Structure and Function of C-CAM1

THE FIRST IMMUNOGLOBULIN DOMAIN IS REQUIRED FOR INTERCELLULAR ADHESION*

(Received for publication, April 22, 1993, and in revised form, July 8, 1993)

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Cell-CAM105 proteins (also called C-CAM) are epithelial cell adhesion molecules of the immunoglobulin (Ig) superfamily. The sequences of C-CAM are highly homologous to those of human carinoembryonic antigen (CEA)-family proteins. In previous studies using baculoviral vectors, we showed that expression of the L-form cell-CAM105 (also called C-CAM1) in insect cells resulted in cell aggregation (Cheung, P. H., Thompson, N. L., Earley, K., Culic, O., Hixson, D., and Lin, S. H. (1993) J. Biol. Chem. 268, 6139–6146). This result indicates that the insect-cell system is suitable for studying the adhesion function of C-CAM. Since C-CAM1 contains four extracellular Ig-domains, the structural features directly responsible for C-CAM1 adhesion function were investigated by site-directed deletion and expression in the baculovirus/insect cell system. Results from these studies indicated that the first Ig domain located in the NH2-terminal of C-CAM plays a crucial role in intercellular adhesion. Site-directed deletion producing mutants lacking the second, third, or fourth Ig domains had no effect on the adhesion function. In addition, adhesion function was retained when both the third and fourth Ig domains were deleted, although the adhesion activity was reduced to half that in control cells. However, simultaneous deletion of the second, third, and fourth domains abolished adhesion, suggesting that these domains affect the accessibility of the binding site localized in the first domain. In our previous studies, we showed that the cytoplasmic domains of C-CAM play a significant role in the isoforms' adhesion activity since expression of a C-CAM isoform containing only 6 instead of 71 amino acids intracellularly failed to show the adhesion phenotype (Cheung, P. H., Culic, O., Qiu, Y., Earley, K., Thompson, N., Hixson, D. C., and Lin, S. H. (1993) Biochem. J. 295, in press). These results together suggest that both the cytoplasmic domain and the first N-terminal Ig-like domain are required for C-CAM-mediated cell adhesion activity.

* This work was supported in part by the National Institutes of Health Grant HL08898 (to Guido Guidotti, Harvard University) and by the National Institutes of Health Grant GM43189 (to S.-H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04963.

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‡‡ The abbreviations used are: Ig, immunoglobulin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; CEA, carinoembryonic antigen; NCA, nonspecific cross-reacting antigen; kb, kilobase(s).

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aries based on previous assignment (Lin et al., 1991). Domain 1 is defined as amino acids 38–139 (QVT ... QFR), domain 2 as 140–232 (VYP ... FNL), domain 3 as 233–317 (DV1 ... KNI), and domain 4 as 318–408 (TVF ... IKL). All deletion mutants were constructed in baculoviral transfer vectors pVL1393 or pVL1395 (Invitrogen). Fig. 1 shows the relative positions of oligonucleotides synthesized for construction of the deletion mutants. Table I shows the nucleotide sequences of the oligonucleotides used for PCR. The nucleotides were numbered according to Lin and Guidotti (1989) by assigning the first nucleotide of the initiation methionine position number 1.

To construct the baculoviral vector containing C-CAM1 from which the first Ig domain was deleted (pVL-AD1), a synthetic oligonucleotide (oligo 9), complementary to nucleotide sequence 95–114 plus 421–445, was used to "loopout" the first domain. A synthetic oligonucleotide (oligo N) containing the 5′ untranslated sequence (–18 to –1) was synthesized to use as a primer for the NHEJ-terminal portion of the sequence. In the first PCR, a 0.38-kb C-CAM1 cDNA fragment (–80 to +313, EcoRI/BamHI fragment) was used as template and amplified by using oligo 9 and oligo N as primers to produce a 157-bp product. This product was isolated and used as a primer in the second PCR. In the second PCR, plasmid containing 1.5-kb C-CAM1 cDNA (+313 to +1857, BamHI fragment) was used as template and amplified by using oligo 11 and oligo 6 (nucleotide sequence 437–454) as primers and the 1.15-kb EcoRI/SacI (–300 to +851) fragment as template and amplified by using oligo 9 and oligo N as primers to produce a 157-bp product. This product was isolated and used as a primer in the second PCR. In the second PCR, plasmid containing 1.5-kb C-CAM1 cDNA (+313 to +1857) was subjected to a filling-in reaction, using T4 DNA polymerase with all four deoxynucleotides, and then was further digested with EcoRI. The resulting 1.5-kb ΔD1 fragment was subcloned into pVL1393 at SmaI/EcoRI sites to generate plasmid pVL-AD1.

