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Lateral Mobility of E-cadherin Enhances Rac1 Response in Epithelial Cells

J. Tsai and L.C. Kam

Department of Biomedical Engineering, Columbia University, New York, NY 10027

Abstract

The fluidity of cellular membranes imparts lateral mobility of proteins across the cell surface. To understand the impact of lateral mobility on cell-cell communication, a protein consisting of the extracellular recognition domains of E-cadherin was associated with the surface of silica beads by either tethering to a bead-supported lipid bilayer or direct adsorption, resulting in laterally mobile and immobile presentations of this protein. These beads were then seeded onto the upper surface of MDCK cells. Functional engagement of these beads was compared by measurement of Rac1 recruitment around the bead. Lateral mobility enhanced recognition of E-cadherin, promoting cell response to the beads at lower per-area concentrations than their immobilized counterparts. A more complete understanding of how lateral mobility of membrane-associated proteins influences molecular recognition, and potentially other downstream responses, could provide new strategies for the design of materials and devices intended to capture the architecture of natural tissues.

Keywords

Membrane Diffusion; Epithelial Cells; E-cadherin

INTRODUCTION

Contact-mediated communication between cells involves the engagement of membrane-associated proteins on the apposed surfaces. The biomolecular architecture of these lipid membranes imparts lateral mobility to these proteins, influencing the interaction and dynamics of these signaling molecules over scales ranging from nanometers to micrometers. However, the full functional implications of this mobility remain incompletely understood. In this report, we use a supported lipid bilayer model to identify such effects in the context of E-cadherin signaling in epithelial cells.

The supported lipid bilayer system consists of a planar assembly of phospholipids in close apposition to a silicon oxide support such as glass or quartz ^{2; 3; 15; 28}. A balance of molecular forces maintains a thin (~ 1 - 3 nm) layer of water that separates the bilayer and support ¹⁷, allowing lateral mobility of the membrane biomolecules while constraining this structure to the substrate surface. Tethering of a protein to these lipids imparts lateral mobility to that biomolecule, providing a model of the cell membrane that has been used predominantly in the study of immune synapse structure but also other cellular systems to a limited extent ^{5; 6; 13; 14; 24; 25; 29; 31}. In the context of epithelial cells, the supported lipid bilayer system offers much promise in capturing the dynamics of cell-cell adhesion that normally leads to formation of barrier structures such as adherens junctions. Functional contact between epithelial cells is

initiated by homophilic binding of E-cadherin between apposed cells, inducing cell ruffling and lamellipodial extensions that encourage further interactions between the membranes^{1; 8} and recruit additional E-cadherin to the developing adhesion site. The role of cadherins in this and other signaling events has been studied extensively by putting cells in contact with either other cells, which capture the dynamic nature of this interface but are compositionally complex, or substrate-attached E-cadherin proteins, which allow precise control over the biomolecular makeup but lack the fluidity of natural membranes^{7; 12; 18; 21; 26}. Notably, cells exert forces on substrates through cadherin-based complexes^{11; 20}. Following the concept of rigidity sensing that is well established for integrin-based signaling, it is plausible that the response of cadherin complexes to cell-generated forces, such as motion along the apposed cell membrane or substrate support, modulates downstream intracellular signaling. In this direction, Perez *et al.* developed a micropatterned protein – lipid bilayer system to present laterally mobile E-cadherin to epithelial cells interacting with a planar substrate²⁵. However, this system does not allow control over either the timing or location of E-cadherin engagement, which is important in separating the influences of multiple factors in these experiments. As an alternative configuration, we explore in this report the use of silica beads to present either mobile (membrane-tethered) or immobile (directly adsorbed) E-cadherin to cells pre-seeded on a cell culture surface (Figure 1).

Engagement of E-cadherin initiates a rich set of downstream cell signaling pathways. This report will focus on Rac1, a member of the Rho family small GTPases that holds a particularly central role as both a downstream result of E-cadherin signaling and also upstream effector of cytoskeletal dynamics regulating cadherin function^{4; 9; 10; 18; 22}. Live cell imaging^{8; 22} showed that Rac1 is present at the edges of developing cell-cell contacts, and we will similarly use the accumulation of this protein around the bead-cell interface as a measure of functional engagement.

