Disulfide Bond Formation Promotes the cis- and trans-Dimerization of the E-cadherin-derived First Repeat*

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Irwan T. Makagiansar†, Phuong D. Nguyen‡, Atsutoshi Ikuesue§, Krzysztof Kucezer¶, William Dentler¶, Jeffrey L. Urbauer¶, Nadezhda Galeva¶, Michail Alterman¶, and Teruna J. Siahaan†∗∗

From the Departments of †Pharmaceutical Chemistry and §Molecular Biosciences and the ¶Biochemical Research Laboratory, University of Kansas, Lawrence, Kansas 66047

Cadherin is a cell adhesion molecule crucial for epithelial and endothelial cell monolayer integrity. The previously solved x-ray crystallographic structure of the E-CAD12 cis-dimer displayed an unpaired Cys9, which protruded away from the Cys9 on the other protomer. To investigate the possible biological function of Cys9 within the first repeat (the E-cadherin-derived N-terminal repeat), E-CAD1 was overexpressed and secreted into the periplasmic space of Escherichia coli cells. Recombinant E-CAD1 produced a mixed monomer and dimer in an equilibrium fashion. The dimer was linked by a disulfide through Cys9 pairing. Analysis by high pressure liquid chromatography and electron microscopy suggested the existence of oligomeric complexes. Mutation at Trp2 appears to indicate that these oligomeric complexes trans-dimerize. Interestingly, mutation of Cys9 affected not only the cis-dimerization, but also the trans-oligomerization of E-CAD1. Accordingly, it is plausible that, under oxidative stress, the homophilic interactions of E-cadherin through E-CAD1 may be promoted and stabilized by this disulfide bond.

E-cadherin, a Ca2+-dependent member of the cadherin family of cell adhesion molecules, is crucial in providing cell polarity, tightness, and integrity of the intercellular junctions (1, 2). In most cancerous tissues, nonfunctional E-cadherin leads to the disturbance of the integrity of the intercellular junctions and consequentially promotes higher mobility and invasiveness of the cancer cells (3–5).

In recent years, considerable information has been gathered on the adhesion mechanism of classical cadherins from both structural and functional studies. The modular architecture of classical cadherins is characterized by the five repeats in the extracellular domain (6). The N-terminal of the extracellular domain is believed to be critical for homophilic cadherin interactions. The structure of the N-terminal fragment derived from the first repeat of the neuronal N-cadherin (N-CAD1) (7) and the epithelial E-cadherin (E-CAD1) (8) displays a resemblance to the structural fold of immunoglobulin.

Biochemical analysis of the first extracellular repeat E-CAD1 revealed only the presence of monomers (8). The solution structure of this monomer form was indeed determined by nuclear magnetic resonance (9, 10). This is in contrast with the crystal lattice structure of N-CAD1 (7), which contains a unique mixture of two different populations. One population consists of monomers interacting closely to form cis-dimers (parallel), which are stabilized by the exchange between the N-terminal β-strand and the intercalation of Trp2 into the partnering hydrophobic core. The other population reveals the pairing between antiparallel-oriented cis-dimers, designated as trans-dimers to reflect the possible head-to-head contacts between two cadherin molecules of the apposing cells. Alternating the cis- and trans-interactions forms a zipper-like structure (7). Crystallographic analysis of E-CAD12 also showed cis-dimers linked together by calcium ions with no evidence for adhesive trans-contact (11). Intriguingly, neither cis- nor trans-dimers were observed in the crystal structure of the extended form of N-CAD12, irrespective of the presence of Ca2+ (12). Thus, the interactions of the molecular repeats of E-CAD and N-CAD remain controversial. Nevertheless, it is basically agreed from in vitro and in vivo studies that lateral cis-dimerization of cadherin molecules is a prerequisite step for the trans-adhesive activity (13–15). Further mutational studies of the E-cadherin ectodomain fused with the pentamerization domain of cartilage oligomeric matrix protein (E-CADCOMP) seem to indicate that Trp2 docks into an intramolecular hydrophobic pocket, and this process appears to be critical for the trans-interaction event (16, 17).

At present, no studies have been performed to determine the role of the conserved Cys9 found in the first repeat of the E-cadherin sequence. Intriguingly, this particular cysteine residue is missing in other classical cadherins. As an initial effort toward understanding the contributory role of Cys9 in the homophilic mechanism of E-cadherin and in contrast to previously published works (8, 11), we have overexpressed and secreted the recombinant E-CAD1 fragment into the oxidizing environment of the periplasmic space of Escherichia coli cells. Our study shows that E-CAD1 is capable of forming disulfide-bonded cis-dimers, followed by their presumably trans-dimerization. These findings have important implications for the proposed mechanism for homophilic trans-interactions occurring at the first repeat of E-cadherin (16, 18–20).