To construct the baculoviral vector containing C-CAM1 from which the second Ig domain was deleted (pVL-ΔD2), synthetic oligonucleotide oligo 10, complementary to nucleotide sequence 400–417 plus 694–719, was used to loopout the second domain. Oligo 10 and oligo N were used in a PCR in which a 0.74-kb XbaI/NcoI (–244 to +467) fragment functioned as the template to produce a 460-bp product. The 460-bp fragment was subcloned into PCR1000 plasmid to produce pCR-ΔD2, and the nucleotide sequence of the double-stranded DNA was determined. No nucleotide substitution was found. Oligo 10 contains a BspEI site that was used to facilitate further subcloning. The 460-bp PCR product was excised from pCR-ΔD2 using EcoRI and BspEI. The resulting 0.46-kb EcoRI/BspEI fragment was ligated with the 0.88-kb BspEI/Nael fragment (the COOH-terminal part of C-CAM1) and subcloned into pVL1393 at EcoRI/PstI sites to generate pVL-ΔD2.

Baculoviral vector containing C-CAM1 with deletion of the third Ig domain (pVL-ΔD3) was constructed as follows. A synthetic oligonucleotide (oligo 11), complementary to nucleotide sequence 676–693 plus 949–970, was used to loopout the third domain. The first PCR fragment was produced by using oligo 11 and oligo 6 (nucleotide sequence 437–454) as primers and the 1.15-kb EcoRI/SacI (–300 to +851) fragment as the template to produce a 278-bp product. The second PCR fragment was produced by using oligo 12 (nucleotide sequence 949–970) and oligo TM (complementary to nucleotide sequence 1252–1281) and the SacI/EcoRI (+851 to +1857) template to produce a 335-bp product. The two PCR fragments were mixed, denatured, annealed, and extended in a third PCR for five cycles at 94 °C for 2 min, 55 °C for 3 min, and 72 °C for 4 min. Without working up, oligo 6 and oligo TM were then added to the reaction mixture of the third PCR, which was subsequently continued for an additional 40 cycles. The 580-bp PCR product was isolated and subcloned into pCR1000 plasmid to produce pCR-ΔD3, and the nucleotide sequence of the double-stranded DNA was determined. One nucleotide substitution, A to T at position 951, was found. However, this substitution did not result in any amino acid change. The 580-bp frag-

Fig. 1. Schematic diagram of mutant C-CAM molecules. Ig-like domains are labeled D1 to D4. Arrows indicate 5' to 3' direction of oligonucleotides used for PCR. sig, signal sequence; TM, transmembrane domain; cyto, cytoplasmic domain.
ment was digested with NcoI and PstI. The resulting 366-bp NcoIPstI fragment was isolated and inserted into a plasmid (pBSfull) containing the full-length C-CAM1 cDNA with deletion in the region between the NcoI and PstI sites. This resulted in plasmid pBS-ΔD3, which contained full-length C-CAM1 cDNA minus the third Ig domain. Plasmid pBS-ΔD3 was digested using Xbal/Nsal and the resulting 1.6-kb fragment isolated and subcloned into pVL1392 at Xbal/PstI sites to generate pVL-ΔD3.

To construct the baculoviral vector containing C-CAM1 from which the fourth Ig domain was deleted (pVL-ΔD4), oligo 13, complementary to nucleotide sequence 931–948 plus 1225–1246, was synthesized. In the first PCR, a 1.4-kb C-CAM1 cDNA fragment (316 to +1088, EcoRI/PstI fragment) was used as the template and amplified with oligo 13 and oligo 4 (387–854) as primers to produce a 134-bp product. This product was then isolated and used as a primer in the second PCR. In the second PCR, a 0.77-kb C-CAM1 cDNA fragment (+1089 to +1857, PstI/EcoRI fragment) was used as the template and amplified by using the 134-bp first PCR product and oligo A (complementary to nucleotide sequence 1745–1766) as primers to generate a 654-bp product. The 0.65-kb PCR product was subcloned into plasmid pCR1000 to produce pCR-ΔD4, which was ligated with a 0.38-kb PstI/BamHI fragment containing the NHE-terminus portion of the sequence to generate plasmid pBS-ΔD234. The resulting C-CAM1 cDNA, with domains 2, 3, and 4 deleted, was excised from pBS-ΔD234 and subcloned into pVL1392 at PstI/EcoRI sites to generate plasmid pVL-ΔD234.

