Cell adhesion activity of the short cytoplasmic domain isoform of C-CAM (C-CAM2) in CHO cells

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Abstract **C-CAM is a Ca^{2+}-independent rat cell adhesion molecule belonging to the CEA gene family of the immunoglobulin superfamily. Two major isoforms that differ in the length of their cytoplasmic domains exist. In previous studies it has been reported that only the long isoform (C-CAM1) but not the short isoform (C-CAM2) can mediate adhesion. However, in the mouse, isoforms with both long and short cytoplasmic domains have been reported to have adhesive activity. In order to analyze this apparent conflict we transfected C-CAM1 or C-CAM2 into CHO Pro5 cells and examined their adhesive phenotype in an aggregation assay. We found that in this cellular system both C-CAM1 and C-CAM2 could mediate cell-cell adhesion in a Ca^{2+}-independent and temperature-independent way. The results suggest that the cellular environment is important for the activity of C-CAM isoforms.**

Key words: Carcinoembryonic antigen family; Cell adhesion; Cell adhesion molecule; Immunoglobulin superfamily; Isoform

1. Introduction

Cell adhesion molecules (CAMs) play a key role in organizing cells into tissues and organs in the development of a multicellular organism [1,2]. The majority of the known CAMs belong to one of the following families: the immunoglobulin superfamily (IgSF), the cadherin family, the integrin family, or the selectin family. C-CAM is a transmembrane glycoprotein belonging to the IgSF, and more specifically to a subfamily within the IgSF, the CEA (carcino-embryonic antigen) gene family [3]. C-CAM can act as a homophilic cell adhesion molecule [4] and it is present in various epithelia, vessel endothelia and hematopoietic cells [5]. So far, two major isoforms, C-CAM1 and C-CAM2, have been isolated. The isoforms, that differ in the length of their cytoplasmic domains (71 amino acids for C-CAM1 versus 10 amino acids for C-CAM2) [6,7,8], are a result of alternative splicing of a single C-CAM gene [9]. Interestingly, the same splicing pattern is conserved between C-CAM and its homologous molecules in mouse (Bgp) and man (BGP) [10,11]. In addition to the differences in the cytoplasmic domains, some sequence variation has been found in the most N-terminal Ig-domain [7,8]. This variation has been observed in C-CAM cDNA isolated from different stocks of outbred rats and is suggested to be the result of an allelic variation of the C-CAM gene [8]. The existence of multiple isoforms raises questions concerning their functions and mechanisms of regulation.

It is generally believed that CAMs not only mediate binding of cells to each other and to the extracellular matrix, but also can induce transmembrane signalling [12–16]. It is possible that adhesion via C-CAM1 and C-CAM2 could generate different cellular responses due to their different cytoplasmic domains. The cytoplasmic domains of both C-CAM isoforms have been shown to bind calmodulin, with two binding sites in C-CAM1 and one in C-CAM2 [17]. The two isoforms can also be differentially phosphorylated on serine and tyrosine residues [7,18–20].

It has been reported that the long cytoplasmic domain of C-CAM1 (71 amino acids) is essential for C-CAM mediated adhesion and that C-CAM2 with the 10 amino acid long cytoplasmic domain, or an incompletely spliced variant of C-CAM1 having a cytoplasmic domain of only six amino acids, are incapable of promoting adhesion [21,22]. However, in mouse and man, Bgp/BGP isoforms with the short cytoplasmic domain have been shown to promote adhesion [11,23–26]. These apparent conflicting results were obtained in different cellular systems. We therefore thought that it would be of interest to further investigate the adhesive properties of C-CAM in another cellular background. In this study we expressed C-CAM1 and C-CAM2 in CHO cells and analyzed their adhesive properties. In contrast to what was previously found in insect cells we now could demonstrate that also C-CAM2 is capable of mediating adhesion when transfected into a proper cellular invironment. This adhesion did not require Ca^{2+} ions or physiological temperature.

