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E-cadherin junction formation involves an active kinetic nucleation process

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Epithelial (E)-cadherin-mediated cell–cell junctions play important roles in the development and maintenance of tissue structure in multicellular organisms. E-cadherin adhesion is thus a key element of the cellular microenvironment that provides both mechanical and biochemical signaling inputs. Here, we report in vitro reconstitution of junction-like structures between native E-cadherin in living cells and the extracellular domain of E-cadherin (E-cad-ECD) in a supported membrane. Junction formation in this hybrid live cell-supported membrane configuration requires both active processes within the living cell and a supported membrane with low E-cad-ECD mobility. The hybrid junctions recruit α-catenin and exhibit remodeled cortical actin. Observations suggest that the initial stages of junction formation in this hybrid system depend on the *trans* but not the *cis* interactions between E-cadherin molecules, and proceed via a nucleation process in which protrusion and retraction of filopodia play a key role.

cadherin | diffusion | adhesion | nucleation | bilayer

There is a growing realization that the cellular microenvironment, including its biochemical composition as well as its geometrical and mechanical characteristics, plays a crucial role in defining the behavior of living cells. For example, growing cells on 2D surfaces patterned with extracellular matrix (ECM) (1), in 3D random ECM networks (2), or confined to microfabricated chambers (3) all reveal that geometry alone can exert specific control over cell fate. Experiments on polymer substrates with controlled rigidity demonstrate that mechanical stiffness of the microenvironment influences key cellular decisions including healthy stem cell differentiation (4) and tumorigenesis in cancer (5). Physical restriction of the clustering or transport of cell surface receptors can alter the transduction of downstream signals (6–8). The ability to control such aspects of the cellular microenvironment in a synthetic setting is key to the advancement of in vitro live cell technologies.

Epithelial (E)-cadherin-mediated adhesion is a prominent feature of epithelial sheets throughout vertebrates and related receptors are similarly used in invertebrates. E-cadherin, a type I cadherin adhesion receptor, is the main component of epithelial adherens junctions and is important for the development and remodeling of epithelial tissues in all Metazoans (9–11). Mutations in the E-cadherin gene can result in the loss of cell adhesion, causing a number of disease phenotypes, including cancer (12). In addition, loss of E-cadherin expression is often used as a marker for epithelial to mesenchymal transition and cancer progression (13, 14). E-cadherin is a type I transmembrane protein that exhibits fluid—albeit somewhat restricted—motion in the cell membrane (15–17). Cadherin adsorbed onto solid substrates is routinely used for in vitro cell studies (18–20). Although solid surface-adsorbed cadherin is capable of binding cadherin on an apposed cell surface, the imposed immobility precludes subsequent assembly into natural structures, and the functional consequences of this remain unclear.

Here, we reconstituted E-cadherin-mediated adhesion between living cells and E-cadherin extracellular domain (E-cad-ECD)-functionalized supported lipid bilayer membranes. This type of hybrid live cell-supported membrane platform has proven to be effective in reconstitution of other juxtacrine signaling systems, in which mobility and spatial assembly of receptors proved to enable important aspects of biological function that are lost in a solid display format (6–8, 21–23). We anticipate similar benefits when the system is applied to cadherins. Supported lipid bilayers allow control over the identity, density, and diffusional characteristics of protein molecules. Significantly, supported membranes also allow dynamic spatial assembly of

Significance

Epithelial (E)-cadherin-based adherens junctions are the basis of epithelial tissue integrity in Metazoans. They are composed of E-cadherin molecules interacting with each other from apposed cells. Using artificial supported lipid bilayers functionalized with the full-length extracellular domain of E-cadherin and live cells, we show that E-cadherin junction formation involves a nucleation process mediated by active filopodia retraction and requiring reduced mobility of E-cadherin on supported lipid bilayers. These results underscore the importance of controlling physical aspects of the cellular microenvironment with synthetic materials for in vitro live cell applications. In this case, tuning the mobility of a viscous fluid display surface enabled functional reconstitution of a cadherin-mediated adhesion junction.