MATERIALS AND METHODS

Protein design and production

This report makes extensive use of Ecad/Fc, a homodimerized fusion protein consisting of the external domains of human E-cadherin appended with the Fc region of human IgG a short spacer, and a 6-histidine sequence. A linker / poly-His cassette was inserted in place of a GPI tether in a previously described pcDNA3.1A(+) vector²⁵ using standard cloning techniques. The vector was stably transfected into HEK 293 cells (and selected using 400 µg/ml G418), and the resultant Ecad/Fc protein purified from the cell supernatant using a cobalt pull-down resin (Pierce). This protein was stored at 1 mg/ml in HEPES buffered saline (HBS; 10 mM HEPES, pH 7.4, 154 mM NaCl, 7.2 mM KCl) at –80° C. SDS-PAGE / Western Blot analysis revealed a strong band a major at ~120 kDa corresponding to the full-length protein as well as a minor band (<10%) at ~ 130 kDa corresponding to an uncleaved E-cadherin precursor. For visualization and quantification purposes, Ecad/Fc protein was labeled with Texas Red (NHS, Molecular Probes) or Cy5 (NHS, Amersham); protein from a single labeling batch was used for quantitative comparison across surfaces.

Substrate preparation and cleaning

Planar glass coverslips (40 mm-diameter round, Biopetechs) were cleaned by immersion into Linbro 7X detergent (ICN Biomedicals, Inc.) diluted 1:3 (v/v) in deionized water, then baked at 450 °C for 4 h. Silica beads of 5 µm diameter (Bangs Laboratories, Inc.) were used as provided from the manufacturer; baking and cleaning of these beads often resulted in less uniform lipid bilayers, and as such, the cleaning step was omitted.

Liposome preparation, bilayer formation, and protein attachment

Stock solutions of small unilamellar vesicles (SUVs) of 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) (Avanti Polar Lipids) were prepared by extrusion 25³ 32 through 50 nm pore membranes (extruder and membranes from Avanti) at 5 mg/mL in vesicle buffer (VB; 10mM HEPES, 140mMNaCl, 5mMKCl, pH 8.5). For tethering of Ecad/Fc, these vesicles were supplemented with 6% (mol/mol) 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS-NTA). Stock solutions were stored at 4° C and used within 1 week. To form bilayers, stock vesicle solutions were diluted 1 : 1 (v/v) with HBS, then applied to the cleaned coverslips or mixed with the silica beads in suspension for at least 5 minutes. Without letting the surfaces dry, the bilayers were rinsed extensively with HBS. To tether Ecad/Fc, bilayers were first loaded with 1 μ M Ni²⁺ in HBS, then incubated with 20 μ g/ml Ecad/Fc in HBS for 30 minutes. Coverslips / beads were then washed extensively with HBS. Surfaces and beads with tethered Ecad/Fc were used within 3 hours of preparation. To prepare beads with immobilized Ecad/Fc, silica beads were incubated with the Ecad/Fc protein at a concentration of 50 μ g/ml for 30 minutes at 4° C. To create beads with lower concentrations of Ecad/Fc, part of the Ecad/Fc was replaced with bovine serum albumin (BSA), maintaining a total of 50 μ g/ml of protein in the coating solution. The specific amount of BSA to be included was determined by measuring the resultant concentration of Ecad/Fc (see below) as a function of BSA dilution. The diffusion coefficient and mobile fraction of bilayer-tethered, Cy5-labeled Ecad/Fc was measured using established Fluorescence Recovery After Photobleaching (FRAP) methods ³².

Protein quantification

The per-area concentration of surface-associated protein, either tethered onto a bilayer or adsorbed onto a bead, was compared between systems by epifluorescence microscopy of Cy5-labeled Ecad/Fc. Relative comparisons were made between beads using protein from the same labeling batch. To obtain an estimate of absolute protein concentration, the fluorescence intensity of membrane-tethered, Texas Red-labeled Ecad/Fc was calibrated against a lipid bilayer containing specified molar fractions (0.03 – 0.14 mol %); we used 3.3×10^6 molecules / μ m² for the total density of lipids, based on the typical cross sectional area of phospholipids, and thus per-area concentrations, of supported lipid bilayers; these membrane provide a highly uniform and reproducible standards that are of appropriate geometry to compare against the tethered proteins ³⁰. Measurement of protein concentration vs. 568-nm extinction coefficient provides an absolute calibration of this method, which we estimate to be reproducible to within 20%.