EXPERIMENTAL PROCEDURES

Construction of E-cadherin-derived Regions—Full-length cDNA for human E-cadherin cloned in pCEP4 was kindly provided by Dr. David

§ Present address: Hisamitsu Pharmaceuticals, Inc., Tsukuba Laboratories, Kannondai 1-25-11, Tsukuba 305, Japan.
** To whom correspondence should be addressed: Dept. of Pharmaceutical Chemistry, University of Kansas, Simons Bldg., 3095 Constant Ave., Lawrence, KS 66047. Tel.: 785-864-7327; Fax: 785-864-5736; E-mail: Siahaan@ku.edu.

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Role of the Disulfide Bond in E-CAD1

Rimm (Department of Pathology, Yale University). The boundary for E-CAD1 is defined as Asp289Asp309 (GenBankTM/EBI Data Bank accession number AA612599). The following oligonucleotide primer pairs, containing a BsuI restriction site at each end and an artificial stop codon (TAG) after the triple base pairs coding for Asp289, were used for PCR amplification: the 8-amino acid streptavidin tag (WSHPQFEK), which is cleaved by the endogenous signal peptidase upon secretion into the periplasmic space, the 5'-agttactccagctgagttactccatc-3' (forward) and 5'-agttactccagctgagttactccatc-3' (reverse). The amplified product was digested with BsuI and ligated to BsuI-cleaved pASK-IBA6 (Sigma) downstream of the DNA sequence encoding the ompA signal peptide (cleaved by the endogenous signal peptidase upon secretion into the periplasmic space), the 8-amino acid streptavidin tag (WSHPQFEK), and the Factor Xa site. The recombinant plasmid DNA (designated as pASKcadEC1) was transformed into competent E. coli BL21 cells. The putative correct clones were screened by PCR using the ompA primer (5'-agttactccagctgagttactccatc-3', forward) and the above reverse primer, and the correct sequence was confirmed by DNA sequencing.

Single point mutations were obtained using the QuikChange mutagenesis kit (Strategene). pASKcadEC1 (20 ng) was used as a template with the following mutated (boldface) primer sets: CA9, 5'-gctgatgggaggaataac-3' (reverse) and W2A, 5'-gcaaaagtaaggagcagctgagttactccatc-3' (forward) and 5'-gctgatgggaggaataac-3' (reverse). For all the mutations, the following PCR parameters were used: 16 cycles of 94°C for 45 s, 50°C for 7 min, 50 s. After digestion with 10 units of DpnI (Stratagene) at 37°C for 1 h, 1 μl of the DpnI-treated DNA was used to transform competent BL21 cells. DNA sequencing identified plasmids containing the desired mutation.

Overexpression of E-CAD1—Cells harboring the plasmids coding for E-CAD1 or E-CAD1 mutants were grown at 37°C in M9 minimal medium (20% d-glucose, 1.3% NaHPO4·7H2O, 0.3% KH2PO4, 0.05% NaCl, 0.1% NH4Cl, 2 mM MgSO4·7H2O, and 0.5 mM CaCl2·2H2O, pH 7.4) supplemented with 1 × 10−3 mM thiamine, 1 × 10−3 mM FeSO4·7H2O, and 100 μg/ml ampicillin. When an A600 of 0.5–0.6 was reached, 2 mg/ml anhydrotetracycline (Sigma) was added to induce protein expression. After 3 h of induction, the cells were harvested by centrifugation at 5000 × g for 15 min at 4°C.

Purification—the pelleted cells were resuspended in 1 ml of pre-cooled periplasmic extraction buffer (100 mM Tris-HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA, and 0.02% (w/v) NaN3) and incubated on ice for 30 min. To remove spheroplasts, the suspension was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant, which contained the periplasmic extract, was carefully transferred into a clean 1.5-ml Eppendorf micro extract, was then lyzed on a DAWN®-DSP laser light scattering photometer (Wyatt Technology Corp.) at 18 different angles. Thus, 450 μl of a 33 μM sample of E-cadherin monomer (E-CAD1m) and dimer (E-CAD1dm) was fractionated on a Superose 6 gel-filtration column (1 × 30 cm, flow rate of 0.3 ml/min; Amersham Biosciences) using buffer containing 20 mM HEPES and 150 mM NaCl with or without 1 mM DTT, pH 8.0. The scattering intensity and refractive index signal of the eluting peaks were monitored. The refractive index signal was used to calculate the protein concentration, using a molar absorption coefficient of 19,580 M−1 cm−1.