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extended structures, as can occur in a physiological situation, but which is not possible with proteins directly adsorbed onto solid substrates.

From the results presented here, it is evident that the mechanism of hybrid junction formation between cells and the E-cad-ECD supported membranes is a dynamic process involving active extension and retraction of filopodia. The resulting hybrid junctions exhibit enrichment of E-cad-ECD in the supported membrane, recruitment of α-catenin (the mechanosensory protein found in adherens junctions) to corresponding regions of the live cell membrane, and remodeled cortical actin. Experiments with chemical inhibitors confirm that junction formation requires actomyosin contraction. A mutation known to destabilize the E-cadherin *cis* interface present in both the cell surface and the bilayer bound species did not significantly impair junction formation; however, deletion of the intracellular domain from the cell surface E-cadherin completely eliminated this process.

A key practical realization from the results presented here is that junction formation proceeds with much greater efficiency using supported membrane lipid compositions with low mobility. Additionally, junction formation is essentially all or nothing. Once successfully initiated, junction formation progresses to completion. Reduced membrane mobility results in two primary physical consequences on cadherin: (*i*) higher resistive forces to actively driven motion and (*ii*) elongated timescales for diffusive dissipation of localized concentrations of E-cad-ECD on the bilayer. The all-or-nothing nature of junction formation combined with the observed sensitivity to supported membrane mobility and active cellular processes indicates an active nucleation step, which can be defeated if the supported membrane E-cadherin exhibits too much freedom of motion. Previous efforts to reconstitute cadherin in supported bilayers have used an Fc-conjugated extracellular domain of E-cadherin linked to the bilayer via either GPI or biotin–streptavidin interaction (24–26). As will be directly evidenced in the results presented here, these early efforts most likely did not achieve the nucleation threshold required to establish extended E-cadherin-mediated junctions.

Overall, E-cadherin is unique among the juxtacrine receptor–ligand systems that so far have been studied in detail in hybrid live cell-supported membrane junctions (7, 8, 27–30). Intermembrane E-cadherin adhesion does not appear to follow simple laws of mass action. That is, the rate of intermembrane bond formation does not exhibit proportionality to receptor concentration as is seen in other systems, even when considering the geometry and mechanics of the intermembrane junction (31–33). Rather, E-cadherin junction formation requires active nucleation before extensive adhesive junctions form. This may reflect a regulatory mechanism that helps to minimize uncontrolled cellular adhesion in vivo.

Results

Biophysical Characterization of E-cadherin on Supported Lipid Bilayers. The mature E-cadherin molecule contains five amino-terminal extracellular cadherin domains in tandem (EC1–5) with multiple Ca^{2+} binding sites, followed by a single transmembrane domain and an intracellular cytoplasmic carboxyl-terminal domain. Crystal structures have revealed two types of interactions between E-cadherin extracellular domains: a *trans* interaction formed by β-strand swapping between the EC1 domains of apposing E-cadherin molecules, and a *cis* interaction formed between regions in the EC1 and EC2 domains of adjacent E-cadherin molecules (34). Disruption of the *trans* interaction interface abrogates cell–cell adhesion whereas disruption of the *cis* interaction interface inhibits localization of E-cadherin to junctions, although *trans* interactions are still observed (34, 35). Further, the formation of *trans*-interacting dimers of E-cadherin is a two-step process involving the formation of an intermediate X-dimer structure, which then converts to the final strand-swapped dimer (36).

To allow for all of these potential interactions in the reconstituted system, the C-terminal His_{12} tagged full E-cad-ECD was expressed in HEK 293F cells and purified using a Ni^{2+}-chelating column (Fig. 1*A*). The construct was engineered to contain a single free Cys residue in the EC5 domain (Cys499 of the mature E-cadherin) (*SI Materials and Methods*), which was used for site-specific fluorescent labeling. The labeled protein eluted as a single peak in size exclusion chromatography (Fig. 1*B*).

