Nanomechanics of the Cadherin Ectodomain

“CANALIZATION” BY Ca\(^{2+}\) BINDING RESULTS IN A NEW MECHANICAL ELEMENT\(^*\)

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Cadherins form a large family of calcium-dependent cell-cell adhesion receptors involved in development, morphogenesis, synaptogenesis, differentiation, and carcinogenesis through signal transduction using an adaptor complex that connects them to the cytoskeleton. However, the molecular mechanisms underlying mechanotransduction through cadherins remain unknown, although their extracellular region (ectodomain) is thought to be critical in this process. By single molecule force spectroscopy, molecular dynamics simulations, and protein engineering, here we have directly examined the nanomechanics of the C-cadherin ectodomain and found it to be strongly dependent on the calcium concentration. In the presence of calcium, the ectodomain extends through a defined (“canalized”) pathway that involves two mechanical resistance elements: a mechanical clamp from the cadherin domains and a novel mechanical component from the interdomain calcium-binding regions (“calcium rivet”) that is abolished by magnesium replacement and in a mutant intended to impede calcium coordination. By contrast, in the absence of calcium, the mechanical response of the ectodomain becomes largely “decanalized” and destabilized. The cadherin ectodomain may therefore behave as a calcium-switched “mechanical antenna” with very different mechanical responses depending on calcium concentration (which would affect its mechanical integrity and force transmission capability). The versatile mechanical design of the cadherin ectodomain and its dependence on extracellular calcium facilitate a variety of mechanical responses that, we hypothesize, could influence the various adhesive properties mediated by cadherins in tissue morphogenesis, synaptic plasticity, and disease. Our work represents the first step toward the mechanical characterization of the cadherin system, opening the door to understanding the mechanical bases of its mechanotransduction.

The acquisition of specific machinery for cell adhesion represented a crucial step in the evolution of metazoan organisms, enabling multicellularity and the development of tissues. There are four major classes of cell adhesion receptors: the immunoglobulin (Ig)-like superfamily, the cadherin superfamily, selectins, and integrins (1). Because of their location at the cell-cell interface, these receptors are often subject to mechanical stress (2, 3). However, to date only the mechanical properties of Ig and fibronectin type III domains have been investigated using atomic force microscopy (AFM)5-based single molecule force spectroscopy (SMFS) and protein engineering (4).

Cadherins can be defined (with some exceptions) as a superfamiliy of calcium (Ca\(^{2+}\))-dependent homophilic cell-cell adhesion proteins that mediate most cell adhesion in vertebrates, playing a crucial role in morphogenesis, synaptogenesis, tissue architecture, tissue repair, and carcinogenesis (5–7). Still, certain cadherins are heterophilic or/and connect two membranes from the same cell. At least in the case of the so-called “classical” cadherins (6, 8), their connection to the cytoskeleton appears to be mediated by several adaptor proteins (Refs. 9–11 and see Fig. 1A). This linkage is responsible for reinforcing cell-cell adhesion through a signaling pathway involving the activation of Rho, Rac, Cdc42, and the Arp2/3 actin nucleator complex (12–15). External mechanical stimuli are among the signals transduced by cadherins, which can induce changes in cell shape and synaptic plasticity (6, 16–19), triggering chemical or electrical (20) signaling. Furthermore, cadherin interactions are known to exert forces that have already been measured (21).

Classical cadherins are single pass transmembrane glycoproteins of 720–750 amino acids with an extracellular (ectodomain or EC) that has a rodlike structure composed of five autonomously folded tandem domains (extracellular cadherin domains EC\(_1\)–EC\(_5\) from the distal N terminus to the proximal C terminus; see Fig. 1A and B). Although it is accepted that these cadherins establish homophilic interactions in cis and trans (22), the quaternary structure of these complexes remains unclear. The resolution of the atomic struct-

5 The abbreviations used are: AFM, atomic force microscopy; SMFS, single molecule force spectroscopy; EC, ectodomain of cadherins; MD, molecular dynamics; SMD, steered MD; Δ\(_{IC}^L\), increase in contour length; WLC, wormlike chain; TM, triple mutant; pN, piconewtons; r.m.s.d., root mean square deviation.
ture of the entire ectodomain of C-cadherin (a classical cadherin; Ref. 23) led to the proposal that trans interactions were based on a “β-strand-swapping” mechanism that only involved EC₁, whereas the cis interactions also involved EC₂. This model was consistent with electron microscopy data (24, 25) and fluorescence resonance energy transfer measurements (26). However, deletion analysis experiments (27, 28) and biophysical experiments using AFM (29), optical tweezers (30), and the surface force apparatus (31) showed that, although the model derived from the atomic structure of the ectodomain could be correct, it appears not to be exclusive as several interactions resulting from different degrees of overlap of the cadherin ectodomains are likely to occur. Indeed, the strongest interaction was detected with a complete overlap. Interestingly, recent evidence points to the existence of an intermediate binding conformation (termed “X-dimer”), which is necessary for the subsequent formation of the mature β-strand-swapping interaction (32, 33). Furthermore, non-classical cadherins seem to use a different mechanism of interaction (34–36).

The fact that cadherins transduce external mechanical stimuli (6, 16–20) makes the study of their nanomechanics a critical task as the first step toward understanding the underlying molecular mechanisms. To this end, unveiling the specific mechanical role of their ectodomain is essential. Related to this, the ectodomain has been postulated to function as a “force sensor” (37, 38), although the force sensitivity of cadherin junctions is still a matter of debate. Thus, in this context, it is highly relevant to study not only its intermolecular interactions (i.e. cis and trans, on which all nanomechanical studies of cadherins have focused so far; Refs. 29–32) but also the intramolecular interactions within the monomer. It is known that mature cadherin interactions are disrupted upon depletion of Ca²⁺ in the extracellular medium (5, 22), and indeed, cadherins were also proposed to function as “Ca²⁺ sensors” (39). Moreover, the cadherin ectodomain loses its rigidity and becomes a very flexible structure at low Ca²⁺ concentrations (40–44).