2. Materials and methods

2.1. PCR primers

The following oligonucleotides, obtained from Scandinavian Gene Synthesis, Köping, Sweden, were used as primers in PCR. F13: 5'TCCAGGAAGACTGGCG3' (1345–1360); F18: 5'TAACCCGGGACTATGGAGCTAGCCTCGGCTCG 3' (−3–20; *Xma*I); B6: 5'TAAGTCGAGCAGGACAGACAATGTCAC 3' (1557–1574; *Sal*I); B13: 5'CCGCCAGTCTTCCTGGAATAAAGG 3' (1338–1361); B19: 5'TAATCTAGACAGGACAGACAATGTCAC 3' (1557–1574; *Xba*I).

The location of each primer (nucleotide position) in the published C-CAM cDNA sequence [6] and its restriction enzyme site (underlined sequences) are specified within the parenthesis. The sequences of F13 (plus strand) and B13 (minus strand) overlap in a stretch corresponding to nucleotide positions 1345–1360.

2.2. Isolation of cDNA and construction of expression vectors

Poly(A) RNA was isolated from a female rat (Sprague–Dawley from B&K, Stockholm, Sweden) using a mRNA purification kit (Promega) and quantified by ultraviolet spectroscopy. cDNA synthesis was performed in 30 μl containing 1.6 μg RNA, 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 100 μM of each dNTP, 45 pmol of oligo(dT) primer, 24 U of AMV (avian myeloblastosis virus) Reverse Transcriptase (Promega) and 30 U of RNAse inhibitor (Promega). The reaction was run in a Perkin Elmer Cetus 480 Thermal Cycler for 40 min at 37°C followed by 30 min at

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42°C and 15 min at 65°C. At the end of the reaction, 270 μl of H_2O was added. 10 μl of this mixture was used in the following PCR reaction which was performed in a total of 50 μl containing 20 mM Tris-HCl, pH 8.2, 10 mM KCl, 0.1% Triton X-100, 2.0 mM $MgCl_2$, 6.0 mM $(NH_4)_2SO_4$, 240 mM of each dNTP, 1.0 μM of each primer (F18/B6) and 2.5 U of Pfu-polymerase (Stratagene). The PCR was carried out with a precycle at 94°C for 4 min, 55°C for 4 min, 72°C for 2 min, followed by 33 cycles at 94°C for 30 s, 51°C for 30 s, 72°C for 1 min and an extension at 72°C for 10 min. The PCR product was electrophoretically purified, cleaved with restriction enzymes *XmaI*/*SalI* and ligated into the pBluescript vector (Stratagene) with T4 DNA ligase (1.5 U; Promega). Sequence analysis by the TaqDyeDeoxy Terminator Cycle Seqencing method (Applied Biosystems Inc.) demonstrated that the cloned cDNA corresponded to C-CAM2. The pBluescript vector with the C-CAM2 insert was used as template in a second PCR reaction as described above, but using primers F18/B19. The PCR product was cleaved with the restriction enzymes *XmaI*/*XbaI* and ligated into the eukaryotic expressionvector pRAX (kindly provided by Dr. Torbjörn Norberg, Pharmacia, Uppsala, Sweden), which contains the RSV LTR promotor, the SV 40 poly(A) site and a cloningbox with unique restriction enzyme sites [27]. Sequencing was performed as above and verified that the pRAX contained an intact C-CAM2 insert. In spite of several attempts only C-CAM2 cDNA was obtained by the RT-PCR method reflecting the fact that C-CAM2 transcripts are more abundant than C-CAM1 transcripts [8].