Supported lipid bilayers were functionalized with fluorescently labeled E-cad-ECD via the kinetic-controlled Ni-nitrilotriacetic acid (NTA)-poly-His chelation (23, 37, 38). Fluorescence recovery after photobleaching (FRAP) experiments revealed large-scale lateral mobility of the membrane-coupled E-cad-ECD in bilayers prepared with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as the base lipid (Fig. 1*C*). Precise Z-scan fluorescence correlation spectroscopy (FCS) measurements (39) revealed lateral diffusion coefficients, D, around 1.6 ± 0.1 $\mu m^2/s$ and molecular densities in the range of ∼100–700 molecules per square micrometer across multiple independently prepared bilayers, depending on incubation conditions (Fig. 1*D*) (37). The diffusion coefficient of E-cad-ECD obtained here is typical of monomeric proteins bound to lipid molecules in supported lipid bilayers by the Ni-NTA His chelation (38, 40), although higher than reported values of E-cadherin on live cell membranes (0.002–0.036 $\mu m^2/s$) (15–17). The surface density of E-cad-ECD on lipid bilayers is comparable to that on livings cells (2.5–16 × 10^4 molecules per cell, which translates to ∼80–500 molecules per square micrometer, assuming the cell to be a sphere with a diameter of 10 μm) (41).

E-cad-ECD on Supported Lipid Bilayers Is Monomeric. The oligomeric state of cell surface cadherins outside of junctions has not been established experimentally, although an interaction between E-cadherin has been proposed based on FRAP experiments on live cell membranes (42), and actin-delimited clusters of E-cadherin have been observed by superresolution imaging (43). A low affinity *cis* interaction observed in extracellular domain crystal

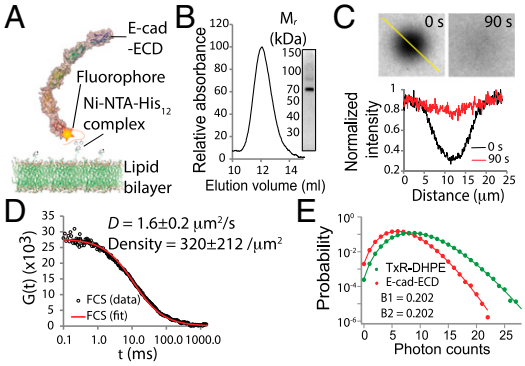

Fig. 1. Biophysical characterization of E-cad-ECD on supported lipid bilayers. (*A*) Schematic representation of E-cad-ECD bound to bilayer via Ni-NTA interaction. Relative position of the fluorophore on the crystal structure of the extracellular domain of E-cadherin (PDB: 3Q2V) is also shown. (*B*) Fluorescently labeled E-cad-ECD was subjected to size exclusion chromatography. *Inset* shows Coomassie stained SDS/PAGE image of E-cad-ECD. (*C*) FRAP analysis of E-cad-ECD functionalized lipid bilayer. (*Top*) Photobleached spot that recovers after 90 s. (*Bottom*) Line scan of the images. (*D*) FCS curve fit to a standard 2D, single-component diffusion model. Diffusion coefficient D and density shown in the box were determined from Z-scan FCS analysis of multiple E-cad-ECD containing bilayers. Values represent mean ± SD (*E*) PCH fit of an E-cad-ECD functionalized bilayer to a two-species model. Identical brightness of the two species obtained from the PCH fit, indicated as B1 and B2, shows that E-cad-ECD is present as single oligomeric species. PCH fit of TexasRed in TexasRed-DHPE containing bilayer is shown as a control.

structures and important for junctional localization could, in principle, allow E-cadherin to assemble into higher oligomeric states (34). FCS measurements of E-cad-ECD on supported lipid bilayers suggest a single diffusive species (Fig. 1*D*). However, the time autocorrelation function analysis used in FCS only provides average mobility information; it does not provide specific information on the distribution of oligomeric states. To resolve this, we performed photon counting histogram (PCH) analysis of the fluorescence intensity fluctuations. We have found PCH, which examines the time-independent brightness distribution of diffusing species, to be an accurate and reliable measure of stoichiometric heterogeneity of proteins on supported lipid bilayers (44). PCH measurements confirmed a single species of E-cad-ECD on the bilayer (Fig. 1*E*). The extremely low affinity of the *cis* interaction in solution ($K_d > 1$ mM in solution) and the formation of a continuous array of *cis*-interacting molecules seen in crystal structures and validated by mutagenesis (10, 34) essentially rule out the possibility that the single species found here is a dimer (45). Thus, E-cad-ECD is a monomer in the absence of *trans* interaction at surface densities similar to those observed in cells (41).

