Evidence for calmodulin binding to the cytoplasmic domains of two C-CAM isoforms

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C-CAM (cell-CAM 105) is a transmembrane cell adhesion molecule, belonging to the immunoglobulin superfamily. It is expressed in epithelia, vessel endothelia and leukocytes, and mediates intercellular adhesion in rat hepatocytes by homophilic binding. Two major isoforms (C-CAM1 and C-CAM2) that differ in their cytoplasmic domains occur A previous study demonstrated that C-CAM can bind calmodulin in a Ca^{2+}-dependent manner. In this study we have expressed the cytoplasmic domains of C-CAM1 and C-CAM2 in fusion proteins and measured calmodulin binding by a gel overlay assay, using ^{125}I-labelled calmodulin. Our results indicate that the cytoplasmic domains of both C-CAM1 and C-CAM2 can bind calmodulin.

Adhesion; Calcium; Calmodulin; C-CAM; Immunoglobulin super-family

1. INTRODUCTION

C-CAM, a cell adhesion molecule belonging to the immunoglobulin superfamily, is expressed in several epithelia, vessel endothelia, platelets and leukocytes [1]. It was originally characterized in rat liver [2,3] and was found to mediate intercellular adhesion between hepatocytes by a Ca^{2+}-independent, homophilic binding mechanism [4]. In rat liver C-CAM invariably appears as two structurally related, highly glycosylated peptide chains with apparent molecular masses of 105 kDa and 110 kDa [3,5]. In recent studies both Culic et al. and our laboratory [6,7] showed that these two forms differ mainly in their cytoplasmic domains. The large form (C-CAM1) has a C-terminal, cytoplasmic domain of 71 amino acids, while that of the small form (C-CAM2) is only 10 amino acids long.

The sequences of liver C-CAM from different outbred rat stocks differ in their extracellular, N-terminal portions, as well [7]. Accordingly, C-CAM in rat liver seems to appear in at least four different isoforms, C-CAM1 (a and b) and C-CAM2 (a and b) [7]. It is likely that even more isoforms in rat await discovery as the homologous molecules in man and mouse, known as biliary glycoprotein, exist in a large number of isoforms [7–9]. Strikingly, all the mouse isoforms and all the

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Abbreviations. BGP, biliary glycoprotein; cDNA, complementary DNA; kDa, kilodalton; MBP, maltose binding protein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcriptase; SDS, sodium dodecyl sulfate.

human transmembrane isoforms, have either of two cytoplasmic domains, both of which are clearly homologous to the two cytoplasmic domains found in the rat liver isoforms of C-CAM.

The question arises, if the large number of isoforms are of importance for the function of C-CAM, and especially if the two different cytoplasmic domains have different properties in the regulation of the molecule. It was previously demonstrated in our laboratory that rat liver C-CAM can bind calmodulin in a Ca^{2+}-dependent manner [10]. Experiments with proteolytically derived peptides from purified C-CAM suggested that the calmodulin binding was to the cytoplasmic domain [10]. In the present study we have explored this binding further by using recombinant fusion proteins containing the cytoplasmic domains of C-CAM1 or C-CAM2. The results indicate that both of these domains can bind calmodulin.

2. MATERIALS AND METHODS

2 1. *Polyacrylamide gel electrophoresis (SDS-PAGE)*

Proteins were electrophoresed under non-reducing or reducing (10 mM DTT with or without boiling for 3 min) conditions on 12% polyacrylamide gels in the presence of SDS, according to Laemmli [11]. The gels were either stained with Coomassie brilliant blue, used for gel overlay assays with ^{125}I-labelled calmodulin, or immunoblotted as previously described [3] using an antibody against the maltose binding protein (MBP) provided by the manufacturer of the pMAL-c2 vector system (New England Biolabs).

2.2. *Gel overlay assay*

Calmodulin (bovine testis, Pharmacia) or RNase A (ribonuclease I 'A' from bovine pancreas, Pharmacia) were labelled with ^{125}I by the lactoperoxidase method and were used in a gel overlay assay, as described by Carlin et al. [12] Briefly, purified rat liver C-CAM [3], or bacterial lysates containing fusion proteins, were subjected to SDS-

polyacrylamide gel electrophoresis, and the gels were fixed in 25% isopropanol and 10% acetic acid. After several washes in binding buffer (0.2 M NaCl, 50 mM Tris-HCl, pH 7.6), containing either 1 mM $CaCl_2$ or 1 mM EGTA, the gels were incubated with 1 mg/ml BSA in binding buffer. They were then incubated with 10 μg of either [^{125}I]calmodulin or [^{125}I]RNase A ($\approx$ 1 × 10⁶cpm/μg protein) in binding buffer with 1 mM $CaCl_2$ or 1 mM EGTA, for 12 h Finally the gels were washed, dried and exposed to X-ray film.

