Calmodulin Binds to Specific Sequences in the Cytoplasmic Domain of C-CAM and Down-regulates C-CAM Self-association*

(Received for publication, June 5, 1995, and in revised form, October 20, 1995)

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C-CAM is a cell adhesion molecule belonging to the immunoglobulin supergene family and is known to mediate calcium-independent homophilic cell-cell binding. Two major isoforms, C-CAM1 and C-CAM2, which differ in their cytoplasmic domains, have been identified. Previous investigations have demonstrated that both cytoplasmic domains can bind calmodulin in a calcium-dependent reaction. In this investigation, peptides corresponding to the cytoplasmic domains of C-CAM were synthesized on cellulose membranes and used to map the binding sites for 125I-labeled calmodulin. Both C-CAM1 and C-CAM2 had one strong calmodulin-binding site in the membrane-proximal region. These binding regions were conserved in C-CAM from rat, mouse, and man. In addition, C-CAM1 from rat and mouse contained a weaker binding site in the distal region of the cytoplasmic domain. Biosensor experiments were performed to determine rate and equilibrium constants of the C-CAM/calmodulin interaction. An association rate constant of 3.3 × 10^5 M^{-1} s^{-1} and two dissociation rate constants of 2.2 × 10^{-2} and 3.1 × 10^{-5} s^{-1} were determined. These correspond to equilibrium dissociation constants of 6.7 × 10^{-8} and 9.4 × 10^{-11} M, respectively. In dot-blot binding experiments, it was found that binding of calmodulin causes a down-regulation of the homophilic self-association of C-CAM. This suggests that calmodulin can regulate the functional activity of C-CAM.

Cell adhesion molecules (CAMs) are important for the organization and integrity of tissues in multicellular organisms (1, 2). Recently, several CAMs have also been demonstrated to be active in transmembrane signaling (3–5). The spatiotemporal expression of many CAMs during development, as well as during various physiological and pathological processes in adult organisms, is highly dynamic (1, 2, 6), which implies that their expression and functional activities are strictly regulated. It has been demonstrated that growth factors, cytokines, hormones, and homeodomain proteins regulate the expression of various CAMs (7–10). It is also well documented that the functional activity of some adhesion proteins can be regulated by phosphorylations of their cytoplasmic domains (3). Several CAMs also interact with other cytoplasmic proteins that influence their function (2, 11–13). However, for most adhesion proteins, our knowledge of interacting cytoplasmic components, their mode of functional regulation, and their participation in signal transduction pathways (14) is still very scanty. Identification and characterization of cytoplasmic components that interact with transmembrane cell adhesion proteins are therefore of great importance as a first step toward understanding how these proteins are regulated.

C-CAM is a cell adhesion molecule that originally was identified in adult rat hepatocytes (15). It is also expressed in several epithelial, vessel endothelial, and hematopoietic cells (16). The homologous molecules in mouse and man are known as Bgp and BGP, respectively (17, 18). C-CAM/Bgp/BGP are members of the carcinoembryonic antigen gene family, which belongs to the immunoglobulin gene superfamily (19–22). Different isoforms of C-CAM exist as a function of alternative splicing and different glycosylation (21, 23, 24). The two major isoforms differ in the length of their cytoplasmic domains, C-CAM1 having a cytoplasmic domain of 75 amino acid residues, whereas that of C-CAM2 only consists of 14 amino acid residues.

C-CAM can mediate cell-cell adhesion via calcium-independent homophilic binding (25). Based on expression in insect cells, it has been reported that the C-CAM1 cytoplasmic domain is necessary for cell adhesion activity (26), but expression studies in other cell types have recently demonstrated that both C-CAM1 and C-CAM2 are effective adhesion molecules (27). In addition to cell adhesion, other activities have also been described for C-CAM. C-CAM1 has been described as an ecto-ATPase (21) and a bile salt transporter (28). In the mouse, one of the allelic variants of Bgp functions as the receptor for mouse hepatitis virus (29). Moreover, C-CAM1 can suppress tumorigenicity of prostate cancer cells (30). Whether this activity depends on its adhesive properties or some other function is not known.