Dissociation Constant of Oligomeric Complexes—A time-dependent study of oligomeric complexes (derived from peak 1) was performed after dilution from 9 to 3.0 μM and incubation at different time intervals during a total period of 24 h. At a given time, 1 ml of the diluted sample was reintegrated onto the size-exclusion column. The integrated peak area (PA) of the oligomeric complex (E-CAD1m) was used to calculate the percentage decrease in the oligomeric complexes using Equation 1.

\[
\% = \left(1 - \left(\frac{(PA_{0} - PA) - PA}{PA_{0}}\right) \times 100 \right)
\]  
\[
(\text{Eq. 1})
\]

A plot of ln[E-CAD1/][E-CAD1] against time was then established (where \(t = \) dissociation time in seconds, \[E-CAD1] = \) concentration of E-CAD1m at time \(t\), and \[E-CAD1] = \) concentration of E-CAD1m at a given time). The dissociation rate constant (\(k_{d}\)) was determined from the slope of this plot (slope = 1/\(k_{d}\)).

Electron Microscopy—Following their purification, E-CAD1m and E-CAD1dm were analyzed immediately by electron microscopy within a 24–48-h period. Electron microscopy solutions containing the protein (0.5 mg/ml) in 20 mM Tris-HCl, pH 7.5, were absorbed to a glow-discharged Formvar-over-carbon support film on a copper grid; the protein was negatively stained with freshly prepared and filtered 1% aqueous uranyl acetate. Images were photographed at magnification ×100,000 using a Joel 12000 EXII transmission electron microscope and were digitized using Adobe Photoshop 5. The mean length and width for each observed structure were determined from 9–12 related particles.

Molecular Dynamics Simulation—The structure of E-CAD12 (mouse E-cadherin, Protein Data Bank code 1FF5) was used in an umbrella sampling simulation employing the stochastic boundary molecular dynamics (SBMD) approach. Hydrogen atoms and missing termini were built in with the CHARMM modeling package. A 20-A sphere was defined, centered at the midpoint between the two sulfur atoms. This sphere contained 85 residues of chain A and 86 residues of chain B. Empty spaces in this sphere were solvated by overlay of a 20-A sphere of TIP3 water molecules (24). After SBMD conditions were set up, a brief energy minimization was performed, followed by a 20-ps molecular dynamics simulation of the water only to relax the solution shell. A second overlay of water was performed to fill any empty spaces in the solvent, followed by another 20-ps equilibration of the solvent and a 20-ps production run of the whole system. The final simulation system contained 2719 fixed atoms and 3904 moving atoms, the latter consisting of the 171 amino acid residues listed above, six calcium ions, and 395 water molecules.

The umbrella sampling simulation was started from the equilibrated SBMD system coordinates. A harmonic constraint potential \(U(x) = f^2(x - x_0)\) was introduced to restrain the disulfide bond distance coordinate \(x\) to a limited region, using a force constant \(f = 20 \text{ kcal/mol A}^2\).

1 The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high pressure liquid chromatography; DTT, dithiothreitol; SBMD, stochastic boundary molecular dynamics.
Initially, the reference value $x_i$ was set at 14.5 Å, close to the 14.76 Å found in the 1FF5 crystal structure. A 100-ps equilibration and a 100-ps data collection simulation were performed. Next, 19 additional simulation windows were generated, each consisting of 50 ps of equilibration and 100 ps of data collection with $x_i = 14.5, 14.0, 13.5$ down to 5.0. Within each window, the biased distribution $P(x)$ of the disulfide bond distance coordinate $x$ was calculated and converted to a potential of mean force $w(x)$ according to Equation 2 (25),

$$w(x) = -kT \ln P(x) - U_i(x) + C$$

where $k$ is the Boltzmann constant, $T$ is the temperature, $U_i$ is the constraint potential, and $C$ is an arbitrary constant. The values of the constants were chosen so that $w(x)$ values from neighboring windows coincided in the region of overlap. The SSMD simulation corresponds approximately to constant pressure and temperature conditions; thus, the differences in $w(x)$ along the path correspond approximately to the Gibbs free energy changes. The simulations used the molecular simulation program CHARMM Version 28 and the CHARMM19 polar atom topology and parameters (26). In this model, only the polar hydrogen atoms are treated explicitly, whereas nonpolar hydrogens are subsumed into the heavy atoms. In all energy evaluations, an atom-based topology and parameters (26). In this model, only the polar hydrogen atoms are treated explicitly, whereas nonpolar hydrogens are subsumed into the heavy atoms. In all energy evaluations, an atom-based topology and parameters (26).

Calculations were performed on an SGI Octane workstation and on the ORIGIN 2400. The Verlet algorithm was used with a 2-fs time step and SHAKE constraints applied to all bonds involving hydrogen atoms. To explore further the feasibility of an intermolecular disulfide bond, a 100-ps equilibration and a 100-ps simulation were performed to demonstrate further that dimerization was mediated by a disulfide bond. The trypsin cleavage point of the N-terminal streptavidin tag fusion sequence (WSHPQFEK). Following its purification by one-step column chromatography on immobilized StrepTactin, the purity of E-CAD1 was assessed by SDS-PAGE. With the exception of fraction 5, the purified fractions gave rise to a single band with an apparent molecular mass of 18 kDa under nonreducing conditions (Fig. 1A). MALDI-TOF spectrometry of an aliquot of this protein revealed a mass at $m/z$ 16,884, the value of which is in close agreement with the predicted size of the fused E-CAD1 (16,897 Da). However, fraction 5 also contained a higher molecular mass band of similar intensity. The apparent molecular mass of the protein in this band was 36 kDa (double that of the lower band). Treatment with the reducing agent β-mercaptoethanol led to the concurrent disappearance of the 36-kDa band and an increase in intensity of the 18-kDa band (Fig. 1B). This upper band therefore appears to represent the dimer form of E-CAD1.