Our working hypothesis is that the nanomechanics of the monomeric cadherin ectodomain may change under different Ca²⁺ concentrations and that this differential response may affect the transduction of the mechanical signals within the cell. We have chosen C-cadherin as a study system as this is the only cadherin for which the atomic structure of the whole ectodomain is available.

C-cadherin is implicated in cell adhesion during the early stages of the development (i.e. the compaction of the morula) of Xenopus laevis (45, 46). This protein contains five EC domains, and each interdomain region can bind three Ca²⁺ ions such that the entire ectodomain is capable of binding 12 Ca²⁺ ions (Ref. 23 and see Fig. 1B). Ca²⁺ binding has a marked effect on the conformation of the protein as the binding of Ca²⁺ induces rigidity, whereas the unbound protein has a flexible structure (23, 40, 43, 44, 47). Although the role of Ca²⁺ in the structure and function of cadherins is well documented, to date no experimental data are available regarding the modulatory effects of Ca²⁺ binding on the nanomechanics of the ectodomain. This is particularly relevant given that these proteins are considered to act as mechanotransducers (3, 12–21, 37, 38, 48).

The cadherin fold, a compact unit with overall dimensions of ~45 × 25 × 25 Å in the presence of Ca²⁺, is a Greek key fold with the N and C termini pointing toward opposite ends of the domain. This topology is remarkably similar to that of the Ig-like fold and superimposes quite well onto Ig I type (“intermediate”; Ref. 49), a class that includes the majority of Ig domains that are present in cell adhesion proteins, surface receptors, and muscle proteins (50). Both folds consist of seven β-strands with antiparallel pairings except for that between the A’ and G strands. These similarities are not likely to be the result of either sequence divergence or functional convergence but rather that of structural convergence (51). The nanomechanics of proteins with similar folds has previously been studied by AFM-based SMFS, revealing a relatively high mechanical stability for all of them (4). Here, using this technique and molecular dynamics (MD) simulations, we have directly measured the nanomechanical properties of the C-cadherin ectodomain and the effects of Ca²⁺ binding. We found that, upon Ca²⁺ binding, this structure rigidifies and “canalizes” its nanomechanical behavior, generating a novel mechanical element (the “Ca²⁺ rivet”).

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of C-cadherin Ectodomain—** The purified recombinant ectodomain of C-cadherin from *X. laevis* (UniProtKB/Swiss-Prot entry P33148) was provided by Dr. Barry Gumbiner (22). The protein was kept frozen (−80 °C) at ~0.5 mg/ml in 20 mM HEPES, 150 mM NaCl (pH 7.5) with either 1 mM CaCl₂ (“presence of Ca²⁺”) or 1 mM EGTA (“absence of Ca²⁺”).

**Construction of Heteropolyproteins—** To construct the heteropolyproteins containing the EC cadherin domains and the I27 marker domains (52), we used the only “ready-to-go” cloning/expression vector available (Ref. 53 and see Fig. 1C). A plasmid encoding the entire ectodomain of C-cadherin (22) was used as a template for the cloning of EC domains.

To construct the (I27)₅-EC₁-₅-I27 polyprotein, the KpnI and MluI sites present in the I27-containing vector (53) were used as cloning sites. In the case of the (I27)₅-EC₁-₂-(I27)₂ and (I27)₅-EC₂-(I27)₂ polyproteins, the KpnI and Spel sites were selected for cloning. To construct the triple mutant (TM) D67A/D100A/D134A, the wild type EC₁-₅ sequence was cloned into the pT7blue vector (Novagen) using KpnI and Spel sites prior to performing site-directed mutagenesis (one point mutation at a time) using the QuikChange kit (Stratagene). This construct was called EC₁-₂TM and was cloned into the KpnI and Spel sites of the aforementioned expression vector, which contains the I27 marker domains (53).

Domain boundaries were chosen according to those deposited in the Swiss-Prot Database (entry P33148) and in the Protein Data Bank (code 1l3w). The EC₁-₅ protein contained residues 1–540, EC₁-₂ residues 1–214, and the EC₂ domain residues 102–214 based on the sequence of the mature C-cadherin. All the sequences were verified by sequencing both strands of the DNA, and the cloning steps were carried out in the Escherichia coli XL1-Blue strain (Stratagene).
Expression and Purification of Heteropolyproteins—Heteropolyproteins were expressed in the E. coli C41(DE3) strain (54). Cells were grown at 37 °C to an A_{600} of 0.6–0.8 after which the expression of the recombinant heteropolyproteins was induced over 4 h by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. We determined the solubility of proteins by immunoblotting the soluble and insoluble fractions. The bacterial cells were lysed by treatment with 1 mg/ml lysozyme and 1% Triton X-100 as described previously (55).

Recombinant heteropolyproteins were purified by Ni^{2+} affinity chromatography using HisTrap HP FPLC columns (GE Healthcare) on an FPLC apparatus (ÄKTera Purifier, GE Healthcare) with a buffer containing 20 mM HEPES, 150 mM NaCl (pH 7.5), which was added with 50 mM imidazole for binding and 500 mM imidazole for elution. Either 1 mM CaCl_{2} or 1 mM EGTA was added to the samples prior to the purification processes but not to the purification buffers (as recommended by GE Healthcare). Afterward, gel filtration purification was performed using a HiLoad 16/60 column (GE Healthcare) with a buffer containing 20 mM HEPES, 150 mM NaCl (pH 7.5) added with either 1 mM CaCl_{2} or 1 mM EGTA (absence of Ca^{2+}). The concentrations of CaCl_{2} and EGTA mentioned above were chosen on the basis of previous reports demonstrating that they saturated all the Ca^{2+} binding sites (40, 42, 43) or prevented C-cadherin interactions (22), respectively. The purified proteins were kept at ~0.5 mg/ml at 4 °C.