We have previously reported that the C-CAM cytodomain binds to the intracellular calcium-regulated protein calmodulin (31, 32). Calmodulin is a major regulator of enzyme activities and cytoskeletal functions in eukaryotic cells. High concentrations of calmodulin are found in submembranous regions coinciding with sites of C-CAM enrichment (33–36), suggesting that it might regulate C-CAM activities. In this investigation, we have used synthetic peptides to determine the binding sites in C-CAM for calmodulin and biosensor technology to determine kinetic and equilibrium binding constants of the C-CAM/calmodulin interaction. We also present evidence indicating that calmodulin regulates the homophilic binding activity of C-CAM.

MATERIALS AND METHODS

Peptide Synthesis—Chemicals and solvents were of the highest purity available. 1-Methyl-2-pyrrolidone and N,N-dimethylformamide were used without further purification. Fmoc, dansyl, 5-dimethylaminonaphthalene-1-sulfonyl, and (9-fluorenyl)methoxycarbonyl were obtained from Novabiochem (Bad Soden, Germany). MOPS, 4-morpholinepropanesulfonic acid, and N,N-dimethylformamide were purchased from Pharmacia Biotechnology AB (Uppsala, Sweden). Phosphatidylcholine, HEPES, bovine serum albumin, horse IgG, 3-acetamidophenanthroline, and calmodulin were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Methyl-2-pyrrolidone and N,N-dimethylformamide were used without further purification.
Calmodulin Binding to C-CAM

**Calmodulin Binding to C-CAM1 cytoplasmic domain peptides.** Cellulose membranes containing 67 individual decapeptides covering the cytoplasmic domain of C-CAM1 were incubated with \(^{125}\text{I}\)-labeled calmodulin (1 µg/ml, 2 × 10^6 cpm/µg of protein). Calmodulin binding was detected by autoradiography (A) or γ-radiation counting (B). A, the peptide numbers (given for the first and last peptide in each row) correspond to the sequences shown in B. B, the amino acid sequence of the long cytoplasmic domain of C-CAM1 is shown in single letter code. TM, transmembrane sequence. Sixty-seven overlapping decapeptides staggered by one amino acid were synthesized as indicated. The peptides are numbered from the amino terminus. The amounts of calmodulin bound to peptides 1–10, 46–58, and 63–67 (the sequences of which are indicated by horizontal lines) are given as cpm values. Each value represents the mean of three individually synthesized peptides. Peptides 11–45 and 59–62 showed no binding activity (see also A).

were purified on a molecular sieve with a pore size of 4 Å (Sigma) according to the instructions of the manufacturer. Fmoc-L-amino acid active esters and activated cellulose membranes (SPOT™ membranes) were purchased from Cambridge Research Biochemical (37, 38). Peptides were synthesized following the protocol of the manufacturer. Briefly, the activated amino acids were dissolved in 1-methyl-2-pyrrolidone and applied to the appropriate spot on the activated membrane. After 15 min of incubation at room temperature, this step was repeated once. Excess chemicals were rinsed away by repeated N,N-dimethylformamide washes, and residual amino groups were blocked by acetylation with acetic anhydride. Protecting Fmoc groups were cleaved by 20% piperidine/N,N-dimethylformamide, and the spots on the membrane were stained with bromophenol blue to facilitate further amino acid additions. The membrane was prepared for the next coupling cycle by repeated N,N-dimethylformamide washes, one wash with methanol, and air-drying. After the last coupling cycle, free amino groups were acetylated, and protecting groups on the amino acid side chains were removed by incubating the membrane in dichloromethane/trifluoroacetic acid (1:1, v/v) for 1 h.

**Amino Acid Analysis**—Amino acid analysis was performed by the Amino Acid Analysis Facility at the Biomedical Center, Uppsala. Spots containing synthesized peptides were cut out, and amino acids were determined by an LKB Model 4151 Alpha Plus amino acid analyzer after hydrolysis of the samples in vacuo with 6 N HCl at 110 °C for 24 h.

