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Intracellular domain contacts contribute to Ecadherin constitutive dimerization in the plasma membrane

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Abstract

Epithelial cadherin (Ecadherin) is responsible for the intercellular cohesion of epithelial tissues. It forms lateral clusters within adherens cell-cell junctions, but its association state outside these clusters is unknown. Here we use a quantitative FRET approach to show that Ecadherin forms constitutive dimers, and that these dimers exist independently of the actin cytoskeleton or cytoplasmic proteins. The dimers are stabilized by inter-molecular contacts that occur along the entire length of Ecadherin, with the intracellular domains having a surprisingly strong favorable contribution. We further show that Ecadherin mutations and calcium depletion induce structural alterations that propagate from the N-terminus all the way to the C-terminus, without destabilizing the dimeric state. These findings provide context for the interpretation of Ecadherin adhesion experiments. They also suggest that early events of adherens junction assembly involve interactions between pre-formed Ecadherin dimers.

Graphical Abstract

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Author contributions

D.S. and K.H. designed the research and wrote the paper. D.S. and S.S. cloned the Ecadherin constructs. D.S. and F.A. performed the FRET experiments in cells. S.S. performed the FRET experiments in vesicles. D.S. and F.A. performed the Western blotting experiments. D.S. analyzed the data.

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INTRODUCTION

Cadherins are a family of cell surface adhesion receptors which play a central role in the maintenance of solid tissues by supporting cell-cell contacts^{1–5}. They are known as the “molecular Velcro” that is responsible for tissue integrity. The specificity of cadherins is a critical factor in the formation of different organs during development. Furthermore, cadherins are involved in tumorigenesis⁶, and contribute to cancer progression and metastasis^{7,8}.

Epithelial cadherin (Ecadherin) is responsible for the intercellular cohesion of epithelial tissues. Ecadherin, like all classical cadherins, contains an adhesive extracellular domain which mediates cell-cell contacts, a single TM domain, and a cytoplasmic domain which contains binding sites for catenins and mediates attachment to the cytoskeleton^{1,3,9}. The Ecadherin extracellular domain is composed of five tandemly arranged beta-barrel repeats. When a cell–cell contact is formed, Ecadherins expressed on neighboring epithelial cells interact through their extracellular domains to form an adhesive bond. The principle adhesive interactions are the ones between the N-terminal extracellular domains 1 (EC1), and the process is known to be calcium dependent^{9–11}. The cytoplasmic domains, at the same time, interact with the cytoskeleton by binding catenins, thus providing a link between adhesion events and intracellular dynamics^{12,13}. Ultimately, via its link to the cytoskeleton, Ecadherin activates signals that regulate cell shape, polarity, proliferation, and differentiation^{3,14}.

In addition to adhesive contacts, cadherins on the cell surface can also engage in lateral (often referred to as “cis”) interactions. In fact, it has been suggested that such lateral contacts are necessary for productive adhesion^{15–18}. It is known that both adhesive and lateral contacts are critical for the formation of the adherens junctions, in which an Ecadherin cluster from one cell engages in adhesive interactions with an Ecadherin cluster on an apposing cell, establishing a robust cell-cell contact^{18,19}. The extracellular domains play a critical role in this process, along with the actin cytoskeleton^{3,5,18,20–22}. The TM domain sequence and the juxtamembrane sequence of the intracellular domain has also been implicated in the clustering process, along with the soluble protein p120^{23–25}.

The association state of Ecadherin far from adherens junctions is debated. Some papers point to the presence of dimers^{15,26–29}, but the propensity for lateral dimerization has never been measured due to the lack of quantitative experimental methods that yield association constants in the plasma membrane. The relative contributions of the different Ecadherin domain to Ecadherin dimerization thermodynamics are also unknown. Yet, most of the basic knowledge about the cadherin adhesive bonds has been deduced from AFM and micropipette manipulation experiments with cadherins far from adherens junctions^{30–34}. It is unclear if these experiments probe cadherin dimers or monomers, or a mixture of different cadherin association states.

Recently, a Forster Resonance Energy Transfer (FRET)-based quantitative technique was established that gives information about the oligomerization state of membrane proteins, and about the strength of their lateral interactions in the membrane³⁵. Here we apply this

technique to study the lateral association of human Ecadherin on the plasma membrane, far from cell-cell junctions.

RESULTS

Full length Ecadherin forms constitutive dimers in cells, far from cell-cell junctions

We sought to characterize the lateral association of full length Ecadherin in the plasma membrane of HEK293T cells using FRET. This cell line was chosen as it does not exhibit significant expression of endogenous Ecadherin, as assessed by Western blotting (Supplementary Figure 1). For the FRET experiments, full length Ecadherin was labeled with the monomeric fluorescent proteins mTurquoise (mTurq) or YFP at the C-terminus, via a (GGG)⁵ flexible linker (Supplementary Figure 2). These two fluorescent proteins constitute a FRET pair ³⁶.

We used the Fully Quantified Spectral Imaging (FSI) – FRET technique ³⁵ which can yield: (i) the number of proteins in an oligomer (i.e. the oligomer order) in the plasma membrane, (ii) the strength of the interactions (i.e., the association constants) and (iii) the value of the so-called Intrinsic FRET or pair-wise FRET efficiency ^{37–39}, which depends on the distance between the fluorophores in the oligomer, and provides insights into the architecture of the oligomer. The basics of the FSI-FRET data acquisition and analysis are shown in Supplementary Figure 3. The FSI method requires the acquisition of complete FRET and acceptor spectra, and employs assumption-free, fully resolved system of equations to calculate FRET efficiencies in the plasma membrane with high precision ³⁵. It can be used to acquire binding (dimerization or oligometization) curves, provided that the two-dimensional concentrations of the proteins in the membrane are known or can be measured. The latter measurement is a challenge in cells, however, since cells possess 2 to 3 times the membrane surface needed to sustain their shape, and their plasma membrane is known to be “wrinkled” (or “ruffled”) ^{40–42}. The complex membrane topology prevents the conversion of effective 3D receptor concentrations, determined by comparing the fluorescence intensities with standard solutions of known concentrations, into 2D receptor concentrations within the plasma membrane ³⁵. However, cells “un-wrinkle” their membranes in a reversible manner when subjected to controlled osmotic stress ⁴³. This controlled osmotic stress leads to disassembly of the caveolae, which are 60–80 nm cup-shaped invaginations of the plasma membrane ⁴³. The application of the reversible osmotic stress does not cause irreversible cell damage, and thus quantitative biophysical experiments can be performed in live cells ^{44,45}.