For the analysis of the (I_{27})_{3}–EC_{1–2}–(I_{27})_{2} polyprotein in 0.1 mM CaCl_{2}, an aliquot of the polyprotein that was purified in the presence of 1 mM CaCl_{2} was dialyzed extensively against a 20 mM HEPES, 150 mM NaCl (pH 7.5) buffer containing no added Ca^{2+}. Finally, several dialysis steps against a 20 mM HEPES, 150 mM NaCl, 0.1 mM CaCl_{2} (pH 7.5) buffer were performed followed by concentration of the polyprotein sample in the same buffer by ultrafiltration using Amicon 10 K filters (Millipore). No Ca^{2+}/EGTA buffer was used to prepare this buffer based on the assumption that the residual Ca^{2+} concentration in Milli-Q water is in the low μM range (56).

To analyze the (I_{27})_{3}–EC_{1–2}–(I_{27})_{2} polyprotein in the presence of magnesium ions (Mg^{2+}), we dialyzed an aliquot of the polyprotein (purified in the presence of EGTA) extensively against a 20 mM HEPES, 150 mM NaCl (pH 7.5) buffer containing no added Ca^{2+}. Finally, several dialysis steps against a 20 mM HEPES, 150 mM NaCl, 0.1 mM CaCl_{2} (pH 7.5) buffer were performed followed by concentration of the polyprotein sample in the same buffer by ultrafiltration using Amicon 10 K filters (Millipore). No Ca^{2+}/EGTA buffer was used to prepare this buffer based on the assumption that the residual Ca^{2+} concentration in Milli-Q water is in the low μM range (56).

The concentration of each protein was estimated by spectrophotometry using its molar extinction coefficient. All the heteropolyproteins constructed using this method end with two C-terminal cysteine residues for their covalent attachment to the gold-coated coverslips used as AFM substrates. However, to avoid rupturing the disulfide bonds present in EC_{9} (23), we only used reducing agents with (I_{27})_{3}–EC_{1–2}–(I_{27})_{2} and (I_{27})_{3}–EC_{2}–(I_{27})_{2} but not with the (I_{27})_{3}–EC_{1–5}–I_{27} polyproteins.

The heteropolyprotein (I_{27})_{3}–EC_{1–5}–I_{27} was highly insoluble, and only small amounts were obtained after purification, hindering the collection of sufficient data for statistical analy-
nates, we formulated the parameter files with LEaP software. The cysteine residues in EC5 were bound, mimicking physiological conditions (23). A pairwise generalized Born model was used utilizing previously described parameters (63), and the surface areas were calculated using the linear combinations of pairwise overlaps model (64) with the default radii set up by LEaP. The time step along these simulations was 2 fs. The potential cutoff was fixed at 12 Å with a switching distance of 10 Å. After minimizing (with a time step of 1 fs) the initial structure with restrained C0, the temperature was increased to 300 K, the restraints were then removed, and free molecular dynamics was performed over 10 ns. Different snapshots were selected every 2 ns as the starting points for the steered MD (SMD) simulations, which were carried out by imposing a restraint to both the N and C termini of the protein and increasing its length at a rate of 1 Å·ps⁻¹ with a constant restraint force of 5 kcal·mol⁻¹·Å⁻². Ca²⁺ ions were also restrained (10 kcal·mol⁻¹·Å⁻²) during the SMD to keep them included in the system. To avoid artificial forces, these restraints were 0 for distances shorter than the linear distance between the coordination residues.

All trajectories were loaded into VMD 1.8.6 (65), which was used to extract the N-C termini distance, backbone r.m.s.d., and dihedral angles. The dihedral angle is defined relative to three domains, and it is the angle between two planes, each one formed by two domains. We used Igor Pro 6 (WaveMetrics) to plot graphs and calculate the force exerted to the protein \( F = -k(N_{\text{measure}} - N_{\text{restrain}}) \).

RESULTS

**Nanomechanics of the Whole C-cadherin Ectodomain**—To characterize the mechanical properties of the C-cadherin ectodomain (EC1–5) at the single molecule level, the recombinant protein was adsorbed onto a gold-coated coverslip and placed into the recording chamber of an atomic force microscope. Random segments of the protein were picked up by the tip of the atomic force microscope cantilever and then stretched to analyze its nanomechanics at a pulling speed of 0.4 nm·ms⁻¹ (Fig. 1D). As for other multimodular proteins previously studied (4), we obtained force extension recordings with a characteristic sawtooth pattern of similarly spaced force peaks (Fig. 2, A and B). Each force peak typically marks the unfolding of a domain, the amplitude of which indicates the mechanical stability (or unfolding force, \( F \)), whereas the spacing between consecutive peaks reflects the gain in length that results after domain unfolding (i.e. increase in contour length, \( \Delta L_c \), after fitting the peaks to the WLC).

We analyzed this protein in the presence of Ca²⁺ (1 mM CaCl₂) and in the absence of this ion (1 mM EGTA). In the presence of Ca²⁺, the ectodomain behaves like a mechanoactive protein, displaying several force peaks that we attribute to the unfolding of the different EC domains \( F = 186 ± 4 \) pN, \( \Delta L_c = 34.2 ± 0.1 \) nm; \( n = 252 \); Fig. 2A). The structural differences among the EC domains in C-cadherin (23) are expected to contribute to some dispersion of \( \Delta L_c \). In the presence of EGTA, the force values fall drastically (83 ± 3 pN, \( n = 119 \); Fig. 2B), indicating that the Ca²⁺ coordination complexes, which are “extradomain” structural elements (Fig. 1B), can modulate the mechanical stability of the EC domains. It must be noted that the coordination complexes also contain residues that belong to the domains themselves as well as residues from the linker regions between domains. In addition to the force peaks produced by the unfolding of the EC domains, presumably resulting from the rupture of their mechanical clamps (4, 57), we were also able to detect several force peaks \( (143 ± 3 \) pN, \( n = 128 \)) with \( \Delta L_c \) values that are consistent with the disruption of the Ca²⁺ coordination complexes \( (2.7 ± 0.1 \) nm). We term this new mechanical element a Ca²⁺ rivet (Fig. 2, A, C, and D).