Interaction of Cells with E-cad-ECD Functionalized and Highly Fluid Supported Lipid Bilayers. We used the MKN-28 epithelial cell line to study E-cadherin junction formation in hybrid live cell-supported lipid bilayer format (Fig. 2*A*). These cells form closely packed colonies in culture and endogenously express E-cadherin and other crucial cell–cell adhesion proteins (46) (Fig. S1 *A* and *B*). In most cases, cells seeded on bilayers containing E-cad-ECD molecules showed no enrichment of the fluorescently labeled E-cad-ECD molecules attached to the bilayer. E-cad-ECD displayed on highly fluid surfaces ($D \geq 1$ μm^2/s) at physiological densities is essentially not competent for adhesion (see *SI Text* for other control experiments and Fig. S2).

However, in less than 1% of cases, strong enrichment and extensive junction formation was observed (Fig. 2*B*, Fig. S3. and Movie S1). Notably, weak or significantly impaired junctions were not observed. The process is essentially all or nothing: Once junction formation is successfully initiated, it goes to completion. This observation suggests that some sort of nucleation event is essential, but is not well reconstituted with the highly fluid supported membrane. We note that adhesion observed in early studies on live cells (26) or giant unilamellar vesicles (47, 48) and supported membranes did not resemble the extensive junction formation we report here, and we suggest that those systems failed to achieve the previously unknown nucleation step described here.

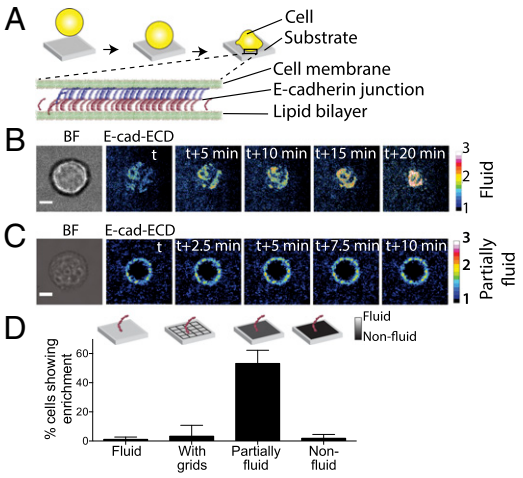

Fig. 2. E-cadherin mediated cell-bilayer junction formation. (*A*) Schematic representation of E-cadherin-mediated hybrid cell–bilayer junction formation. (*B* and *C*) Representative time series of epifluorescence images of E-cad-ECD showing enrichment of E-cad-ECD by MKN-28 cells on either fluid (*B*) or partially fluid (*C*) bilayers. Color bars represent fold increase in E-cad-ECD surface density. (Scale bar, 5 μm.) (*D*) Percentage of MKN28 cells showing E-cadherin enrichment on bilayers with different properties. Values represent mean ± SD from multiple experiments. *Inset* is a schematic representation of bilayer fluidity in gray scale.

E-cadherin Junctions Form Efficiently with Low-Mobility Supported Lipid Bilayers. Unlike the supported bilayer system where E-cadherin exhibits high diffusive mobility, E-cadherin on the surface of live cells exhibits reduced mobility, with a fraction of molecules confined laterally (15, 43, 49). We, therefore, attempted to systematically test if altering E-cad-ECD mobility on bilayers could increase the probability of junction nucleation and formation.