Cell culture

MDCK cells stably transfected with both GFP-Rac1 and DsRed-Ecadherin (a gift from W. James Nelson, Stanford University) were cultured in low glucose DMEM supplemented with 10% Fetal Bovine Serum, L-glutamine and penicillin/streptomycin. Cells were maintained using standard cell culture conditions, and split every 3–4 days. The inclusion of these two fluorescently-labeled proteins did not result in any obvious large-scale changes in cell behavior compared to the non-transfected counterparts.

Bead engagement assays

The interaction of bead-associated Ecad/Fc with MDCK cells was monitored using live-cell microscopy approaches, using a Bioptechs FC2 chamber mounted on an Olympus IX70 inverted fluorescence microscope. Coverslips (cleaned, 40-mm borosilicate glass) were mounted in an open-top FC2 and coated with 10 μ g/ml collagen I (rat, Sigma) for 10 minutes. The collagen solution was removed, and the substrates sterilized by exposure to UV light for

2 hours (until dry). The surfaces were rinsed extensively with phosphate-buffered saline (PBS, pH 7.4, Invitrogen).

GFP-Rac1 / DsRed E-cadherin – transfected MDCK cells were seeded at 1×10^6 cells/mL (8×10^3 cells/mm²) onto these surfaces for 1 hour in DMEM supplemented with 10% FBS to promote spreading. Prior to introducing beads, media was replaced with warm (37°C) phenol red- and serum-free DMEM w/ HEPES (no bicarbonate), and the chamber mounted on the microscope. Beads were then seeded onto these cells, and imaged 20 minutes later.

Microscopy

All microscopy was carried out in epifluorescence mode, using an Olympus IX71 inverted microscope. A 100x, 1.45 NA, oil-immersion objective was used throughout this study; this high magnification was required to resolve the interaction of cells with these small-diameter beads. A Hamamatsu C9100-02 EMCCD camera was used for recording of images.

RESULTS

Mobility of membrane tethered Ecad/Fc

To capture the lateral mobility of E-cadherin on cell surfaces, Ecad/Fc (containing a poly-His tail) was tethered to supported lipid bilayers containing Ni/NTA. Five-micrometer beads were chosen as a bilayer support for presentation to cells, given the control over the initiation of Ecad/Fc contact with the cell provided by this system (Figure 1). However, it is difficult to accurately measure the diffusive properties of Ecad/Fc in this format. Instead, mobility was first characterized on planar coverslips, using an image-based Fluorescence Recovery After Photobleaching (FRAP) technique we described earlier³². Figure 2a illustrates a bilayer presenting tethered, Cy5-labeled Ecad/Fc immediately and 20-minutes after photobleaching of an octagonal spot (defined by an internal aperture) in the middle of the field of view; outside of this spot, the bilayer can be seen to be uniform and free of long-range structure. Recovery of signal in the center of the spot demonstrates the mobility of tethered Ecad/Fc. Quantitative comparison of fluorescence before and after recovery indicated that Ecad/Fc has a mobile fraction (percent of the total signal that is mobile) of typically > 80%. Analysis of a series of images taken at 2 minute intervals³² indicated that tethered Ecad/Fc exhibits a diffusion coefficient of approximately $0.3 \mu\text{m}^2/\text{sec}$, which is less than that associated with lipids in a supported bilayer ($1 \mu\text{m}^2/\text{sec}$), but larger than that associated with E-cadherin on the cell surface undergoing simple Brownian diffusion ($0.01 - 0.1 \mu\text{m}^2/\text{sec}$)¹⁶. We note that an analogous molecule based on the extracellular domain of canine E-cadherin, tethered to Ni/NTA-containing bilayers, was essentially immobile; for this reason, we pair MDCK cells with bilayer-tethered human E-cadherin (and observe functional, cross species activation). To obtain an estimate of the absolute per-area concentration of tethered protein, Texas-Red labeled Ecad/Fc was compared against a set of standards consisting of a series of lipid bilayers supplemented with specific concentrations of Texas-Red labeled lipids³⁰. From this approach, we estimate that this specific bilayer tethering and Ecad/Fc-capture approach yields ~ 300 molecules (dimers) per square micrometer of area.

