Evidence for regulated dimerization of cell–cell adhesion molecule (C-CAM) in epithelial cells

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C-CAM is a Ca^{2+}-independent cell adhesion molecule (CAM) belonging to the immunoglobulin superfamily. Addition of chemical cross-linkers to isolated rat liver plasma membranes, intact epithelial cells and purified preparations of C-CAM stabilized one major C-CAM-containing product whose apparent molecular mass was approximately twice that of the C-CAM monomer. The failure to detect additional proteins after cleavage of the cross-linked species demonstrated that C-CAM exists as non-covalently linked dimers both in solution and on the cell surface. Dimerization occurred to the same extent in adherent monolayers and in single cell populations, indicating that dimer formation was the result of *cis*- interactions within the membranes of individual cells. Using isoform-specific anti-peptide antibodies, both C-CAM1 and C-CAM2 were found to be involved in dimerization, forming predominantly homo-dimeric species. Both calmodulin and Ca^{2+} ionophore modulated the level of dimer formation, suggesting a role for regulated self-association in the functional activity of C-CAM.

INTRODUCTION

Cell adhesion is a spatially and temporally dynamic process, essential for the normal development and function of multicellular organisms. Specific cell–cell adhesion is mediated by a number of cell adhesion molecules (CAMs), whose regulated expression is believed to direct the fundamental processes determining tissue morphology [1–3].

C-CAM is a transmembrane glycoprotein, belonging to the immunoglobulin superfamily, originally identified as mediating intercellular adhesion of isolated rat hepatocytes [4]. The two major isoforms, C-CAM1, whose cytoplasmic domain is 71 amino acids in length and C-CAM2, which has a short cytoplasmic domain of only 10 amino acids [5–7], are alternatively spliced products of the C-CAM gene [8]. Both isoforms have been shown to support Ca^{2+}-independent homotypic cell aggregation [9–13]. While recent studies [12,13] have demonstrated a homophilic interaction between C-CAM2 molecules on opposing cell membranes, the basis for C-CAM1-mediated adhesion remains to be established.

In addition to mediating cell–cell adhesion, C-CAM1 acts as a bile acid transporter [14] and has been suggested to be an ecto-ATPase [5], although recent evidence indicates that this enzymic activity is due to a co-purifying protein [15]. Both C-CAM1 [16] and the homologous murine protein [17] have been shown to suppress tumour growth.

There is increasing evidence that CAMs are important transducers of cellular signals [18–20]. Consistent with this, the cytoplasmic domain of C-CAM1 contains two tyrosine residues, absent from C-CAM2, and is a substrate for the insulin receptor tyrosine kinase [21]. The two tyrosine residues of C-CAM1 are present within a tyrosine-based activation motif (TAM), a motif originally recognized within the cytoplasmic domains of components of the B and T cell antigen receptors [22], and which has been demonstrated to act as the basic signalling module of these receptors by its ability to bind tyrosine kinases of the src and syk/ZAP 70 families [23]. Interestingly, the human homologue of C-CAM1 is also phosphorylated on tyrosine residues [24,25] and binds $pp60^{c\text{-}src}$ [25]. The cytoplasmic domains of both C-CAM1 and C-CAM2 bind to calmodulin [26–28], a regulatory protein which functions as an important mediator of Ca^{2+}-dependent signalling pathways [29]. In addition, C-CAM has been shown to co-purify with a number of as yet unidentified cellular proteins [30–32].

Despite its ability to interact with a number of proteins, the molecular organization of C-CAM within the cell remains unknown. Using chemical cross-linkers to stabilize close protein–protein interactions we now report that both C-CAM1 and C-CAM2 have the ability to self-associate forming dimers by *cis*-interactions within the membranes of single cells and that the extent of dimer formation can be regulated by intracellular Ca^{2+} and calmodulin.

EXPERIMENTAL

Preparation of membranes and purification of C-CAM

Plasma membranes were prepared from rat liver [4], and C-CAM was purified [31] and quantified [33] as described previously.

Cell culture

The rat bladder carcinoma cell line, NBT II, was cultured as described previously [34]. These cells undergo epithelial to

Abbreviations used: CAM, cell adhesion molecule; DSP, dithiobis(succinimidyl) propionate; DTSSP, 3,3′dithiobis(sulphosuccinimidyl) propionate; DTT, dithiothreitol; FCS, fetal calf serum; KLH, keyhole-limpet haemocyanin; MBS, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester; TAM, tyrosine-based activation motif.

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mesenchymal transitions in response to a number of growth factors [35], and during routine culture, apparently in response to growth in a particular batch of fetal calf serum (FCS), a number of cells became dissociated, some having a more elongated, fibroblast-like morphology. After cloning by limited dilution, four sub-clones (1e, 6c, 7d and 9f), selected on the basis of their altered morphology, were chosen for further study and were cultured under identical conditions to those of the parent cells. For metabolic labelling, adherent NBT II cells, grown to 80% confluence in 10 cm-diam. culture dishes, were rinsed twice with Dulbecco's modified Eagle's medium lacking cysteine and methionine and were incubated overnight at 37 °C in the same medium containing 10% dialysed FCS (Gibco), 250 μCi of [^{35}S]cysteine and 250 μCi [^{35}S]methionine (Amersham). Adherent cells were briefly treated with 0.05% (w/v) trypsin and 1 mM EDTA in PBS, pipetted gently to cause dissociation and were then suspended in complete growth medium.

The hepatocyte cell line RALA255-10G was obtained from Dr. J. Chou (NIH, Bethesda, MD, U.S.A.) and cultured as described [36] at the permissive temperature of 33 °C. CHO cells, stably transfected with either C-CAM1 or C-CAM2, were grown as described previously [11].