In order to obtain cDNA for C-CAM1 an overlapping PCR technique was applied. To generate the extracellular part of C-CAM1 we used the forward primer F18 and the backward primer B13 with the pRAX/C-CAM2 vector as a template. The transmembrane and intracellular parts of C-CAM1 were generated by PCR utilizing a cDNA-clone (clone 4) previously isolated in our laboratory [8] as template and F13 and B19 as primers. The PCR conditions were as above with optimal cycle programs empirically specified for each reaction. The PCR fragments, which overlap in the region of the F13 and B13 primers, were electrophoretically purified, mixed and used in an extension cycle reaction for 5 min at 94°C and 30 min at 55°C. This reaction was performed in a volume of 50 μl containing 20 mM Tris-HCl, pH 8.2, 10 mM KCl, 0.1% Triton X-100, 2.0 mM $MgCl_2$, 6.0 mM $(NH_4)_2SO_4$, 240 μM of each dNTP, 120 ng of each PCR fragment and 2.5 U of Pfu-polymerase. Directly from this mixture, 1 μl was used as template in a new PCR reaction using primer combination F18/B19. This generated an approximately 1575 basepair long fragment that was cleaved with restriction enzymes *XmaI*/*XbaI* and ligated into pRAX with T4 DNA ligase. Sequence analysis performed as described above verified that the pRAX vector contained an intact C-CAM1 insert.

2.3. Cell culture and transfection

CHO Pro5 cells (American Tissue Culture Collection) were grown at 37°C and 5% CO_2 in monolayer cultures in α-MEM (Gibco BRL, Life Technologies) supplemented with 8% fetal bovine serum (JRH Biosciences), penicillin (100 U/ml)/streptomycin (100 μg/ml (Gibco BRL, Life Technologies). The cells were transfected by the calcium phosphate precipitation method [28] using 5 μg of the expression vector together with 0.5 μg of a vector containing the gene for neomycin resistance [29]. Selection was made by growth in G-418 (0.6 mg/ml Sigma) for 14 days and surviving cells were cloned by limiting dilution.

2.4. Immunoblotting

Cells were rinsed twice with PBS, scraped and pelleted by centrifugation. The cell pellets were extracted with PBS containing 1% Triton X-100 and 0.5 mM AEBSF (aminoethylbenzenesulfonyl fluoride) for 1 h at 4°C. The samples were centrifuged at 14,000 × *g* for 15 min and the supernatants were mixed with an equal volume of 2 × SDS sample buffer [30] and heated at 100°C for 5 min. SDS-PAGE was performed on 7.5% polyacrylamide gels [30] followed by transfer to nitrocellulose filters [31]. The filters were blocked with TBS, pH 7.4, containing 5% non-fat dry milk powder and 0.05% Tween-20 and incubated with rabbit polyclonal anti-C-CAM antibodies [19] overnight at 4°C. After washing with TBS, the filters were incubated with alkaline phosphatase-conjugated swine anti-rabbit antibody (Dako A/S, Denmark) in TBS containing 1% non-fat dry milk powder and 0.05% Tween-20 for 2 h at room temperature. The filters were washed with TBS and developed with Nitroblue tetrazolium (NBT) and 5-bromo-chloro-indolylphosphate (BCIP) [32]. Alternatively, for quantitative analyses the ECL (enhanced chemiluminescence) Western blotting kit (Amersham) was used for development. The films were analysed by scanning densitometry on a Shimadzu CS-930 scanner.

2.5. Immunofluorescence and FACS analysis

Cells were grown on glass coverslips and fixed in Buffer 3 (137 mM NaCl, 4.7 mM KCl, 0.6 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing 2% paraformaldehyde. The cells were then incubated for 30 min in Buffer 3/0.1 M glycine (to quench reactive aldehyde groups) in the absence or presence (to permeabilize the cells) of 0.1% Triton X-100. The specimens were washed extensively and incubated with rabbit affinity-purified polyclonal antibodies [19] against rat liver C-CAM over night at 4°C, followed by FITC-conjugated swine anti-rabbit secondary antibodies (Dako A/S, Denmark) for 1 h at room temperature. The coverslips were mounted on glass slides, examined and photographed using a Nikon Labophot epifluorescence microscope.

The relative amounts of C-CAM expressed on the cell surface was determined by FACS analysis. Briefly, the cells were trypsinized to yield single cells, and 4×10^6 cells were suspended in 400 μl of PBS containing 0.5% bovine serum albumin and 40 μl of anti-C-CAM rabbit antiserum [19] or non-immune serum for 1 h at 4°C. The cells were then washed three times and incubated with FITC-conjugated swine anti-rabbit secondary antibodies (Dako A/S, Denmark) for 30 min at 4°C. The cells were washed in PBS and left overnight at 4°C. The following day the cells were examined in a fluorescence-activated cell sorter (FacScan, Becton Dickinson).

