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Purification and spectroscopic characterization of a recombinant amino-terminal polypeptide fragment of mouse epithelial cadherin

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Abstract Cadherins are a family of Ca^{2+}-dependent cell adhesion molecules containing four extracellular tandem repeats each of 110 amino acids. The most amino-terminal repeat is believed to confer the specificity of cell adhesion. A polypeptide containing the amino-terminal repeat of mouse epithelial cadherin has been over-expressed in *E. coli* and purified to homogeneity. This polypeptide binds Ca^{2+} with a dissociation constant of 1.6×10^{-4} M. CD and NMR experiments indicate that the polypeptide adopts a predominantly β-sheet conformation and that binding of Ca^{2+} induces only small conformational changes.

Key words: Cadherin; Cell adhesion molecule; Calcium binding; Circular dichroism; NMR

1. Introduction

Cadherins are a family of cell–cell adhesion molecules that are crucial for the physical association of vertebrate cells (for reviews see [1–4]). Cadherins mediate Ca^{2+}-dependent cell adhesion through homophilic binding interactions. For example, epithelial (E-) cadherin binds selectively to E-cadherin, and neural (N-) cadherin recognizes N-cadherin. These homophilic interactions have been implicated in the preferential adhesion and sorting of cells during morphogenesis. The molecular basis of cadherin-mediated cell adhesion specificity is poorly understood, but depends on structural characteristics of the extracellular regions of cadherins.

The N-terminal extracellular region of cadherins consists of four cadherin or CAD repeats, each of approximately 110 amino acid residues, followed by a cysteine-rich domain (Fig. 1). Analyses using chimeric cadherins [5] showed that the most N-terminal CAD repeat (CAD1) is crucial for cadherin specificity. In particular, a region containing the conserved HAV tripeptide (residues 80–82) has been suggested to be responsible for binding specificity [6]. Each CAD repeat contains Asp/Glu clusters of similar size and distribution which constitute putative Ca^{2+}-binding sequences differing from the Ca^{2+}-binding motif of the EF-hand superfamily of proteins [7]. Ringwald et al. [8] reported that an 84 kDa tryptic fragment consisting of almost the entire extracellular region of uvomorulin (E-cadherin) indeed binds Ca^{2+}. Ozawa et al. [9] demonstrated that a single amino acid substitution in a putative Ca^{2+}-binding site inactivates the adhesive function of E-cadherin. These and other [1,8,9] results clearly demonstrate the absolute requirement for Ca^{2+} ions in cadherin-mediated cell adhesion. CAD repeats have been identified in many other integral membrane proteins, including desmosomal cadherins [10,11], the proto-oncoprotein tyrosine kinase RET [12,13], and the *Drosophila* tumour suppressor protein FAT [14].

Despite the growing interest in cadherin-mediated cell adhesion, little is known about the structural and biophysical properties of cadherins. Herein we present the purification, Ca^{2+}-binding properties, and CD and NMR structural characterizations of the N-terminal 145-residue polypeptide fragment (referred to as E-CAD1) of mouse E-cadherin [15,16]. This E-CAD1 fragment (Fig. 1) comprises the entire sequence of the CAD1 repeat, including putative Ca^{2+}-binding sites and the proposed adhesion recognition site [5,6], providing an excellent candidate for studying the structural basis of Ca^{2+}-dependent cell adhesion mediated by E-cadherin.

2. Materials and methods

2.1. Plasmid construction and over-expression

The plasmid for over-expression of E-CAD1 of mouse E-cadherin was constructed by inserting the portion of the cDNA encoding amino acids R1–A145 [15], as well as an N-terminal methionine into the pAS expression vector [17] using standard PCR procedures. The sense primer used for the PCR included a *Bsp*HI site and the antisense primer included a termination codon and an *Xba*I site. The amplified product was first inserted at the *Sma*I site of pTZ18R (Pharmacia) and then removed from the resulting plasmid at *Bsp*HI and *Xba*I. This DNA fragment was inserted into the pAS expression vector at the *Nco*I and *Xba*I sites by uni-directional ligation (Fig. 1). The resulting plasmid, pmE-CAD1, contains the $\lambda P_L P_R$ promoter and is under control of the thermosensitive λcI repressor.