**Expression and Purification—**E-CAD1 was expressed in the cytoplasm as a fusion fragment with both the streptavidin-derived sequence and the ompA signaling peptide. When secreted into the periplasmic space of E. coli cells, the signaling peptide was cleaved proteolytically by the host cell. Thus, the resulting recombinant E-CAD1 protein incorporated only an N-terminal streptavidin tag fusion sequence (WSHPQFEK).

**HPLC/Dynamic Light Scattering of the Monomer/Dimer Fraction—**To confirm further the presence of the oligomeric complex in peak 1, 2 or 3 was cross-linked with dimethyl pimelimidamide dibydrochloride, only peak 1 revealed a high molecular mass smeared band of 180–210 kDa by SDS-PAGE (Fig. 4C). The cross-linked sample from either peak 2 or 3 did not increase in molecular size and therefore remained as a dimer and monomer, respectively. These data suggest that the oligomeric complexes in peak 1 must be derived from the self-association of dimers. At this stage, the oligomeric complexes in peak 1 were designated as E-CAD1HER. Furthermore, MALDI-TOF analysis of E-CAD1HER derived from both peaks 1 and 2 under nonreducing conditions revealed prominent dimer bands (E-CAD1DM). On the other hand, fractions from peak 3 contained only the monomer band (E-CAD1M) (Fig. 4B). This pattern indicates that the proteins in all three eluted peaks were derived from E-CAD1 and excludes the possibility that peak 1 was due to a contaminating protein.

**Role of the Disulfide Bond in E-CAD1**

The tryptic mass spectrum of the nonreduced dimer displayed an increase in intensity of the 18-kDa band (Fig. 1A). Treatment with the reducing agent thiothymine for noncovalent protein-protein studies (22, 23) led to the concurrent disappearance of the 36-kDa band and an increase in intensity of the 18-kDa band (Fig. 1A). From the calculated Debye plot (Fig. 6B) the molar mass across the size-exclusion column was also detected and analyzed by direct on-line dynamic light scattering. The light scattering trace showed a broadened peak 1 (Fig. 6A). From the calculated Debye plot (Fig. 6B) the molar mass across peak 1 was not constant, as the molecular mass varied from 140 to 300 kDa (Table I). This polydisperse distribution strongly indicates that peak 1 contained oligomeric molecules. Thus, it would be fairly speculative to assign a given oligomeric state for the molecules that eluted in peak 1. In contrast, the distribution of molar mass across peak 2 or 3 was constant, indicating a monodisperse distribution (i.e. a homogeneous molecule) for each peak with molecular masses of 36.1 kDa (E-CAD1DM) and 17.6 kDa (E-CAD1M), respectively.

**Importance of the Disulfide Bond**

To explore further the feasibility of an intermolecular disulfide bond in E-cadherin, molecular dynamics simulations were performed based on the coordinates derived from the previously solved x-ray crystallography of the E-CAD1 dimer. A 20-Å sphere centered at the midpoint of the two sulfur atoms was defined. The protein atoms in the sphere were solvated and allowed to move according to the CHARMM molecular mechanics force field, whereas atoms outside the sphere remained fixed. A harmonic umbrella potential was applied to the S–S distance coordinate, centered at progressively decreasing distances. As a result, the potential of mean force for changing the S–S distance was generated in the range between 15.0 and 5.0 Å. Interestingly, changing the distance from 14.8 Å (found in the E-CAD1 dimer crystal structure) to 5.0 Å produced an increase in the Gibbs free energy of only 1.3 kcal/mol. The simulation stopped when the region of non-bonded repulsion between the sulfurs was reached.

**Oligomeric State of E-CAD1**

When an aliquot derived from peak 1, 2, or 3 was cross-linked with dimethyl pimelimidamide dibhydrochloride, only peak 1 revealed a high molecular mass smeared band of 180–210 kDa by SDS-PAGE (Fig. 4C). The cross-linked sample from either peak 2 or 3 did not increase in molecular size and therefore remained as a dimer and monomer, respectively. These data suggest that the oligomeric complexes in peak 1 must be derived from the self-association of dimers. At this stage, the oligomeric complexes in peak 1 were designated as E-CAD1HER.