2.6. Aggregation assay

$6–7 \times 10^6$ cells were seeded in 10-cm tissue culture dishes 24 h prior to the aggregation experiment. The next day the confluent cells were dissociated by incubation for 20 min at room temperature in 2 ml of Ca^{2+}/Mg^{2+}-free PBS (PBS-A)/0.5% trypsin/5.4 mM EDTA (Gibco) with gentle pipetting at two occasions during this incubation period. The trypsin treatment was stopped by adding 8% fetal calf serum in PBS-A. After centrifugation the cells were washed twice with PBS-A. Suspensions of single cells at a concentration of 2×10^6 cells/ml in 0.5 ml of Buffer 3/glucose(1 mg/ml)/DNase(50 μg/ml) were incubated in BSA-coated 16-mm dishes in a rotary shaker at 80 rpm at 37°C or 4°C. Samples were taken after various times (routinely after 60 min), and the degree of aggregation was determined by counting in a hemocytometer. Cells in aggregates containing more than three cells were scored as aggregated, from which the percent of aggregated cells was calculated. All experiments were performed in duplicate samples and several countings were performed for each sample with a minimum of 600 cells counted each time.

In some aggregation experiments the effects of anti-C-CAM or non-immune Ig Fab fragments, prepared as previously described [36], were analyzed. The cells were preincubated for 30 min on ice in the presence of the Fab fragments in Buffer 3/glucose/DNase and the aggregation

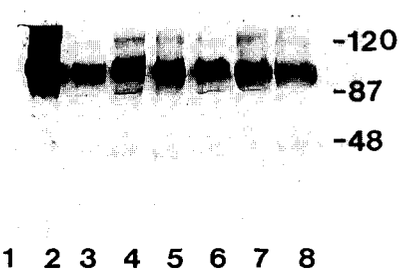

Fig. 1. Immunoblot analysis of C-CAM-transfected CHO cells. Detergent extracts were subjected to SDS-PAGE, transferred to nitrocellulose filters and developed with a polyclonal anti-C-CAM antibody. Lane 1 = C-CAM purified from rat liver; lane 2 = clone H8 transfected with C-CAM1; lane 3 = clone G8C11 transfected with C-CAM1; lane 4 = clone E7 transfected with C-CAM1; lane 5 = clone F12 transfected with C-CAM1; lane 6 = clone G5H4 transfected with C-CAM2; lane 7 = clone 3G9 transfected with C-CAM2; lane 8 = untransfected, parental CHO Pro5 cells. Migration positions of molecular weight markers ($M_r \times 10^{-3}$) are indicated to the right.