We thus started our investigations into Ecadherin lateral interactions by asking whether Ecadherin self-associates in the plasma membranes of HEK293T cells under reversible osmotic stress. The cells were co-transfected with plasmids encoding Ecadherin-mTurq and Ecadherin-YFP in three different ratios (3: 1, 1:1 and 1:3). The cells were starved for 12 hours and then subjected to reversible osmotic stress, following the protocol described in ⁴³. They were imaged in a spectrally resolved two-photon microscope equipped with the OptiMiS detection system ^{38,46}. In all cases, single membranes that were far from cell-cell junctions were chosen for the analysis (Supplementary Figure 3B). Figure 1A shows the FRET efficiency as a function of the acceptor concentration, while Figure 1B shows the donor concentration as a function of the acceptor concentration, for the three DNA

transfection ratios used. Each data point in Figure 1A and B represents a single membrane region of a swollen cell (Supplementary Figure 3B).

The FRET efficiency in such experiments is known to occur due to both (i) specific interactions between the membrane proteins and (ii) stochastic or “proximity FRET” that arises due to the random approach of donors and acceptors within 10 nm in the two dimensional membrane, even in the absence of specific interactions^{47,48}. The proximity FRET depends mainly on the concentration of the acceptor, but also on the oligomer order and on the strength of the interaction, i.e., on the critical unknowns in the experiment⁴⁸. Following the FSI-FRET protocol, different oligomeric models (monomer, dimer, trimer, tetramer, pentamer, and hexamer) were fitted to the data in Figure 1A and 1B while accounting for the relevant proximity contribution associated with these models. This fitting procedure was recently tested and verified⁴⁹. The mean square error (MSE), calculated for all the models, is shown in Figure 1C. The MSE is the lowest in the dimer case (Figure 1C), demonstrating that full-length Ecadherin forms dimers in swollen cells. The black line in Figure 1A represents the optimized proximity FRET for these dimers.

For dimers, the ratio of (i) the FRET efficiency after proximity correction and (ii) the acceptor fraction gives the dimeric fraction of the receptors in a cell, f_d , multiplied by the so-called Intrinsic FRET or ($\tilde{E}$) (Equation 6). Intrinsic FRET depends only on the structure of the dimer, i.e., on the relative positioning of the two fluorophores in the dimer, and is a constant. The values of $f_d \times \tilde{E}$ are plotted versus the total Ecadherin concentration in Figure 1D. We see that these data do not depend on the concentration of Ecadherin. This is indicative of constitutive dimerization, rather than the existence of measurable populations of monomers and dimers. The value of $f_d \times \tilde{E}$ is constant at 0.16 over the entire range of concentrations in the experiment. The value of $\tilde{E}$ is therefore $\sim 0.16 \pm 0.01$ (Table 1), and the dimeric fraction is $\sim 100\%$ for all concentrations. While the exact dimerization free energy cannot be determined in this case, it can be estimated based on having 100% dimers at the lowest Ecadherin concentration that is accessible in our experiments. We thus estimate that the free energy is more favorable than -8 kcal/mole, the measurable limit in the experiments. Over-all, this analysis shows that Ecadherin is constitutively dimeric in cells under reversible osmotic stress, down to the lowest concentrations that are accessible in our experiments.

To confirm the formation of Ecadherin dimers and not higher order oligomers in cells under reversible osmotic stress, we examined the dependence of the FRET efficiency on the acceptor fraction. FRET efficiencies were compared for three different DNA transfection ratios of Ecadherin-mTurq to Ecadherin-YFP: 3:1, 1:1, and 1:3. Histograms of FRET efficiencies and acceptor fractions, for the three different transfection ratios, are shown in Supplementary Figure 4, and are fitted to Gaussian functions to obtain the averages and the standard errors. The average FRET efficiencies obtained from the histograms are plotted versus the average acceptor ratios in Figure 1E for the three DNA transfection ratios (red symbols). The dependence of the FRET efficiency on the acceptor fraction is known to be linear for a constitutive dimer and nonlinear for higher order oligomers^{50–53}. The data in Figure 1E are well described by a linear function ($R^2=0.97$), and thus support the conclusion that Ecadherin forms dimers in HEK293T cells under reversible osmotic stress.

Next we asked whether the application of osmotic stress affects the FRET efficiencies. Since FRET efficiencies can be measured precisely even in the absence of osmotic stress³⁵ (unlike the concentrations), we performed FRET experiments in intact cells that are co-transfected with genes encoding Ecadherin-mTurq and Ecadherin-YFP, at three different ratios (3:1, 1:1 and 1:3). Figure 2A shows the FRET efficiency as a function of the “apparent” acceptor concentration, which depends on both acceptor concentration and the local “ruffling” of the membrane.

Figure 2B compares the histograms of measured FRET efficiencies in intact and swollen cells, for the three transfection ratios. The distributions in Figure 2B are identical, indicating that the FRET efficiencies are the same with and without cell swelling. This comparison demonstrates that the interactions between Ecadherin molecules are not affected by the application of reversible osmotic stress. In Figure 1E, we plot the average FRET efficiencies measured in unperturbed cells as a function of the averaged acceptor fractions, for the three transfection ratios. The linear dependence in intact cells further confirms that E-cadherin forms dimers in cells.