**Table I**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Position</th>
<th>Nucleotide sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo A</td>
<td>1745–1766 (antisense)</td>
<td>TTTAATTAACAGCGAGACGTCT</td>
</tr>
<tr>
<td>Oligo N</td>
<td>–18 to –1 (sense)</td>
<td>TACGAGAACAGCAGAAGCT</td>
</tr>
<tr>
<td>Oligo TM</td>
<td>1252–1281 (antisense)</td>
<td>TGGCAATTGCGCTTTAAGGAGCTAG</td>
</tr>
<tr>
<td>Oligo 4</td>
<td>837–854 (sense)</td>
<td>ATCCCTCCAAAGCATCTT</td>
</tr>
<tr>
<td>Oligo 7</td>
<td>437–454 (antisense)</td>
<td>GGGCAACTGTCACGAGGT</td>
</tr>
<tr>
<td>Oligo 9</td>
<td>237–254 (sense)</td>
<td>TCCAGATATATAAGTAC</td>
</tr>
<tr>
<td>Oligo 10</td>
<td>95–114+421–445 (antisense)</td>
<td>CGTGAGCTCTCTAGTGCAATGGCACC</td>
</tr>
<tr>
<td>Oligo 11</td>
<td>400–417+694–719 (antisense)</td>
<td>GTGTTGGATCAGGTATTACGTCAATGTTCTTGACTGTGGTGAGCT</td>
</tr>
<tr>
<td>Oligo 13</td>
<td>678–693+949–970 (antisense)</td>
<td>TTGAGGATGGGATGGAGGTTT</td>
</tr>
<tr>
<td>Oligo 12</td>
<td>949–970 (sense)</td>
<td>AGGTGCTCAATGGCACC</td>
</tr>
<tr>
<td>Oligo 14</td>
<td>931–948+1225–1246 (antisense)</td>
<td>GTGTTGGATCAGGTATTACGTCAATGTTCTTGACTGTGGTGAGCT</td>
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</tbody>
</table>

**Structure and Function of C-CAM1**

**Immunoblot Analysis of C-CAM1 Mutant Proteins Expressed in Sf9 Cells**—Aliquots of cell lysate from infected or uninfected Sf9 cells were boiled in sodium dodecyl sulfate sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Western immunoblotting using Ab669 (Lin et al., 1991) was performed as previously described (C-CAM1 cDNA, 1993).

**Immunofluorescence**—Sf9 cells grown on coverslips coated with polylysine (Sigma) were infected with recombinant viruses. After 48 h, the cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. The cells were then treated in PBS with or without 0.1% Triton X-100 for 10 min. The cells on coverslips were incubated with Ab669 at 1:300 dilution in PBS, 0.1% bovine serum albumin. The coverslips were washed three times with 5 ml each in PBS. The coverslips were then incubated with fluorescein-conjugated goat anti-rabbit secondary antibody for 45 min at room temperature. The coverslips were washed three times with 5 ml each in PBS, 0.1% bovine serum albumin. Staining of Sf9 cells with antibodies without fixation, Sf9 cells grown on coverslips were infected with recombinant viruses. After 48 h, the cells were incubated with Ab669 at 1:300 dilution in insect-cell medium containing 10% fetal bovine serum for 45 min at room temperature. The cells were washed three times with the insect-cell medium and further

**Adhesion Assays**—Cell-adhesion assays were performed by two methods. In the first method, Sf9 cells in monolayer culture were infected with recombinant viruses or wild-type baculovirus. Formation of cell aggregates was examined at 72 h after infection. In the second method, adhesion was quantified as decreases in the number of single cells. Sf9 cells in suspension were infected with recombinant viruses or wild-type baculovirus. The virus-infected Sf9 cells were cultured in a test tube with constant mixing at room temperature. In control experiments, we have observed that the constant mixing did not prevent aggregation during the time permitted for expression. The adhesion in the suspension culture occurred before the removal of aliquots for analysis. At various time intervals, aliquots of control Sf9 cells or virus-infected Sf9 cells were removed and treated with 50 units/ml of DNase (Promega, Madison, WI) to digest the DNA released during cell lysis. The numbers of single cells in these aliquots were counted using a hemocytometer.

The effect of anti-C-CAM antibody on adhesion was tested by addition of different amounts of IgG from control or Ab669. The IgG fraction of the serum was prepared by passing the anti serum through a DEAE-Affi-Gel blue (Bio-Rad) column according to the procedures provided by the manufacturer. The concentration of IgG was determined by the method of Peterson (1977) using bovine serum albumin as a standard. Different amounts of the IgG from Ab669 or control serum were added at 24 h post-infection, and the number of single cells were determined at 72 h post-infection.
incubated with fluorescein-conjugated goat anti-rabbit secondary antibody (1:250 dilution) for 45 min at room temperature. The antibodies were removed and trypan blue (1:300 dilution) was added to the cells. The excess trypan blue was removed, and fluorescence photomicrographs were taken using the Nikon fluorescence microscope with two Nikon photo cubes, B2E and G2E.