**Evidence of a Disulfide-linked Dimer**—In-gel trypsinization of this E-CAD1 dimer and MALDI-TOF spectrometry were performed to demonstrate further that dimerization was mediated by a disulfide bond. The trypsin cleavage point of the N-terminal cysteine-containing sequence is shown in Fig. 2A. The tryptic mass spectrum of the nonreduced dimer displayed molecular masses at $m/z$ 3776 and 4303, which correspond to the calculated masses of the disulfide-linked fragments, respectively (Fig. 2B). In contrast, when the putative dimer was reduced and alkylated with iodoacetamide, the two high molecular mass bands disappeared and gave rise to molecular masses at $m/z$ 1683 and 2210, which correspond to the predicted masses of the cysteine-alkylated fragments (Fig. 2C).
elution buffer resulted in the elimination of both peaks 1 and 2 (Fig. 7A and Table I). Thus, DTT affected the dimerization (peak 2) and oligomerization (peak 1) of E-CAD1. To assess further the importance of the disulfide bond, Cys9 was substituted with alanine to yield a recombinant E-CAD1 C9A fragment. Following its expression and purification, E-CAD1 C9A was subjected to size-exclusion gel chromatography. As expected, the elution profile of the single peak is identical (data not shown) to the chromatographic profile obtained with E-CAD1 under reducing conditions. SDS-PAGE of the eluted peak fractions showed a single band corresponding to the monomer form (Fig. 7B). This observation implies the importance of the disulfide...
not only in promoting dimer formation, but also, at the same time, in stabilizing formation of the oligomeric complex.

Dissociation Rate Constant of E-CAD1 oc—A time-dependent study of E-CAD1 oc stability by gel filtration showed a rapid decrease in E-CAD1 oc and the appearance of E-CAD1 dm. An equilibrium between the oligomeric complex and dimer forms was reached after 12 h at 22 °C (Fig. 8A). The E-CAD1 oc dissociation rate constant ($k_d$) was calculated to be $23 \times 10^{-4}$ s$^{-1}$ at 22 °C (Fig. 8B).

Electron Microscopy—The electron micrographs of negatively stained E-CAD1 m/E-CAD1 dm showed particles of heterogeneous sizes (Fig. 9A). Many prominent small particles revealed one or two bead-like particles with average sizes of 80.6 ± 0.6 Å (Fig. 9B, row 1) or 254 ± 2.3 × 118 ± 1.2 Å (row 2), respectively. Much longer particles of different lengths were also observed when the sample from peak 1 (E-CAD1 oc) was negatively stained (Fig. 9B, row 3). Because these particles appeared as nonlinear bead-like particles, their size could not be measured accurately. Electron microscopy of samples from both peaks 2 and 3 revealed no particles (data not shown), probably because they were too minute for detection.

Orientation of Dimer Interactions—Previous studies with Ca$^{2+}$-dependent E-CAD12 suggest that Trp 2 plays a crucial role in initiating the homophilic trans-interaction of one dimer with another (17). To determine whether the bead-like structures observed in the electron micrographs are made up of cis-E-CAD1 dm (parallel) or trans-E-CAD1 dm (antiparallel), Trp2 was substituted with alanine to yield the recombinant E-CAD1 W2A fragment. Following its expression and purification, E-CAD1 W2A was further fractionated by size-exclusion gel chromatography. Interestingly, the chromatogram from the size-exclusion column revealed three peaks with the same profile as wild-type E-CAD1, but this time the percentage area of peak 1 from E-CAD1 W2A decreased significantly compared with that from wild-type E-CAD1 (Table II). Thus, although the Trp2 mutation did not completely annul the formation of E-CAD1 oc, it can be inferred that E-CAD1 dm trans-dimerizes to form E-CAD1 oc.
DISCUSSION

Previously, the structure of N-CAD1 revealed a dimer (9) as opposed to the crystallographic structure E-CAD1 (7), whereas the cooperative binding of Ca\(^{2+}\) enhanced the cis-dimerization of E-CAD12 (11, 17). It was found that the Ca\(^{2+}\)-binding inter-
face located between E-CAD1 and E-CAD2 mediates this dimer arrangement (27). In this present work, we further show the existence of a novel E-CAD1 dimer linked by a disulfide bond via its Cys\(^9\). The same disulfide bond was also formed when we overexpressed the extended E-CAD12 (the second repeat does...
TABLE I  
Molecular mass determination of eluted E-CAD1m/E-CAD1dm by Superose 6 HPLC/light scattering

For reducing conditions, E-CAD1 was treated with 1 mM DTT for 20 min before sample loading and resolved in standard buffer containing 1 mM DTT. Values in parentheses represent the peak area percentage.

| Peak No. | Mass range observed (kDa) | Average mass for entire peak (kDa) | Monodisperse peak |
|---------|---------------------------|-----------------------------------|-------------------|
| −DTT    | 140–300 (19.1%)           | 214                               | No                |
|         | 35–37.5 (28.6%)           | 36.1                              | Yes               |
| +DTT    | 17.2–17.8 (52.3%)         | 17.6                              | Yes               |