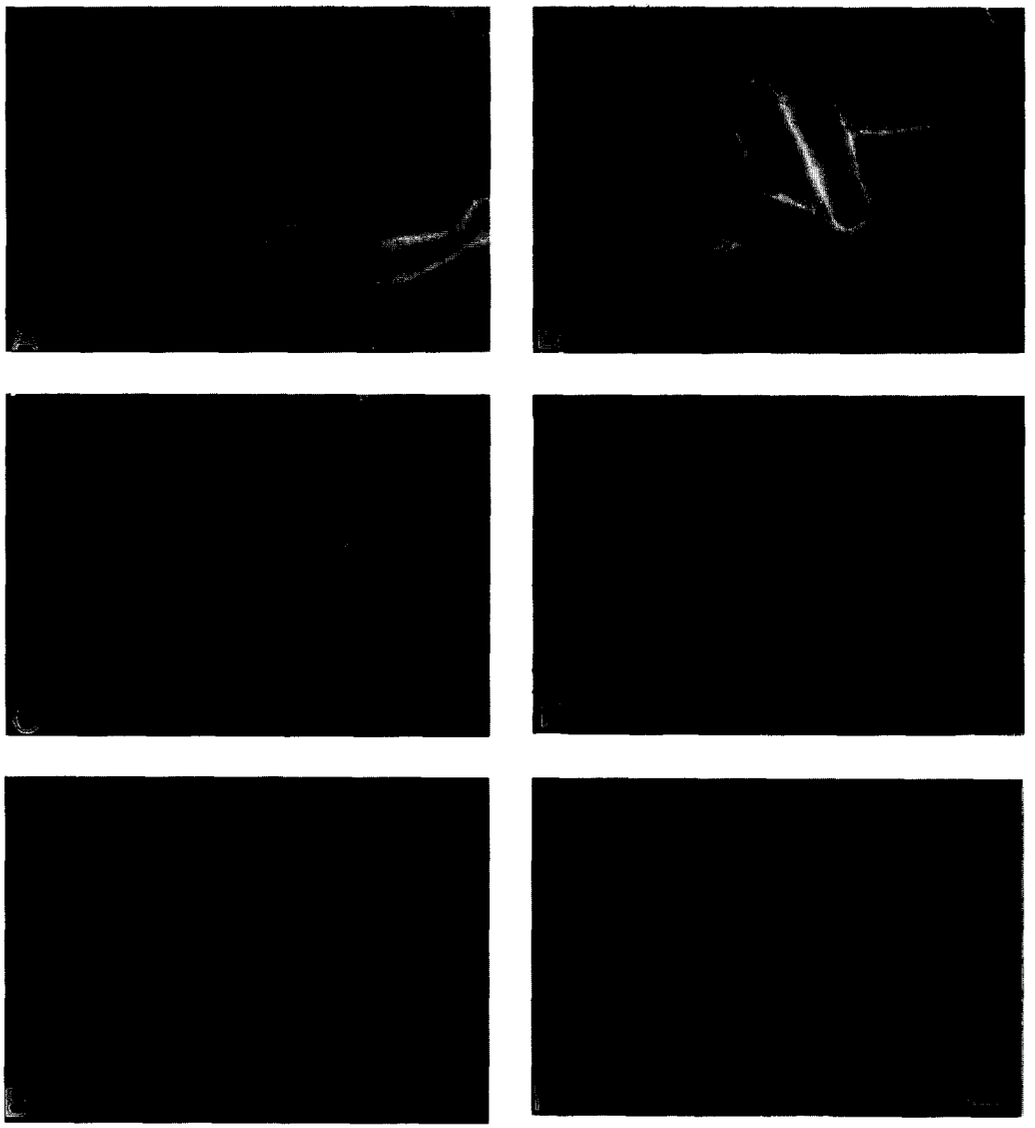

Fig. 2. Immunolocalization of C-CAM in transfected CHO cells. The cells were fixed and subjected to indirect immunofluorescence microscopy with affinity-purified anti-C-CAM antibodies (A–E) or non-immune IgG (F). (A) Clone H8 transfected with C-CAM1. (B) Clone F12 transfected with C-CAM1. (C) Clone G5H4 transfected with C-CAM2. (D) Clone 3G9 transfected with C-CAM2. (E) Untransfected parental CHO Pro5 cells. (F) Clone F12 transfected with C-CAM1. Bar = 20 μm.

was then assayed in the continued presence of the Fab fragments as described above.

The influence of Ca^{2+}-ions on cell aggregation was determined by suspending the dissociated cells in Buffer 3/glucose/DNase with or without Ca^{2+}-ions. Aggregation was then determined as described above.

3. Results

3.1. Expression of C-CAM in the transfected CHO cells

Immunoblot analysis demonstrated that the transfected CHO cells expressed C-CAM (Fig. 1). The apparent molecular masses of the expressed proteins correlated well with that of C-CAM isolated from rat liver [19], indicating that the C-CAM molecules produced by the CHO cells were similarly glycosylated as C-CAM in rat liver.

Using indirect immunofluorescence staining we could demonstrate that both C-CAM1 and C-CAM2 were targeted to the surface of the transfected cells (Fig. 2). Both isoforms were predominantly localized to the lateral cell membranes. A weaker staining was also found on the apical as well as basal cell surfaces. This pattern of localization of C-CAM has also been found in cultured primary hepatocytes [33] and in cell lines expressing C-CAM [34]. Parental untransfected cells or cells transfected with the empty vector did not show any specific staining with anti-C-CAM antibodies. Non-immune antibodies failed to stain both parental and transfected cells. The staining pattern of non-permeabilized and permeabilized cells were very similar, indicating that the C-CAM molecules were exposed on the extracellular face of the cell surface.

