1:1000; (b) anti-C1 antibody diluted 1:200; (c) anti-C2 antibody diluted 1:200; (d) anti-C3 antibody diluted 1:200.

Figure 4 Aggregation of C-CAM1 mutants in suspension culture

Sf9 cells cultured in suspension were infected with wild-type baculovirus or recombinant virus as indicated. At various times, aliquots were removed from the cultures and the numbers of single cells were determined. The numbers of cells are presented as the percentage of the numbers infected at time 0.

anti-C1, while CAM1-K465 and CAM1-H458 were not detected with any of the anti-(C-terminal peptide) antibodies.

Effect of mutation of Tyr-488

To study the role of Tyr-488, a potential tyrosine kinase phosphorylation site, in C-CAM1-mediated adhesion, a mutant C-CAM1 protein in which Tyr-488 was replaced with Phe (CAM1-F488) was generated. As shown in Figure 4, changing

Tyr to Phe did not significantly affect aggregation. This result suggests that Tyr-488 does not play a significant role in C-CAM1-mediated aggregation activity.

Effect of deletion of the A-kinase phosphorylation site

The possible involvement of A-kinase in C-CAM1-mediated adhesion was examined by deletion of the potential A-kinase phosphorylation site. Mutant CAM1-K499, which lacks the C-terminal 20 amino acids (which include the potential A-kinase phosphorylation site, KRPTS) produced aggregation similar to that of wild-type C-CAM1 (Figure 4), suggesting that the A-kinase phosphorylation site is not critical for aggregation activity.

Effects of deletions of exons 7, 8 and 9

The C-CAM1 cytoplasmic domain is encoded by exons 7, 8 and 9 [13]. It is possible that each exon encodes information specific for a unique function of the molecule. Therefore, we constructed a mutant containing a deletion of exon 9 (CAM1-K482) and a mutant containing a deletion of both exons 8 and 9 (CAM1-N472) to examine the contributions of exons 8 and 9 to adhesion. As shown in Figure 4, expression of CAM1-K482 and CAM1-N472 caused Sf9 cell aggregation. These results suggest that sequences encoded by exons 8 or 9 are not critical for C-CAM1-mediated adhesion.

In our previous study, we isolated a cDNA for a C-CAM isoform, C-CAM3, from a rat intestinal cDNA library. The C-CAM3 cDNA encodes a polypeptide with a truncated cytoplasmic domain that lacks the 65 most C-terminal amino acids of C-CAM1. Genomic sequence analysis showed that the C-CAM3 transcript contains an unspliced intron after exon 6. Because of the presence of the unspliced intron, which contains a stop codon immediately after the regular splice junction, translation of C-CAM3 terminates after exon 6 [13]. As a result, C-CAM3 lacks C-CAM1 exons 7, 8 and 9. We had previously shown that expression of C-CAM3, which has six intracellular amino acids, in Sf9 cells did not mediate cell adhesion. This result indicated that the cytoplasmic domain of C-CAM1 is required for C-CAM1-mediated adhesion function [13]. As deletion of both exons 8 and 9 did not abolish adhesion, it is possible that exon 7, which codes for 18 amino acids, plays a critical role for the C-CAM1-mediated adhesion activity.

Examining the peptide sequence encoded by exon 7 (GSDHRDLTEHKPSTSSHN), we noticed that the sequence is Ser- and Thr-rich (six of 18 amino acids). Furthermore, there is a Ser/Thr-rich region in the C-terminus of this peptide, PSTSSHN. It is possible that this region is a kinase phosphorylation site and plays a role in mediating adhesion. Therefore, we constructed two more deletion mutants: CAM1-K465, which contains a deletion of the Ser/Thr-rich region leaving 17 intracellular amino acids, and CAM1-H458, which lacks another Thr, thus having only 10 intracellular amino acids. In Sf9 cells, CAM1-K465 and CAM1-H458 both induced cell aggregation, although CAM1-H458 caused slightly less aggregation than did the wild-type protein. These results indicate that although the sequence encoded by exon 7 is required for cell adhesion, expression of a C-CAM with only 10 intracellular amino acids is sufficient to elicit this function.

Phosphorylation of C-CAM1 mutants

To study whether the C-terminal sequences of C-CAM1 were differentially phosphorylated, Sf9 cells were infected with C-CAM1 or mutant recombinant viruses for 48 h. The cells were then labelled *in vivo* by incubating with [32 P]phosphate for 16 h.