For oxidizing conditions, E-CAD1 was oxidized with 1 mM DTT for 20 min before sample loading and resolved in standard buffer containing 1 mM DTT. Values in parentheses represent the peak area percentage.

| Peak No. | Mass range observed (kDa) | Average mass for entire peak (kDa) | Monodisperse peak |
|---------|---------------------------|-----------------------------------|-------------------|
| 1       | 120–400 (3.6%)            | 200                               | No                |
| 2       | 16.5–18.0 (96.4%)         | 17.3                              | Yes               |

Fig. 7. Disulfide bond involvement in dimerization and oligomerization. A, when 33 µM E-CAD1m/E-CAD1dm was further resolved across a Superose 6 column in buffer containing the reducing agent DTT, both peaks 1 and 2 disappeared, leaving the monomer as the only eluted peak (peak 3). B, analysis by SDS-PAGE of eluted E-CAD1m showed a sole monomer band. MW, molecular weight.

not contain cysteine). This peculiar phenomenon was not previously observed with N-CAD1, E-CAD1, or E-CAD12. Thus, dimerization of our E-CAD1 appears to be promoted by the disulfide bond, regardless of the presence of Ca^{2+}. The assumption that dimerization can occur with E-CAD1 only because of the Ca^{2+}-binding interface located between the first and second repeats therefore may not hold true.

It is interesting to note that the x-ray structure of the E-CAD12 dimer displays each thiol group protruding inwardly with the sulfur atoms separated by 14.8 Å, which appears to sterically hinder the formation of the disulfide bond. Our results suggest that, in solution, the βα-region of E-CAD1dm undertakes a less rigid conformation, rendering the thiol group more exposed for pairing to form the disulfide bond. Indeed, our thermodynamic simulation using the coordinates from the x-ray structure of the E-CAD12 dimer demonstrates that a disulfide bond can be optimally formed at a pairing distance of 2.1 Å without the necessity of large-scale changes in the protein. In relation to this finding, it remains unclear what mechanical force causes N-CAD1 to dimerize in the absence of a disulfide (7) given the fact that N-CAD1 does not contain cysteine (28).

The absence of disulfide formation in previous E-CAD1 and E-CAD12 structures may arise because of the expression system used to produce these recombinant fragments. The latter were overexpressed in the cytoplasmic compartment of E. coli cells (8, 11), a condition that prevents formation of structural disulfide bonds due to its reducing environment (29, 30). In contrast, our present E-CAD1 was expressed and exported into the E. coli periplasm, the oxidizing environment of which promotes disulfide formation (28, 29). More importantly, the presence of thiol-disulfide oxidoreductases (i.e. DsbA and DsbC) (31–34) in the periplasmic compartment may contribute significantly to the favorable arrangement and pairing of the neighboring thiol side chain from each E-CAD1 monomer to promote dimerization.

Intriguingly, the E-CAD1 dimer can, in fact, form oligomeric complexes. MALDI-TOF analysis of E-CAD1m/E-CAD1dm using the less acidic matrix condition for noncovalent protein-protein studies revealed masses at m/z 66,585 and 133,257, which are calculated to correspond to a tetramer (66,585/16,910 = 3.94) and an octamer (133,257/16,910 = 7.90), respectively. No higher masses were observed, which was due presumably to the instability of the higher oligomeric complexes under this condition. Indeed, when the dimer band in peak 1 was cross-linked, a smeared band with apparent molecular masses in the range of 180–210 kDa was observed. Furthermore, light scattering of peak 1 revealed a mixture of species of different masses, with a molecular mass distribution ranging from 140 to 300 kDa. This observation strongly suggests that this peak is polydisperse and contains different oligomeric complexes. The E-CAD1oc dissociation rate constant (k_d) was calculated to be 23 × 10^{-4} s^{-1} at 22 °C, which indicates that the complexes may not be too stable.

Additionally, the oligomerization of E-CAD1 appears to be stabilized by the formation of the disulfide bond. In the presence of the reducing agent DTT, peak 1 was eliminated. Similarly, when Cys^9 was replaced with alanine (E-CAD1C9A), the same chromatographic profile as obtained with DTT was produced.

Electron microscopic images of E-CAD1m/E-CAD1dm showed particles of various sizes, consisting mainly of single or longer bead-like molecules. However, these particles did not clearly show whether they adopted a cis-orientation (parallel) or a trans-orientation (antiparallel). Studies with E-CADCOMP demonstrated that docking of the second tryptophan residue (Trp^2) into the hydrophobic cavity (7θHAVA^8) of the same molecule is important for adhesive trans-interactions (17, 27). Mutation of Trp^2 in E-CAD12COMP abolished the trans-dimer interaction, but not the cis-dimerization. In our case, substituting Trp^2 with alanine (E-CAD1W2A) led to the partial reduction of oligomeric complexes without affecting dimerization. Full abrogation of the E-CAD1W2A oligomeric complexes was not observed, probably due to other intermolecular forces influencing the trans-dimerization. Among such factors is the presence of the disulfide bond, which may substantially alleviate the mutational effect of tryptophan. Taken together, however, our data support the presumption that the oligomeric complex of E-CAD1 adopts an antiparallel orientation.