For over-expression of E-CAD1, *E. coli* strain AR58 [17] was transformed with pmE-CAD1 and grown at 37°C in M9 minimal medium (0.5% (w/v throughout) D-glucose, 0.1% NH_4Cl, 0.05% NaCl, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 7.4) supplemented with 2×10^{-4}% thiamine, 2×10^{-4}% D-biotin, and 100 μg/ml ampicillin. When an OD_{600} of 0.8 was reached, the temperature

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Abbreviations: CD, circular dichroism; DTT, dithiothreitol; E-CAD1, mouse E-cadherin polypeptide spanning the first extracellular repeat (residues R1-A145 plus a methionine residue at the amino-terminus); EDTA, ethylenediamine tetraacetic acid; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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was increased to 42°C to induce the production of the E-CAD1 fragment. After 3 h of induction, the cells were harvested by centrifugation at 6000 × g for 20 min and then stored at −70°C. For ^{15}N-labelling of E-CAD1, $^{15}NH_4Cl$ (Isotec) was used as the sole nitrogen source in M9 minimal medium.

2.2. Purification

All purification steps were carried out at 4°C. The frozen cells were suspended in 3/80 (v/v) of the original culture volume using buffer A (50 mM Tris-HCl, pH 7.9 containing 5% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), 50 mM NaCl, 0.01 mg/ml pepstatin, 0.01 mg/ml leupeptin, 1%(v/v) aprotinin, 10 μg/ml DNase I, 0.1%(v/v) Nonidet P40. The suspended cells were lysed by sonication on ice using a Branson Sonifier Model 450 sonicator. Cell debris was removed by ultracentrifugation at 46,500 × g for 20 min. Approximately 80% of E-CAD1 remained in the soluble fraction, as judged by ^{45}Ca blot assay (see below). An equal volume of buffer A was added to the supernatant prior to loading onto a DEAE-Sepharose (Pharmacia) column (2.5 × 15 cm). The column was washed extensively with buffer A containing 25 mM NaCl and the adsorbed proteins were eluted using a NaCl gradient (25–125 mM) in buffer A. E-CAD1 eluted at 85–100 mM NaCl. Fractions containing E-CAD1 were pooled and concentrated using Centriprep 10 concentrators (Amicon). The concentrated solution was applied to a Sephacryl S-100HR (Pharmacia) column (2.5 × 88 cm) equilibrated with 0.2 M NaCl in buffer A. Fractions containing E-CAD1 (Fig. 2A) were pooled and concentrated again as before. The protein sample was further purified using FPLC (fast protein liquid chromatography) (Pharmacia) with a Mono Q HR 5/5 column and eluted using a NaCl gradient (25–125 mM) in buffer A, over 50 ml at a flow rate of 0.5 ml/min. The E-CAD1 polypeptide purified in this manner was at least 95% homogeneous, as judged by tricine 15% SDS-PAGE (Fig. 2B) and one- and two-dimensional NMR spectra. The identity of the purified product was checked by amino acid analysis and electrospray ionization mass spectrometry. The yield of E-CAD1 was approximately 5 mg per l of M9 minimal medium.

2.3. Ca^{2+} binding and chemical cross-linking

The ^{45}Ca blot assay was performed by transferring electrophoretically separated proteins onto a polyvinylidene difluoride membrane (DuPont) followed by labelling with $^{45}CaCl_2$ (DuPont) and subsequent autoradiography [18].

Ca^{2+}-binding measurements were performed at 23°C using a home-built flow dialysis apparatus. The experimental details are the same as described previously [19]. The dialysis solution contained 1.0 mM protein in 20 mM Tris-HCl, 0.1 M KCl, 10 mM DTT, pH 7.0. The experimental data was fitted to a polynomial curve using extensions of the non-linear regression computer program Enzfitter [20], and a midpoint of the calculated curve is reported as the dissociation constant.

Chemical cross-linking analysis was performed both in the absence and in the presence of 10 mM Ca^{2+} using the procedure described previously [21]. The purified E-CAD1 (30, 150, 500 μM) was incubated in the absence or presence of 0.001% or 0.01% glutaraldehyde for 15 min at 37°C. The results were analyzed using tricine SDS-PAGE and subsequent ^{45}Ca blot assay as described above. Comparable amounts of ribonuclease A, bovine serum albumin, and a fragment of the p53 oligomerization domain (Y. Yin and C. Arrowsmith, unpublished) were used as negative (the former one) and positive (the letter two) controls.

2.4. CD and NMR measurements

CD measurements were performed at 22°C using a Jasco J-720 spectropolarimeter and 1.0 mm path length cells with the following parameters: scan speed 50 nm/min, response time 1 s, and bandwidth of 1 nm. A standard noise reduction routine was employed. The concentration of the protein was 8 μM in H_2O (pH 7.0) in the presence or absence of 5 mM $CaCl_2$.