We could only observe up to four main force peaks because EC5 is natively disulfide-bonded in a way that seems to reinforce its A'-G mechanical clamp (23), and therefore, it should not be unfolded in these experiments (because no reducing agent was used). Interestingly, the Ca²⁺ rivet always precedes the unfolding of an EC domain (showing lower forces than the latter). The \( \Delta L_c \) values attributable to domain unfolding correspond very well with those expected for the folded domains, assuming a stretched length of 0.4 nm per amino acid (Ref. 66; the length of the force-hidden region after the mechanical clamp of the different ECs ranges from 81 to 88 amino acids, which would result in a nominal \( \Delta L_c \) ranging from 32 to 35 nm approximately). In the absence of Ca²⁺, no Ca²⁺ rivet is present, and the \( \Delta L_c \) values were somehow randomly distributed, suggesting that the protein becomes less stable and that its unfolding pathway is less defined. Indeed, no correlation was observed in \( F \) versus \( \Delta L_c \) scatter plots (data not shown). This is in accordance with previous thermal and chemical denaturation data from E-cadherin EC1–2 domains, which become very unstable in the absence of Ca²⁺. This loss of stability is probably due to electrostatic repulsion effects resulting from the large number of negatively charged residues located in the regions between the EC domains (67, 68). In the presence of Ca²⁺ ions, this electrostatic repulsion is counterbalanced by the coordination of these ions. These considerations suggest that the extra force peaks observed experimentally originate from the forced disruption of the Ca²⁺ coordination complexes (the Ca²⁺ rivet). Furthermore, although the Ca²⁺ rivet may represent the rupture of residues that belong to the interdomain region and the domain itself, it seems to be both structurally and mechanically independent of the mechanical clamp of the EC domains. Although the modulation of the nanomechanics of a protein by ligand and ion binding has already been demonstrated (69–71), to our knowledge, this is the first time that ion binding has been shown to produce an autonomous mechanical feature.

We interpret our results in terms of a decrease in the number of mechanical unfolding pathways of the cadherin ectodomain in the presence of Ca²⁺, a novel effect that we describe in terms of a “canalization” of the mechanical unfolding pathway. Thus, whereas in the presence of Ca²⁺ the mechanical unfolding pathway of the ectodomain is canalized into Ca²⁺ rivets and mechanical clamps of the EC domains (4, 57), in its absence, these pathways are numerous and variable, suggesting a “decanalization” effect on the energy landscape, which increases the number of mechanical pathways associated with a variety of barriers during the process of protein stretching.
FIGURE 1. Cadherin system and experimental setup. A, classical cadherins are connected to the actin cytoskeleton through several adaptor proteins (9–11). Recent findings indicate that epithelial protein lost in neoplasm (EPLIN) appears to be the missing link connecting the system to the actin cytoskeleton (11). B, EC₅ (which contains the N terminus of the protein) is the cadherin domain most distal to the membrane, whereas EC₁ is the most proximal. Ca²⁺ ions (represented by light blue spheres) are bound in groups of three per interdomain region. The residues that form the coordination complexes (drawn in bond representation in VMD terminology; Ref. 65) not only belong to the linker between the EC domains but also to the domains themselves. The bars indicate the different protein regions included in our constructs. The structure of C-cadherin (Protein Data Bank code 1l3w) is represented by VMD 1.8.6 (65). C, scheme of the proteins and polyproteins analyzed in this study. D, schematic representation of an SMFS experiment. A drop of the sample is deposited on top of a substrate (a gold-coated coverslip) that can be moved in any direction with subnanometer resolution by a piezoelectric device (57). Through the movement of the piezoelectric device in the z axis, the substrate is moved up toward the tip so it can pick up a molecule and, upon retraction, stretch it.
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FIGURE 2. Nanomechanics of the entire C-cadherin ectodomain. A and B, typical SMFS recording of the entire C-cadherin ectodomain in 1 mM Ca\textsuperscript{2+} (A) and 1 mM EGTA (B). In the presence of Ca\textsuperscript{2+}, we observe several extra peaks or humps (light blue) identified as a deviation from the domain unfolding peak (dark blue; Ref. 4). Only the extra peaks or humps that precede domain unfolding peaks are included in our analysis. In the absence of Ca\textsuperscript{2+}, the extra peaks were never seen, and the magnitude of the forces was always lower than that in the presence of Ca\textsuperscript{2+}. Furthermore, the distance between peaks is not as well defined as in the presence of Ca\textsuperscript{2+}. This color code will be followed in the rest of the figures. C, force histograms showing that the forces are lower in the absence of Ca\textsuperscript{2+} (83 ± 3 pN) than in its presence (143 ± 3 pN for the extra peaks and 186 ± 4 pN for domain unfolding). D, ΔL, histograms of the protein in the two conditions. In the presence of Ca\textsuperscript{2+}, the extra peaks show a mean value of 2.7 ± 0.1 nm, and the domain unfolding peaks show a mean value of 34.2 ± 0.1 nm. However, we cannot determine whether the extra peaks correspond to the rupture of individual coordination complexes or to the accumulation of several of them breaking simultaneously. In the absence of Ca\textsuperscript{2+}, there is a wide dispersion of the distribution. All histograms are normalized. Norm. freq., normalized frequency. E and F, typical SMFS recordings of [(I\textsuperscript{2})\textsubscript{2}-EC\textsubscript{1–5}-I\textsuperscript{27}. In the presence of Ca\textsuperscript{2+} (E), this recording shows the same features as the ectodomain alone, displaying relatively high forces and extra peaks. We were unable to obtain recordings with more than three EC peaks. In the absence of Ca\textsuperscript{2+} (F), a fully undetermined pattern of ΔL and lower unfolding forces were observed.