**Calmodulin Binding to Synthetic Peptides**—Calmodulin from bovine testes was purchased from Pharmacia Biosensor AB (Uppsala) or Sigma and labeled with \(^{125}\text{I}\) by a lactoperoxidase-catalyzed reaction as described previously (39). Cellulose membranes with synthesized peptides were blocked overnight with a protein solution provided by the manufacturer (Cambridge Research Biochemical). The membranes were washed for 1 h in reaction buffer (50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 1 mM CaCl\(_2\)) and incubated with rocking for 2 h at room temperature with \(^{125}\text{I}\)-calmodulin included in the buffer (1 µg/ml, 2 × 10^6 cpm/µg of protein). After additional washing with reaction buffer, the filters were dried, and bound calmodulin was detected by autoradiography on Fuji RX x-ray films. Bound \(^{125}\text{I}\)-calmodulin was quantified by cutting out individual spots and counting in a γ-spectrometer. For analysis of calcium dependence, binding and washings were performed in calcium/EGTA buffer (10 mM MOPS, pH 7.0, 100 mM KCl, 2 mM EGTA, CaCl\(_2\) to the desired concentration); calcium concentrations (10⁻³ to 10⁻⁵ M) were determined with a calcium-sensitive electrode. The sequence of the myosin light chain kinase peptide used in these studies was based on the amino acid sequence of the chicken smooth muscle myosin light chain kinase calmodulin-binding domain (40).

**Biosensor Analysis**—Real-time analysis of the binding between calmodulin and C-CAM was performed with a BIAcore biosensor instrument (Pharmacia Biosensor AB) (41, 42). CMS sensor chips and amine coupling kits were obtained from Pharmacia Biosensor AB. Calmodulin was immobilized on the CMS sensor chip as recommended by the manufacturer. Briefly, the pumps and flow chambers were equilibrated with 10 mM Hepes, pH 7.4, 0.15 M NaCl, 0.05% surfactant P-20; and a continuous flow was maintained at 5 µl/min. The dextran layer of the sensor chip was activated by injecting 35 µl of 0.05 M N-hydroxysuccinimide and 0.05 M N-ethyl-N-(3-diethylaminopropyl)carbodiimide. Next, 100 µg of calmodulin in 35 µl of 10 mM formate buffer, pH 3.7, was injected. Excess reactive groups were blocked by injection of 35 µl of ethanolamine.

Binding experiments were performed at 25 °C by injecting 35–40-µl portions of various concentrations of C-CAM (0.6–63 nM) dissolved in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% octyl glucoside, and 1 mM CaCl\(_2\) or 2 mM EGTA at a continuous flow maintained at 5 or 10 µl/min. In some experiments, calmodulin (0.5 µM) was included in the flow buffer during the dissociation phase to prevent rebinding of C-CAM to the sensor surface. The sensor chip was regenerated by washing with reaction buffer. C-CAM was purified from rat liver, and its concentration was determined by radioimmunoassay as described previously (43).
Calcium Dependence of the C-CAM/Calmodulin Interaction—Calcium ions are required for calmodulin to bind to most of its ligands (45). By varying the Ca\(^{2+}\) concentration between 10\(^{-8}\) and 10\(^{-3}\) M, we found that maximal binding of calmodulin to C-CAM peptides occurred at a calcium level of 10\(^{-6}\) M Ca\(^{2+}\). Binding of calmodulin to C-CAM was determined by preincubating the \(^{125}\)I-C-CAM with calmodulin (1 \(\mu\)g/ml) for 30 min before addition to the filters.

RESULTS

Binding of Calmodulin to Synthetic Peptides—Radiolabeled calmodulin and peptides synthesized on cellulose filters were used to map the calmodulin-binding sequences in the cytoplasmic domains of the C-CAM isoforms. Sixty-seven decapeptides staggered by one amino acid residue were synthesized to represent the entire 75-amino acid residue cytoplasmic domain of C-CAM1; six peptides covered the 14-amino acid residue long C-CAM2 cytoplasmic domain. The long cytoplasmic domain was found to have one strongly binding region (peptides 2-5) and two regions with weaker binding activities (peptides 47-57 and 66-67) (Fig. 1, A and B). The peptides covering the cytodomain of C-CAM2, the amino acid sequence of which is identical to that of C-CAM1 in the most membrane-proximal residues, also showed strong calmodulin binding (peptides 2-6) (Fig. 2).

Since the coupling efficiency might differ for different amino acids in the peptide synthesis, it was important to investigate if the observed differences in calmodulin binding between the different peptides reflected differences in peptide content on the filters rather than sequence-specific differences. Analyses of the amino acid contents in the different spots on the filters showed no correlation between the amount of peptide in each spot and the degree of bound calmodulin (data not shown). Thus, the different calmodulin binding activities are sequence-related.