NMR experiments were carried out with a sample containing approximately 1.5 mM unlabelled or uniformly ^{15}N-enriched E-CAD1 in 95%/5% H_2O/D_2O containing 20 mM [$^2H_{11}$]Tris-HCl (MSD Isotopes), pH 7.0, 0.1 M KCl, 10 mM [$^2H_{10}$]DTT (MSD Isotopes), and 0.1 mM NaN_3. One-dimensional ^1H and sensitivity-enhanced ^1H ^{15}N HSQC [22] two-dimensional NMR spectra were recorded on Varian UNITY-600 and UNITY-plus 500 spectrometers.

3. Results and discussion

With the aim of obtaining large quantities of individual cadherin repeats for biophysical and structural characterization, several truncated cDNAs were cloned into the pAS expression vector [17]. Among those tested in our laboratory, E-CAD1 (Fig. 1) gave high level expression and high solubility in *E. coli* (see section 2). The procedure used for the purification of E-CAD1 consists of three column chromatography steps. E-CAD1 was first purified to approximately 80% by DEAE-Sepharose ion-exchange followed by Sephacryl S-100HR gel-filtration. The elution profile of the S-100HR column is shown in Fig. 2. The E-CAD1 fractions contained some minor components of lower molecular weight, which were removed by FPLC using a Mono Q column. This procedure resulted in E-CAD1 of >95% purity. The purified product showed a single SDS-PAGE band which contained Ca^{2+}-binding activity, as judged by the ^{45}Ca blot assay (Fig. 2). It should be noted that although E-CAD1 migrated at 20 kDa, the calculated M_r of 16,135 was confirmed by electrospray ionization mass spectrometry.

In order to assess the possibility that E-CAD1 forms a dimer or a multimer, native gel-electrophoresis and chemical cross-linking experiments were performed (data not shown). The native gel (both in the absence and in the presence of 10 mM Ca^{2+}) yielded a single band at various E-CAD1 concentrations between 10 and 500 μM. Chemical cross-linking experiments of E-CAD1 (30–500 μM) with glutaraldehyde, both in the absence and in the presence of 10 mM Ca^{2+}, yielded no higher molecular weight products. These results suggest that E-CAD1 is predominantly monomeric in solution. This was further supported by ^{15}N linewidth measurements using ^1H ^{15}N HSQC spectroscopy, in which the overall rotational correlation time

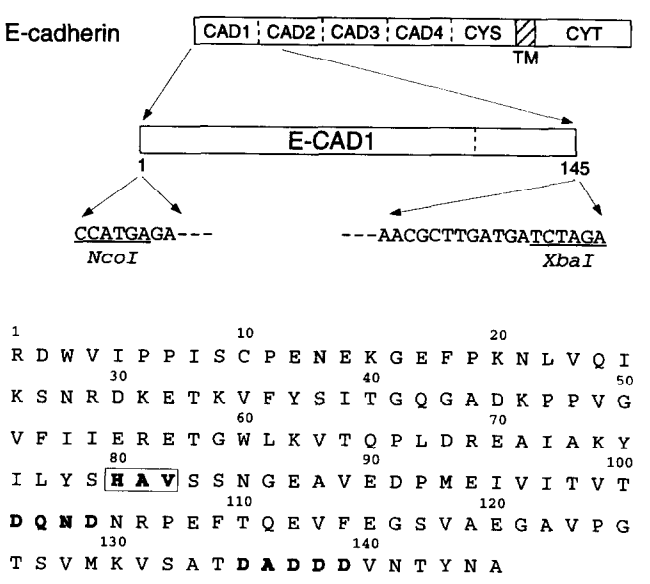

Fig. 1. A schematic diagram of E-CAD1 and the modular structure of E-cadherin, together with the amino acid sequence of E-CAD1 [1]. The extracellular cadherin repeats are denoted as CAD1, CAD2, CAD3, and CAD4. The cysteine-rich, membrane spanning, and cytoplasmic regions are denoted as CYS, TM, CYT, respectively. The cloning sites which join the truncated E-CAD1 gene [15] to the pAS expression vector [17] are indicated. The putative Ca^{2+}-binding sites [1,8] are highlighted by bold letters and the consensus HAV sequence [1,6] by bold letters and a box.