To be able to interpret the FRET data comparison, we asked if the actin cortex is preserved in HEK293T cells under osmotic stress. Previously, the flattening of the plasma membrane due to the dissolution of the caveolae in response to osmotic stress has been shown to occur independently of cortical actin⁴³. To confirm or refute these prior findings, we co-transfected HEK293T cells with genes encoding both Ecadherin-mCherry and Lifeact-Citrine, and imaged them in a confocal microscope. Lifeact is a 17-amino-acid peptide which binds to filamentous actin. The confocal images of cells and cells under reversible osmotic stress are shown in Figure 3A and 3B. The left panels show the distribution of Ecadherin-mCherry, which can be found on the plasma membrane. The middle panels show the distribution of Lifeact-citrine, which can be found just below the membrane in both cells and swollen cells. These images confirm that the cortical actin is preserved upon the application of osmotic stress, as reported previously⁴³.

Yet another model system that can be used to characterize membrane protein lateral interactions in quantitative terms using FRET is plasma membrane derived vesicles^{54,55}. These vesicles bud off CHO cells upon their incubation with an osmotic stress buffer. They have membrane composition that is similar to the plasma membrane. However, they lack cell cytoplasm, attested by our findings that both soluble and membrane-bound cytoplasmic proteins of molecular weight of at least 200 kDa are not retained inside the vesicles⁵⁶. Here we used Lifeact-Citrine to assess the effect of the osmotic stress vesiculation treatment on the actin cortex of CHO cells. Confocal images shown in Figure 3C reveal that the cortical actin is destroyed in the cells upon incubation with the vesiculation buffer. As a result, the produced vesicles lack the actin cortex and offer an opportunity to examine if Ecadherin dimerization requires cortical actin.

We thus performed quantitative imaging FRET experiments in plasma membrane vesicles derived from CHO cells (see^{37,55}, and Supplementary Figure 5). The raw FRET data for full length Ecadherin, for the three different donor to acceptor transfection ratios are shown in Supplementary Figure 6 with the navy symbols. In Figure 1E, we show the average FRET

efficiencies for the three transfection ratios, as a function of the average acceptor fractions. Data in vesicles and cells are very similar. The dependence in vesicles is linear, indicative of dimer formation. The only difference that we observe is in the value of the structural parameter Intrinsic FRET, which depends on the separation and the relative orientation of the fluorescent proteins attached to the C-terminus of Ecadherin. It can be argued that the binding of soluble proteins, such as α and β catenins, close to the C-terminus of Ecadherin, may affect Intrinsic FRET. These soluble proteins are present in HEK293T cells, but are not present in the “ghost” plasma membrane derived vesicles that lack cytoplasmic content⁵⁶. Thus it is possible that the measured difference in Intrinsic FRET is due to the presence and binding of soluble proteins to Ecadherin in cells but not in vesicles.

Contribution of Ecadherin domains to Ecadherin lateral dimerization

Next, we sought to determine the contributions of the individual Ecadherin domains to Ecadherin dimerization. Towards this goal, we generated three truncated versions of this receptor. In one truncated version (EC+TM), the intracellular domains were removed and the fluorescent proteins were attached to the cytoplasmic end of the TM domains via flexible, 15 residue (GGS)⁵ linkers (see Figure 4A). In the second truncated version (TM+IC), the EC domains were removed and the fluorescent proteins were attached at the C-terminus via the same flexible (GGS)⁵ linkers (Figure 4A). In the third truncated version (TM), both the IC and EC domains were removed, so that these constructs only contained the TM domains attached to the fluorescent proteins via the (GGS)⁵ linkers (Figure 3A).

The raw FRET data for the truncated EC+TM, TM and TM+IC constructs are shown in Supplementary Figure 7. The FRET efficiency versus acceptor fraction is shown in Supplementary Figure 8. The dependence is linear for all truncated Ecadherins, suggesting that they also form dimers^{44,50,52}. The dimerization constant (and the respective dimerization free energy), and the value of Intrinsic FRET were determined in a two-parameter fit using equation (9), while accounting for proximity FRET as described in detail previously⁴⁹. The dimerization curves for the truncated Ecadherins in swollen cells are shown in Figure 4B, along with the results for the full-length Ecadherin for comparison. The dimerization constants, free energies of dimerization and intrinsic FRET values for all the variants are shown in Table 1.

These results reveal that the TM domains alone form dimers, with dimerization free energy of -5.15 ± 0.32 kcal mol⁻¹. Second, EC+TM Ecadherin has even higher propensity to form dimers, with dimerization free energy of -6.03 ± 0.46 kcal mol⁻¹. Thus, the contribution of the EC domains to E-cadherin dimerization is favorable and is -0.88 ± 0.56 kcal mol⁻¹. Third, we see that TM+IC Ecadherin forms constitutive dimers, just as the full-length, down to the lowest concentration that is accessible in our experiments. The exact dimerization free energies cannot be measured for TM+IC Ecadherin, but they can be estimated as more favorable than -8 kcal/mole, the limit that is measurable with the FSI technique. Thus, the favorable contribution of the IC domains to E-cadherin dimerization must exceed about -3 kcal/mole. Therefore, we find that lateral contacts between all the domains contribute to Ecadherin dimer stability. We also find that the contribution of the IC domain to Ecadherin

dimerization is more favorable than the contributions of the EC domain, a fundamentally novel finding.

We also investigated the association of these truncated constructs of Ecadherin in plasma membrane derived vesicles of CHO cells. The raw FRET data for all constructs are shown in Supplementary Figure 6. The dependence of the FRET efficiency on acceptor fraction is shown in Supplementary Figure 9. The dimerization curves in cells and in vesicles are compared in Supplementary Figure 10. All three truncated constructs form dimers in the vesicles, and their dimerization propensities are the same in vesicles and in cells. Just as in cells, the presence of the IC domains correlates with 100% dimerization. Since there are no soluble proteins or actin in the vesicles, these results suggest that the Ecadherin dimer is stabilized by direct Ecadherin-Ecadherin interactions involving the IC domain.

Effect of calcium on E-cadherin dimerization

The EC domain of Ecadherin has multiple calcium binding sites (see Figure 4A). Since calcium is known to be critically important for cadherin function^{3,5}, we next examined the role of calcium on the dimerization propensity of full length Ecadherin and EC+TM Ecadherin, the two proteins that contain the EC domain (the site of calcium binding). HEK293T cells expressing these proteins were incubated in starvation medium containing 5mM EGTA for four hours. Prior to imaging, the starvation medium was replaced with swelling medium with 5mM EGTA. The FRET data for full length and EC+TM Ecadherin with and without calcium are compared in Supplementary Figure 11.