From the x-ray crystallography of N-CAD1, a dimer size of 38 × 38 Å was obtained (7), whereas the size for the E-CAD1 dimer derived from the x-ray crystallography of E-CAD12 was 46 × 48 Å (11). Given the condition under which two E-CAD1

\[^{2}\text{I. T. Makagiansar, P. D. Nguyen, and T. J. Siahaan, unpublished data.}\]
We speculate that the smaller width may be due to the presence of the disulfide, which brings the interfacial surfaces of the two monomers closer to one another. It is to be noted, however, that neither the monomeric nor the dimeric E-CAD1 particle was detected, probably due to its minute size. Meanwhile, a particle size of $254 \times 118 \text{Å}$ appears to correspond to six dimers $\text{trans}$-interacting (hence, a 200-kDa dodecamer). Much longer bead-like structures of different lengths were also detected by electron microscopy. Because of their nonlinear elongated shapes, measurement of these dimensions could not be carried out accurately. Nevertheless, by estimating the tetrameric repeats contained in these long bead-like particles, an apparent molecular mass range of $-240-340 \text{kDa}$ was deduced.

Thus, the overall molecular mass range observed by electron microscopy is consistent with both the MALDI-TOF results and the molecular mass distribution of peak 1 obtained by the HPLC/dynamic light scattering method. This information provides strong evidence for the existence of $\text{trans}$-oligomeric complexes of E-CAD1.

Despite the fact that molecular force measurements between antiparallel oriented monolayers of the Xenopus C-cadherin extracellular repeats may indicate that cadherin binding involves distinct multiple repeats for $\text{trans}$-interactions (35–37), several lines of evidence, including our present data, suggest the possible homophilic cadherin $\text{trans}$-interaction taking place at the N terminus to form a zipper-like molecule (7, 19, 20, 39, 40). Indeed, a cryoelectron microscopy study by rapid-freeze deep-etching of the adherens junction in retinal pigment epithelium showed that the extracellular domain of E-cadherin forms zipper-like molecules within the intercellular space (41). These molecules consist of combined rods and globules, the rod being the dimerized E-cadherin and the globule being the enlarged $\text{trans}$-contact regions between the first or second repeat of E-cadherin.

The biological implication of this disulfide bond suggests an alternative means of regulation of E-cadherin by a redox mechanism. Redox modulation at specific cysteine residues was observed to influence the function of hemoglobin (42), the $\text{N}$-methyl-D-aspartate receptor (43), and integrin $\alpha II\beta 5$ (38). Under oxidative stress, it may be possible that a redox switch from an unpaired Cys$^8$ to a paired cysteine affects the conformation of the extracellular domains, stabilizing further E-cadherin cell-mediated adhesion. In retrospect, more work will be required to confirm this possibility.

In summary, our data provide clear evidence supporting the idea that the first repeat is involved in the $\text{trans}$-dimerization of E-cadherin. It is further demonstrated that a disulfide bond not only appears to be a decisive factor in promoting the dimerization of the first repeat, but also acts as a stabilizing force toward formation of $\text{trans}$-dimeric complexes of E-CAD1.
Role of the Disulfide Bond in E-CAD1