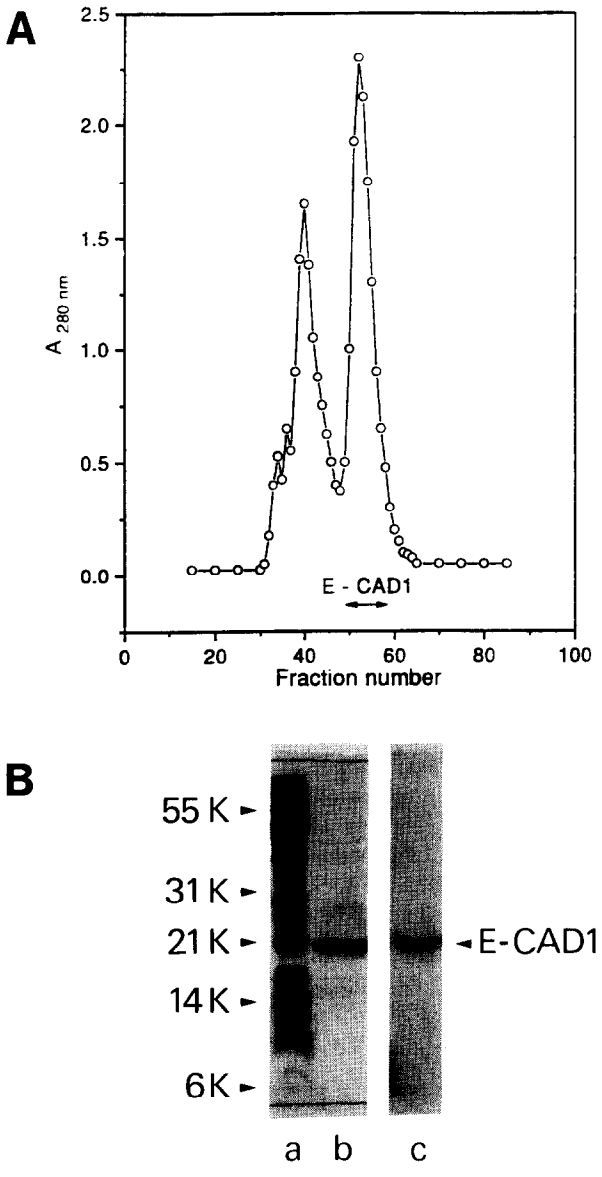

Fig. 2. (A) Sephacryl S-100HR gel-filtration column chromatography of E-CAD1. The horizontal bar with arrows indicates the E-CAD1 fractions. The flow rate was 15 ml/h and the fraction size was 4 ml/tube. (B) SDS-PAGE of E-CAD1. A 15% gel in Tris-Tricine buffer system was used. (a) Total soluble extract from *E. coli* after 3 h induction at 42°C; (b) E-CAD1 fraction purified using the procedure described in section 2; (c) autoradiogram of a ^{45}Ca blot of the same fraction as that in lane b.

of E-CAD1 was estimated to be that of a protein of 15–20 kDa. This correlation time did not change dramatically upon the addition of 10 mM Ca^{2+}, suggesting no Ca^{2+}-dependent multimerization by E-CAD1.

The Ca^{2+} binding properties of E-CAD1 were studied by the flow dialysis method [19]. The Ca^{2+} binding curve of E-CAD1 obtained by this method is shown in Fig. 3. The dissociation constant (K_d) for Ca^{2+} was found to be 1.6×10^{-4} M. This value is lower than the value (2×10^{-3} M) previously obtained with a 13-residue synthetic peptide comprising a putative Ca^{2+} binding site from E-cadherin (uvomorulin) [7,23], suggesting that E-CAD1 forms a structurally stable conformation better suited to Ca^{2+} ligation than the 13-residue peptide. On the other hand, the K_d value obtained for E-CAD1 is higher than those (10^{-5}–10^{-7} M) of the intracellular EF-hand Ca^{2+}-binding proteins, consistent with a millimolar concentration of extracellular Ca^{2+}. Due to the technical limitation associated with high dissociation constants in flow dialysis, the exact stoichiometry of Ca^{2+} bound to E-CAD1 remains to be determined by other methods. The amino acid sequence suggests that E-CAD1 contains at least two conserved putative Ca^{2+}-binding regions [1,8], DQNDN (residues 102–106) and DADDD (residues 136–140). It is possible that these regions form two separate sites or that both together contribute to a single Ca^{2+}-binding site.