FACS analysis and ECL were used to quantitate the expression of C-CAM by the various clones (Table 1). The relation between signal intensity and expression level between these two methods is not directly comparable. However, both methods indicated that the different clones expressed somewhat varying

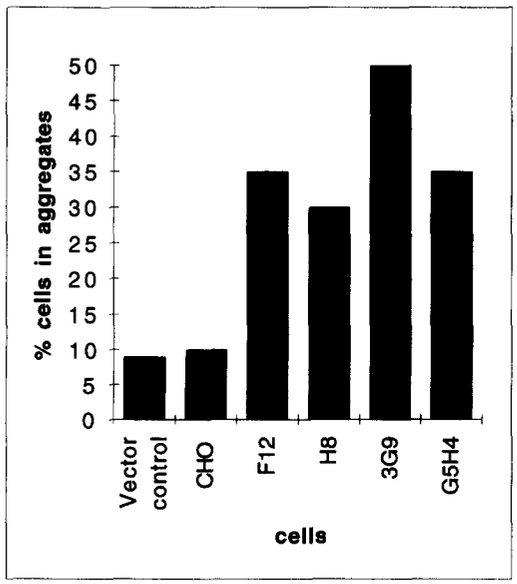

Fig. 3. Aggregation of C-CAM-transfected CHO cells. Cells were allowed to aggregate in suspension for 60 min. The figure shows results obtained with two C-CAM1-transfected clones (F12 and H8), two C-CAM2-transfected clones (3G9 and G5H4), cells transfected with the empty vector (vector control) and untransfected cells (CHO Pro5).

amounts of C-CAM, and that C-CAM2-transfected cells seemed to express slightly more C-CAM than the C-CAM1-transfected cells.

3.2. Adhesive activity of C-CAM in the transfected CHO cells

The adhesive properties of the transfected CHO cells were analyzed by an aggregation assay. Under the experimental conditions used, the background aggregation of untransfected cells and cells transfected with the empty vector was low. In contrast both C-CAM1-transfected and C-CAM-2-transfected cells aggregated significantly (Fig. 3). The C-CAM2-transfected cells in general aggregated to a somewhat larger extent than the C-CAM1 cells, which most likely reflects the slightly higher level of expression of C-CAM2. Since adhesion mediated by C-CAM1 but not by C-CAM2 has been described before, the latter was investigated in more detail. Fab fragments of antibodies against C-CAM could inhibit the aggregation of C-CAM1-transfected cells (data not shown) as well as of C-CAM2-transfected cells (Fig. 4), demonstrating that the adhesion of the transfected cells indeed was mediated by C-CAM.

The C-CAM-transfected cells aggregated to the same extent both at 37°C and 4°C, indicating that there was no temperature dependence of the C-CAM mediated adhesion reaction (data not shown). The dependence on extracellular Ca^{2+}-ions was also investigated by performing aggregation experiments in the presence and absence of Ca^{2+}-ions in the medium. As shown in Fig. 5 the C-CAM-transfected CHO cells aggregated equally well both in the presence and the absence of Ca^{2+}-ions.

4. Discussion

To address the question whether C-CAM isoforms differing in their cytoplasmic domains have different adhesive properties we used a system in which cells, that do not express endogenous C-CAM, were transfected with the individual isoforms. We found, as has also been described by others [21,22], that C-CAM1 could mediate adhesion in vitro. We also found that the adhesive property is not unique to this particular isoform, but that also C-CAM2 could mediate intercellular adhesion when transfected into CHO cells. The adhesive phenotype of the CHO cells clearly depended on the expression of C-CAM2 as shown by the fact that aggregation could be abrogated by antibody treatment. Our results are thus contrasting with previous reports where it was postulated that the long cytoplasmic domain is required for C-CAM-mediated adhesion [21,22].