2.3. Oligonucleotide primers

The malE primer (5'-GGTCGTCAGACTGTCGATGAAGCC-3') was purchased from New England Biolabs. The following oligonucleotides, corresponding to sense (F) or antisense (B) sequences of the cytoplasmic domains of C-CAM, were synthesized by Scandinavian Gene Synthesis:

F13   5'-TCCAGGAAGACTGGCG-3'
F14   5'-GCATACTTCCTTTATTCCAGG-3'
F16   5'-TTCAATGCCCAGCAATCC-3'
B6   5'-TAAGTCGACCAGGACAGACAATGTCAC-3'

The underlined sequence in B6 is a SalI restriction site.

2.4. Isolation of cDNA

The cDNA used for the construction of the fusion proteins was produced either by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) with rat liver poly (A⁺) RNA [7] as a template, or by PCR with a Lambda Zap (Stratagene) rat liver cDNA library as a template. 0.5 μg of poly (A⁺) RNA was transcribed at 42°C for 60 min with 25 U of AMV reverse transcriptase (Promega), 10 μM of B6 as a primer and 0.2 mM of each of the four dNTPs in a total reaction volume of 20 μl. The reaction was stopped by heating for 5 min at 95°C, and the cDNA product (1 ml of reaction mixture) was amplified by PCR. All PCR reactions were performed in a 50 μl reaction mixture containing 1 μl of template (RT-reaction mixture or cDNA library), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 mM of each of the dNTPs, 0.5 mM of each primer and 2.5 U of Pfu polymerase (Stratagene). The primer combinations F13/B6, F14/B6 and F16/B6 were used. All PCR reactions ran for 30 seconds at 94°C, 30 s at 55°C and 1 min at 72°C. 5 μl of the products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide.

2.5. Production and characterization of fusion proteins

After cleavage of the pMAL-c2 vector (New England Biolabs) with XmnI and SalI, and cleavage of the purified cDNA fragments with SalI, the cDNA fragments were ligated into the cloning box of the vector. Recombinant plasmids were amplified in the TBI host bacteria (New England Biolabs). Positive clones were selected by PCR screening, with primer combinations appropriate for each cDNA insert. The nucleotide sequences of the recombinant plasmids of each of the isolated clones were determined by the Taq DyeDeoxy Terminator Cycle Sequencing method (Applied Biosystem Inc.), using an Applied Biosystems Model 373A DNA sequence analyser and the vector specific primer malE. Fusion protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG), when bacterial suspensions had reached an optical density of $A_{600} = 0.5$. Two hours later bacterial lysates were prepared by repeated freeze-thawing and lysozyme digestion. The fusion proteins in the lysates were analyzed by SDS-PAGE and immunoblotting.

3. RESULTS

3.1. Construction and characterization of fusion proteins

Three fusion proteins containing different portions of the cytoplasmic domains of C-CAM were produced (Fig. 1). C1/71 contained the entire 71-amino acid cytoplasmic domain of C-CAM1, C1/26 contained the 26 most C-terminal amino acids of the cytoplasmic domain

of C-CAM1, and C2/10 contained the entire 10-amino acid cytoplasmic domain of C-CAM2. A fourth fusion protein, C0/22, served as a control. This fusion protein contained a stretch of 22 amino acids, of which the 7 most N-terminal corresponded to 5 of the transmembrane and 2 of the proximal cytoplasmic amino acids of C-CAM. The remaining 15 amino acids were completely unrelated to any C-CAM sequence. The sequences of all four fusion proteins were verified by nucleotide sequence determination of the recombinant plasmids.