The dimeric fractions measured in the presence and absence of calcium are shown in Figures 5A and 5B, for full-length Ecadherin and the EC+TM Ecadherin, respectively. Full length Ecadherin forms constitutive dimers both in the presence and absence of calcium, down to the lowest concentration that is accessible in our experiments. The dimerization of the EC +TM construct, however, is decreased in the absence of calcium by 1.66 ± 0.47 kcal/mole. Thus, the stabilizing effect of calcium on the Ecadherin dimer can be estimated as -1.66 ± 0.47 kcal mol⁻¹ (Figure 5B and Table 1). This effect is significant, as it is larger than the contribution of the EC domain, -0.88 ± 0.56 kcal mol⁻¹ (but not the IC domain, > -3 kcal mol⁻¹) to dimerization. However, since the full length Ecadherin dimer is very stable, a reduction in dimeric fraction is not observable for the full-length Ecadherin which remains 100% dimer even in the absence of calcium.

The Intrinsic FRET values in the absence and presence of calcium are reported in Table 1. For the full-length dimer, we observe a small effect due to calcium. The effect observed for the truncated dimer is significant, suggesting that the configuration of the TM domain is altered in the absence of calcium.

Effect of the pathogenic V678M mutation on Ecadherin dimerization

The V678M mutation in the cytoplasmic domain of Ecadherin has been linked to gastric cancer. While some believe that this mutation decreases the binding of catenin to Ecadherin⁵⁷, others question this effect⁵⁸. Since in this work we observed that the IC domain contributes significantly to Ecadherin dimer stabilization, we asked if this mutation may also affect the lateral interaction between E-cadherin molecules.

To answer this question, we engineered the V678M mutation in the full-length and TM+IC Ecadherins, the two constructs that contain the IC domain. The FRET data for these mutants are shown in Supplementary Figure 12. The dimerization curves are shown in Figure 6A and Figure 6B, respectively, along with the dimerization curves of the wild-type constructs for comparison. Both the full length and TM+IC Ecadherins form constitutive dimers in the presence of this mutation down to the lowest concentration accessible in our experiments. The Intrinsic FRET values for the full-length and TM+IC constructs, shown in Table 1, are increased modestly due to the mutation. Since the Intrinsic FRET depends on the separation and the orientation of the fluorescent proteins in the Ecadherin dimer, and since the catenin binding site is close to the Cterminus, it is possible that catenin binding affects Intrinsic FRET. A reduction in catenin binding in the mutant⁵⁷, therefore, can provide an explanation for the observed change in Intrinsic FRET.

Next, we investigated the effect of calcium on the dimerization propensity of these mutants. Since the calcium binding EC domain is not present in the TM+IC construct of E-cadherin, we performed this experiment with the full-length Ecadherin mutant only. The FRET data are shown in Supplementary Figure 11 in the absence and presence of calcium. We see a significant decrease in dimer stability (> 1.3 kcal/mole) for the full-length mutant in the absence of calcium (Figure 7C), an effect that we did not observe in the full-length wild-type (Figure 5A). This interesting observation suggests that the mutation makes Ecadherin dimerization more sensitive to calcium removal.

Effect of the D1A and W2A mutation on Ecadherin dimerization

It is well known that the N-terminal cadherin Ig-like domain (also called extracellular domain 1, EC1) is critical for cadherin adhesion. The tryptophan in position 2 (W2) is a key functional residue, as its mutagenesis is known to interfere with cadherin adhesion^{59–61}. The functional importance of W2 was first deduced from a crystal structure of the EC1 domain in 1995¹¹. In this structure, a few amino acids at the N-terminus insert into the neighboring EC1 domain, and W2 engages in hydrogen bonding interactions with the neighbor. Furthermore, the neighbors are oriented in the same directions, “as if they were emanating from the same cell surface”, suggesting that cadherins might “protrude from the cell surface as dimers”¹¹. A question therefore arises if the N-terminal cadherin residues and W2 in particular play a role in the lateral dimerization that we observe in this work. To answer this question, here we mutated W2, as well as D1, to Ala, and we measured the effect of these two mutations on dimerization. The raw FRET data for D1A and W2A mutants of full-length are shown in supplementary Figure 14. Supplementary Figure 15 shows the raw data for the EC+TM version of Ecadherin bearing the D1A and W2A mutations.

The dimerization curves for the D1A and W2A mutants of full length Ecadherin are shown in Figure 7A. For comparison we have also included the dimerization curve for wild type in Figure 7A. The dimerization curves for the D1A and W2A mutants of the EC+TM construct of Ecadherin are shown in Figure 7B along with the dimerization curve of wild-type EC+TM Ecadherin for comparison. We see that the W2A substitution destabilizes the EC+TM dimer by 0.80 ± 0.55 kcal/mole, while the D1A substitution destabilizes the Ecadherin dimer

by 1.41 ± 0.48 kcal/mol (Table 1). However, the destabilizing effects due to these two mutations are not strong enough to decrease the 100% dimeric population of full-length Ecadherin, even at the lowest concentration accessible in our experiments. Thus, the mutants are constitutive dimers, but they exhibit a lower Intrinsic FRET (Figure 7A,B). Finally, calcium depletion does not change the association state of the full-length mutant, but leads to an increase in Intrinsic FRET (Figure 7C and Table 1).

DISCUSSION

Some believe that Ecadherin lateral interactions are very weak and become significant only in the presence of adhesive “trans” interactions²⁰. Others have speculated that Ecadherin may exist as a preformed dimer on the cell surface^{18,24,62,63}. Here we provide direct evidence that Ecadherin, expressed on the surface of HEK293T cells, forms constitutive dimers in the absence of trans interactions, far from adherens junctions. We first demonstrate that dimerization occurs in cells under reversible osmotic stress using a rigorous quantitative approach which requires the knowledge of two dimensional protein concentrations³⁵. We then confirm the results in unperturbed HEK 293T cells. Ecadherin is invariably 100% dimeric, even at the lowest Ecadherin concentrations that we can reach in our experiments. Previous work has estimated the expression of Ecadherin in MDCK cells as ~20 molecules per μm^2 ³³, similar to our lowest concentrations. Thus, our work shows that cadherin is a constitutive dimer under physiological expressions.