RESULTS

Length of 5' Noncoding Sequence Does Not Significantly Affect the Expression—Because the restriction sites used for the construction of C-CAM1 mutant varied, the 5'-untranslated regions in these constructs contained different numbers of nucleotides. To test whether the differing lengths of these 5'-untranslated sequences affected the efficiency of protein expression, two baculoviral transfer vectors containing C-CAM1 cDNA sequences with different 5'-noncoding regions were constructed. In the first construct, the full-length C-CAM1 cDNA was excised from plasmid pBS/full using restriction enzymes XbaI/NcoI. This full-length cDNA contains 230 bp of nucleotides in front of the initiation codon. In this 230-bp region, there exist three out-of-frame ATG sequences, which may or may not interfere with the expression efficiency. In the second construct, the 5'-untranslated sequence (Lin and Guidotti, 1989) was deleted at an NheI site and replaced with an oligonucleotide containing an NheI site and a methionine codon. As a result, the three out-of-frame ATGs contained in the first construct were deleted in the second construct and the initiation ATG codon in the second construct was brought to close proximity of the polyhedrin start site. Recombinant viruses were generated from these plasmid constructs by cotransfection of the plasmids with wild-type baculoviral DNA. Sf9 cells infected with the two recombinant viruses expressed C-CAM1 proteins and exhibited a similar phenotype (data not shown). This result indicates that the presence of the 230-bp noncoding sequence, which contains three out-of-frame ATG sequences upstream of the initiation ATG codon, does not significantly affect protein expression.

C-CAM1-mediated Cell Adhesion Does Not Require Ca\(^{2+}\) —The adhesive activity of C-CAMS was previously investigated using isolated hepatocytes (Ocklind and Obrink, 1982) and found to be Ca\(^{2+}\) independent (Ocklind et al., 1984). In contrast, the adhesion functions of closely related members of the same carcinoembryonic antigen (CEA) family, i.e., mouse mmCGM2 (Turbide et al., 1991) and human BGPl (Rojas et al., 1990), were studied with proteins expressed on the mammalian cells and found to be calcium dependent. The discrepancy in the calcium dependence of these closely related proteins could be due to their intrinsic properties or due to the different systems used. It therefore was of interest to determine whether C-CAM1 expressed in a heterologous system had calcium-dependent adhesion function.

To study the role of calcium in C-CAM1-mediated adhesion, we used EGTA to control the free calcium in the medium. However, we found that if EGTA was present in the insect-cell medium prior to viral infection the cells were not infected by either the wild-type or C-CAM1 recombinant viruses (data not shown). Thus, EGTA was added to the medium at 24 h after infection. The concentration of EGTA used was 1.5 times that of the calcium in the medium to ensure complete removal of free calcium. This concentration of EGTA did not interfere with the expression of C-CAM1 (data not shown). More important, C-CAM1-mediated cell aggregation was not affected by the presence of EGTA, indicating that it was calcium independent. This observation is consistent with results obtained in tests using hepatocytes (Ocklind et al., 1984). Therefore, the adhesion functions of C-CAM1 and its human homolog BGPl and mouse homolog mmCGM2 are different regarding their cal-

the possibility that the variations in mobility may have been due to differential glycosylation of certain sequences instead.

In western immunoblotting using anti-NH$_2$-terminal antibody (anti-N) (Lin et al., 1991), which was generated against a peptide derived from the first Ig domain, only ΔD1 failed to react with the antibody, while C-CAM1, ΔD2, ΔD3, and ΔD4 all did so. All four deletion mutants, however, reacted with anti-C1, an antibody against a peptide derived from the cytoplasmic domain of C-CAM1, confirming the presence of cytoplasmic domains in the mutants (data not shown). These results confirm that the desired deletion mutants were produced in the insect cells.

We previously showed that production of C-CAM1 protein in insect cells is first detectable on day 2 after infection and reaches maximum on day 4. Consistent with the time course of protein expression, C-CAM1-mediated cell aggregation is first detected at 48 h after infection (Cheung et al., 1993). In the adhesion assays of deletion mutants, Sf9 cells infected with recombinant viruses ΔD2, ΔD3, and ΔD4 showed aggregations comparable to that obtained using the full-length C-CAM1 in both monolayer (Fig. 3) and suspension (Fig. 4) aggregation assays at 48 h after infection. When infected with recombinant viruses C-CAM1, ΔD2, ΔD3, or ΔD4, the insect cells in culture dishes showed strong interaction with adjacent cells and formed multiple cell aggregates with continuous contours (Fig. 3). This aggregation phenotype could be easily distinguished from that of control wild-type infected cells. In contrast, ΔD1 recombinant virus-infected cells showed no aggregation and had a phenotype indistinguishable from that of the wild type (Fig. 3). To better quantify the extent of aggregation, Sf9 cells grown in suspension were infected in separate experiments with the C-CAM1, ΔD1, ΔD2, ΔD3, and ΔD4 recombinant viruses. Cell aggregation occurred among the C-CAM1-, ΔD2-, ΔD3-, and ΔD4-infected cells 48 h after infection. In contrast, no aggregation was observed among Sf9 cells infected with ΔD1, even after prolonged culture (>120 h) (data not shown).