As for mouse Bgp, there seem to exist two allelic variants of C-CAM, that have been denoted a and b, respectively [8,11]. Both variants occur with long or short cytoplasmic domains, giving rise to C-CAM1a, C-CAM1b, and C-CAM2a, C-

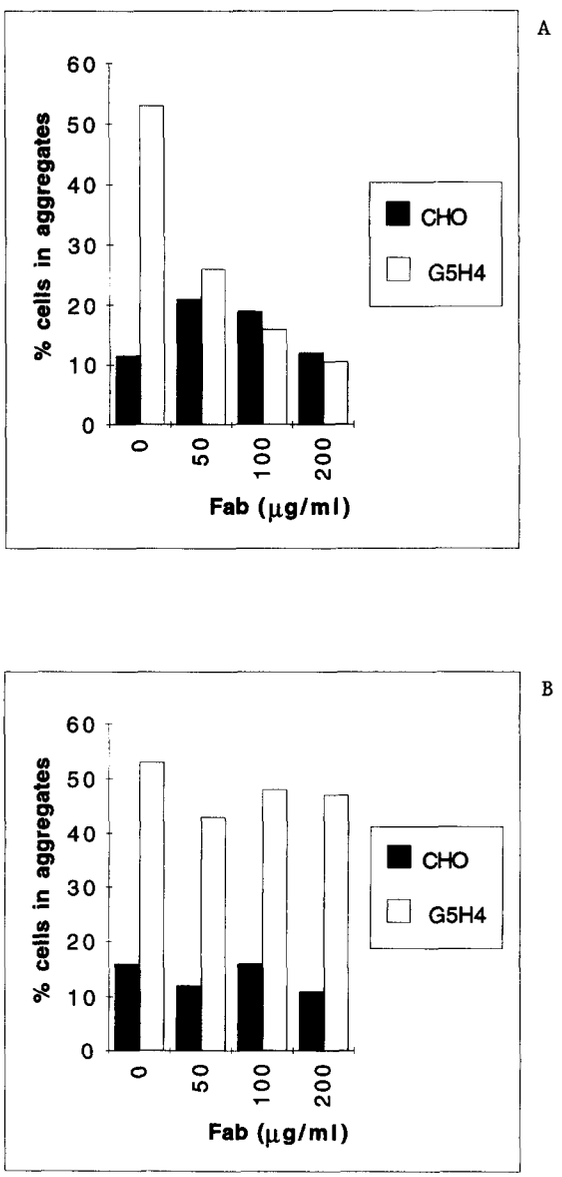

Fig. 4. Effect of Fab fragments on the C-CAM-mediated aggregation of CHO cells. The C-CAM2-transfected clone G5H4 and untransfected CHO cells were allowed to aggregate for 60 min in the presence of various concentrations of Fab fragments of polyclonal anti-C-CAM antibodies (A) or non-immune IgG (B).

CAM2b, respectively [8]. In the present investigation we analyzed C-CAM1a and C-CAM2a. The two allelic variants of mouse Bgp isoforms show a similar variation in the amino acid sequence of the first Ig-domain as was found for the C-CAM variants. Cheung et al previously showed that C-CAM with the long cytoplasmic domain, but not C-CAM with the short cytoplasmic domain can promote cell adhesion in Sf9 insect cells [21]. However, the long isoform that they analyzed was C-CAM1a and their short isoform was C-CAM2b. Thus, one possible explanation to our contradictory results would be that the amino acid differences in the N-terminal Ig-domain in the two variants could have functional consequences leading to a loss of adhesion promoting activity in C-CAM2b. This does not seem be the most likely explanation, however, since an incompletely spliced variant of C-CAM1a containing a cytoplasmic domain of only six amino acids also was unable to mediate adhesion when expressed in insect cells [22]. Furthermore, both allelic variants of mouse Bgp have been demonstrated to have adhesive properties [24,25]. A more plausible possibility is that the different results reflect the different cellular systems that were used.