Figure 5 Phosphorylation of C-CAM1 mutants expressed in Sf9 cells

Sf9 cells were infected with recombinant viruses as indicated. At 48 h post-infection, the culture medium was changed to phosphate-free insect cell medium containing [32 P]phosphate and cultures were maintained at 27 °C for 16 h. The cells were washed, lysed and immunoprecipitated with immobilized Ab669 as described in the Experimental section. The materials bound to the immobilized Ab669 were analysed by (a) fluorography, and (b) Western immunoblot with Ab669.

Figure 6 Tyrosine phosphorylation of C-CAM1 mutants expressed in Sf9 cells

Sf9 cells were infected with recombinant viruses as indicated. At 48 h post-infection, sodium vanadate at a concentration of 100 μ M was added to the medium. The cells were harvested at 72 h post-infection. The cells were washed, lysed and immunoprecipitated with immobilized Ab669 as described in the Experimental section. The materials bound to the immobilized Ab669 were analysed by Western immunoblot using (a) anti-phosphotyrosine antibody, and (b) Ab669.

Immunoprecipitation with Ab669 was carried out on the detergent extracts of Sf9 cells. The samples were separated on SDS/polyacrylamide gel and transferred on to a nitrocellulose membrane. Fluorography of the blot revealed several radiolabelled proteins (Figure 5a). However, only the one with an apparent molecular mass around 75 kDa had a mobility corresponding to that of C-CAM1 proteins, was detected only in cells infected with C-CAM1 recombinant viruses and was reactive

with Ab669 (Figure 5b). These observations indicated that the 75 kDa radiolabelled protein was C-CAM1 or its C-terminal deletion mutants. This result also suggested that C-CAM1 and the mutant proteins were phosphorylated *in vivo*. Western immunoblot of the same filter with monoclonal antibody against phosphotyrosine did not reveal any reactivity (results not shown), suggesting that these phosphoproteins were not phosphorylated at Tyr under the conditions used to label the C-CAM1 proteins. This is consistent with results from phosphoamino acid analysis of the 75 kDa radiolabelled proteins, which were found to contain only phosphoserine and phosphothreonine after hydrolysis with acid and analysis with one-dimensional chromatography as described by Cooper et al. [20] (results not shown). Interestingly, mutant CAM1-H458, which has only 10 amino acids intracellularly, was also phosphorylated. This observation suggests that the Ser/Thr-rich region proximal to the membrane was phosphorylated.

The failure to detect phosphorylation at tyrosine residues may be due to the presence of phosphatases in the cells. To investigate this possibility, C-CAM1 or its mutants were expressed in Sf9 cells in the presence of a phosphatase inhibitor. At 48 h post-infection, sodium vanadate at a concentration of 100 μ M was added to the medium to inhibit the phosphatase activity. The cells were harvested at 72 h post-infection and extracted on ice for 30 min with lysis buffer. The solubilized fractions were immunoprecipitated with Ab669 immobilized on Affi-gel 10 beads. Bound components were eluted with SDS/PAGE sample buffer, separated on SDS/PAGE, and immunoblotted with anti-phosphotyrosine antibody. As shown in Figure 6(a), only C-CAM1 and CAM1-K499 showed positive reactivities with anti-phosphotyrosine antibody. In contrast, CAM1-F488, CAM1-K482, CAM1-N472, CAM1-K465 and CAM1-H458 were negative. These results showed that C-CAM1 expressed in insect cells could be phosphorylated at tyrosine residues and this phosphorylation was detectable only if the tyrosine phosphatase activities present in the cell were inhibited by vanadate prior to cell lysis. It is interesting to note that CAM1-K499, which contains Tyr-488 but not Tyr-513 in its cytoplasmic domain, was reactive with anti-phosphotyrosine antibody. In contrast, CAM1-F488, which contains Tyr-513 but not Tyr-488 in its cytoplasmic domain, was not reactive with anti-phosphotyrosine antibody. These observations suggest that among the two tyrosines present in the cytoplasmic domain, only Tyr-488 is phosphorylated.

Taken together, these results suggest that C-CAM1 expressed in Sf9 cells is phosphorylated at both serine/threonine and tyrosine residues.

DISCUSSION

We examined the role of C-CAM1 C-terminal domain in adhesive function by studying the ability of mutant proteins to confer the adhesive phenotype to Sf9 insect cells. In addition, involvement of potential phosphorylation sites in C-CAM-mediated adhesion was also investigated. Results presented here indicate that phosphorylations of these potential sites are not required for the adhesion function of C-CAM1. Thus, phosphorylation regulation might be involved in other functions (e.g. signal transduction) of C-CAM1. In a previous report [13], the importance of the cytoplasmic domain was demonstrated by the absence of aggregation after expression of C-CAM3, an isoform containing only the first six amino acids of the C-CAM1 cytoplasmic domain. This suggested that the C-terminal 65 amino acids were functionally important. In the present studies, examination of several additional cytoplasmic domain mutants of C-CAM1 indicated that the first 10 amino acids of the cytoplasmic domain

Table 2 Sequence comparison of the cytoplasmic domains of several C-CAM/BGP proteins

Abbreviations: TM, transmembrane; cyto, cytoplasmic.