Antibodies

Polyclonal antibodies against C-CAM were prepared and affinity purified [4,31]. For the preparation of anti-peptide antibodies, peptides corresponding to sequences present in the cytoplasmic domains of C-CAM1 (QSKRPTSASSSPTERVYSVVKKK) and C-CAM2 (TGGSGSF) were synthesized (Sawaday Technology) with an additional cysteine residue at the N-terminus to act as a linker to the carrier protein, keyhole-limpet haemocyanin (KLH). KLH in 10 mM phosphate buffer, pH 7.2, was reacted with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) (Sigma), and stirred for 30 min at room temperature. After removal of free MBS by chromatography on a Sephadex G-25 column, activated KLH was incubated with peptides for 4 h at room temperature. Rabbits were immunized by subcutaneous injection with 100 μg of KLH-coupled peptide in Titer-Max (1:1, by vol.) (CytRx Corp.) boosted with 100 μg KLH–peptide without adjuvant on day 30, with 50 μg of KLH–peptide in Titer-Max (1:1) after a further 7 days and were bled 10 days later. The anti-peptide antibodies, L2 (raised against a peptide corresponding to sequences present in C-CAM1) and S1 (raised against a peptide corresponding to sequences present in C-CAM2), were purified using affinity chromatography by overnight incubation with peptide-coupled cellulose, eluted with 0.1 M glycine, pH 2.5, and after neutralization with 1/5 vol. of 1 M Tris/HCl, pH 8.0, stored at −70 °C.

Chemical cross-linking

Prior to cross-linking, plasma membranes and cells were washed twice with PBS or buffer 3 (10 mM Hepes containing 137 mM NaCl, 4.7 mM KCl, 0.6 mM $MgSO_4$, 1.2 mM $CaCl_2$, pH 7.4), and plasma membranes and dissociated cells were resuspended in, or adherent cells were overlayed with, the same buffer. Cross-linking was initiated by the addition of either dithiobis(succinimidyl) propionate (DSP) or 3,3′dithiobis-(sulphosuccinimidyl) propionate (DTSSP) (Pierce Chemical Company) at the indicated concentrations. After 30 min at room temperature, the reaction was quenched by the addition of Tris/HCl, pH 7.4 at a final concentration of 50 mM. Cell counts before and after cross-linking, confirmed that no aggregation of suspended cells had occurred. Plasma membranes and cells were extracted with 10 mM Tris/HCl, 150 mM NaCl, pH 7.4, containing 1% (v/v) Triton X-100, 1 mM PMSF and 1000 kallikrein inhibitory units/ml Trasylol for 30 min at 4 °C and the protein concentration of the supernatants was estimated by a modified Lowry micro-assay [37].

Purified C-CAM in 0.1% (v/v) Triton X-100, PBS was cross-linked with a 10- or 50-fold molar excess of DTSSP as described above. To examine the effects of calmodulin, purified C-CAM in 25 mM Hepes, pH 7.4, containing 0.1% (w/v) *n*-octyl-Δ-glucopyranoside, 0.1 M NaCl and either 1 mM $CaCl_2$ or 2 mM EGTA was incubated with 5 μM calmodulin (from bovine testis; Pharmacia) for 2 h at room temperature, prior to cross-linking with a 50-fold molar excess of DTSSP as described above.

Biotinylation

In some experiments, plasma membranes, cross-linked and quenched as described above, were biotinylated by resuspension in PBS containing 0.5 mg/ml sulpho-NHS-biotin (Pierce). After 15 min at room temperature, the membranes were pelleted and resuspended twice in 50 mM Tris/HCl, pH 7.4, prior to extraction as described above.

Ionophore treatment

Confluent cultures of NBT II cells were incubated in buffer 3 or buffer 3 lacking bivalent cations, together with 10 μM ionomycin or A23187 (Sigma), or vehicle (DMSO) alone. After incubation at 37 °C for varying periods of time, the cells were cross-linked as described above. In some experiments, the ionophore was removed prior to cross-linking, without noticeable effect on the results.

Immunoaffinity chromatography, immunoprecipitation and immunodepletion

Detergent extracts of biotinylated, cross-linked plasma membranes were incubated with anti-C-CAM–Sepharose and the C-CAM-containing species were eluted [31]. For immunoprecipitation, purified C-CAM or detergent extracts of plasma membranes or cells were incubated with antibodies overnight at 4 °C. Immunoprecipitates were collected after the addition of Protein-A–Sepharose CL-4B beads for 2 h at 4 °C and the bound proteins were eluted by boiling in SDS sample buffer [38] in the presence or absence of 10 mM dithiothreitol (DTT). Purified C-CAM preparations were depleted of the C-CAM1 isoform by repeated immunoprecipitation with the L2 anti-peptide antibody, before cross-linking as described above.

Electrophoresis and immunoblotting

Non-ionic detergent extracts and detergent-insoluble pellets of plasma membranes and cells were boiled in non-reducing SDS sample buffer [38] prior to SDS/PAGE. Western blotting was performed as described previously [34], and immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham). Biotinylated proteins were detected using horseradish peroxidase-conjugated streptavidin before development with ECL. Films were scanned using a Shimadzu CS-930 scanner equipped with an automatic integrator. For two-dimensional electrophoresis, slices from gels run under non-reducing conditions were equilibrated for 30 min at room temperature in 125 mM Tris/HCl, pH 6.8, containing 0.1% (w/v) SDS and 50 mM DTT before SDS/PAGE in the second dimension.

Immunofluorescence

Staining of paraformaldehyde-fixed monolayers of NBT II cells and subclones with affinity purified polyclonal antibodies against rat liver C-CAM was performed exactly as described previously [34].