To investigate if the mouse and human homologues of rat C-CAM, Bgp and BGP, respectively, would also bind calmodulin, peptides corresponding to the cytoplasmic domains of Bgp and BGP were synthesized and tested. The membrane-proximal regions of both the long and short isoforms of Bgp and BGP were depleted of calcium ions by extensive washing in an EGTA-containing buffer (10 mM MOPS, pH 7.0, 100 mM KCl, 2 mM EGTA). The membrane-proximal domains of the C-CAM isoforms. Sixty-seven decapeptides corresponding to all of the calmodulin-binding regions of C-CAM were synthesized and tested. The membrane-proximal portions of the long and short cytoplasmic domains of C-CAM, BGP, and Bgp were synthesized and subjected to \(^{125}\)I-calmodulin binding. Binding was detected by autoradiography. The amino acid sequences (shown in single letter code) represent homologous regions of C-CAM, BGP, and Bgp.

![Calmodulin Binding to C-CAM](image)

**Fig. 3.** Calmodulin binding to BGP and Bgp cytoplasmic domain peptides. Homologous peptides corresponding to the membrane-proximal portions of the long and short cytoplasmic domains of C-CAM, BGP, and Bgp were synthesized and subjected to \(^{125}\)I-calmodulin binding. Binding was detected by autoradiography. The amino acid sequences (shown in single letter code) represent homologous regions of C-CAM, BGP, and Bgp.

![Calmodulin Binding to C-CAM](image)

**Fig. 2.** Calmodulin binding to C-CAM2 cytoplasmic domain peptides. Six overlapping decapeptides covering the short cytoplasmic domain of C-CAM2 were synthesized and subjected to \(^{125}\)I-calmodulin binding. For further details, see the legend to Fig. 1. TM, transmembrane sequence.

Evaluation and calculation of binding parameters were done according to the manual provided by Pharmacia Biosensor AB, using the following relationship: dR/dt = k\(_a\)R - k\(_d\)R, where k\(_a\) is the association rate constant, k\(_d\) is the dissociation rate constant, and R is the binding expressed in relative units, and k\(_\text{app}\) = k\(_a\) + k\(_d\) is the maximum binding that theoretically can be obtained (44). Plots of dR/dt versus relative response (R) gave linear relationships. The negative values of the slopes (k\(_d\)) at each concentration of C-CAM were replotted against the C-CAM concentration (C). This gave a straight line, from the slope of which the association constant (k\(_a\)) could be determined. The equilibrium dissociation constant (K\(_d\)) was determined from the equilibrium binding levels (R\(_\text{app}\)) by Scatchard plot analysis using the following relationship: R\(_\text{app}\)/C = K\(_d\)R\(_\text{app}\) - K\(_d\)K\(_a\), where K\(_d\) is the equilibrium association constant (K\(_a\) = 1/K\(_d\)). K\(_d\) was also calculated from the relationship between the rate constants (K\(_d\) = k\(_d\)/k\(_a\)).

Calmodulin Binding to Liver Membranes—Plasma membranes were isolated from rat livers as described previously (43). The membranes were depleted of calcium ions by extensive washing in an EGTA-containing buffer (10 mM MOPS, pH 7.0, 100 mM KCl, 2 mM EGTA). The membranes were then washed twice in the same solutions supplemented with Ca\(^{2+}\) to Ca\(^{2+}\) concentrations of 10\(^{-5}\) or 10\(^{-4}\) M. Binding of calmodulin was determined in a total reaction volume of 100 \(\mu\)l containing buffer (10 mM MOPS, pH 7.0, 100 mM KCl, 2 mM EGTA). The membrane-proximal region covered by the short cytoplasmic domain of C-CAM, Bgp, and BGP showed no binding (data not shown). The membrane-proximal regions of both the long and short isoforms of Bgp and BGP were synthesized and tested. The membrane-proximal regions of both the long and short isoforms of Bgp and BGP were depleted of calcium ions by extensive washing in an EGTA-containing buffer (10 mM MOPS, pH 7.0, 100 mM KCl, 2 mM EGTA). The membrane-proximal domain of C-CAM, Bgp, and BGP showed weak binding, whereas that of BGP showed no binding (data not shown).
Calmodulin Binding to C-CAM

The well characterized calmodulin-binding protein myosin light chain kinase bound calmodulin with a similar Ca\(^{2+}\) dependence (Fig. 4B).