Sequence		Species	Isoform	Adhesion	Reference
TM domain	Cyto domain				
FLHF	GKTGSSGPLQ	Human	BGPZ	Not determined	[24]
FLHF	GKTGRASDQ..	Human	BGP _a and BGP _b	+	[41]
FLYS	RKTGGGSDH..	Rat	C-CAM1	+	[14]
FLYS	RKTGGGSDH	Rat	C-CAM1-H458	+	This study
FLYS	RKTGGSGSF	Rat	C-CAM2	+	*
FLYS	RKTGG	Rat	C-CAM3	—	[13]
FLYS	RKSGGSGSF	Mouse	mmCGM1a and 1b	+	[21,22]

* (S.-H. Lin and D. C. Hixson, unpublished work).

were sufficient to confer the adhesive phenotype to Sf9 cells, suggesting that one or more of the four amino acids between Gly-454 and Arg-459 is important for the adhesion function.

The importance of these four amino acids in conferring an adhesive phenotype is consistent with previous studies demonstrating the adhesive activities of mouse BGPs, mmCGM1a and mmCGM1b, which also have 10-amino-acid intracellular domains [21,22]. Interestingly, C-CAM2, which has a cytoplasmic domain identical to that of mmCGM1a and mmCGM1b (Table 2) was unable to induce adhesion in insect cells [14]. Although the reason for this apparent discrepancy is not clear, we recently found that C-CAM2 was able to induce cell aggregation when expressed in COS or human PC3 prostate carcinoma cells (D. C. Hixson and S.-H. Lin, unpublished work).

Clues to the possible significance of the four amino acids that distinguish C-CAM3 and the mutant C-CAM1-H458 are suggested by comparison in Table 2 of the cytoplasmic domain sequences of rat C-CAM [13,23], human BGP [24], and mouse BGP [21,25]. Each of the four sequences contains an (A/G)S dipeptide downstream from one or more basic amino acids, a configuration that constitutes a potential phosphorylation site. On the N-terminal side of the (A/G)S dipeptide is a highly conserved triplet, K(T/S)G, which is another possible site for phosphorylation. We had previously found that C-CAM2 was phosphorylated in hepatocytes [23], indicating that one or both of these potential sites is phosphorylated. Taken together, these observations suggest that the terminal four amino acids of mutant C-CAM1-H458 may either contain an essential site of phosphorylation or may influence phosphorylation at Thr-452.

Shaw et al. [26] identified several residues crucial for the antigen-presenting or signal-transducing activities of mIgM. These investigators showed that both activities were lost after deletion of the 3-amino-acid cytoplasmic domain of mIgM and suggested this might reflect an inability of mIgM to associate with IgM β/γ dimers. By analogy, the loss of the GSDH sequence from the C-CAM1 cytoplasmic domain may directly or indirectly affect its interactions with cytoplasmic accessory molecules. Relevant in this regard is a recent report by Edlund and Obrink [11] that the cytoplasmic domains of both C-CAM1 and C-CAM2 can bind calmodulin. In addition, Lim et al. [27] reported associations between C-CAM and a 65 kDa cytoplasmic protein in the annexin family of calcium-binding proteins. In addition to interactions with cytoplasmic accessory molecules, CAMs may also interact with cytoskeletons. Interactions between the 72-amino-acid C-terminal domains of cadherins and cytoskeletal proteins have been well documented and removal of the

cytoplasmic domains of cadherins abolishes cell adhesion [28]. However, linkage with the cytoskeleton does not appear to be a general requirement for CAMs. Deletion of the cytoplasmic domain, for example, does not compromise cell-cell adhesion by homophilic binding of receptor tyrosine phosphatases [29,30]. This also appears to be true for neural CAM (N-CAM), an immunoglobulin-like CAM that exists in both transmembrane and phospholipid-linked forms [31], and for several CAMs in the CEA family that are attached to the cell surface by a phospholipid linkage [32,33].