RESULTS

Characterization of anti-peptide antibodies

In addition to the major C-CAM1 (110 kDa) and C-CAM2 (105 kDa) isoforms, several non-reducible polypeptides having an apparent molecular mass of 150, 200–215 or > 300 kDa were recognized by affinity-purified anti-C-CAM antibodies in extracts of rat liver plasma membranes (Figure 1, lane 1). While C-CAM monomers were efficiently extracted from plasma membranes, the high-molecular-mass C-CAM-containing species were found predominantly in the non-ionic-detergent-insoluble fraction (Figure 1, lane 2), suggesting that they were more tightly bound to the cytoskeleton.

The anti-peptide antibody L2, recognized the C-CAM1 monomer and several higher-molecular-mass species present in plasma membranes, giving a particularly strong reaction with a band of about 205 kDa (Figure 1, lane 3), while the S1 antibody reacted with only the C-CAM2 monomer (Figure 1, lane 4). Immunoprecipitation of plasma-membrane extracts with L2 (Figure 1, lane 5) or S1 (Figure 1, lane 6) and subsequent blotting with anti-C-CAM antibodies confirmed the specificity of these anti-peptide antibodies in addition to the presence of a number of high molecular-mass L2-reactive polypeptides in plasma membranes. Baum et al. [39] have recently reported similar results.

While purified C-CAM consisted of both C-CAM1 (Figure 1, lane 8) and C-CAM2 (Figure 1, lane 9) monomers, the presence of the higher molecular mass components was no longer detectable by blotting with either anti-C-CAM (Figure 1, lane 7) or L2 antibodies (Figure 1, lane 8).

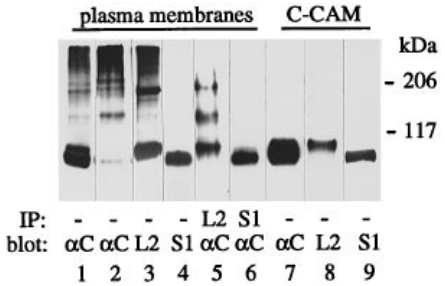


Figure 1 Specificity of anti-peptide antibodies

Triton X-100 extracts (lanes 1, 3 and 4) or insoluble pellets (lane 2) of plasma membranes were resolved by SDS/PAGE and immunoblotted with anti-C-CAM (αC) (lanes 1 and 2), L2 (lane 3) or S1 (lane 4) antibodies. Detergent extracts of plasma membranes, immunoprecipitated with antibodies L2 (lane 5) or S1 (lane 6) were immunoblotted with anti-C-CAM (αC) antibodies. Purified C-CAM was immunoblotted with anti-C-CAM (αC) (lane 7), L2 (lane 8) or S1 (lane 9) antibodies. The molecular masses (kDa) are shown on the right.

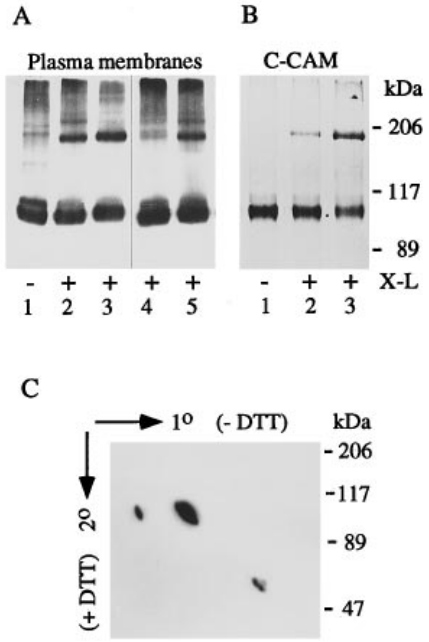


Figure 2 Chemical cross-linking of rat liver plasma membranes and isolated C-CAM

(**A**) Untreated (— X-L) plasma membranes (lane 1) or membranes treated (+ X-L) with 0.1 mM or 1 mM DSP (lanes 2 and 3) or with 0.1 mM or 1 mM DTSSP (lanes 4 and 5) were extracted, subjected to SDS/PAGE and immunoblotted with anti-C-CAM antibodies. (**B**) Purified C-CAM (lane 1), treated with a 10- (lane 2) or 50-fold (lane 3) molar excess of DTSSP and analysed as in (**A**). (**C**) Detergent extracts of cross-linked, biotinylated plasma membranes, purified on anti-C-CAM Sepharose, were separated on 6% (w/v) acrylamide gels under non-reducing conditions (— DTT) in the first dimension (1^0) and on 7.5% (w/v) acrylamide gels under reducing conditions (+ DTT) in the second dimension (2^o). Biotinylated proteins were detected with peroxidase-conjugated streptavidin. The molecular masses (kDa) are shown on the right.

C-CAM exists as dimers in plasma membranes and in solution

In an attempt to determine the molecular organization of C-CAM, we used chemical cross-linkers to stabilize interactions between C-CAM and cellular proteins with which it is in close molecular contact. Using a similar approach, Löster et al. [40] have detected a large number of, so far, undefined C-CAM-containing aggregates.

Treatment of plasma membranes with the reducible, homobifunctional cross-linkers DSP (Figure 2A, lanes 2 and 3) and DTSSP (Figure 2A, lanes 4 and 5) resulted in a decrease in the intensity of both the C-CAM1 and C-CAM2 monomers and the stabilization of one major species with an apparent molecular mass of 200 kDa, suggesting that it may represent dimers of C-CAM.

In support of this notion, cross-linking of purified C-CAM (Figure 2B) resulted in the stabilization of one major species with an apparent molecular mass of 200 kDa, indicating a dimeric form of C-CAM. Thus while we cannot completely exclude that a protein of identical molecular mass co-purifies with C-CAM, the results are consistent with the suggestion that C-CAM exists as non-covalently linked dimers within plasma membranes. The presence of additional, higher-molecular-mass cross-linked products in plasma membranes (Figure 2A, lanes 2–5) with no counterparts in cross-linked C-CAM preparations (Figure 2B, lanes 2–3), indicates, however, that C-CAM is also involved in a number of heterophilic interactions *in vivo*.