Mutational Analysis of Binding Peptides—No consensus binding sequence exists, but in many calmodulin-binding proteins, the binding sites adopt a special structure of a basic, amphipathic \(\alpha\)-helix (46). There was no strong prediction of an \(\alpha\)-helical structure in C-CAM using the Garnier algorithm, although it is possible that binding of calmodulin could induce an \(\alpha\)-helical conformation. The C-CAM peptides that bound calmodulin, however, shared some common characteristics in that they contained both positively charged amino acids and several hydroxylated as well as hydrophobic amino acid residues.

To identify amino acid residues that are needed for calmodulin binding, we systematically changed all amino acids in the peptide sequences. The binding of calmodulin to each series of peptides is given in relative figures, where binding to the unmodified peptide was set to 100%.

| Peptide sequence          | Mutation  | Binding |
|---------------------------|-----------|---------|
| C-CAM isofrm              |           |         |
| C-CAM1                    | LYSRKTGGGS| 100     |
| R → A                     | 46        |
| K → A                     | 58        |
| RK → AA                   | 22        |
| C-CAM1                    | LNFNAQQSKR| 100     |
| K → A                     | 74        |
| R → A                     | 115       |
| KR → AA                   | 61        |
| C-CAM1                    | QQSKRPTSA| 100     |
| K → A                     | 44        |
| R → A                     | 56        |
| KR → AA                   | 30        |
| C-CAM2                    | SRKTGGSSSF| 100     |
| R → A                     | 40        |
| K → A                     | 79        |
| RK → AA                   | 31        |

The Scatchard data could not be used to calculate the stoichiometry of binding, as shown in Fig. 6. This is in reasonable agreement with the \(K_D\) values obtained from the kinetic data. However, it is obvious from Fig. 6 that the slopes of the lines, due to the limited number of data points, do not correspond exactly to the limiting slopes of the smooth curve to which the experimental values were fitted. Thus, the calculated value of the larger \(K_D\) (3.8 × 10\(^{-9}\) M) is too small, and that of the smaller \(K_D\) (1.8 × 10\(^{-10}\) M) is too large. Accordingly, there is good agreement between the kinetic and equilibrium analyses.

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Calmodulin Binding to C-CAM

**Fig. 6.** Scatchard plot analysis. The equilibrium binding of C-CAM to the calmodulin biosensor chip was evaluated by Scatchard plot analysis. C-CAM concentrations ranged from 0.6 to 12.7 nM. Relative responses expressed as equilibrium binding values (R_{eq}) and C-CAM concentrations (C) were plotted as indicated. The broken line represents a calculated curve fit of the experimental values. The reciprocal of the slopes of the solid lines gave values for the dissociation constants (K_{d}) of 3.8 × 10^{-9} and 1.8 × 10^{-10} M. RU, relative units.

**Fig. 5.** Binding kinetics of C-CAM to immobilized calmodulin. Representative overlaid sensograms illustrating the real-time binding of C-CAM at various concentrations (0.6, 3.2, 6.4, and 12.7 nM, from bottom to top) to calmodulin immobilized on a biosensor chip are shown. The inset shows dissociation, with and without 0.5 μM calmodulin included in the buffer during the dissociation phase, after injection of 12.7 nM C-CAM. Arrows indicate phases of association (A), equilibrium (B), and dissociation (C). RU, relative units.

**DISCUSSION**

In a previous study, we demonstrated that the cytoplasmic domains of both C-CAM1 and C-CAM2 could bind calmodulin (32). The data were compatible with two binding sites in C-CAM1 and one binding site in C-CAM2. In the present investigation, we have mapped the calmodulin-binding sites in the cytoplasmic domains of C-CAM1 and C-CAM2 more closely, utilizing synthetic peptides attached to a cellulose filter. The membrane-proximal regions of both C-CAM isoforms bound calmodulin effectively; a weaker binding site was also found in the distal portion of the C-CAM1 cytoplasmic domain. All peptide sequences, as well as C-CAM intercalated in plasma membranes, bound calmodulin in a strictly calcium-dependent manner. Biosensor analyses demonstrated that two classes of binding interactions with different dissociation rates existed. We could also demonstrate that calmodulin binding caused a down-regulation of the self-association of C-CAM.