We further perform experiments in ghost plasma membrane derived vesicles which lack cytoskeleton or soluble proteins⁵⁶. In the ghost plasma membrane derived vesicles, just as in cells, Ecadherin is a constitutive dimer. These results demonstrate that Ecadherin dimers form independently from the actin cytoskeleton or cytoplasmic proteins.

One goal of this work was to measure the contributions of the different domains to dimerization. Our results suggest that the Ecadherin dimer is stabilized by inter-molecular contacts that occur along the entire length of the protein. Indeed, we observed stabilizing interactions between all the domains (Figure 4). Previously, the EC domains have been implicated in Ecadherin lateral interactions¹⁸. We confirm that the IC domains contribute to dimer stabilization and measure their thermodynamic contributions. We see that the isolated TM domains also can form a dimer. We further reveal that the IC domains have a surprisingly favorable contribution to dimer stability. Since the interactions between Ecadherin molecules are very strong, we could not determine the exact thermodynamic contribution of the IC domain to dimer stability, G_{IC} . We could, however, estimate a lower bound of this contribution, $G_{IC} < -3$ kcal/mole. It is likely that this significant favorable energetic contribution ensures that Ecadherin remains 100% dimeric even in the presence of factors perturbing the interactions, such as mutations or calcium depletion.

We further show that the two N-terminal residues of Ecadherin, located in the first Ig-like domain, EC1, play a role in dimer stabilization. We show that calcium binding also contributes to dimer stability. We reveal an interesting interplay between the effect of calcium and the V678M pathogenic mutation, as the mutation makes the dimerization more

susceptible to calcium depletion. Yet, the stability of the full-length Ecadherin dimer is sufficiently high, such that all mutants are 100% dimeric under physiological conditions.

Currently, it is not known if the interactions involved in Ecadherin dimerization that we reveal here also play a role in clustering. The Ecadherin dimer is very stable, however, so it is possible that it remains unchanged and the clusters form from the pre-formed dimers that we observe here. It is now well established that interactions between the EC domains contribute to clustering^{20,21,62}. A 94 amino acid stretch in the cytoplasmic portion, the so-called juxtamembrane domain, has also been suggested to play a role in the clustering²³. Thus, both the extracellular and the cytoplasmic domains appear to be important for both dimerization and clustering.

Clustering has been shown to depend on actin and on cytoplasmic proteins such as p120 which interact with the cytoplasmic juxtamembrane region²³. It can be therefore hypothesized that these soluble proteins play a critical role in the clustering of the pre-formed dimers observed in this work. On the other hand, the dimerization that we observed in this work is likely mediated by direct contacts in the cytoplasmic portion. Indeed, the presence of the IC domain correlates with 100% dimeric Ecadherin, both in cells and in ghost plasma membrane derived vesicles. Thus, the stabilizing contribution of the IC domain does not depend on the presence of soluble proteins or the actin cortex, in the dimer case.

The new knowledge that we gain through the use of FRET measurements in cell and in cell derived vesicles can be now used to refine models about cadherin adhesion and function. Our findings of strong Ecadherin dimerization on the cell surface provides new context for the interpretation of Ecadherin adhesion experiments. In particular, they suggest that experiments that probe the kinetics of endogenous cadherin-mediated binding between two cells^{30–34} interrogate Ecadherin dimers and not monomers.

MATERIALS AND METHODS

Plasmid Constructs

The YFP plasmid was a generous gift from Prof. Beatenbaugh (Johns Hopkins University) and the A206K mutation was introduced in YFP to render it monomeric. The plasmid encoding the fluorescent protein mTurquoise (mTurq) was obtained from Prof. Paul S. Park (Case Western University). The Ecadherin gene was a gift from Prof. Wirtz (Johns Hopkins University).

All Ecadherin plasmid constructs were subcloned into the pcDNA3.1(+) vector (Invitrogen). For the cloning, we started with an a plasmid construct that has been previously described, pcDNA3.1(+) FGFR1-15aa-mCherry⁶⁴. In pcDNA3.1(+)FGFR1-15aa-mCherry, FGFR1 is positioned between the restriction sites Hind III and Kpn I; the 15 amino acid (GGS)₅ linker is between the restriction sites KpnI and AgeI; and YFP is between the restriction sites AgeI and XbaI. The Ecadherin gene contains the KpnI and the AgeI restriction sites, which we eliminated based on the degeneracy of nucleotide sequences. We amplified the Ecadherin gene using PCR and double digested it using the Hind III and Kpn I restriction enzymes. We then inserted it into the pcDNA3.1(+)FGFR1-15aa-mCherry

plasmid in place of FGFR1. The YFP and the mTurquoise genes were amplified and inserted into pcDNA3.1(+)-Ecadherin-15aa-mCherry in place of mCherry to obtain pcDNA3.1(+)-Ecadherin-15aa-mTurq and pcDNA3.1(+)-Ecadherin-15aa-YFP.

The sequences encoding for the EC and the TM domains of Ecadherin were amplified by PCR, double digested using Hind III and KpnI restriction enzymes and used to replace Ecadherin in the plasmid constructs pcDNA3.1(+) Ecadherin-(GGS)₅-mTurq, pcDNA3.1(+) Ecadherin-(GGS)₅-YFP, and pcDNA3.1(+) Ecadherin-(GGS)₅-mCherry to obtain the plasmid constructs pcDNA3.1(+) EC+TM-Ecadherin-(GGS)₅-mTurq, pcDNA3.1(+) EC+TM-Ecadherin-(GGS)₅-YFP, and pcDNA3.1(+) EC+TM-Ecadherin-(GGS)₅-mCherry.