To more directly assess the composition of the major 200 kDa cross-linked species, detergent extracts of biotinylated, cross-linked plasma membranes were purified on anti-C-CAM–Sepharose and subjected to SDS/PAGE under non-reducing conditions, in the first dimension. Since the cross-linkers used in

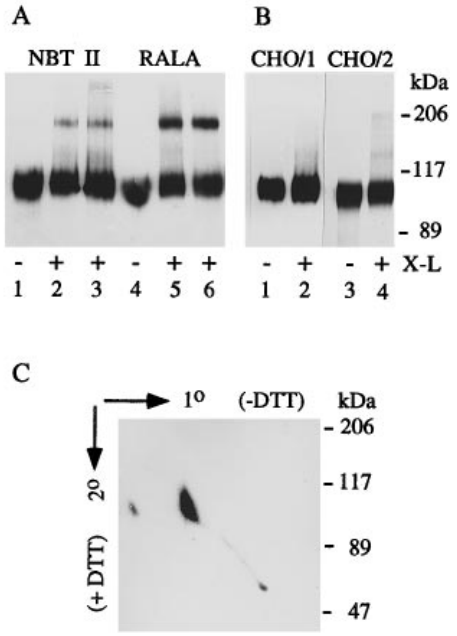
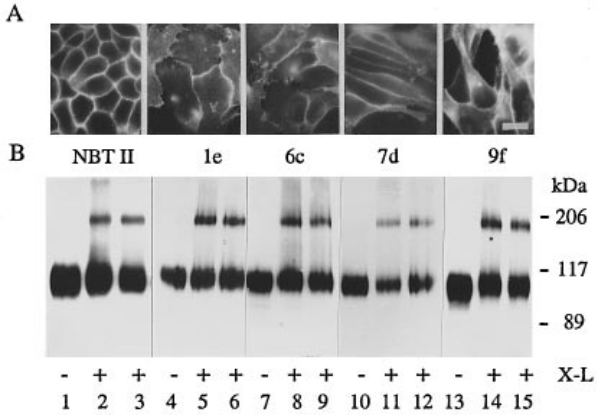


Figure 3 Chemical cross-linking of intact epithelial cells

(**A**) Adherent monolayers of NBT II (lanes 1–3) and RALA255-10G (lanes 4–6) cells, either untreated (— X-L) (lanes 1 and 4) or treated (+ X-L) with 1 mM DSP (lanes 2 and 5) or 1 mM DTSSP (lanes 3 and 6) were analysed as described in the legend to Figure 2(**A**). (**B**) Adherent monolayers of CHO cells, transfected with C-CAM1 (lanes 1 and 2) or C-CAM2 (lanes 3 and 4), either untreated (— X-L) (lanes 1 and 3) or treated (+ X-L) with 1 mM DTSSP (lanes 2 and 4) were analysed as described in the legend to Figure 2(**A**). (**C**) Extracts of metabolically labelled, cross-linked NBT II cells were immunoprecipitated with anti-C-CAM antibodies, before two-dimensional SDS/PAGE as described in Figure 2(**C**). Radiolabelled proteins were detected by fluorography. Molecular masses (kDa) are shown on the right.



C

Cell type	% Dimer	
	adherent	trypsinized
NBT II	14.6	15.1
1e	33.0	31.2
6c	31.0	27.0
7d	17.7	18.4
9f	25.5	26.1

Figure 4 Cross-linking of adherent monolayers and single cell suspensions of NBT II cells

(**A**) NBTII cells and sub-clones 1e, 6c, 7d and 9f, grown on glass coverslips, were fixed and stained with anti-C-CAM antibodies. Bar = 20 μm. (**B**) Adherent monolayers (lanes 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14) and suspended cells (lanes 3, 6, 9, 12 and 15), either untreated (— X-L) or treated (+ X-L) with 1 mM DSP were analysed as described in the legend to Figure 2(**A**). (**C**) The extent of dimer formation was determined by scanning densitometry and expressed as a percentage of total C-CAM protein (% Dimer). Molecular masses (kDa) are shown on the right.

this study contained an internal disulphide bond, the constituents of the cross-linked products were released and separated in the second-dimension PAGE, run under reducing conditions. As shown in Figure 2(C), the major 200 kDa cross-linked product of plasma membranes was reduced to a single spot, whose mobility was indistinguishable from that of monomeric C-CAM.

Taken together, these results provide strong support for the conclusion that C-CAM, at least in part, exists as dimers, and that this represents the native structure of the protein, since it is found not only in solution but also in rat liver plasma membranes.

C-CAM dimers are present on the surface of epithelial cells

While providing evidence for the existence of C-CAM dimers, our studies with isolated C-CAM and rat liver plasma membranes cannot distinguish between *cis*- and *trans*-interactions. We, therefore, examined the possibility that dimers were present within the plasma membranes of cell lines expressing C-CAM.

We have demonstrated previously that the NBT II [34] and RALA255-10G [41] cell lines express both C-CAM1 and C-CAM2, which appear as a broad band with an apparent molecular mass of 110–115 kDa (Figure 3A, lanes 1 and 4). Treatment with chemical cross-linkers stabilized one major species in both cell lines (Figure 3A, lanes 2 and 3, and lanes 5 and 6), with an apparent molecular mass twice that of the C-CAM monomers. There was little difference in the relative amount of cross-linked product in cells treated with DSP (Figure 3A, lanes 2 and 5) or its membrane-impermeable analogue DTSSP (Figure 3A, lanes 3 and 6), indicating that cross-linking occurred between C-CAM and proteins exposed on the extracellular surface of these cells. In contrast, we have failed to identify any major cross-linked species in CHO cells transfected with either C-CAM1 (Figure 3B, lanes 1 and 2) or C-CAM2 (Figure 3B, lanes 3 and 4), suggesting that in these cells C-CAM has a different membrane organization.