Attached peptides, directly synthesized on cellulose membranes, have several advantages over soluble, conventionally synthesized peptides in ligand binding studies. They allow screening of large sequences in a rapid and relatively inexpensive way. Furthermore, binding of various ligands to the cellulose membranes is easy to determine. With soluble peptides, a different monitoring assay has to be used. One approach that has been used for calmodulin is to determine the change in fluorescence intensity of dansyl-labeled calmodulin that occurs, due to conformational changes, when large peptides (20 amino acids or more) bind (47). We tried this approach with a soluble decamptide corresponding to the cytoplasmic domain of C-CAM2. However, we did not observe any change in the fluorescence intensity, probably because a decamptide is too short to induce the conformational change of the calmodulin. On the other hand, a decamptide corresponding to the identified cal-

chirometry of the C-CAM-calmodulin complex since it was not known how much of the surface-bound calmodulin was available for interaction with C-CAM. Since calmodulin is a much smaller molecule than C-CAM, it penetrates the hydrogel of the sensor chip more effectively and accordingly is withdrawn from binding interactions with C-CAM. Furthermore, some of the calmodulin might be inactivated as a result of the covalent coupling to the hydrogel.

Calmodulin Binding to Liver Cell Membranes-To investigate if calmodulin binds to C-CAM that is intercalated in the plasma membrane, binding experiments with isolated rat liver plasma membranes were performed. We have previously demonstrated that C-CAM is one of four major calmodulin-binding proteins in such membranes (31). Three of these proteins, including C-CAM, need calcium, whereas one of them can bind calmodulin in the absence of calcium. As demonstrated in Fig. 7, ^{125}I-calmodulin bound to the plasma membranes in the absence of calcium ions, but the binding increased 2.6 times when calcium was added. Antibodies against C-CAM reduced the binding when Ca^{2+} was present, but not when it was absent. Nonimmune immunoglobulins did not influence the binding under either condition. Since we used polyclonal antibodies, it is not known if the inhibitory effect was due to a direct competition with calmodulin or to an effect on the conformation of C-CAM that affected calmodulin binding. The important thing is that antibodies specifically recognizing C-CAM could inhibit calmodulin binding, demonstrating that calmodulin can bind to C-CAM naturally located in the plasma membrane.

Effect of Calmodulin on C-CAM Homophilic Binding—One of the known functional properties of C-CAM is that it can bind to itself, thereby mediating homophilic cell adhesion. To investigate if calmodulin could modulate the homophilic binding properties of C-CAM, we used an assay in which binding of ^{125}I-labeled C-CAM to C-CAM spotted onto filters was determined. In agreement with previous results (25, 31), we found that C-CAM can bind to itself in a calcium-independent manner (Fig. 8). Calmodulin in the presence of calcium greatly reduced this binding. No obvious effect of calmodulin was seen in the absence of calcium.

**Fig. 7.** Arrows indicate phases of association (A), equilibrium (B), and dissociation (C). RU, relative units.

**Fig. 5.** Binding kinetics of C-CAM to immobilized calmodulin. Representative overlaid sensograms illustrating the real-time binding of C-CAM at various concentrations (0.6, 3.2, 6.4, and 12.7 nM, from bottom to top) to calmodulin immobilized on a biosensor chip are shown. The inset shows dissociation, with and without 0.5 μM calmodulin included in the buffer during the dissociation phase, after injection of 12.7 nM C-CAM. Arrows indicate phases of association (A), equilibrium (B), and dissociation (C). RU, relative units.
modulin-binding sequence of myosin light chain kinase, synthesized by the SPOT™ method, bound calmodulin with the expected calcium dependence. These results clearly demonstrate the superiority of membrane-bound peptides to soluble peptides for determination of binding sequences for various ligands.

Binding experiments with mutated C-CAM peptides showed that the positively charged amino acids arginine and lysine are important for calmodulin binding. That amino acids other than arginine and lysine are also important for calmodulin binding was clearly demonstrated by the fact that the binding activities of two overlapping peptides, FLYSRKTTGS and AYFLYS- RKTG, differed 5-fold even though both contained the two basic residues. These results are in good agreement with the characteristics of binding sequences in other calmodulin-binding proteins (48).