We manipulated the mobility of E-cad-ECD in supported lipid bilayers in two different ways. In the first approach, supported lipid bilayers were formed on substrates that were prepatterned with grids of metal lines ranging from 0.5 μm to 4 μm in spacing (7, 8, 50). In this configuration, local mobility of the E-cad-ECD within individual corrals is unchanged, while long-range transport across substrate-imposed barriers is blocked. However, confining E-cad-ECD on metal grids failed to induce cells to efficiently form junctions (Fig. 2*D*).

In the second approach, membrane composition was manipulated to uniformly increase the average viscosity of the membrane. E-cad-ECD displayed on low-mobility bilayers prepared using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) doped with either 0.1 or 1 mole % acyl chain labeled 1-acyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) and 4 mole % 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] nickel salt (Ni-NTA-DOGS) enabled cells to form junctions efficiently (Fig. 2 *C* and *D*). The bulky fluorescent NBD group at the C12 position in the acyl chain of the phosphatidylcholine is known to disrupt molecular ordering, and thus reduce the melting temperature of DPPC bilayers (51); these membranes are not in a fully gel phase at the experimental temperature (37 °C) and are not expected to be phase separated due to the low density of Ni-NTA-DOGS used. FRAP experiments on these bilayers allowed an estimation of the diffusion coefficient of 0.04 μm^2/s for the NBD-PC lipid molecules (Fig. S4). However, E-cad-ECD on the same bilayer did not show a significant recovery even after 30 min of bleaching (Figs. S4 and S5), indicating an even lower effective mobility, which may overlap with the range of reported apparent diffusion coefficients of E-cadherin on live cell membranes (0.002–0.036 μm^2/s) (15–17). It is important to realize that for membrane systems with such low mobility, the diffusion coefficient is not generally a well-defined physical parameter. Clustering, frictional coupling, and interactions with fixed obstacles all contribute to the effective mobility (52–59). We note that although E-cad-ECD on these bilayers does not show diffusive motion, they are not immobilized on the surface. Thus, application of force by living cells resulted in some rearrangement of E-cad-ECD on these bilayers, which was not seen with bilayers containing only DPPC (gel state under experimental conditions) and 4 mole % Ni-NTA-DOGS at 37 °C (Fig. 2*D*). The difference in the morphologies of E-cadherin junctions formed on these and the fluid DOPC bilayers (see Fig. 2*B*) was due to the difference in the diffusive mobility of the *trans*-interacting molecules. That is, when cells manage to start a junction on a fluid membrane, the already adhered cadherin continues to move around freely and, therefore, fill the interior of the junction (Movie S1).

Reduction of the intrinsic mobility of E-cadherin creates two distinct physical effects. First, any actively driven transport of cadherin is met by larger opposing forces (*SI Text*) (60), and thus could conceivably activate a force-sensitive catch bond or mechanosensory system. Single-molecule experiments suggesting

force-dependent catch bond formation by a mutant E-cadherin that forms the intermediate X-dimer have been reported; however, no such catch bond has been observed for the wild type E-cadherin construct used in the experiments we report here (61). Second, the timescale for diffusive dissipation of any localized concentration of cadherin is elongated. As will be detailed below, this is directly visible in our experiments. Thus, local concentration-mediated kinetic nucleation of stable intermembrane E-cadherin interaction appears to play a role in junction formation.

Both cellular E-cadherin and α-catenin were found to colocalize with enriched zones of E-cad-ECD on the bilayer, and successful junction formation required Ca^{2+} (Fig. 3 *A* and *B*). Phalloidin staining of cells revealed a cortical actin ring around zones of E-cad-ECD enrichment, which is a characteristic of adherens junctions between living cells (62) (Fig. 3*E*). Similar results were observed in the rare cases where cells formed junctions on fluid bilayers, suggesting that once nucleated, the cadherin junction is no longer sensitive to membrane lipid mobility (Fig. S6*C*).