Two-dimensional SDS/PAGE analysis of anti-C-CAM immunoprecipitates of metabolically-labelled, cross-linked NBT II cells (Figure 3C) demonstrated that, under reducing conditions, the 200 kDa cross-linked species migrated as a single spot, with an apparent molecular mass identical with that of monomeric C-CAM. These results indicate that, in NBT II cells, as in rat liver plasma membranes, C-CAM exists as non-covalently associated dimers.

C-CAM dimers are formed by *cis*- interactions

As described previously [34], NBT II cells formed typical epithelial colonies of closely associated cells in which C-CAM was localized at sites of cell–cell contact (Figure 4A, NBT II). While 10–15% of NBT II cells remained in the form of small aggregates after trypsinization, the subclones 1e, 6c, 7d and 9f gave rise to $>95\%$ single cell populations. The sub-clones showed little tendency to form colonies at low cell density and C-CAM was distributed over the entire cell surface. At high cell density, C-CAM became localized in areas of cell–cell contact (Figure 4A, 1e, 6c, 7d and 9f).

Treatment with cross-linker stabilized C-CAM dimers in both adherent monolayers (Figure 4B, lanes 2, 5, 8, 11 and 14) and

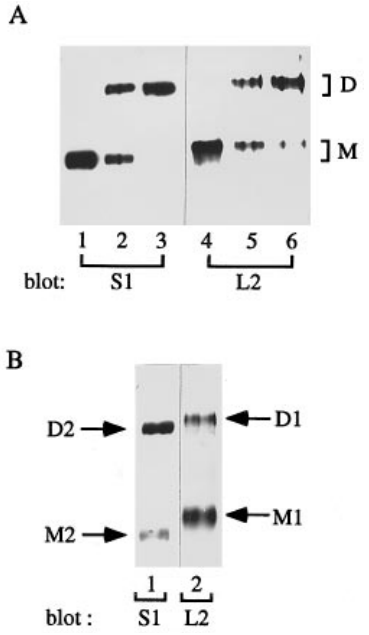


Figure 5 Isoform analysis of cross-linked C-CAM

(**A**) Purified C-CAM, either untreated (lanes 1 and 4) or cross-linked with a 10- (lanes 2 and 5) or 50-fold (lanes 3 and 6) molar excess of DTSSP, was subjected to SDS/PAGE and immunoblotted with antibodies S1 (lanes 1–3) or L2 (lanes 4–6). The monomer (M) and dimer (D) bands are indicated. (**B**) Cross-linked C-CAM was subjected to prolonged electrophoresis and blotted with antibodies S1 (lane 1) or L2 (lane 2). Monomeric (M1 and M2) and dimeric (D1 and D2) forms of C-CAM1 and C-CAM2 respectively are indicated.

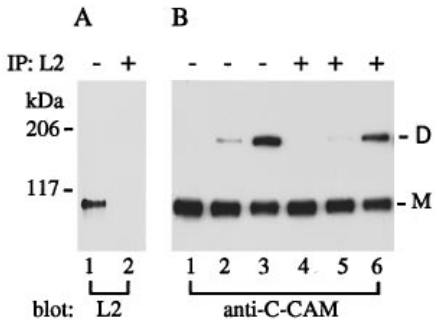


Figure 6 Cross-linking of immunodepleted C-CAM

(**A**) Purified preparations of C-CAM, before (IP:L2 —, lane 1) and after (IP:L2 +, lane 2) immunodepletion of the C-CAM1 isoform, were resolved by SDS/PAGE and immunoblotted with the L2 antibody. (**B**) Total (IP:L2 —, lanes 1–3) and immunodepleted C-CAM preparations (IP:L2 +, lanes 4–6) were incubated without (lanes 1 and 4) or with a 10- (lanes 2 and 5) or 50-fold (lanes 3 and 6) molar excess of DTSSP and analysed as described in the legend to Figure 2(**A**). The monomer (M) and dimer (D) bands are indicated.

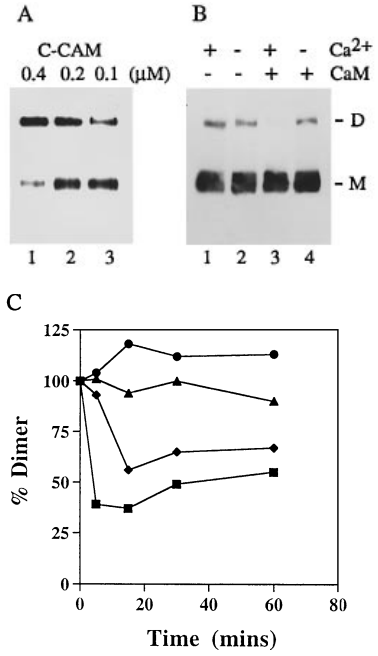


Figure 7 Effects of calmodulin and increased intracellular Ca^{2+} on C-CAM dimerization