Mouse Bgp and human BGP, homologous molecules to rat C-CAM, were also able to bind calmodulin. For mouse Bgp, the binding is not surprising as mouse Bgp and rat C-CAM show a high degree of sequence identity in their cytoplasmic domains. The only difference between the short isoforms is that a threonine residue in the rat molecule is a serine residue in the mouse molecule. The short form of mouse Bgp bound calmodulin similarly compared with the corresponding sequence of C-CAM2. The long cytoplasmic domain of Bgp, the sequence of which is 84% identical (93% conserved) to the corresponding part of C-CAM1, exhibited the same two calmodulin-binding regions as C-CAM1. The human BGP cytoplasmic sequences are not conserved to the same extent. The overall sequence identity between the long cytoplasmic domains of the human and rat proteins is only 60% (83% conserved), and the similarity is even less in the region corresponding to the short cytoplasmic domain (50% identity and 71% conserved). These differences notwithstanding, calmodulin bound effectively also to sequences corresponding to the membrane-proximal portion of both cytoplasmic domain variants of human BGP. This can be explained if BGP and C-CAM adopt an α-helical conformation, which makes the distribution of positively charged and hydrophobic amino acids strikingly similar in the two molecules. Calmodulin generally seems to bind to α-helical stretches (46, 49). Thus, it seems as if the ability to bind calmodulin to the short isoform and to the membrane-proximal part of the long isoform has been conserved in spite of the relatively large sequence difference. Unlike the rat and mouse proteins, peptides from the distal part of the long isoform of human BGP did not bind calmodulin. Although we cannot exclude the possibility that longer peptides would reveal distal binding sites in the human cytodomain, this result suggests that the proximal binding site may be more significant than the other ones in the native proteins.

The activation of calmodulin in vivo is caused by an increased intracellular calcium concentration (50), which leads to binding to its target proteins (45). Therefore, the finding that calmodulin binding to the synthetic C-CAM peptides was regulated by variations of the calcium concentration in the physiological range (10−7 to 10−5 M) supports the notion that the interaction is of physiological importance.

The biosensor analyses revealed both a weak (K_D = 6.7 × 10−9 M) and a strong (K_D = 9.4 × 10−11 M) binding interaction between calmodulin and intact rat liver C-CAM, which contains both C-CAM1 and C-CAM2. Thus, the two classes of binding sites might correspond to the membrane-proximal and membrane-distal binding sites that were discovered by the peptide binding experiments. Alternatively, the two classes of binding sites might reflect the presence of different oligomeric forms of C-CAM, which has been found to occur as both monomers and dimers in the plasma membrane.2 Whereas both classes of binding sites had the same rate of association, their dissociation rates differed. The association rate was relatively fast, which might be of physiological importance because it would allow a rapid association of calmodulin with C-CAM in

2I. Hunter and B. Öbrink, unpublished observations.
Transient increase in the intracellular concentration of Ca\(^{2+}\) is caused by ligand/receptor interactions at the cell surface, which trigger a different C-CAM molecules. In any case, it seems plausible that C-CAM molecules within the same membrane or between opposing membranes can lead to increased binding of calmodulin to the cytoplasmic domains of C-CAM. This could then lead to reduced homophilic C-CAM binding, which could be either between opposed membranes or between C-CAM molecules within the same membrane.

Acknowledgments—We thank Monica Lindh for expert help with purification and concentration determination of C-CAM and preparation of liver plasma membranes. Professor Arne Holmgren is gratefully acknowledged for making the BiACore instrument available to us.

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27. Sonic binding (i.e. C-CAM-mediated binding between opposed membranes) or cis-binding (i.e. binding between C-CAM molecules within the same membrane) between different C-CAM molecules. In any case, it seems plausible that ligand/receptor interactions at the cell surface, which trigger a transient increase in the intracellular concentration of Ca\(^{2+}\), can lead to increased binding of calmodulin to the cytoplasmic domains of C-CAM. This could then lead to reduced homophilic C-CAM binding, which could be either between opposed membranes or between C-CAM molecules within the same membrane.