Intracellular Domain Contacts Contribute to Ecadherin Constitutive Dimerization in the Plasma Membrane

Deo R. Singh, Fozia Ahmed, Sarvenaz Sarabipour and Kalina Hristova

Department of Materials Science and Engineering and Institute of NanoBioTechnology, *Johns Hopkins University, 3400 Charles Street, Baltimore, MD 21218, USA*

Correspondence to Kalina Hristova: *kh@jhu.edu*
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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 Forster resonance energy transfer (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 intermolecular 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 from preformed Ecadherin dimers.

Introduction

Cadherins are a family of cell surface adhesion receptors that 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 tumorigenesis [6] 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 that mediates cell–cell contacts, a single transmembrane (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 (EC) 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 have 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 domains to Ecadherin dimerization thermodynamics are also unknown. Yet, most of the basic knowledge about the cadherin adhesive bonds has been deduced from atomic force microscopy 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 membrane [35]. 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 endogeneous Ecadherin, as assessed by Western blotting (Supplementary Fig. 1). For the FRET experiments, full-length Ecadherin was labeled with the monomeric fluorescent proteins mTurquoise (mTurq) or yellow fluorescent protein (YFP) at the C terminus, via a $(GGS)_5$ flexible linker (Supplementary Fig. 2). These two fluorescent proteins constitute a FRET pair [36].

We used the fully quantified spectral imaging (FSI)-FRET technique [35] that 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 Fig. 3. The FSI method requires the acquision 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 (2D) 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 two to three 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- to 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 h and then subjected to reversible osmotic stress, following the protocol described in Ref. [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 Fig. 3B). Fig. 1a shows the FRET efficiency as a function of the acceptor concentration, while Fig. 1b shows the donor concentration as a function of the acceptor concentration, for the three DNA transfection ratios used. Each data point in Fig. 1a and b represents a single membrane region of a swollen cell (Supplementary Fig. 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 2D membrane, even in the absence of specific interactions [47,48]. The proximity FRET depends mainly on the concentration of the acceptor and also on the oligomer order and the strength of the interaction, that is, on the critical unknowns in the experiment [48]. Following the FSI-FRET protocol, different oligomeric models (monomer, dimer, trimer, tetramer, pentamer, and hexamer) were fitted to the data in Fig. 1a and b while accounting for the relevant proximity contribution associated with these