(**A**) Purified C-CAM at a concentration of 0.4 μM (lane 1), 0.2 μM (lane 2) or 0.1 μM (lane 3) was cross-linked and analysed as described in the legend to Figure 2(**A**). The monomer (M) and dimer (D) bands are indicated. (**B**) C-CAM (final concentration 0.04 μM) in the presence of 1 mM Ca^{2+} (lanes 1 and 3) or 2 mM EGTA (lanes 2 and 4) was incubated without (lanes 1 and 2) or with (lanes 3 and 4) 5 μM calmodulin (CaM) for 2 h, cross-linked and analysed as described in the legend to Figure 2(**A**). (**C**) Adherent monolayers of NBT II cells were incubated with 10 μM ionomycin (▲, ■) or A23187 (◆) or vehicle (DMSO) alone (●), in buffer 3 (■, ◆, ●) or in buffer 3 lacking bivalent cations (▲) for the times indicated. The cells were cross-linked and analysed as described in the legend to Figure 2(**A**). The extent of dimer formation was determined by scanning densitometry and the relative amount of dimer was expressed as a percentage of the value at zero time (100%).

single cell suspensions (Figure 4B, lanes 3, 6, 9, 12 and 15) in all cell lines. Since the extent of dimerization was similar in both adherent and suspended cells (Figure 4C), we conclude that C-CAM dimerization occurs predominantly through *cis*- interactions within the membranes of individual cells. That C-CAM dimers are formed by *cis*- rather than *trans*- interactions is further supported by the absence of dimers in transfected CHO cells, although C-CAM in these cells has been shown to mediate *trans*- homophilic binding [11–13]. Interestingly, the level of dimer formation was found to vary in different clones of NBT II cells, suggesting that dimerization may be under cellular regulation.

Both C-CAM1 and C-CAM2 form dimers

The anti-peptide antibodies S1 (Figure 5A, lanes 1–3) and L2 (Figure 5A, lanes 4–6) recognized both their respective monomers and the dimer band in cross-linked samples of purified C-CAM, and on prolonged electrophoresis the L2- and S1-reactive dimeric species were largely resolved (Figure 5B). C-CAM preparations depleted of the C-CAM1 isoform (Figure 6A) showed only a slight reduction in the extent of dimer formation (Figure 6B, compare lanes 1–3 and 4–6).

Taken together, these results indicate that both C-CAM1 and C-CAM2 have the potential to form homo-dimers. Since *trans*- homophilic interactions would be expected to yield a mixture of homo- and hetero-dimers, our results also suggest that in solution, as on the cell surface, C-CAM forms *cis*-dimers. The lack of reactivity of anti-peptide antibodies with extracts of cultured cells has, however, prevented us from assessing whether homo-dimerization occurs in intact cells.

C-CAM dimerization is regulated by intracellular Ca^{2+} and calmodulin

For C-CAM in solution, the extent of dimerization is dependent upon protein concentration (Figure 7A, lanes 1–3), indicating that monomers and dimers are in equilibrium. Variations in the level of dimerization found in different subclones of NBT II cells (Figure 4B) and the absence of dimers in transfected CHO cells (Figure 3B), suggests, however, that in a cellular context dimerization is a regulated process.

Previous studies from this laboratory [26,27] have demonstrated that calmodulin binds to the cytoplasmic domains of both C-CAM1 and C-CAM2 and inhibits C-CAM self-association in a solid-phase binding assay [28]. We have, therefore, investigated the possibility that calmodulin may be involved in the regulation of C-CAM dimerization. In the absence of calmodulin, cross-linking of C-CAM in buffers containing either Ca^{2+} (Figure 7B, lane 1) or the Ca^{2+} chelator, EGTA (Figure 7B, lane 2) resulted in the stabilization of C-CAM dimers. While the presence of calmodulin had no effect on C-CAM dimer formation in EGTA-containing buffers (Figure 7B, lane 4), in buffers containing Ca^{2+}, dimer formation was abrogated (Figure 7B, lane 3).

Since calmodulin regulates the activities of a large number of target proteins in response to transient increases in Ca^{2+}, we investigated if an increase in the level of intracellular Ca^{2+} modulates C-CAM dimerization in NBT II cells. Treatment of NBT II cells with Ca^{2+} ionophore resulted in a decrease in the extent of C-CAM dimer formation (Figure 7C), which was maximal after 15 min (Figure 7C), when it fell to about 40–50 % of the untreated level. In buffer lacking bivalent cations, Ca^{2+} ionophore had no effect on the extent of C-CAM dimerization (Figure 7C).

The results of experiments *in vivo* and *in vitro*, described here, indicate that increased levels of intracellular Ca^{2+} inhibit C-CAM dimerization and suggest that this may be mediated by the activation of calmodulin.

DISCUSSION

Group-specific chemical cross-linkers have proved to be useful tools in delineating the higher order structure of many cellular proteins. In the present study we have used the reducible cross-linker DSP, and its membrane-impermeable analogue DTSSP, to investigate the supramolecular organization of C-CAM and have identified one major cross-linked species in isolated rat liver plasma membranes, in intact epithelial cells and in solution. The apparent molecular mass of the cross-linked species, which was twice that of monomeric C-CAM, and the failure to detect additional proteins after cross-linker cleavage strongly suggests that C-CAM exists as non-covalently linked dimers. The extent of dimerization was almost identical in both adherent monolayers and in single cell populations, indicating that dimerization occurs almost entirely by *cis*- interactions within the membranes of individual cells.

Interestingly, computer modelling of CEA [42], a member of the subgroup of the Ig superfamily to which C-CAM belongs, had predicted a 3-dimensional structure in which the Ig domains were aligned in parallel, projecting from the cell surface as dimers. The results of the present study and other studies [43,44] suggesting that I-CAM1 is a cell surface homo-dimer, provide experimental evidence in support of this proposal. Recent NMR and crystallographic analyses [45–47] demonstrating the remarkable structural similarity of the extracellular domains of the cadherins to the Ig fold and their interaction to form *cis*-dimers suggests that this organization may be a feature of many adhesion receptors.