Active Cytoskeletal Processes Are Required for Cell–Cell Junction Formation. Active filopodia extension and retraction were clearly visible by reflection interference contrast microscopy (RICM) during junction formation on the supported bilayers. Additionally, localized concentrations of E-cad-ECD occurred under these filopodia and could be seen to move back toward the cell periphery along with filopodia retraction (Fig. 4*A* and Movie S2). Pretreatment of cells with a Cdc42 inhibitor (ML 141) resulted in a loss of filopodia and the E-cad-ECD enrichment (Fig. 4*B* and Fig. S6*D*). Pretreatment of cells with blebbistatin, which is an inhibitor of myosin II, or with antimycin A in combination with 2'-deoxy glucose, which deplete ATP, also reduced the frequencies of cells forming junctions (Fig. 4*B*). In contrast, treatment of cells with calyculin A (63), a phosphatase inhibitor that increases the cellular actomyosin contractility (64), resulted in an increase in the frequency of successful junction formation (Fig. 4*B*).

The Intracellular Domain of E-Cadherin, but Not the *cis* Interaction, Is Essential to Initiate Junction Formation. Structural studies have revealed a *cis* interface between the E-cadherin monomers from the same cell, and ablation of the *cis* interaction by site-directed mutagenesis (V81D L175D) has been found to significantly alter the ordering and dynamics of E-cadherin clustering and

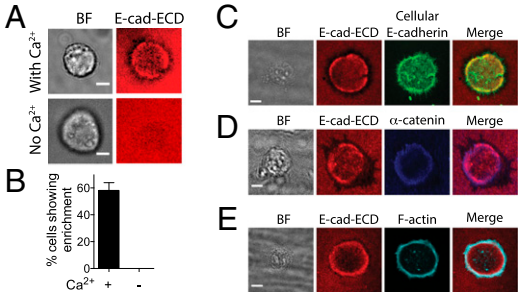

Fig. 3. Characterization of hybrid cell–bilayer junctions. (*A*) Bright field images of MKN28 cells seeded on partially fluid bilayers functionalized with E-cad-ECD in the presence or absence of Ca^{2+} and epifluorescence images of E-cad-ECD on bilayers showing the formation or lack of a junction. (*B*) Percentage of MKN28 cells showing E-cad-ECD enrichment on partially fluid bilayers in the presence or absence of Ca^{2+}. Values represent mean ± SD from multiple experiments. (Scale bar, 5 μm.) (*C*–*E*) MKN-28 cells forming junctions on partially fluid bilayers were either immunostained for cellular E-cadherin using an antibody raised against the intracellular part of E-cadherin (*C*) and an antibody against α-catenin (*D*) or stained with phalloidin (*E*). Cellular E-cadherin and α-catenin colocalize with E-cad-ECD molecules on bilayer whereas F-actin is remodeled to form a ring around the hybrid junction. (Scale bar, 5 μm.)

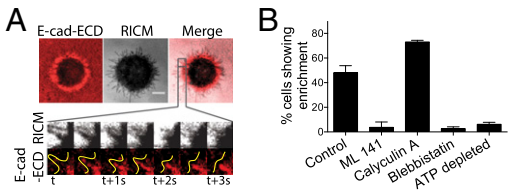

Fig. 4. E-cadherin clustering and junction formation is an actomyosin-dependent process. (*A*) Epifluorescence and RICM imaging of the cell shown in Fig. 2*C* revealing a tight contact with the bilayer around the zone of E-cad-ECD enrichment. Zoomed-in view of a retracting filopodia shows clustering of E-cad-ECD molecules. A trace of the retracting filopodia is overlapped on the epifluorescence image of E-cad-ECD on the bilayer. Numbers indicate time in minutes, with initial time assumed to be *t*. (*B*) Percentage of MKN28 cells, either untreated or treated with ML 141, calyculin A, blebbistatin, or a combination of antimycin A and 2′-deoxy glucose, showing enrichment of E-cad-ECD on partially fluid bilayers. Values represent mean ± SD from multiple experiments. (Scale bar, 5 μm.)

localization of E-cadherin to adherens junctions (34, 35). To test the role of the *cis* interface in the nucleation of E-cadherin, we used the cadherin-negative variant of the E-cad expressing A-431 epithelial cells, denoted A-431D (34, 65). A-431D cells expressing the *cis*-mutant E-cadherin seeded on low-mobility bilayers functionalized with the *cis*-mutant E-cad-ECD showed a similar frequency of junction formation as was found with A-431D cells expressing wild-type E-cadherin on wild-type E-cad-ECD functionalized low-mobility bilayers (Fig. 5*A* and Fig. S6*E*), suggesting that the *cis* interaction is not necessary for the formation of junction-like structures in these bilayer assays. We note that this lack of difference in the frequency of junction formation is not a result of a difference in the expression levels of the wild-type and *cis*-mutant E-cadherin, as these cells express the proteins at similar levels (35).