We considered that ΔD1 recombinant virus-infected cells may have failed to aggregate because of decreased protein expression. This possibility was investigated by comparing the amount of ΔD1 protein expression with expression of the other mutants and of the wild-type C-CAM1 using Ab669. At 96 hrs after infection, the level of protein expressed from AD1 recombinant virus-infected Sf9 cells was indeed comparable to that in cells infected by the other mutants and C-CAM1 (data not shown). This result suggests that the lack-of-aggregation phenotype of the ΔD1-infected cells was not due to a lower level of protein production.

An alternative explanation for the failure of the ΔD1 mutant to show the aggregation phenotype is that it was not properly targeted to the cell surface. It is well documented that point mutation or deletion of a plasma-membrane protein can lead to misfolding and retention of the mutant protein in the endoplasmic reticulum. To investigate this possibility, Sf9 cells infected with ΔD1 were first fixed with 4% formaldehyde with or without Triton X-100 permeabilization. These fixed cells were then stained with Ab669. Strong immunofluorescent staining by Ab669 was seen in all ΔD1 recombinant virus-infected cells (Fig. 5, A and B). In contrast, anti-C1, which recognizes the cytoplasmic domain, showed staining in ΔD1-expressing cells only after Triton X-100 permeabilization (Fig. 5, C and D). Without Triton X-100 permeabilization, anti-C1 stained very few cells; those cells which did stain in the absence of permeabilization were likely among the small percentage of ΔD1 recombinant virus-infected cells which were dead. These observations suggest that fixation with formaldehyde alone did not make cells permeable to antibodies. Therefore, that Ab669 could immunostain ΔD1-expressing cells without Triton X-100 permeabilization indicated that the protein was expressed on the cell's surface. Similar results were obtained for cells not subjected to formaldehyde fixation; those infected with the ΔD1 recombinant virus stained with Ab669 but not with anti-C1 (Fig. 6, A and B). These observations further support the notion that the ΔD1 mutant was expressed on the cell surface. Therefore, the lack of cell aggregation observed for ΔD1-expressing cells suggests that the first Ig domain of C-CAM1 is essential for adhesion function.

**Fig. 3. Morphology of cell cultures at 48 h after infection.** Sf9 cells grown in six-well culture dishes were infected with wild-type or recombinant virus as indicated.
with X-100. Immunostaining was performed with antibody Ab669 virus. After 48 h of incubation, the cells were fixed by formaldehyde treatment with (Panels B and D) or without (Panels A and C) Triton X-100. Immunostaining was performed with antibody Ab669 (A and B) or antibody anti-C1 (C and D).

**Fig. 5. Immunostaining of insect cells infected with ΔD1 recombinant virus.** Sf9 cells grown on coverslips were infected with the virus. After 48 h of incubation, the cells were fixed by formaldehyde treatment with (Panels B and D) or without (Panels A and C) Triton X-100. Immunostaining was performed with antibody Ab669 (A and B) or antibody anti-C1 (C and D).

ΔD2 recombinant virus-infected cells were also stained positive with Ab669 but not with anti-C1 in the absence of formaldehyde fixation (Fig. 6, C and D); however, significant cell aggregation occurred (Fig. 6C). These results further indicate that the first Ig domain is important for adhesion function. In addition, they show that C-CAM1 containing only three Ig-like domains can mediate cell aggregation as long as the first Ig domain is present.