To clone the TM and TM+IC Ecadherin constructs, we inserted Xho I restriction sites just after the pro-peptide (see Supplementary Figure 2). We amplified the TM or the TM+IC domains of Ecadherin by PCR, double digested with the XhoI and KpnI restriction enzymes, and inserted them in place of Ecadherin in the plasmid constructs pcDNA3.1(+) Ecadherin-(GGS)₅-mTurq, pcDNA3.1(+) Ecadherin-(GGS)₅-YFP, and pcDNA3.1(+) Ecadherin-(GGS)₅-mCherry to obtain the plasmid constructs pcDNA3.1(+) TM-Ecadherin-(GGS)₅-mTurq, pcDNA3.1(+) TM-Ecadherin-(GGS)₅-YFP, pcDNA3.1(+) TM-Ecadherin-(GGS)₅-mCherry, pcDNA3.1(+) TM+IC-Ecadherin-(GGS)₅-mTurq, pcDNA3.1(+) TM+IC-Ecadherin-(GGS)₅-YFP, and pcDNA3.1(+) TM+IC-Ecadherin-(GGS)₅-mCherry.

We also generated the D1A and W2A mutants of the full length and the EC+TM Ecadherin constructs as well as the V678M mutant of full length and TM+IC Ecadherin. These mutants were created using the Quik Change II XL Site-Directed Mutagenesis kit (Stratagene, CA). All primers used in this work are given in Supplementary Table 2.

Cell culture and transfection

HEK293T cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Hyclone), 3.5g/L D-glucose (19.4mM) and 1.5g/L (17.9mM) sodium bicarbonate in 35 mm glass bottom dishes (MatTek Corporation, MA). After twenty four hours, the cells were co-transfected with 10 ng to 4 µg of DNA encoding Ecadherins tagged with either mTurq or YFP using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

Swelling of HEK293T under reversible osmotic stress

12 hours post transfection, the cells were serum-starved for 12 hours. Prior to imaging, the starvation medium was replaced with hypo-osmotic medium to induce osmotic stress and swelling under reversible conditions as described in ⁴³. The cells were allowed to settle down for 10 minutes and imaged for two hours.

Removal of Calcium

To remove the calcium, the starvation medium was replaced with starvation medium containing 5mM of EGTA. The cells were incubated for 4 hours at 37°C. Before imaging, the starvation medium containing 5mM EGTA was replaced with swelling medium containing 5mM EGTA.

Production of CHO plasma membrane vesicles

CHO cells were cultured at 37 °C with 5% CO₂ for 24 h. Cells were co-transfected with 3–7 µg of DNA encoding receptors tagged with either YFP or mCherry using Fugene HD (Roche Applied Sciences). Vesiculation was performed using a chloride salt vesiculation buffer consisting of 200 mM NaCl, 5 mM KCl, 0.5 mM MgSO₄, 0.75 mM CaCl₂, 100 mM bicine and protease inhibitor cocktail (complete mini EDTA-free tabs, Roche Applied Science) adjusted to pH of 8.5⁵⁴. CHO cells were rinsed twice with 30% PBS (pH 7.4), and incubated with 1 ml of chloride salt vesiculation buffer overnight at 37 °C. The vesicles were transferred into four-well Nunc Lab-Tek II chambered coverslips for imaging. The cytoplasm in the vesicles is lost during vesicle production, as attested by the fact that soluble proteins of molecular weight up to 200 kDa are not retained inside the vesicles⁵⁶.

Two photon and confocal microscopy

Imaging was performed with a spectrally resolved two photon microscope with line-scanning capabilities^{38,46}. An ultrashort-pulse laser (MaiTai™, Spectra-Physics, Santa Clara), which generates femtosecond mode locked pulses at wavelengths between 690 nm to 1040 nm, was used as the excitation source for the fluorophores. Details of the microscope have been discussed previously^{38,46}. The swollen cells were imaged at 800 nm and 960 nm as described³⁵.

The plasma membrane derived vesicles were imaged in a Nikon confocal microscope as described previously^{37,55}.

FRET data analysis

Two scans are performed: (i) a FRET scan at wavelength λ_1 , which excites primarily the donor, and (ii) an acceptor scan at wavelength λ_2 which excites primarily the acceptor. These two scans and calibration curves of fluorescence versus known concentrations of donor and acceptor “solution standards” are used to calculate the FRET efficiency, the donor concentration, and the acceptor concentration using the following equations³⁵:

$$E_{app} = 1 - \frac{F_{\lambda_1}^{DA}}{F_{\lambda_1}^D} \quad (1)$$

$$[D] = \frac{F_{\lambda_1}^D}{i_{D,\lambda_1}} \quad (2)$$

$$[A] = \frac{F_{\lambda_2}^A}{i_{A,\lambda_2}} \quad (3)$$

Here, $F_{\lambda_1}^{DA}$ is the fluorescence of the donor in the presence of acceptor when the donor is excited at λ_1 , and $F_{\lambda_1}^D$ is the fluorescence of the donor when excited at λ_1 in the absence of

acceptor. $F_{\lambda_2}^A$ is the fluorescence of the acceptor when excited at λ_2 in the absence of the donor. i_{D,λ_1} is the slope of the calibration curve (fluorescence versus concentration) for the donor solution standards at wavelength λ_1 and i_{A,λ_2} is the slope of the calibration curve (fluorescence versus concentration) for the acceptor solution standards at wavelength λ_2 . $F_{\lambda_1}^D$ and $F_{\lambda_2}^A$ can be written in terms of experimentally measured parameters as follows³⁵:

$$F_{\lambda_1}^D = F_{\lambda_1}^{DA} + \frac{Q^D}{Q^A} \left(F_{\lambda_1}^{AD} - \frac{i_{A,\lambda_1}}{i_{A,\lambda_2}} F_{\lambda_2}^A \right) \quad (4)$$

$$F_{\lambda_2}^A = \left(F_{\lambda_2}^{AD} - \frac{i_{D,\lambda_2}}{i_{D,\lambda_1}} F_{\lambda_1}^{AD} \right) \left(1 - \frac{i_{A,\lambda_1}}{i_{A,\lambda_2}} \frac{i_{D,\lambda_2}}{i_{D,\lambda_1}} \right)^{-1} \quad (5)$$

where i_{A,λ_1} and i_{A,λ_2} are concentration calibration constants, namely the slopes of the acceptor calibration curves.