Using isoform-specific antibodies, we and others [39] have identified several high molecular mass, non-reducible, C-CAM1-containing species in rat liver plasma membranes, indicating that C-CAM1 most likely interacts with a number of cellular proteins to form large complexes. This finding is of particular interest in view of the diverse functions attributed to this isoform [5,14,16], and suggests that through a variety of cellular interactions C-CAM may participate in or regulate a number of seemingly disparate cellular processes. While the nature of these interactions remains to be established, the presence of a group of proteins of about 200 kDa in untreated plasma membranes raises the possibility that a population of C-CAM dimers becomes covalently linked, perhaps by the activity of transglutaminase, an enzyme which catalyses the formation of γ-glutamyl-ϵ-lysine bonds in a restricted number of cellular proteins [48]. While there is no consensus sequence for transglutaminase catalysed cross-linking, the cytoplasmic domain of C-CAM1 contains two immediately adjacent glutamine residues in close proximity to a number of charged and polar amino acids, features often found in known transglutaminase substrates [49]. While monomeric C-CAM1 is generally expressed at lower levels than C-CAM2 in all tissues examined [50], the presence of multiple C-CAM1-containing adducts such as those detected in liver, would lead to a significant increase in the amount of this long cytoplasmic tail isoform and may be of considerable functional importance, since, as is shown here, these species appear to be more tightly associated with the cytoskeleton. Since C-CAM1 and C-CAM2 differ only in their cytoplasmic domains, the formation of homo-dimers suggests that sequences within these domains may be involved in isoform recognition, with dimers subsequently stabilized by interactions of the transmembrane and/or extracellular domains. Indeed, a role for the transmembrane domain in dimerization is suggested by the presence of a number of glycine residues, whose distribution would lead to their alignment along one face of the transmembrane α-helix [7], a motif found in a number of proteins, including I-CAM-1, that are known to form homo- or hetero-dimers [51]. The extracellular domains of C-CAM are also in close apposition when judged by their efficient stabilization by the membrane-impermeable cross-linker, DTSSP.

An alternative possibility is that homo-dimerization may result from a restricted cellular distribution of C-CAM isoforms. Physical measurements of the lateral movement of proteins have clearly demonstrated that the plasma membrane consists of distinct domains varying in composition and function, with proteins often restricted to particular areas as a result of tethering to the cytoskeleton [52]. We have previously demonstrated that in NBT II cells, a population of C-CAM molecules is associated with the actin cytoskeleton [34]. The identification in the present study of a number of C-CAM1-containing species, which were enriched in the detergent-insoluble pellet of plasma membranes, suggests that it may be this isoform which preferentially interacts with actin filaments, and that this in turn could result in restricted distribution in the membrane.

The functional significance of C-CAM dimerization is currently unknown. For the cadherins [46,47] and for CEA [42] it has been proposed that *cis*-dimerization will lead to strengthened adhesion and, indeed, dimeric forms of I-CAM-1 show enhanced binding to LFA-1 [43,44]. The absence of dimers in CHO cells transfected with C-CAM isoforms indicates, however, that dimer formation is not a prerequisite for adhesion, since both isoforms support homotypic aggregation of CHO cells [11,13]. The finding that the extent of C-CAM dimer formation is similar in both adherent monolayers and in single cell populations and that cells expressing

higher relative amounts of dimer have a less well-organized epithelial morphology also argues against the necessity of dimerization for cell adhesion. The alternative possibility, that dimerization may be involved in C-CAM-mediated signalling is currently under investigation.

While monomeric and dimeric forms of C-CAM are in equilibrium in solution, the extent of dimerization varies in different cell lines suggesting that this process is under cellular control. The demonstration that calmodulin inhibits C-CAM dimerization in solution in a Ca^{2+}-dependent manner and that treatment with Ca^{2+} ionophore has a similar effect in intact NBT II cells, implicates calmodulin in the control of the dimerization process. Calmodulin has been shown to bind to the cytoplasmic domains of both C-CAM1 and C-CAM2 [27], but whether it functions to directly inhibit association or induces conformational changes in domains required for dimerization remains to be established. Although monomeric, calmodulin has two binding sites, raising the possibility that it could interact with adjacent C-CAM molecules to regulate dimer formation. Calmodulin–ligand interactions can be modulated by post-translational modification. The differential phosphorylation of the cytoplasmic domains of the different C-CAM isoforms [6,21,31] and the phosphorylation of calmodulin itself [53] thus provide a multiplicity of mechanisms by which the dimerization state and presumably the function of C-CAM may be regulated. The challenge now is to understand the functional correlates of the regulated process of C-CAM dimerization identified here.