**Requirement of at Least Two Ig Domains for C-CAM1 Function**—Although C-CAM contains four Ig domains, it is clear from the above studies that not all of them are required for adhesion function. The minimum number required for C-CAM1 adhesion function was further investigated using mutant C-CAM1 that lacked two or three Ig domains. Since the first Ig domain is essential for adhesion function, C-CAM1 mutants with either two (ΔD34) or three (ΔD234) Ig domains deleted were constructed for this study. The apparent molecular masses of C-CAM1 mutant proteins expressed from insect cells infected with the ΔD34 and ΔD234 recombinant viruses were around 50 and 26 kDa, respectively (Fig. 2B). The decrease in apparent molecular weight is consistent with the molecular masses predicted from the DNA sequence analysis. When the insect cells in monolayer culture were infected with ΔD34, small aggregates with continuous contours could be seen (Fig. 3). In contrast, similar cells infected with ΔD234 showed no sign of aggregation (Fig. 3). Further analysis of the extent of aggregation by using cells in suspension culture showed that the adhesive activity of cells infected with the ΔD34 mutant was retained but reduced to half that of control cells (Fig. 4, A and B). These results indicate that retention of the first and second domains of C-CAM1 is sufficient for adhesive activity. However, simultaneous deletion of the second, third, and fourth domains (ΔD234) abolished the adhesive activity completely (Figs. 3 and 4). Since the levels of ΔD34 and ΔD234 protein expressed at 96 h after infection were comparable to those of other C-CAM1 mutants (data not shown), the attenuated (ΔD34) and negative (ΔD234) adhesion phenotypes were not due to reduction in protein expression. Furthermore, the ΔD234-expressing cells stained positive with Ab669 in immunofluorescence studies with or without formaldehyde fixation (data not shown), suggesting that the mutant protein was expressed on the cell surface. Therefore, the loss of adhesive activity conferred by the ΔD234 mutant was not due to failure of the mutant protein to appear on the cell surface. These results suggest that simultaneous deletion of the second, third, and fourth Ig domains may affect the accessibility or conformation of the first domain.

**Inhibition of Aggregation by Antibody Against C-CAM**—The adhesion activity observed upon expression of C-CAM1 and several C-CAM1 deletion mutants may be due to direct involvement of C-CAM in the adhesion. However, it is also possible that expression of C-CAM serves to transmit a signal to activate an insect cell specific adhesion system. In the latter case, C-CAM is not directly involved in the adhesion process. In an attempt to elucidate the mechanism by which C-CAM1 and its deletion mutants mediate the adhesion function, the effect of anti-C-CAM antibody on cell aggregation was investigated. In the first experiment, insect cells grown in monolayer were infected with C-CAM1 recombinant viruses. After 24 h, IgG fractions (75 µg of protein) prepared from either control or Ab669 serum were added to the cells. While the cells treated with control IgG exhibited normal aggregation phenotype, the aggregation phenotype was inhibited in cells treated with Ab669 IgG. This observation suggests that Ab669 can neutralize the aggregation activity of C-CAM1. To better quantify the inhibition by Ab669, cells in suspension were infected with C-CAM1 recombinant viruses and the IgG fractions from Ab669 or control serum were added at 24 h post-infection. No aggregation was observed at 24 h post-infection (Fig. 4B). The infection was allowed to continue and the amount of single cells were determined at 72 h post-infection. As shown in Fig. 7, addition of Ab669 IgG caused inhibition of cell aggregation in a dose-dependent manner as evidenced by the increase of single cells, while control IgG did not significantly affect cell aggregation. The possibility that addition of IgGs may affect C-CAM1 expression was examined by western blot analysis using Ab669. C-CAM1 expression was not affected by the addition of either control or Ab669 IgG (data not shown). Since antibody against C-CAM1 can inhibit the aggregation phenotype, C-CAM1 is involved in the adhesion process.

**DISCUSSION**

This study reports structure and function relations in C-CAM1 as determined by using site-directed deletion and the insect-cell expression system. We have demonstrated that C-CAM-mediated adhesive activity is Ca²⁺ independent and that the first extracellular Ig domain is essential for adhesion function. The necessity of the first Ig domain for adhesion function is supported by two results. First, in the deletion study, only the mutant with the first Ig domain deleted showed a loss of adhesion phenotype. Second, the C-CAM1 deletion mutant that retained only the first and second Ig domains also retains the
important for adhesion interaction is our previous  
observation
ily isoform that differs  from C-CAM1 in both the  first  Ig domain
that C-CAM2 (also called S-form cell-CAM1OFi), a C-CAM-fam-
lied by minor structural differences. This phenomenon is very
highly homologous suggests  that  the specificity can be modu-
notype when expressed in  insect cells (Cheung

Consistent  with  the conclusion that  the  first  Ig V-like domain is
operate  in antibody and  antigen recognition also apparently  is
portant for recognition. Thus, a mechanism similar to that

The fact that  the  Ig V-like domains of NCA,  CEA, and W272 are
determined by the  first  Ig V-like domain  (Oikawa

with themselves and  in a heterophilic  fashion with each other
adhesion activities  between CEA and CEA-related molecules