We transfected mammalian cells (CHO cells) with rat C-CAM cDNA, whereas Cheung et al used a baculovirus system expressed in Sf9 insect cells. It is well known that the specificity and efficiency of glycosylation are different in mammalian and insect cells. The C-CAM molecules expressed in the baculovirus system had apparent molecular masses of 70 and 80 kDa, compared to the C-CAM molecules produced by the CHO cells which had apparent molecular masses of about 100–110 kDa. Since about one-third of the mass of rat liver C-CAM, that has the same size as C-CAM in CHO cells, is made up by carbohydrate [19], it is possible that the glycosylation is of importance for the overall structure and function of C-CAM. An abberrant glycosylation might accordingly make the protein nonfunctional. However, a more likely explanation is that the mammalian cells might express cytoplasmic or membrane components required for the functional activity of C-CAM2, that are not expressed by insect cells.

In the present investigation we found that the C-CAM-mediated aggregation of CHO cells was both Ca^{2+}- and temperature-independent. Also primary rat hepatocytes [35] and the rat bladder carcinoma cell line NBT II [34] exhibit Ca^{2+}-independent C-CAM-mediated cell adhesion. However, conflicting results have been reported for transfected BGPa, which is the human counterpart of C-CAM1. One study found a requirement for extracellular calcium ions [23], whereas in another study adhesion did not require calcium ions [36]. For mouse Bgp isoforms both Ca^{2+}-dependent [24] and Ca^{2+}-independent adhesion [25] have been reported. Similar variations in the temperature dependence of C-CAM/Bgp/BGP-mediated adhesion have been found. In the present investigation we found no dependence on the temperature. However, human BGP-mediated adhesion has been found to be temperature dependent [23], whereas both temperature dependent [24] and temperature independent [25] adhesion has been found for mouse Bgp isoforms. The mosty likely explanation to these differences in the Ca^{2+}- and temperature-dependence of C-CAM/Bgp/BGP-mediated cell adhesion of transfected cells is that different cell lines have been used in the different laboratories.

The results of the present investigation demonstrate that both the long and the short cytoplasmic domain isoforms of C-CAM can mediate cell adhesion if expressed in a proper cellular environment. They also make likely that other cellular factors are important for regulating the functional activity of the different C-CAM isoforms. The identification of such factors is a challenge for future analysis.

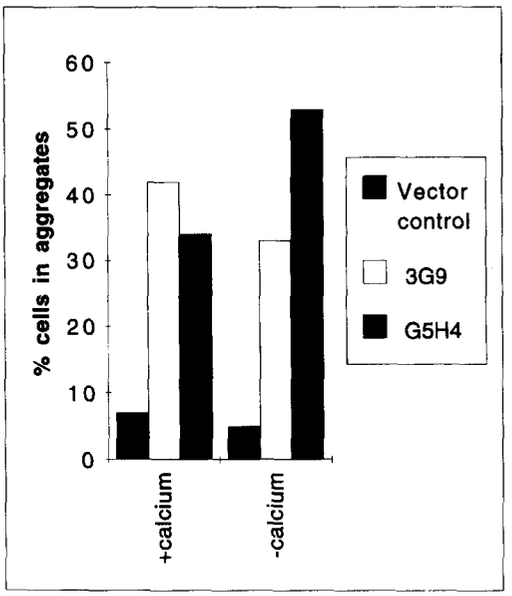

Fig. 5. Effect of calcium on the C-CAM-mediated aggregation of CHO cells. Two C-CAM2-expressing clones, 3G9 and G5H4, as well as a clone transfected with the empty vector (vector control) were allowed to aggregate for 60 min in the presence (1.2 mM) or absence of Ca^{2+}-ions.

Table 1
Comparison of C-CAM-expression in different transfected cell clones

Cell clone	Relative expression	
	FACS	ECL
H8 (C-CAM1)	45	86
F12 (C-CAM1)	49	71
3G9 (C-CAM2)	100	100
G5H4 (C-CAM2)	87	87
CHO Pro5	2	0

The expression was determined by FACS analysis, or by ECL followed by scanning densitometry. The values are given in relative units normalized to the C-CAM expression of the transfected clone 3G9, which was set to 100%.

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