To study the conformational properties of E-CAD1 in response to Ca^{2+} binding, CD and NMR experiments have been performed. The CD spectra of E-CAD1 in the absence and in the presence of Ca^{2+} are shown in Fig. 4A. The positive ellipticity at 234 nm in the spectra indicates that E-CAD1 contains a large percentage of β-sheet conformation. The addition of Ca^{2+} yielded no significant change at 234 nm but some perturbation in the range 190–220 nm. These results suggest that most of the β-sheet conformation is present both in the absence and in the presence of Ca^{2+}, but subtle structural changes may be occurring upon binding of Ca^{2+}. This is consistent with Ca^{2+} titration data using fluorescence spectroscopy in which tryptophan fluorescence intensity changes only ~0.5% upon the addition of Ca^{2+} (J. Ames, unpublished results).

The ^1H NMR spectrum of E-CAD1 in the presence of 10 mM Ca^{2+} is shown in Fig. 4B. During the course of NMR experiments, it was found that E-CAD1 is unstable at temperatures higher than 30°C. Therefore, NMR experiments were performed at 23°C, at which temperature E-CAD1 retained a folded conformation for 2–3 weeks. There are a number of high-field shifted proton resonances from −1.1 to 0.5 ppm and also downfield shifted amide proton resonances from 9.0 to 10.2 ppm, indicating that the protein has a defined tertiary structure under the conditions used for the experiment. The presence of several downfield-shifted Hα proton resonances, to the left-hand side of the strong water signal (4.8 ppm), is char-

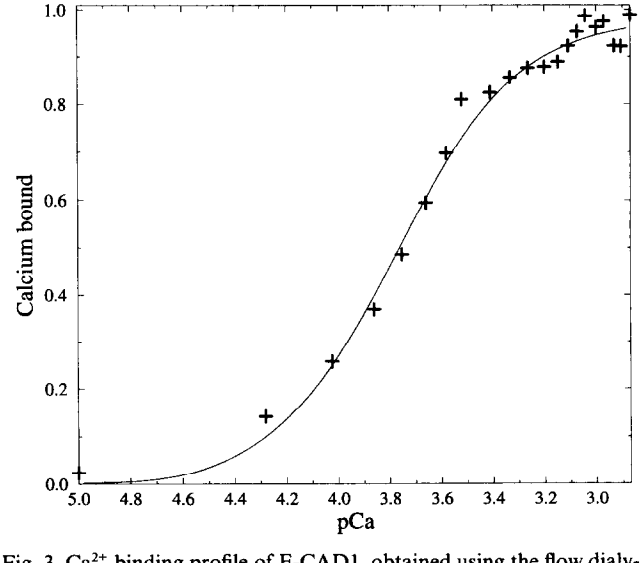

Fig. 3. Ca^{2+}-binding profile of E-CAD1, obtained using the flow dialysis method. The vertical scale for Ca^{2+} bound to E-CAD1 is normalized to unity. The experimental details are given in section 2.