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REFERENCES

1 Edelman, G. M. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 1460–1464
2 Edelman, G. M. and Crossin, K. L. (1991) Annu. Rev. Biochem. **60**, 155–190
3 Takeichi, M. (1991) Science **251**, 1451–1455
4 Ocklind, K. and Öbrink, B. (1982) J. Biol. Chem. **257**, 6788–6795
5 Lin, S.-H. and Guidotti, G. (1989) J. Biol. Chem. **264**, 14408–14414
6 Culic, O., Huang, Q.-H., Flanagan, D., Hixson, D. C. and Lin, S.-H. (1992) Biochem. J. **285**, 47–53
7 Edlund, M., Gaardsvoll, H., Bock, E. and Öbrink, B. (1993) Eur. J. Biochem. **213**, 1109–1116
8 Najjar, S. M., Accili, D., Phillipe, N., Jernberg, J., Margolis, R. and Taylor, S. (1993) J. Biol. Chem. **268**, 1201–1206
9 Tingström, A., Blikstad, I., Aurivillius, M. and Öbrink, B. (1990) J. Cell Sci. **96**, 17–25
10 Cheung, P., Culic, O., Qiu, Y., Earley, K., Thomson, N., Hixson, D. C. and Lin, S.-H. (1993) Biochem J. **295**, 427–435
11 Olsson, H., Wikström, K., Kjellström, G. and Öbrink, B. (1995) FEBS Lett. **365**, 51–56
12 Lucka, L., Cichocka, I., Bäumler, K. and Reutter, W. (1995) Eur. J. Biochem. **234**, 527–535
13 Wikström, K., Kjellström, G. and Öbrink, B. (1996) Exp. Cell Res. **227**, 360–366
14 Sippel, J., Suchy, F. J., Ananthanarayanan, M. and Perlmutter, D. H. (1993) J. Biol. Chem. **268**, 2083–2091
15 Stout, J. G., Strobel, R. S. and Kirley, T. L. (1995) J. Biol. Chem. **270**, 11845–11850
16 Hsieh, J. T., Luo, W., Song, W., Wang, Y., Kleinerman, D. I., Van, N. T. and Lin, S.-H. (1995) Cancer Res. **55**, 190–197
17 Kunath, T., Ordonez-Garcia, C., Turbide, C. and Beauchemin, N. (1995) Oncogene **11**, 2375–2382
18 Gumbiner, B. M. (1996) Cell **84**, 345–357
19 Kirkpatrick, C. and Peifer, M. (1995) Curr. Opin. Gen. Dev. **5**, 56–65
20 Yamada, K. M. and Miyamoto, S. (1995) Curr. Opin. Cell Biol. **7**, 681–689
21 Margolis, R. N., Schell, M. J., Taylor, S. and Hubbard, A. (1990) Biochem. Biophys. Res. Commun. **166**, 562–566
22 Reth, M. (1994) Curr. Opin. Immunol. **6**, 3–8
23 DeFranco, A. L. (1995) Curr. Opin. Cell Biol. **7**, 163–175
24 Afar, D. E. H., Stanners, C. P. and Bell, J. C. (1993) Biochim. Biophys. Acta **1134**, 46–52
25 Brümmer, J., Neumaier, M., Gopfert, C. and Wagener, C. (1995) Oncogene **11**, 1649–1655
26 Blikstad, I., Wikström, T., Aurivillius, M. and Öbrink, B. (1992) FEBS Lett. **302**, 26–30
27 Edlund, M. and Öbrink, B. (1993) FEBS Lett. **327**, 90–94
28 Edlund, M., Blikstad, I. and Öbrink, B. (1996) J. Biol. Chem. **271**,1393–1399
29 James, P., Vorherr, T. and Carafoli, E. (1995) Trends Biochem. Sci. **20**, 38–42
30 Hansson, M., Blikstad, I. and Öbrink, B. (1989) Exp. Cell Res.**181**, 63–74 **11**, 2375–2382
31 Odin, P., Tingström, A. and Öbrink, B. (1986) Biochem. J. **236**, 559–568
32 Lim, Y.-P., Callanan, H., Lin, S.-H., Thomson, N. L. and Hixson, D. C. (1993) Anal. Biochem. **214**, 156–164
33 Odin, P. and Öbrink, B. (1986) Exp. Cell Res. **171**, 1–15
34 Hunter, I., Lindh, M. and Öbrink, B. (1994) J. Cell Sci. **107**, 1205–1216
35 Valles, A. M., Tucker, G. C., Thiery, J. P. and Boyer, B. (1990) Cell Regul. **1**, 975–988
36 Chou, J. Y. (1983) Mol. Cell Biol. **3**, 1013–1020
37 Findlay, J. B. C. (1990) in Biological Membranes, A Practical Approach, (Findlay, J. B. C. and Evans, W. H., eds.), pp. 179–217, IRL Press, Oxford
38 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
39 Baum, O., Reutter, W., Flanagan, D., Callanan, H., Lim, Y.-P., Lin, S.-H. and Hixson, D. C. (1995) Eur. J. Biochem. **228**, 316–322
40 Löster, K., Baum, O., Hofmann, W. and Reutter, W. (1995) J. Chromatogr. **711**, 187–199
41 Olsson, H. (1995) M.Sc. Thesis, Karolinska Institute, Stockholm
42 Bates, P. A., Luo, J. and Sternberg, M. J. E. (1992) FEBS Lett. **2**, 207–214
43 Miller, J., Knorr, R., Ferrone, M., Houdei, R., Carron, C. P. and Dustin, M. L. J. (1995) Exp. Med. **182**, 1231–1241
44 Reilly, P. L., Waska, J. R., Jeanfavre, D. D., McNally, E., Rothlein, R. and Bormann, B.-J. (1995) J. Immunol. **155**, 529–532
45 Overduin, M., Harvey, T., Bagby, S., Tong, K. I., Yau, P., Takeichi, M. and Ikura, M. (1995) Science **267**, 386–389
46 Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lemann, M. S., Grubel, G., Legrand, J.-F., Als-Nielson, J., Colman, D. R. and Hendrickson, W. (1995) Nature (London) **374**, 327–337
47 Nagar, B., Overduin, M., Ikura, M. and Rini, J. M. (1996) Nature (London) **380**, 360–364
48 Greenberg, C. S., Birckbichler, P. J. and Rice, R. H. (1991) FASEB J. **5**, 3071–3077
49 Aeschlimann, D., Paulsson, M. and Mann, K. J. (1990) Biol. Chem. **267**, 11316–11321
50 Cheung, P. H., Thomson, N. L., Earley, K., Culic, O., Hixson, D. C. and Lin, S.-H. (1993) J. Biol. Chem. **268**, 6139–6146
51 Bormann, B. J. and Engelman, D. M. (1992) Annu. Rev. Biophys. Biomol. Struct. **21**, 223–242
52 Edidin, M. (1993) J. Cell Sci. **17**, 165–169
53 Quadroni, M., James, P. and Carafoli, E. (1994) J. Biol. Chem. **269**, 16116–16122

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