Cytoplasmic domains are also known to play a central role in sorting of membrane proteins into vesicles that are transported to basolateral or apical membrane domains and in correct targeting of proteins following endocytosis. In hepatocytes, both of these processes are critical during the transcytosis of proteins destined for the apical membrane domain. Recent evidence indicates that delivery to the correct membrane domain depends on specific sorting signals [34–37]. For polymeric Ig receptor (pIgR), a major site of serine phosphorylation within the cytoplasmic domain is thought to be a critical determinant for entry into the transcytotic pathway [35]. Unphosphorylated receptors or receptors with an Ala-for-Ser substitution at this site bind ligand and are internalized with the same kinetics as phosphorylated receptors but do not get transported efficiently to apical membranes and remain stranded in cytoplasmic transport vesicles. If the serine in the terminal four amino acids of the C-CAM1-H458 mutant and its counterparts in C-CAM2 and BGP perform a similar role, loss of these four amino acids may result in endocytosis without recycling, thereby effectively reducing the surface levels of C-CAM or the stability of any homophilic contacts that may form. This possibility is consistent with the high levels of immunoreactive C-CAM3 observed in the cytoplasm of some infected Sf9 cells that have been permeabilized before staining (S.-H. Lin, unpublished work).

Although evidence presented here clearly shows that an intact cytoplasmic domain is not required for adhesion, demonstration of both Ser/Thr and Tyr phosphorylations suggests that the cytoplasmic domain of C-CAM1 may play a role in transmitting signals from the extracellular domain. The presence of an antigen recognition activation motif (ARH1, also named ARAM or TAM) is of particular significance in this regard because this motif is found in many of the non-antigen binding components of the T-cell receptor and B-cell receptor antigen recognition complexes [38]. Mutational analysis has shown that this motif is both necessary and sufficient for coupling these receptors to early and late signalling events, most likely by acting as a tyrosine

kinase docking site or a site for tyrosine phosphorylation [38,39]. We have previously shown with pulse-chase studies that C-CAM1 was rapidly phosphorylated on both Ser/Thr and Tyr residues (Figures 5 and 6) [23]. Results obtained in the current study using C-CAM mutants corroborate this finding. Interestingly, although phosphorylations at Ser/Thr and Tyr residues do occur *in vivo*, mutations at these phosphorylation sites did not significantly affect the adhesion function. These results show that there is an apparent distinction at the structural level between the cytoplasmic subdomains involved in signal transduction and in cell adhesion, a finding that parallels the dissociation in the structural requirement for antigen presentation and signal transduction by the B-cell receptor [26].

The end points for post-adhesion signals transmitted by the cytoplasmic domain of C-CAM1 remain to be determined. Studies by Jaffe et al. [6] showed that the cytoplasmic domains of CAMs such as L-CAM and N-CAM are important for 'sorting out' of cells but not for their aggregation. In their studies, Jaffe et al. [6] found that chimeric adhesion molecules containing the L-CAM extracellular domain linked to the N-CAM cytoplasmic domain were able to induce aggregation but were unable to direct the sorting out of transfected and untransfected cells, a process that was carried out at high efficiency by L-CAM transfectants. Whether the C-CAM1 cytoplasmic domain plays a similar role remains to be determined. On the basis of extensive immunohistochemical analysis at the light- and electron-microscopic levels, Mowery and Hixson [40] speculated that C-CAM1 and 2 were directly involved in the early stages of bile canalicular morphogenesis, a functional role analogous to that proposed for uvomorulin and one that would be likely to rely on post-adhesion signalling to initiate the subsequent cascade of events involved in morphogenesis. We also recently showed that C-CAM1 expression suppressed the tumorigenicity of prostate carcinoma cells [12], a process that also requires the initiation of signal cascades affecting cell growth and proliferation.

Although the insect expression system is a valuable model for testing the structural requirements for adhesive activity, the analysis of post-adhesion events involving signal transduction such as membrane polarization and decreased cell growth or tumour suppression requires the induction of stable expression in mammalian cells. Optimal levels of expression and cell viability occur in baculovirus-infected insect cells between 48 and 72 h after infection, followed by a rapid rise in cytotoxicity and cell death 72–96 h after infection, which precludes the possibility of studying long-term interactions. In addition, it is not clear whether insect cells contain the factors required for C-CAM1-mediated signal transduction.

In summary, this report extends our previous studies of C-CAM3, showing the importance of the cytoplasmic domain for C-CAM1 adhesive activity. The results demonstrate a structural distinction between the subdomains essential for adhesion and those needed for signal transduction. In addition, we identified a 4-amino-acid segment that appears to be necessary for adhesion and, based on analogies to other receptors, delineated several additional sites in the cytoplasmic domain, Thr-452 and Ser-456, for more detailed mutational mapping.

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