The measured FRET efficiencies were corrected for proximity FRET that arises due to random approach between donors and acceptors within 100 Å. This correction was necessary for the fluorophores confined in two-dimensional cell membrane of swollen cells and is discussed in detail elsewhere⁴⁸. For dimers, FRET due to specific interactions, E_D , is given by:

$$E_D = f_D x_A \tilde{E} \quad (6)$$

In Equation (6) f_D is the dimeric fraction, x_A is the acceptor fraction and $\tilde{E}$ is the structural parameter called “Intrinsic FRET”, which depends on the dimer structure, in particular on the distance d between the fluorescent proteins in the dimer and is defined according to:

$$E = \frac{1}{1 + \left(\frac{d}{R_0} \right)^6} \quad (7)$$

Here R_0 is the Förster radius of the FRET pair. For mTurq and YFP, R_0 is 54.5 Å. For YFP and mCherry, R_0 is 53.1 Å. This equation assumes free rotation of the fluorescent proteins in our experiments, justified by the fact that the fluorescent proteins are attached to the receptors via long flexible linkers.

Based the law of mass action, the dimeric fraction can be written in terms of total receptor concentration, $[T]$, and the dimerization constant, K , according to Equation 8:

$$f_D = \frac{1}{[T]} \left([T] - \frac{1}{4K} \left(\sqrt{1 + 8[T]K} - 1 \right) \right) \quad (8)$$

Substitution of Equation (8) in (6) yields:

$$\frac{E_D}{x_A} = \frac{1}{[T]} \left([T] - \frac{1}{4K} \left(\sqrt{1 + 8[T]K} - 1 \right) \right) \tilde{E} \quad (9)$$

Equation (9) is used to fit monomer-dimer model to the measured $\frac{E_D}{x_A}$ while optimizing for two adjustable parameters: the dimerization constant K , and the Intrinsic FRET, $\tilde{E}$, while taking into account the proximity FRET correction as described.

The dissociation constant $K_{diss}=1/K$ is reported in receptors/ μm^2 in Table 1. The dimerization free energy ΔG is calculated from the dissociation constant K_{diss} . The standard state is defined as $\text{nm}^2.\text{receptor}^{-1}$ ³⁷ and therefore:

$$\Delta G = -RT \ln \left(\frac{10^6}{K_{diss}} \right) \quad (10)$$

Western blots

Twenty four hours following transfection, the cells were lysed in lysis buffer (25mM TrisHCl, 0.5% TritonX-100, 20mM NaCl, 2mM EDTA, 2mM Na_3VO_4 , and Roche Applied Science protease and phosphatase inhibitor). The lysed samples were collected and centrifuged at 14,000g for 15 minutes at 4°C. The lysates were collected and stored at -20°C. The total protein concentrations in the lysates were measured with the BCA protein assay kit (Bio-Rad, CA). The lysates were loaded into 3–8% NuPAGE^HNovex^HTris-Acetate mini gels (Invitrogen, CA). The proteins were transferred onto a nitrocellulose membrane, and blocked using 5% non-fat milk in TBST. E-cadherin, β -Actin, α -Catenin, β -Catenin were detected using anti-E-cadherin antibodies (Cell Signaling (5296S), MA), anti- β -Actin antibodies (Cell Signaling (4967S), MA), anti- β -Catenin antibodies and anti- α -Catenin antibodies (Cell Signaling (9562S, 3236S), MA), respectively. Anti-mouse HRP conjugated antibodies (Promega (W402B), WI) were used as secondary antibodies for E-cadherin while anti-rabbit HRP conjugated antibodies (Promega (W401B), WI) were used as secondary antibodies for β -actin, α -catenin, and β -catenin. The membranes were incubated with Amersham ECL PlusTM Western Blotting Detection Reagent (GE HealthCare Life Sciences, PA) for 2 minutes and then exposed for 1 to 60 seconds in a Chemidoc molecular imager (Bio-Rad, CA) to detect the chemiluminescent bands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Ecadherin is a constitutive dimer under physiological expression conditions

Ecadherin dimer is stabilized by contacts along the entire length of the protein

The intracellular domains have a large favorable contribution to dimer stability

The intracellular domain contribution is independent of soluble proteins or actin

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Figure 1. Ecadherin forms constitutive dimers in the plasma membrane

(A) FRET efficiency as a function of acceptor concentration in HEK 293T cells under reversible osmotic stress when transfected with DNA encoding Ecadherin-mTurq (FRET donor) and Ecadherin-YFP (FRET acceptor) in 3:1, 1:1, and 1:3 ratios. Each data point is obtained from a membrane region of a cell under reversible osmotic stress (see Supplementary Figure 3B). The black line represents the proximity FRET that arise due to random approach of donors and acceptors with 10nm in plasma membrane plane (see text). (B) Acceptor concentration as a function of donor concentration in HEK 293T cells under reversible osmotic stress, for the three transfection ratios. (C) Means square error (MSE) versus oligomer order. MSE has a minimum for $n=2$, a dimer. (D) Dimeric fraction (f_D) $\times$ intrinsic FRET as a function of total concentration. There is no significant dependence on Ecadherin concentration, indicative of constitutive dimerization. (E) FRET as a function of acceptor fraction in HEK293T cells under reversible osmotic stress (red), intact HEK293T cells (green), and plasma membrane derived vesicles (black). FRET as a function of acceptor fraction is a straight line, indicative of dimer formation.

Figure 2. Comparisons of FRET efficiencies in HEK293T cells and HEK293T cells under reversible osmotic stress

(A) FRET efficiency as a function of the apparent receptor concentration when cells when transfected with DNA encoding Ecadherin-mTurq (FRET donor) and Ecadherin-YFP (FRET acceptor) in 3:1, 1:1, and 1:3 ratios and imaged without the application of osmotic stress. (B) Histograms of measured FRET efficiencies in cells and in cells under reversible osmotic stress. The application of osmotic stress does not change the FRET efficiencies, and therefore the interactions between the Ecadherin molecules.