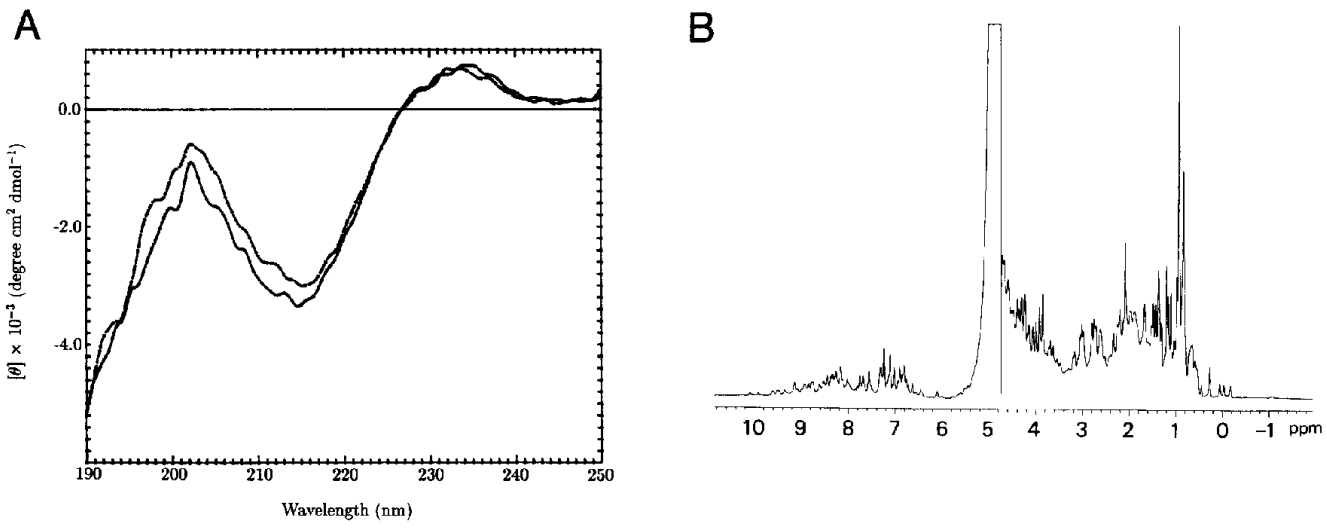

Fig. 4. (A) CD spectrum of E-CAD1 in the absence (solid line) and in the presence (broken line) of 5 mM $CaCl_2$, pH 7.0. (B) 500 MHz ^1H NMR spectrum of E-CAD1 in 95% H_2O/5% D_2O, 10 mM $CaCl_2$, pH 7.0. A presaturation field was used to reduce the water signal. Other conditions are described in section 2.

acteristic of β-sheet conformation, consistent with the CD data described above. The ^1H NMR spectrum of Ca^{2+}-free E-CAD1 (data not shown) was very similar to that shown in Fig. 4B, suggesting that binding of Ca^{2+} does not induces major conformational changes, again in agreement with the CD and fluorescence data.

^1H ^{15}N HSQC spectra have been recorded with ^{15}N-enriched E-CAD1 in the absence and in the presence of Ca^{2+} (data not shown). The spectra are very similar to each other, although 6 out of 134 cross-peaks are significantly shifted upon the addition of Ca^{2+}. This observation suggests that Ca^{2+}-dependent conformational changes are localized, presumably close to the Ca^{2+}-binding site(s). These small changes in the NMR spectra induced by Ca^{2+} contrasts with results from other Ca^{2+}-binding proteins, such as calmodulin [24,25], troponin C [26], calbindin D_{9k} [27], and the bacterial spore coat Ca^{2+}-binding protein S [28,29], all of which show more dramatic Ca^{2+}-induced spectral changes. Protein S is a soluble extracellular Ca^{2+}-binding protein from *Myxococcus xanthus*, known to be involved in Ca^{2+}-dependent spore–spore interactions [30]. It is interesting to note that, like E-CAD1, this extracellular Ca^{2+}-binding protein consists mainly of β-sheet conformation [28,29], in contrast with the intracellular α-helical, EF-hand Ca^{2+}-binding proteins such as calmodulin, troponin C, and calbindin D_{9k}.

4. Conclusions

Until now, it has been unclear whether or not a single CAD repeat forms a structural domain and binds Ca^{2+}. The present study reveals that the N-terminal CAD1 repeat of E-cadherin indeed forms an independent structural domain and has a moderate Ca^{2+}-binding activity ($K_d = 1.6 \times 10^{-4}$ M), sufficient to bind Ca^{2+} under physiological conditions in the extracellular matrix. Furthermore, CD and NMR data provide evidence that the structure of this single cadherin repeat consists predominantly of a β-sheet conformation.

It has been reported [1,3,6] that the CAD1 domain plays an important role in determining the specificity of cadherin-mediated cell adhesion. The monomeric state of the isolated E-CAD1 repeat, however, suggests that cell–cell interaction mediated by E-cadherin may involve more than one extracellular repeat. Dimerization might have been expected if molecules of a single CAD repeat were able to self-associate. Interestingly, isolated single adhesive domains of other cell adhesion molecules such as CD2 [31] and CD4 [32] were also found to be monomeric in solution. Furthermore, a number of previous observations [1–4] indicate that other portions of the cadherin molecule are necessary for cell adhesive function. For example, tight cell–cell junctions mediated by cadherin interactions may require anchoring to a complex of cytoskeletal proteins through the cytoplasmic domain and also possibly molecular clustering via lateral interactions in the plasma membrane [33,34]. The present study supports this view in that a single CAD repeat itself does not form a stable dimer or an oligomer.

The availability of large amounts of E-CAD1 will allow determination of the three-dimensional structure of this important domain using NMR or X-ray crystallography. The present biochemical and biophysical studies establish a starting point towards our ultimate goal of detailed structural and functional characterization of E-CAD1, and consequent insights into the molecular mechanism of cadherin-mediated cell adhesion.

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