To introduce a single molecule marker in our experiments, which allows us to unequivocally select single molecule recordings and disregard nonspecific interactions, multimers, aggregates, or other species present in the sample, we constructed heteropolyproteins with an internal marker of known mechanical stability (I\textsuperscript{27} domain of human cardiac titin; see “Experimental Procedures” and Fig. 1C). This strategy also provided us with an internal control to test the possibility that the chelating agent (EGTA) might also affect the mechanical stability of the marker. We found the same behavior of the cadherin EC domains in this construct, [(I\textsuperscript{27})\textsubscript{3}-EC\textsubscript{1–5}-I\textsuperscript{27}, as in the recombinant full-length ectodomain, and the I\textsuperscript{27} marker domains showed their typical mechanical pattern in both experimental conditions (Ref. 4 and see Fig. 2, E and F). However, this protein yielded only a few full-length recordings, which made it unsuitable for statistical analysis.

Nanomechanics of EC\textsubscript{1–2}: Ca\textsuperscript{2+} Dependence, Mg\textsuperscript{2+} Replacement, and Site-directed Mutagenesis—To examine in detail these changes in the mechanical stability due to Ca\textsuperscript{2+} binding, we constructed an additional recombinant polypeptide containing a single set of Ca\textsuperscript{2+} binding sites located in the interdomain region (Fig. 1C). This protein contained EC domains 1 and 2 and one set of three Ca\textsuperscript{2+} binding sites: (I\textsuperscript{27})\textsubscript{1}-EC\textsubscript{1–2}- (I\textsuperscript{27}). We found mechanical properties in the ECs similar to those described for the entire ectodomain (Fig. 3). In 1 mM Ca\textsuperscript{2+}, we observed force peaks that presumably originated from the rupture of the Ca\textsuperscript{2+} coordination complexes (the Ca\textsuperscript{2+} rivet; ΔL\textsubscript{c} = 3.0 ± 0.2 nm, F = 90 ± 5 pN, n = 26), which preceded, sometimes as a “hump,” one of the two main peaks that originated from the unfolding of the EC domains (ΔL\textsubscript{c} = 34.6 ± 0.2 nm, F = 102 ± 5 pN, n = 50; Fig. 3, A–C, top). Using this polypeptide, we performed experiments in 0.1 mM Ca\textsuperscript{2+}, which is a Ca\textsuperscript{2+} concentration that, although still permitting residual cellular interactions (17), is not high enough to promote the conformational changes in classical cadherins that occur with 1 mM Ca\textsuperscript{2+} (Refs. 42 and 43 and see Fig. 3, A–C, middle). In this condition, the protein exhibited a decanalized unfolding pathway (without preferential ΔL\textsubscript{c} values) with lower unfolding force values than those shown in 1 mM Ca\textsuperscript{2+} (F = 81 ± 7 pN, n = 69) and no Ca\textsuperscript{2+} rivet. A very similar behavior was observed in the absence of Ca\textsuperscript{2+} (Fig. 3, A–C, bottom) where a decanalized unfolding pathway and low F values (84 ± 5 pN, n = 66) were found. For this construct, like the ectodomain in the absence of Ca\textsuperscript{2+}, no correlation was shown in F versus ΔL\textsubscript{c} scatter plots for both the absence or presence of low Ca\textsuperscript{2+} concentration (data not shown). This confirms that, at low Ca\textsuperscript{2+} concentrations, the EC domains present an undefined unfolding pattern and become mechanically less stable.

We performed further studies with this polypeptide in the presence of Mg\textsuperscript{2+}, the divalent cation closest in size to Ca\textsuperscript{2+}.  

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In the presence of 1 mM Mg$^{2+}$ (Fig. 4), two different classes of recordings were found: one with canalized domain unfolding (Fig. 4A, right; $\Delta L_c = 34.9 \pm 0.1$ nm, $F = 72 \pm 4$ pN, $n = 34$) and the other with decanalized domain unfolding (Fig. 4A, left; $F = 56 \pm 5$ pN, $n = 48$). However, we did not observe a putative “Mg$^{2+}$ rivet.” This is in agreement with previous results showing that at this concentration, Mg$^{2+}$ binding is insufficient to promote the conformational changes seen in E-cadherin EC$_{1–2}$ domains at the equivalent Ca$^{2+}$ concentration (42), and it seems just to counterbalance the electrostatic repulsion effects, which can destabilize the EC domains. Considering that Mg$^{2+}$ coordination is less thermodynamically stable than that of Ca$^{2+}$ (42, 72), we interpret these results assuming that some of the ion binding sites occasionally bind Mg$^{2+}$, whereas others do not, remaining mechanically less stable. Indeed, the absence of Mg$^{2+}$ rivets (Fig. 4C, middle) and the lower $F$ values (compared with Ca$^{2+}$; Fig. 3B) observed in the canalized class (Fig. 4B) suggest that Mg$^{2+}$ binding is less stable than that of Ca$^{2+}$.