Although the  first  Ig domain is crucial for adhesive activity,

adhesive activity. Interestingly, among the four extracellular Ig
domains, the second, third, and fourth Ig-like domains of CAM1 have characteristics of a C-type immunoglobulin,
whereas the first Ig domain has the characteristics of a V-type
immunoglobulin (Williams and Barclay, 1988). However, unlike
immunoglobulins, the Ig V-like domains in C-CAM and other
CEA-family proteins do not contain the cysteine pair typical of
an immunoglobulin domain. The paired cysteine residues in an
Ig V-like domain form a disulfide bond, which is believed to
stabilize the intradomain β-sheets formed within the domain.
In the first Ig domain of CEA family proteins, this disulfide
bond is probably replaced by a salt bridge between arginine at position 98 (GRETI) and aspartic acid at position 116 (TDERA)
(Thompson et al., 1989). This Ig V-like domain also plays a
pivotal role in mediating both the homophilic and heterophilic
adhesion activities between CEA and CEA-related molecules
(Oikawa et al., 1991). Among the human CEA family proteins,
CEA and NCA can mediate adhesion in a homophilic fashion with themselves and in a heterophilic fashion with each other
(Oikawa et al., 1989). However, W272, another CEA family
protein, exhibits only heterophilic adhesion activity. Further-
more, W272 can interact with only NCA, not CEA (Oikawa et
al., 1991). It was previously shown that these specificities are
determined by the first Ig V-like domain (Oikawa et al., 1991).
The fact that the Ig V-like domains of NCA, CEA, and W272 are
highly homologous suggests that the specificity can be modu-
lated by minor structural differences. This phenomenon is very
similar to that pertaining to immunoglobulins in which the
V-type domains show considerable variation and are most
important for recognition. Thus, a mechanism similar to that
operate in antibody and antigen recognition also apparently is
used in recognition between C-CAM isoforms or their ligands.
Consistent with the conclusion that the first Ig V-like domain
is important for adhesion function is our previous observation that C-CAM2 (also called S-form cell-CAM105), a C-CAM-family
isoform that differs from C-CAM1 in both the first Ig domain
and the cytoplasmic domain, did not confer the adhesion pheno-
type when expressed in insect cells (Cheung et al., 1993).

Although the first Ig domain is crucial for adhesive activity,
it is not the sole determinant of that function. The deletion
mutant ΔD234, containing only the first domain, did not show
adhesive activity suggesting that the other Ig domains may
affect the first domain's accessibility to other molecules. Simi-
lar observations have been reported for other cell-adhesion
molecules. In the case of intercellular adhesion I (I-CAM1), for
example, the binding of its ligand, lymphocyte function-associated
antigen-1 (LFA-1), to the first Ig domain was reduced as the
length between the first Ig domain and the transmembrane
domain was shortened (Staunton et al., 1990). The binding of
rhinovirus to 1-CAM1 was similarly affected (Staunton et al.,
1990). It has also been observed that the homophilic adhesive
activity of CEA is stronger than that of NCA (Oikawa et al.,
1989). Besides the first Ig V-like domain, CEA has six and NCA
has two Ig C-like domains extracellularly and it is possible that
CEA's greater adhesive activity may result from its longer
extracellular domain which may have less steric hindrance and
hence have better interaction.

The nature of the adhesion conferred by C-CAM was not very
clear. Results of studies using liposomes incorporated with the
purified C-CAM led to the hypothesis that adhesion mediated
by C-CAM is homophilic (Tingstrom et al., 1990). In the studies
using deletion mutants, we have shown that C-CAM1 with the
D3 and D4 domains deleted retains adhesion function. This
observation suggests that the D1 domain, which is essential for
function, does not require the D3 or D4 domain for interaction.
Therefore, if C-CAM-mediated adhesion is homophilic, i.e. C-
CAM interacts with C-CAM, it is possible that D1 interacts
with D1 or D2. Since we did not test the adhesive activity of
ΔD23 and ΔD24, we have not eliminated the possibility that D1
can interact with either Ig domain without stringent specificity.
However, it is more likely that D1 interacts with a molecule,
which is not identified yet, present on the surface of Sf9 cells.
This last possibility is supported by the fact that Sf9 cells,
labeled with fluorescent dye, can bind to C-CAM1-expressing
cells but not to wild-type virus-infected cells. 3 The insect-cell
surface, therefore, contains a "ligand" for C-CAM1. Whether
such a ligand is also present in rat hepatocytes, from which C-
CAM was derived, is not clear. That C-CAM1 may be able to
interact with molecules other than itself is consistent with the
behavior of other members of the Ig superfamily. Adhesion
mediated by several Ig-superfamily adhesion molecules has
been found to be heterophilic, i.e. they bind to structurally
distinct counter-receptors. The intercellular adhesion mol-
ecules 1-CAM1, 1-CAM2, and 1-CAM3, which are members of
the Ig superfamily, bind to LFA-1, a member of the integrin
family, as their counter-receptor. The adhesive activity medi-
ated by neuronal cell adhesion molecule (N-CAM), also a mem-
ber of the Ig superfamily, is both homophilic and heterophilic.
In the latter case, N-CAM can specifically bind to cell-surface
proteoglycan heparan phosphate (Cole et al., 1986). The iden-
tification and localization of such a ligand for C-CAM should
add to our understanding of C-CAM function in vivo.