The intracellular domain of E-cadherin both regulates various signaling proteins by its multiple interaction sites and forms a linkage with the actin cytoskeleton through β- and α-catenins, as well as other proteins (42, 66–70). This link has been suggested to stabilize cadherin *cis* interactions, thereby stabilizing the entire cadherin adhesive structure (35). However, the intracellular domain is not essential for the formation of some junction-like structures as cells expressing only the extracellular domain form such structures, which depend on both the *cis* and *trans* interaction interfaces of E-cadherin (43, 65). How exactly the intracellular domain of E-cadherin and its interaction with the cytoskeleton cooperate with the extracellular domain of E-cadherin in the process of junction formation is not completely understood.

We tested the role of the intracellular domain of E-cadherin in junction formation by comparing wild-type E-cadherin with its cytoplasmic domain-deleted mutant. Unlike A-431D cells expressing wild-type E-cadherin that formed junctions, A-431D cells expressing the intracellular domain-deleted E-cadherin with an intact extracellular domain failed to form junctions on partially fluid bilayers (Fig. 5*A* and Fig. S6*E*) (34, 65).

The fact that the intracellular domain-deleted E-cadherin failed to form junctions raised the possibility that E-cadherin engagement with the actin cytoskeleton is required for junction formation. It is possible that the low frequency of junction formation on the fluid bilayers is caused by inability of cells in this condition to appropriately interconnect cadherin with the actin cytoskeleton. To test this possibility, we characterized junction formation by MKN28 cells expressing a fusion protein containing the actin-binding domain of α-catenin fused to the C terminus of E-cadherin (Fig. S6*G*). This strategy has been used previously to constitutively link E-cadherin to the actin cytoskeleton (35, 71). However, even such direct engagement of E-cadherin with the

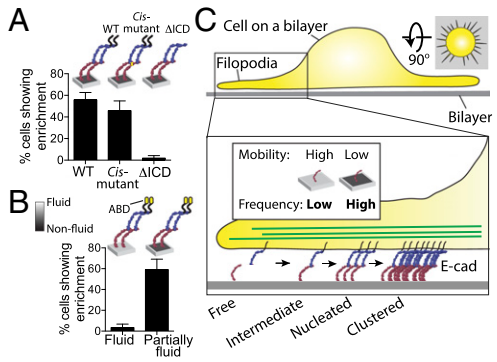

Fig. 5. Mechanism of E-cadherin junction formation. (*A*, *Bottom*) Percentage of A-431D cells expressing wild-type, *cis*-mutant, or intracellular domain-deleted E-cadherin showing enrichment of fluorescently labeled E-cad-ECD on partially fluid bilayers functionalized with wild-type, *cis*-mutant, or wild-type E-cad-ECD, respectively. (*Top*) Schematic representation of junction formation by wild-type, *cis*-mutant, or intracellular domain-deleted E-cadherin on partially fluid bilayers. (*B*, *Bottom*) Percentage of MKN28 cells expressing E-cadherin fused with the actin-binding domain of α-catenin showing E-cad-ECD enrichment on fluid or partially fluid bilayers functionalized with the wild-type E-cad-ECD. (*Top*) Schematic representation of junction formation by E-cadherin fused to the actin-binding domain of α-catenin on fluid and partially fluid bilayers. Values represent mean ± SD from multiple experiments. (Scale bar, 5 μm.) (*C*) Schematic representation of E-cadherin junction formation on a supported lipid bilayer involving filopodia retraction-mediated local increase in E-cad-ECD concentration.

actin cytoskeleton did not increase the frequency of junction formation on fluid bilayers (Fig. 5*B* and Fig. S6*F*).