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REFERENCES

1. Takeichi, M. (1991) Science 251, 1451–1455
2. Larue, L., Ohsugi, M., Hirchenhain, J., and Kemler, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8263–8267
3. Beavon, I. R. (2000) Eur. J. Cancer 36, 1607–1620
4. Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. (1998) Nature 392, 190–193
5. Masur, K., Lang, K., Niggemann, B., Zanker, K. S., and Entschladen, F. (2001) Mol. Biol. Cell 12, 1973–1982
6. Pokutta, S., Herrenknecht, K., Kemler, R., and Engel, J. (1994) Eur. J. Biochem. 223, 1019–1026
7. Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grubel, G., Legrand, J. F., Als-Nielsen, J., Colman, D. R., and Hendrickson, W. A. (1995) Nature 374, 327–337
8. Tong, K. I., Yau, P., Overduin, M., Bagby, S., Takeichi, M., and Ikura, M. (1994) FEBS Lett. 352, 311–322
9. Overduin, M., Harvey, T. S., Bagby, S., Tong, K. I., Yau, P., Takeichi, M., and Ikura, M. (1995) Science 267, 386–389
10. Overduin, M., Tong, K. I., Kay, C. M., and Ikura, M. (1996) J. Biol. Chem. 271, 173–189
11. Nagar, R., Overduin, M., Ikura M., and Rini, J. M. (1998) Nature 390, 366–364
12. Tamura, K., Shan, W. S., Hendrickson, W. A., Colman, D. R., and Shapiro, L. (1998) Neuron 20, 1153–1163
13. Yap, A. S., Brieher, W. M., Pruskey, M., and Gumbiner, B. M. (1997) Curr. Biol. 7, 308–315
14. Alatia, J. R., Ames, J. B., Porumb, T., Tong, K. I., Heng, Y. M., Ottensmeyer, P., Kay, C. M., and Ikura, M. (1997) FEBS Lett. 417, 405–408
15. Murase, S., Hirano, S., Wang, X., Kitagawa, M., Natori, M., Takeichi, S., and Suzuki, T. S. (2000) Biochem. Biophys. Res. Commun. 267, 1191–1198
16. Tomochi, A., Fauser, C., Landwehr, R., and Engel, J. (1996) EMBO J. 15, 6197–6202
17. Pertz, O., Bozic, D., Koch, A. W., Fauser, C., Brancaccio, A., and Engel, J. (1999) JMB 295, 1738–1747
18. Blaschuk, O. W., Sullivan, R., David, S., and Poulist, Y. (1990) Devel. Biol. 139, 227–229
19. Nose, A., Tsuji, K., and Takeichi, M. (1990) Cell 61, 147–155
20. Kitagawa, M., Natori, M., Murase, S., Hirano, S., Takeichi, S., and Suzuki, S. T. (2000) Biochem. Biophys. Res. Commun. 271, 358–363
21. Mach, H., Volkin, D. B., Burke, C. J., and Middaugh, C. R. (1995) Methods Mol. Biol. 40, 91–114
22. Farmer, T. B., and Capriotti, R. M. (1998) J. Mass Spectrom. 33, 697–704
23. Woods, A. S., and Huestis, M. A. (2001) J. Am. Soc. Mass Spectrom. 12, 88–96
24. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impy, R. W., and Klein, M. L. (1983) J. Chem. Phys. 79, 926–935
25. Brooks, C. L., III, Karplus, M., and Pettitt, B. M. (1988) Proteins: A Theoretical Perspective of Dynamics, Structure, and Thermodynamics, 2nd Ed., John Wiley & Sons, Inc., New York
26. Brooks, B. R., Brucoleri, R., Olafson, B., States, D., Swaminathan, S., and Karplus, M. (1983) J. Comp. Chem. 4, 187–217
27. Koch, A. W., Bozic, D., Pertz, O., and Engel, J. (1999) Curr. Opin. Struct. Biol. 9, 275–281
28. Reid, R. A., and Hemperly, J. J. (1990) Nucleic Acids Res. 18, 5896
29. Skerra, A., and Pluckthun, A. (1988) Science 240, 1039–1041
30. Serudin, M. A., and Beckwith, J. (1991) J. Bacteriol. 173, 719–722
31. Wunderlich, M., Jaenicke, R., and Glockshuber, R. (1995) J. Mol. Biol. 233, 559–566
32. Wunderlich, M., and Glockshuber, R. (1993) J. Biol. Chem. 268, 24547–24550
33. Jouda, S., Huber-Wunderlich, M., Glockshuber, R., and Moksony E. (1999) EMBO J. 18, 3271–3281
34. Zapun, A., Missiakas, D., Raina, S., and Creighton, T. E. (1995) Biochemistry 34, 5075–5089
35. Corada, M., Liao, F., Lindgren, M., Lampugnani, M. G., Breviario, F., Frank, R., Muller, W. A., Hicklin, D. J., Bohlen, P., and Dejana, E. (2001) Blood 97, 1679–1684
36. Chappuis-Flament, S., Wong, E., Hicks, L. D., Kay, C. M., and Gumbiner, B. M. (2001) J. Cell Biol. 154, 231–243
37. Sivasanakar, S., Gumbiner, B., and Leckband, D. (2001) Biochemistry 1038–1041
38. Yan, B., and Smith, J. W. (2000) J. Biol. Chem. 275, 39964–39972
39. Tomochi, A., Shinmyama, Y., Nagatsu, A., and Hirohashi, S. (1999) Nat. Struct. Biol. 6, 310–312
40. Makagiansar, I. T., Avery, M., Hu, Y., Audus, K. L., and Slihaan, T. J. (2001) Pharm. Res. 18, 446–453
41. Miyaguchi, K. (2000) J. Struct. Biol. 132, 169–176
42. Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996) Nature 380, 221–226
43. Kim, W. K., Choi, Y. B., Rayudu, P. V., Das, P., Aasaad, W., Aroelle, D. R., Stamler, J. S., and Lipton, S. A. (1999) Neuron 24, 461–469
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