In previous studies, we found that an expressed C-CAM iso-
form (C-CAM3) containing only 6 instead of 71 amino acids
intracellulary failed to show adhesion activity, suggesting that
the cytoplasmic domains of C-CAM also play a significant role
in their adhesion properties. 4 This is in contrast to some other
Ig-superfamily cell-adhesion molecules, whose cytoplasmic
domains are either not necessary for function or the function of
them is not defined. In the case of N-CAM, expression of both
the transmembrane and the glycolipid-linked isoforms has
been shown to induce neuronal outgrowth (Doherty et al.,
1989), suggesting that the cytoplasmic domain is not necessary
for the function. Expression of CEA or NCA, neither of which

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has a cytoplasmic domain, in mammalian cells was shown to be sufficient for inducing cell aggregation (Benchimol et al., 1989; Oikawa et al., 1989, 1991). Conversely, association of the cytoplasmic domain with a group of cytoplasmic proteins named catenins is essential for cadherin-mediated adhesion function (Ozawa et al., 1989; McNeill et al., 1990). It is possible that the cytoplasmic domain of C-CAM, like that of cadherin, also interacts with cytosolic or cytoskeletal factors that modulate the intercellular adhesion mediated by the extracellular domain. At least two reports suggest that calcium-binding proteins are among the most likely factors. Blikstad et al. (1992) reported that calmodulin could bind to C-CAM polypeptides immobilized on nitrocellulose filters suggesting that calmodulin may be the cytosolic protein that interacts with the C-CAM cytoplasmic domain. Using immunoprecipitation, Lim et al. (1990) found that a 65- or 67-kDa calcium-binding protein is associated with C-CAM. Whether these molecules modulate C-CAM adhesion in vivo requires further investigation.

There are several mechanisms which can explain the adhesive activity observed upon expression of C-CAM and its mutants on the insect cells. In the first mechanism, the C-CAM protein does not directly mediate cell adhesion. Rather, the cytoplasmic domain of C-CAM1 is required for adhesion-promoting carbohydrate moieties. The first Ig-domain of C-CAM provides attachment sites for adhesion molecules on the insect cells. In this model, the C-CAM protein does not directly mediate cell adhesion. Rather, the first Ig-domain of C-CAM provides attachment sites for adhesion-promoting carbohydrate moieties. The third mechanism is that C-CAM serves to transmit a signal to activate an adhesion-promoting carbohydrate moiety on the insect cells. The second mechanism is that the adhesion is due to interactions between carbohydrate moieties on the first Ig-domain and lectin-like cell adhesion molecules on the insect cells. In this model, the C-CAM protein does not directly mediate cell adhesion. Rather, the first Ig-domain of C-CAM provides attachment sites for adhesion-promoting carbohydrate moieties. The third mechanism is that C-CAM serves to transmit a signal to activate an insect cell specific adhesion system. We have previously shown that the cytoplasmic domain of C-CAM1 is required for adhesive activity. Therefore, it is possible that the cytoplasmic domain may play a role in signal transduction. Although inhibition of aggregation by anti-C-CAM antibody (Fig. 7) suggests that C-CAM protein is directly or indirectly involved in the adhesion process, this result does not differentiate between these mechanisms. If C-CAM1 is the adhesion molecule, antibody is expected to block the adhesion. However, blocking may be also observed if antibody interferes with the accessibility of carbohydrate moieties or with the ligand binding site for signal transduction. To differentiate between these mechanisms, further studies are required. For example, if synthetic peptides containing sequences of the first Ig-domain could block the adhesion function, it will be unlikely that the carbohydrate moieties are involved. Similarly, site-directed mutagenesis studies of the glycosylation sites in the first Ig-domain or the phosphorylation sites in the cytoplasmic domain may shed light on the possible involvement of glycosylation or signal transduction in the adhesion process. Results from the current study nevertheless point to the importance of the first Ig-domain of C-CAM1 in the adhesion function. These results together with our previous finding suggest that both the NH2-terminal domain and the cytoplasmic domain are required for C-CAM1 adhesion activity.

REFERENCES