To test our hypothesis that this new mechanical component (the Ca$^{2+}$ rivet) is due to the rupture of the Ca$^{2+}$ coordination complexes, we modified such complexes by site-directed mutagenesis to prevent ion coordination. Based on the crystallographic structure of the ectodomain (23), we constructed a TM in which three (of eight) negatively charged residues were replaced by alanine residues (D67A/D100A/D134A), which should prevent Ca$^{2+}$ binding in between the EC$_{1–2}$ domains (Fig. 5A). Accordingly, we expected that the Ca$^{2+}$ rivet should be affected when this mutant protein is stretched in the presence of Ca$^{2+}$. Furthermore, as these mutations reduced the number of negatively charged residues in the linker region between the EC$_{1–2}$ domains from eight to five, there would be a reduction in the possible electrostatic repulsion effect experienced in this region in the absence of Ca$^{2+}$. For nanomechanical analysis, we flanked this mutant with repeats of the I27 marker as in the wild type: (I27)$_3$-EC$_{1–2}$TM-(I27)$_2$ (Fig. 1C). Although we did not directly demonstrate that this protein could coordinate Ca$^{2+}$, a previous report on a single mutant that included one of the selected positions (D134A) showed it to be more sensitive to trypsin degradation and that it was unable to mediate adhesion, which was taken as indirect evidence that Ca$^{2+}$ binding was disrupted in this mutant (73). No Ca$^{2+}$ rivet was observed for this mutant protein in the presence of 1 mM Ca$^{2+}$ ($\Delta L_c = 35.6 \pm 0.5$ nm, $n = 44$; Fig. 5, B–D, bottom). Interestingly, the magnitude of the force peaks that could be attributed to EC domain unfolding was slightly higher than that of the wild type protein ($118 \pm 11$ pN). Furthermore, $\Delta L_c$ for this mutant was slightly higher than that of the wild type. These differences may indicate that the perturbation of the conformation of the linker region in this mutant may also have slightly changed the mechanical clamp of the EC domains (4, 57). In the absence of Ca$^{2+}$, the nanomechanics of the domains was similar to that observed in its presence, showing comparable values ($F = 120 \pm 8$ pN, $\Delta L_c = 35.6 \pm 0.4$ nm, $n = 65$; Fig. 5, B–D, top). Thus, the nanomechanics of this
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FIGURE 4. Mg2+ effects on the nanomechanics of cadherin EC domains. A, on the left, we show a recording of a molecule with low mechanical stability peaks and decanalized domain unfolding (light green; no fitting to a homogeneous family of WLC curves is possible here; Ref. 4), whereas on the right, we show a recording of a molecule with higher mechanical stability peaks and canalized domain unfolding (dark green; we show the fitting to the WLC). B, the F histograms show lower unfolding force values than those seen in the presence of Ca2+ (Fig. 3B). In the bottom distribution, all the force data are plotted (n = 82). The distribution in the middle shows the F for those molecules with canalized domain unfolding, whereas that on the top shows the F for those with decanalized domain unfolding (A, left; 56 ± 5 pN). C, the corresponding ΔLc histograms show that this protein can eventually be stabilized by Mg2+ coordination. The ΔLc distribution of all the data from the EC domains under these conditions is shown at the bottom, demonstrating the two possible conformations (canalized and decanalized). The distribution in the middle shows the ΔLc values for the mechanostable EC domains (A, right; 34.9 ± 0.1 nm). No Mg2+ rivets were seen here, meaning that the rupture of the Mg2+ coordination complexes was not stable enough to be resolved in our experiments. In the distribution on the top, we show the ΔLc values resulting from the unfolding of the EC domains with decanalized unfolding (A, left). Norm. freq., normalized frequency.

A mutant was not sensitive to the presence of Ca2+, and it did not show electrostatic repulsion effects in the linker region.

Nanomechanics of Single EC Domain—To examine the mechanical properties of a single EC domain in isolation without the linker region involved in Ca2+ binding, we constructed a polyprotein in which several I27 markers flanked the EC2 domain: (I27)3-EC2-(I27)2 (Fig. 1C). As expected, this domain behaved similarly in the presence or absence of Ca2+ (Fig. 6), and no Ca2+ rivet was found. The unfolding forces in the presence of Ca2+ were similar to those in its absence (88 ± 4 pN in Ca2+ and 82 ± 5 pN in EGTA, nCa2+=24, nEGTA=25). The ΔLc values corresponded well with the size of the domain, considering that each stretched residue contributes 0.4 nm to the length (66): 35.2 ± 0.2 nm in Ca2+ and 34.9 ± 0.2 nm in EGTA. The expected size of the force-hidden region of the folded EC2 domain is 87 amino acids, which represents 34.8 nm (87 × 0.4 nm), which is in close agreement with the results. Finally, these ΔLc values were similar to those measured for the ECs in the other constructs studied. These results provide additional support to the proposal that the extra peaks observed in the presence of Ca2+ originate from an extradomain structure, i.e. the Ca2+ rivet.

SMD Simulations of the Whole Ectodomain—To access the atomic details of the structural changes taking place in the process, we performed MD simulations of the stretching of the entire ectodomain. Previous MD simulations focused on the rupture of the cadherin-cadherin interaction (74) and on the conformational changes that the ectodomain experiences upon Ca2+ binding (47, 75). Although several regions of the ectodomain have also been stretched from their termini by MD (35, 47, 76–78), the entire ectodomain has never been stretched before. Also, the extent of the stretching was somewhat short because of computational limitations. We performed our analysis using the generalized Born surface area approximation as described previously (57). This approach allowed us to run 10 ns of free MD and to perform five stretching simulations of the entire ectodomain at larger extensions, which allowed us to stretch all the domains. Similar to previous results (47), we observed much larger fluctuations in the entire ectodomain in the absence of Ca2+ with the fold of the individual EC domains remaining intact (Fig. 7, A and B). EC2 still showed more backbone deviation in the absence of Ca2+ than the other domains. These results confirm the rigidification effect experienced by the cadherin ectodomain upon Ca2+ binding (40–44, 47, 75). We also monitored the dihedral angles between domains during all free MD simulations (see “Experimental Procedures” and Fig. 7, C and D).

Interestingly, Ca2+ binding triggered very different effects in different regions of the ectodomain. In the presence of Ca2+, the EC123 angle was around 90° (varying from 27° to 137°), whereas the EC345 angle was around 180° (with a minimum of 126°). In the absence of Ca2+, the angle became about 180° for EC123 (fluctuating from 140° to 180°), and it fluctuated highly for EC343 and EC345 (varying from 27° to 172° and from 59° to 175°, respectively). These results suggest a switching mechanism in which the presence of Ca2+ rotates the distal region of the ectodomain and aligns the proximal region, whereas in its absence, the distal region remains aligned, and the proximal region becomes more motile.