Conclusion

Based on the composite of observations described above, we propose the following mechanism for E-cadherin junction formation in the hybrid live cell-supported membrane system (Fig. 5*C*). Junction formation is an active process requiring actomyosin-driven extension and retraction of filopodia. Cellular E-cadherin molecules interact with the actin cytoskeleton via the intracellular domain and are enriched in the dense F-actin networks present in the filopodia (70). E-cadherin molecules present on the surface of the filopodia interact with bilayer bound E-cadherin, resulting in the formation of the intermediate cadherin–cadherin bond (36). Localized increase in the concentration of the cadherin is achieved as bound cadherin are dragged back, and laterally compressed, with the retracting filopodium. On a liquid-disordered bilayer displaying high-mobility E-cadherin, this localized concentration of the E-cadherin dissipates too quickly by diffusion, even in the presence of an intact *cis* interaction interface, to enable development of the stable strand-swapped dimer needed for stable *trans* interactions. In contrast to this, local enrichments of E-cad-ECD in low-mobility supported membranes are dissipated much more slowly, thus enabling efficient formation of the stable strand-swapped dimer and clustering. A longer interaction time achieved in these low-mobility bilayers may allow E-cadherin to convert to the stable, strand-swapped *trans* dimer in a way that was seen in single-molecule force spectroscopy experiments (61). However, failure of the cells to form junctions when filopodia formation is inhibited (Fig. 4*B*) suggests that this may not be the sole factor controlling the frequency of cell–bilayer junction formation. Kinetic stabilization provided by the low-mobility supported membrane is sufficient to overcome the need for *cis* interactions. However, the junctions formed with the *cis*-mutant E-cadherin may not be assembled into 2D lattices as seen in cell–cell interfaces but, rather, may represent a concentrated assembly driven entirely by *trans* interactions. We speculate that subtle differences in the mobility of free cadherin on the cell surface, along with competition from other cell surface molecules, may create a reaction environment where the added effects of *cis* interactions are required for junction formation.

Materials and Methods

Full details are available in *SI Materials and Methods*. Briefly, wild-type or V81D L175D *cis*-mutant (34) E-cad-ECD (domains EC1–5 comprising residues Asp1 to Asp533 of the mature, furin-processed protein) engineered to contain a single cysteine residue were expressed and purified from HEK293 cells, and site-specifically labeled with fluorescent dye to observe junction formation. All bilayers were prepared using vesicle fusion method (40). Bilayers were functionalized with proteins via Ni-NTA-poly-His chelation (37). FRAP was performed by illuminating a small field of view (15–30 μm diameter) at high intensity followed by continued imaging to observe recovery of fluorescence. FCS was performed by illuminating a diffraction-limited spot using a pulsed super continuum laser (SuperK Extreme; NKT Photonics) bilayers through a 100× oil objective, and data were analyzed using homebuilt codes in Matlab. Hybrid cell–bilayer junctions were reconstituted by seeding cadherin-expressing cells on bilayers, and enrichment of E-cad-ECD fluorescence on the bilayer was observed by epifluorescence. Frequency of junction formation was determined manually. Substrates with chromium grids were prepared by nanoimprinting (7). Diffusion of E-cad-ECD on bilayers was reduced by changing the constituent lipid compositions from DOPC to DPPC+NBD-PC. Cells were stained with specific antibodies or phalloidin and imaged by confocal microscopy to confirm the composition of junctions. Cells were treated with ML 141 to inhibit filopodia formation, calyculin A to increase and blebbistatin to decrease actomyosin tension, and a combination of 2′-deoxy glucose and antimycin A to deplete ATP in cells. A431-D cells expressing wild-type, cis-mutant, and intracellular domain-deleted mutant have been previously described (34, 65). FRAP, FCS, and imaging were performed with a 100× objective in an Eclipse Ti inverted microscope (Nikon).

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