The four fusion proteins were successfully expressed by the TBI bacteria, as shown in Fig. 2A and B. They were identified by immunoblotting with an antibody against the maltose binding protein (Fig. 2B). Stainings of whole lysates revealed that the fusion proteins were overexpressed relative to the bacterial protein background (Fig. 2A). The molecular masses of the wildtype fusion protein MBP/GAL and of the MBP part of the fusion proteins are 52 kDa and 42 kDa, respectively. The fusion proteins C1/26, C2/10 and C0/22 had the expected molecular masses (Fig. 2, lanes 2, 3 and 6). Fusion protein C1/71, however, appeared as two components (Fig. 2, lane 1). The largest component, which was the most abundant one, had the expected size. The smaller component did not disappear after chemical reduction and probably resulted from proteolytic degradation or alternate folding. The same two components appeared again when the construction and expression of C1/71 was repeated in an independent experiment (data not shown).

3.2. Calmodulin binding to fusion proteins

The maltose binding protein binds specifically to amylose. However, all four recombinant fusion proteins C1/71, C1/26, C2/10 and C0/22 had lost this affinity and could accordingly not be purified by affinity-chromatography on amylose. Therefore, we used whole bacterial lysates for calmodulin binding experiments with the gel overlay assay. Purified liver C-CAM was included in the binding experiments as a positive control. As demonstrated in Fig. 2C, fusion proteins C1/71, C1/26 and C2/10, but not MBP/GAL or C0/22 bound calmodulin in the presence of Ca^{2+}. Calmodulin binding to any other bacterial protein was negligible. Independent constructions of C1/71 and C2/10 also bound calmodulin (data not shown). No calmodulin binding occurred in the presence of the calcium chelator EGTA (data not shown). The binding experiments shown in Fig. 2C were performed with non-reduced proteins. Binding also occurred after mild reduction (no boiling, data not shown). Reduction and boiling of the bacterial lysates caused heavy precipitation, which prevented binding experiments under these conditions. As a separate control, gel overlay binding experiments were performed with [^{125}I]RNase A. No binding was observed either in the presence or absence of Ca^{2+} (data not shown).

A

B

C

Fig. 1. Construction of fusion proteins. (A) The linear structure of C-CAM showing the extracellular, the transmembrane (TM) and the intracellular domains is shown. The intracellular domains of C-CAM1 and C-CAM2 contain 71 and 10 amino acids, respectively. (B) DNA species corresponding to different parts of the cytoplasmic domains of C-CAM were made by PCR with the following primer combinations: F13/B6, the entire cytoplasmic domain of C-CAM1 = C1/71, F16/B6, the distal 26 C-terminal amino acids of the cytoplasmic domain of C-CAM1 = C1/26, F13/B6, the entire cytoplasmic domain of C-CAM2 = C2/10; F14/B6, which resulted in DNA unrelated to C-CAM was used to construct fusion protein C0/22 (see Fig. 1C) After cleavage with SalI and ligation into the pMAL-c2 vector, bacterial translation yielded fusion proteins C1/71 (C-CAM1 amino acid residues 449–519), C1/26 (C-CAM1 amino acid residues 494–519), C2/10 (C-CAM2 amino acid residues 449–458), and C0/22, respectively. (C) The amino acid sequences (single letter code) deduced from the four DNA species C1/71, C1/26, C2/10 and C0/22, respectively.