Before addressing the atomic details of the stretching process, an obvious prerequisite is that the simulations should correlate with our experimental observations. Indeed, the simulations of the stretching of the ectodomain closely reproduced the experimental results qualitatively both in the presence and absence of Ca2+ (Fig. 8, A–D). In the presence of Ca2+, we observed extra peaks (i.e. putative Ca2+ rivets) that could be attributed to the rupture of the different Ca2+-binding complexes. The criterion we used to assign Ca2+-related events was to monitor the release of Ca2+ ions and correlate it to the location of events in the curve (Fig. 8A). Although we could not identify specific force peaks a priori as Ca2+-rivets (by just observing the curve) as they were often masked in the noise, we could assign them by using the above criterion. In Fig. 8A, we only marked as extra peaks those that could be assigned unambiguously. Only four unfolding peaks were observed because in the simulations we maintained the disulfide bonds that are natively formed in EC3 and covalently lock its putative A´-G mechanical clamp (23). We observed that most of the time these Ca2+-rivets correlated with immediate domain unfolding, similar to the SMFS experiments. In contrast, we detected several EC unfolding intermediates in this condition (Fig. 8, A and E) that were not observed in the experi-
ments. This difference may result from the different pulling speeds used in each method (simulations were done at 1 Å/ps⁻¹, 8 orders of magnitude faster than the AFM experiments that are performed at 0.4 nm/ms⁻¹). In the absence of Ca²⁺, as in the experiments, the unfolding forces were lower, whereas the ΔLc values did not follow any preferential unfolding pathway, remaining decanalized (Fig. 8B). Also, the unfolding hierarchy of the domains seems to be less well defined (Fig. 8, E and F).

Finally, we should note that our pulling simulations were in close agreement with previous simulations of specific cadherin domains (35, 47, 77, 78), which also predicted force peaks originating from the rupture of the Ca²⁺ coordination complexes (Ca²⁺ rivets) and higher unfolding forces for the EC domains than those seen in the absence of Ca²⁺. However, none of these studies reported the mechanical canalization we have shown in the presence of Ca²⁺, probably due to the fact that those simulations only examined a single domain rather than the complete ectodomain.

**DISCUSSION**

Taken together, our findings led us to conclude that the cadherin ectodomain is a mechanostable structure highly dependent on the Ca²⁺ concentration. In the absence of Ca²⁺, its mechanical unfolding pathway appears to be decanalized, whereas in the presence of Ca²⁺, we observed a canalized unfolding pathway showing domain unfolding preceded by specific Ca²⁺ rupture events (Fig. 9A). Although the influence of Ca²⁺ on the structure, conformation, and function of cadherins is well documented, the role of Ca²⁺ as a mechanical
stabilizer of these proteins has not been experimentally demonstrated before. Here we present the first evidence that the nanomechanics of these proteins is strongly dependent on Ca\(^{2+}\) binding. Therefore, the ectodomain behaves as a “Ca\(^{2+}\)-sensitive” mechanostable structure. Furthermore, we have discovered a novel element of mechanical resistance in proteins comprising an ion coordination complex, which behaves as an “interdomain” clamp: the Ca\(^{2+}\) rivet. Hence, we demonstrate that Ca\(^{2+}\) ions have two different effects: they provide EC domains themselves with defined mechanical resistance such that they show true mechanical clamps (i.e. modularly autonomous resistance elements), and they generate extradomain mechanical resistance elements that seem to protect the mechanical clamps.

Based on our findings (i.e. the ectodomain as a Ca\(^{2+}\)-dependent mechanostable structure) and considering that residual adhesion in low Ca\(^{2+}\) concentration has already been reported (17, 29), we postulate that the ectodomain behaves as a “Ca\(^{2+}\) switch” in which Ca\(^{2+}\) binding determines its nanomechanical properties, including its mechanical integrity. The biological relevance of our findings can be fully appreciated considering that the local concentration of extracellular Ca\(^{2+}\) in specific microenvironments varies widely during development and adulthood in many physiological processes (48, 79–82). For instance, after electrical depolarization of the synapse, it is known that the Ca\(^{2+}\) levels in the synaptic cleft can drop from basal levels (around 1–2 mM) to 0.1 mM (48, 83). Interestingly, considering that these changes are not expected to completely abolish cadherin adhesion (17, 84) and that cadherin has been proposed as a physiological reader (i.e sensor) of external Ca\(^{2+}\) fluctuations (39), the mechanical response of the ectodomain to Ca\(^{2+}\) changes clearly appears to be critical (Fig. 9, B and C).

We have shown here that in the absence of Ca\(^{2+}\) the ectodomain displays higher conformational plasticity than in its presence, which not only dramatically affects its conformation but also its mechanical behavior. This would in turn determine the way it transmits mechanical stimuli (Fig. 9, B and C). Considering that the Ca\(^{2+}\) binding sites in the ectodomain are not equivalent and that the range of their affinity constants is rather large (40–43), the possibility of the existence of differential mechanical properties along the ectodomain remains to