Figure 3. Visualization of actin in (A) HEK 293T cells, (B) HEK 293T cells under reversible osmotic stress, and (C) CHO cells incubated with a vesiculation buffer. Cells were co-transfected with genes encoding both Ecadherin-mCherry and Lifeact, and imaged in a confocal microscope. (B) The application of osmotic stress does not destroy the cortical actin network. (C) The vesiculation buffer destroys the cortical actin network and the vesicles lack an actin cytoskeleton.

Figure 4. Contributions of Ecadherin domains to dimerization

(A) A Schematic of the full length, EC+TM, TM and TM+IC Ecadherin. EC: extracellular domain; TM: transmembrane domain; IC: intracellular domain. The fluorescent proteins are attached to the C-terminus via 15 amino acid flexible linkers. The calcium ions are shown as red stars (B) Dimerization curves for the full-length (red), EC+TM (purple), TM (dark cyan) and TM+IC (blue) Ecadherins. The measured dimeric fractions are binned and are shown with symbols, along with the standard errors. The solid lines are the best fits of a monomer–dimer equilibrium model to the single-membrane region data. All domains stabilize the dimer.

Figure 5. Effect of calcium on the dimerization of full length and EC+TM Ecadherin
 (a) Dimerization curves of full length Ecadherin in the presence (red) and in the absence (black) of calcium. E-cadherin forms constitutive dimers both in the presence and in the absence of calcium. (b) Dimerization curves of EC+TM Ecadherin in the presence (purple) and in the absence (pink) of calcium. Calcium promotes the dimerization of EC+TM Ecadherin.

Figure 6. Effect of the pathogenic V678M mutation on Ecadherin lateral dimerization
 (A) Dimerization of full length Ecadherin (red) and the V678M Ecadherin mutant (olive). Both the wild-type and the mutant form constitutive dimers. (B) Dimerization propensity of TM+IC Ecadherin (cyan) and V678M TM+IC Ecadherin (magenta). Both constructs form constitutive dimers. (C) Comparison of V678M E-cadherin dimerization curves, with and without calcium. The lack of calcium destabilizes the V678M Ecadherin dimer.

Figure 7. Effect of the D1A and W2A mutations on Ecadherin lateral dimerization

(A) Dimerization of the full length Ecadherin (purple), the D1A Ecadherin mutant (pink), and the W2A Ecadherin mutant (cyan). The wild type and the mutants form constitutive dimers. (B) Dimerization of EC+TM Ecadherin (purple), D1A EC+TM Ecadherin (pink), and W2A EC+TM Ecadherin (cyan). Both mutations destabilize the EC+TM Ecadherin dimer. (C) Dimerization curves with and without calcium for the full-length Ecadherin mutants: D1A Ecadherin, calcium (light green); D1A Ecadherin, no calcium (dark green); W2A Ecadherin, calcium (gray); W2A Ecadherin, no calcium (black).

Table 1
Parameters describing the dimerization of Ecadherin in cells under reversible osmotic stress

The parameters were obtained from least-square fits of a dimer model to the FRET data. Shown are the best fits, along with the 95% confidence intervals. The values shown in parenthesis are the lower and upper bounds for the confidence intervals, determined from the fit. . In the text, these uncertainties are symmetrized. G: dimerization free energies (dimer stabilities) calculated using equation (9), K_{diss} : two-dimensional dissociation constants, d: effective distance between fluorescent proteins in a dimer, calculated from the intrinsic FRET efficiencies under the assumption of free rotation using equation (7).

Ecadherins	G (kcal/mol)	K_{diss} (receptors. μm^{-2})	Intrinsic FRET	d (Å)
full length	100% dimer (<-8)		0.16 (0.15 to 0.17)	71.8 (72.8 to 71.0)
EC+TM	-6.03 (-5.79 to -6.45)	38 (18 to 57)	0.26 (0.25 to 0.27)	64.9 (65.5 to 64.3)
TM	-5.15 (-5.77 to -4.85)	168 (59 to 278)	0.48 (0.42 to 0.53)	55.2 (57.5 to 53.4)
TM+IC	100% dimer (<-8)		0.10 (0.09 to 0.11)	78.6 (80.1 to 77.2)
full length, no Ca^{2+}	100% dimer (<-8)		0.13 (0.12 to 0.14)	74.8 (76.0 to 73.8)
EC+TM, no Ca^{2+}	-4.37 (-4.47 to -4.27)	624 (521 to 726)	0.69 (0.65 to 0.72)	47.7 (49.2 to 46.6)
V678M full length	100% dimer (<-8)		0.19 (0.18 to 0.20)	69.4 (70.2 to 68.7)
V678M TM+IC	100% dimer (<-8)		0.16 (0.15 to 0.17)	71.8 (72.8 to 71.0)
V678M, no Ca^{2+}	-6.62 (-7.28 to -6.31)	14 (5 to 23)	0.21 (0.20 to 0.22)	68.0 (68.7 to 67.3)
W2A full length	100% dimer (<-8)		0.07 (0.06 to 0.08)	83.9 (86.2 to 81.9)
W2A full length, no Ca^{2+}	100% dimer (<-8)		0.18 (0.17 to 0.19)	70.2 (71.0 to 69.4)
W2A EC+TM	-5.23 (-4.90 to -5.80)	146 (59 to 233)	0.26 (0.23 to 0.29)	64.9 (66.6 to 63.3)
D1A full length	100% dimer (<-8)		0.12 (0.11 to 0.13)	76.0 (77.2 to 74.8)
D1A full length, no Ca^{2+}	100% dimer (<-8)		0.13 (0.12 to 0.14)	74.8 (76.0 to 73.8)
D1A EC+TM	-4.62 (-4.36 to -5.08)	406 (185 to 626)	0.19 (0.17 to 0.22)	69.4 (71.0 to 67.3)