4. DISCUSSION

In the present study we have demonstrated that fusion proteins containing sequences corresponding to the cytoplasmic domains of C-CAM1 and C-CAM2 can bind calmodulin. The binding was strictly Ca^{2+}-dependent and quite specific. Very little calmodulin binding occurred to any other bacterial proteins, and radiolabelled RNase A did not bind. Since neither the wildtype fusion protein MBP/GAL nor the control fusion protein C0/22 exhibited any significant calmodulin binding, C-CAM-specific sequences seem to be responsible for the observed binding. These results strengthen the conclusions of our previous study of calmodulin binding to proteolytically derived peptides of liver C-CAM [10], and suggest that both the large and the small cytoplasmic domains of C-CAM contain calmodulin binding motifs.
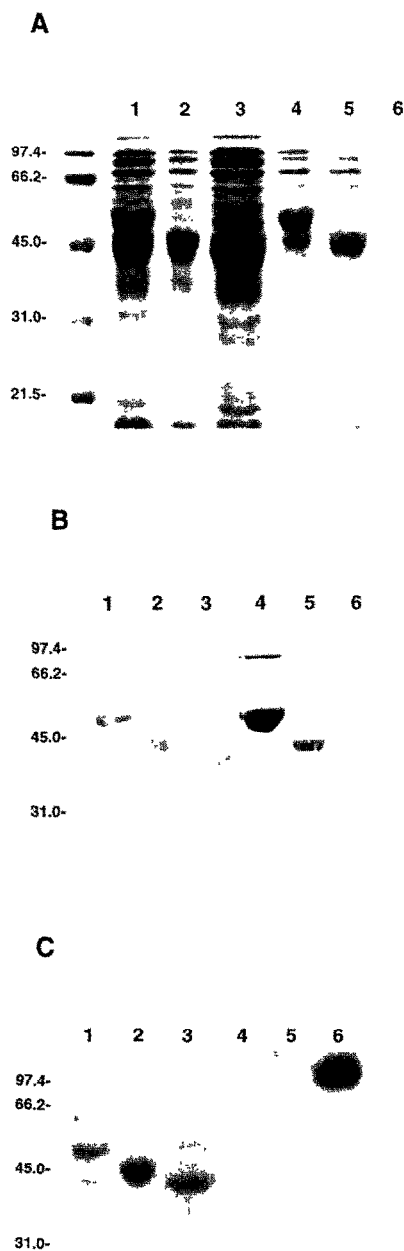
A

B

C

Fig. 2. SDS-PAGE and calmodulin binding of fusion proteins. Total bacterial lysates were subjected to SDS-PAGE under non-reducing conditions. The gels were stained with Coomassie blue (A), immunoblotted with an antibody against the maltose binding protein (B), or subjected to gel overlay assay with ^{125}I-labelled calmodulin in the presence of CaCl$_2$ (C). Lane 1, bacteria expressing C1/71; lane 2, bacteria expressing C1/26; lane 3, bacteria expressing C2/10; lane 4, bacteria expressing the wildtype MBP/GAL protein; lane 5, bacteria expressing C0/22; lane 6, purified rat liver C-CAM. Molecular weight marker proteins are shown to the left in (A). Pure C-CAM stains poorly with Coomassie blue and is therefore not detected in (A), lane 6.

The sequence of the six membrane-proximal amino acids is identical in C-CAM1 and C-CAM2, suggesting

that this sequence is essential for calmodulin binding. However, since calmodulin bound also to the fusion protein C1/26, that contains the 26 C-terminal distal amino acids of C-CAM1, a second binding site is suggested in the cytoplasmic domain of C-CAM1.

Calmodulin is known to bind to a variety of intracellular proteins. No consensus binding sequence has been found, but in all of the characterized calmodulin binding sites both positively charged and hydrophobic amino acids are abundant [13,14]. Many of these sequences probably form basic amphiphilic alpha helices. The putative calmodulin binding sequences in the cytoplasmic domains of C-CAM1 and C-CAM2 fulfil some of these requirements in that they contain both positively charged and hydrophobic amino acids.

Calcium-regulated calmodulin binding influences the activities of several different enzymes, membrane transport proteins and cytoskeletal proteins [13,15–17]. It is thus tempting to speculate that the calcium-dependent binding of calmodulin to the cytoplasmic domains of C-CAM could control the functional activity of this transmembrane protein. Indeed, it has recently been demonstrated that the cytoplasmic domains of C-CAM can have a regulatory role in that C-CAM1 with the large cytoplasmic domain, but not C-CAM2 with the small cytoplasmic domain, conferred intercellular adhesion activity to insect Sf9 cells [18]. Furthermore, the cytoplasmic domains of C-CAM can be phosphorylated on both serine and tyrosine residues [3,19], several of which are contained in the putative calmodulin binding regions. Phosphorylation of these residues might alter the binding of calmodulin, with possible consequences for the extracellular function of C-CAM. Conversely, changes in the extracellular, C-CAM-mediated adhesion status of the cell could regulate calmodulin binding to C-CAM, thereby affecting the activities of other cytoplasmic, calmodulin-regulated proteins.

In conclusion, our results indicate that the cytoplasmic domains of both C-CAM1 and C-CAM2 can bind calmodulin in a calcium-dependent manner. The elucidation of the location, structure and regulation of the binding sites, as well as of the possible effect of changes in calmodulin binding on the function of C-CAM, remains for future studies.

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