**FIGURE 7. Free MD simulations of C-cadherin ectodomain.** A and B, r.m.s.d. of the C-cadherin ectodomain in the presence (A) or absence (B) of Ca\(^{2+}\) during 10 ns of free MD. At the bottom of the graphs, we show the r.m.s.d. of the individual domains, the most distal to the membrane represented in lighter colors (this color code will be maintained in the rest of the panels of this figure and in Fig. 8). The structure of the individual domains is preserved in both conditions (with slightly larger deviations in the absence of Ca\(^{2+}\)) with an r.m.s.d. in the range of 1–3.5 Å with Ca\(^{2+}\) and 2–5 Å without Ca\(^{2+}\). The tertiary structure of the ectodomain in the absence of Ca\(^{2+}\) shows much larger deviations in the r.m.s.d. (values up to 18 Å) than in its presence (r.m.s.d. close to 12 Å), indicating a greater flexibility of the structure in these conditions. C, dihedral angles during the free MD in the presence of Ca\(^{2+}\). The proximal region (close to the membrane) of the ectodomain remains aligned (≈180°) during the 10 ns of free MD, whereas the distal region rotates 90°. D, dihedral angles in the absence of Ca\(^{2+}\). In these conditions, the evolution of the free MD is completely different with the more distal region (EC\(_{123}\)) aligned, whereas the more proximal dihedral angles (EC\(_{234}\) and EC\(_{345}\)) display a stochastic behavior.
be explored. This heterogeneity would add an additional layer of complexity to the proposed role in force transmission. It is known that when extracellular levels of Ca\textsuperscript{2+} are depleted, mature cadherin homophilic interactions are disrupted (5, 22). However, recent evidence demonstrates that a local and temporal depletion in extracellular Ca\textsuperscript{2+} levels does not necessarily imply a complete disruption of cellular adhesion. This could later facilitate plastic rearrangements in the cell junctions (17, 18, 32, 33, 39, 48, 84). Thus, certain Ca\textsuperscript{2+} concentrations may still allow the cadherin-cadherin interaction to occur while affecting some mechanical properties of the ectodomain. Such an effect would alter the manner in which it transmits mechanical signals (Fig. 9, B and C) and may in turn affect the strength of the adhesion contact. Indeed, the unfolding of protein domains may not only regulate the range but also the life span of a single adhesion bond, acting as a molecular “shock absorber” (85, 86). It is also of interest to test whether the Ca\textsuperscript{2+} rivet, the new element of resistance described here, appears during the stretching of a single cadherin-cadherin complex. This type of experiment is hard to implement but would allow us to build a more complete mechanical picture of the system so we can begin to understand how these mechanical signals are transduced through the cadherin-catenin system, promoting different cell responses.

To establish the biological relevance of the ectodomain nanomechanics in cell-cell adhesion and mechanotransduction, it is critical to compare the mechanical properties of the
ectodomain with the mechanical strength of the cadherin-cadherin interaction. Although the latter has not yet been unequivocally measured (i.e. using single bond markers), a previous report estimated this strength in vitro to be ~40 pN (at a comparable pulling speed; Ref. 29). Furthermore, several experiments on live cells showed comparable rupture values for single cadherin interactions, although these measurements were strongly dependent on the catenin-actin system, which could alter the force of the interaction through unknown conformational effects on the cadherin system triggered from inside the cell (12–15, 17, 87, 88). The magnitude of these rupture forces is clearly below that of both Ca²⁺ rivets and EC mechanical clamps in the presence of Ca²⁺. This may imply that the cadherin ectodomain is not the “force-sensing” element of the cadherin-catenin system (Fig. 9, B and C).

In summary, the ectodomain can show two extreme mechanical behaviors: (i) a fairly rigid and stable structure, the integrity of which would assure the adequate transmission of mechanical stimuli (at high Ca²⁺ concentrations) and (ii) a compliant structure that may affect the mechanical transmission (at low Ca²⁺ concentrations). Thus, the force sensor of the system should be another component with a mechanical stability below the magnitude of adhesion forces mentioned above (working on the range of up to ~40 pN; Fig. 9, B and C). Both β- (48)⁶ or α-catenin (38) seem to be better candidates for being the force-sensing elements of this system in a manner analogous to that of the talin rod in the integrin system; Refs. 38, 48, 89, and 90⁶ whose mechanical properties are still to be determined (represented by question marks).

Our hypothesis of the Ca²⁺ switch would only be valid if residual adhesion is present in the system at low Ca²⁺ concentrations. Otherwise, adhesion would be impaired, and the mechanical properties of the ectodomain would be physiologically irrelevant (i.e. a mere epiphenomenon). If this were the case, the mechanical properties of the ectodomain at high Ca²⁺ concentrations would represent the only properties physiolog-

FIGURE 9. Nanomechanics of the ectodomain and the Ca²⁺ switch hypothesis. A, diagram showing the mechanical folding energy (E) landscape of an EC domain in low and high Ca²⁺ concentration. At high Ca²⁺ concentrations, the mechanical unfolding of an EC domain is trapped into two energy minima: that of the Ca²⁺ rivet and that of the mechanical clamp of the domain. Landscapes are represented schematically in a one-dimensional cross-section. The color code is the same used in the rest of the figures. B, a high Ca²⁺ concentration rigidifies and canalizes the unfolding pathway of the ectodomain and adds a novel mechanical component (the Ca²⁺ rivet). This structure may therefore act as a rigid force transmitter (i.e. without significant force damping). C, a low Ca²⁺ concentration decanalizes and destabilizes the ectodomain, also removing the Ca²⁺ rivet. This may allow for structural rearrangements, which should in turn affect the force transduction mechanism. Our results suggest that the cadherin ectodomain may be the force transmitter of the system, whereas the actual force sensor may be an intracellular component of the cadherin system (analogous to the talin rod in the integrin system; Refs. 38, 48, 89, and 90)⁶ whose mechanical properties are still to be determined (represented by question marks).
ally relevant as they would assure the integrity of the structure and the adequate transmission of mechanical signals.

This hypothesis and that of the shock absorber effect of the ectodomain (85, 86) could be tested in the future by in vitro SMFS experiments using an unequivocal design to measure the interaction in whole ectodomains (i.e. by measuring the relationship between the mechanical stability and lifetime of this interaction with the compliance of the ectodomain). Furthermore, SMFS experiments with cells (87) in which the Ca²⁺ concentration is varied could also provide valuable information in this regard. However, in both cases, a single bond marker should first be developed for unambiguous analysis. Nevertheless, our results represent an important first step required for the interpretation of future experiments of this type.

Future experiments should also explore intermediate concentrations of Ca²⁺ to determine whether the “all-or-none” behavior we report here (1 versus 0.1 mM or sub-μM Ca²⁺) represents the whole picture or whether a gradual response is rather present.

Furthermore, it is interesting to note that a number of mutations in the cadherin ectodomain do not affect the cadherin-cadherin interaction have been described in relation to pathologies, including several gastric cancers (7) as well as familial deafness and the Usher syndrome (20, 35, 91–93). However, in both cases, a single bond marker should first be developed for unambiguous analysis. Nevertheless, our results represent an important first step required for the interpretation of future experiments of this type.

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