Mobility of Ecad/Fc on silica beads was examined by partial photobleaching of the bead surface; the geometry and small size of individual beads is incompatible with a well-defined FRAP experiment such as was done on the planar coverslips. Figure 2b compares recovery of fluorescently-labeled Ecad/Fc on bead doublets (which partially alleviate the problems posed by the small size of individual beads) presenting bilayer-tethered (mobile, top row) and directly adsorbed (immobile, bottom). The lower right portion of each bead was photobleached by partially closing the microscope aperture; images of the beads before and immediately after ($t=0$) photobleaching illustrate this process. In addition, a background level of Ecad/Fc, more

evident on the tethered-Ecad/Fc bead owing to the lower concentration of protein relative to directly adsorbed protein (discussed in more detail below), illustrates the edge of the photobleached region. Over the ten minutes following photobleaching, bilayer-tethered Ecad/Fc was observed to diffuse back across the bead (Figure 2b), while the immobile, directly-adsorbed counterpart did not show this recovery. Fluorescence intensity profiles taken before, immediately after, and ten minutes after photobleaching further illustrate this recovery (the green star indicates recovery at the bead edge for the mobile sample). The curved geometry and small dimensions of the beads made accurate estimation of diffusion coefficient on these beads impractical, and we adopt the values obtained using the planar surfaces.

Recruitment of Rac1

Functional engagement of E-cadherin was evaluated on the basis of recruitment of Rac1 to the bead-cell interface. To minimize potential disruption of this interface (as would be incurred by fixation and immunohistochemical staining), recruitment was measured using GFP-tagged Rac1. MDCK epithelial cells were dual-transfected with vectors encoding this protein as well as DsRed-E-cadherin, both of which have been independently described^{8; 23}. As illustrated in Figure 3a (top row), presenting mobile, membrane-tethered Ecad/Fc to MDCK cells resulted in significant recruitment of both Rac1 and cellular E-cadherin to the site of engagement. These rings were apparent within 5 minutes after introduction of the beads, and stable by the 15 minute timepoint illustrated in Figure 2.

To test the influence of mobility in promoting effective engagement, Ecad/Fc was immobilized onto beads by direct adsorption; the use of a poly-His-tagged Ecad/Fc was chosen to allow mobile (tethered) and immobile (adsorbed) preparations using the same protein construct. Directly-adsorbed Ecad/Fc exhibited qualitatively similar recruitment of Rac1 and E-cadherin to the sites of bead / cell interaction, as illustrated in the second row of Figure 3a. However, comparison of Cy5-labeled Ecad/Fc indicated that the surface concentration of this protein directly adsorbed to the silica beads is 4 times higher than that of Ecad/Fc tethered to bead-supported bilayers, corresponding to ~ 1200 molecules / μm^2 . To more directly compare cell response, the surface concentration of adsorbed Ecad/Fc was controlled by diluting this protein with bovine serum albumin; a 3 : 1 mass ratio of BSA : Ecad/Fc resulted in a surface concentration of ~ 300 molecules / μm^2 , similar to that tethered to the Ni/NTA containing bilayers.

In contrast to bilayer-tethered proteins, MDCK cells lacked functional engagement of beads containing immobile, adsorbed protein of similar surface concentration (low, last row of Figure 3a). Thus, lateral mobility of E-cadherin-based proteins reduces the concentration required to achieve functional engagement, and is thus more active than their immobile counterparts.

Figure 4 shows a quantitative comparison of Rac1 recruitment. The basal level of GFP-Rac1, as well as DsRed-E-cadherin, varied from cell to cell. As such, we normalized the average intensity observed in the area adjacent to each bead (just outside the dark void region corresponding to the beads), corresponding to the rings illustrated in Figure 3A, against that measured on an open surface of the cell just outside these rings (Figure 4a). This is reported as percentage increase in Figure 4b. In agreement with the qualitative data of Figure 3, beads presenting mobile Ecad/Fc induced a significant recruitment of Rac1. Beads presenting a high concentration of immobilized Ecad/Fc promoted a similar amount of recruitment, indeed a level that was statistically similar to that attained with the mobile counterpart. Beads presenting a lower concentration of Ecad/Fc which is similar to that presented on bilayers (approximately 300 molecules / μm^2) exhibited a lower level of Rac1 recruitment. As a further control, beads presenting lipid bilayers of DOPC alone (offering no site for Ecad/Fc tethering) were exposed to cells, and exhibited no recruitment of Rac1.

DISCUSSION

Cell-cell communication proteins such as cadherins have important roles in coordinating the assembly of cells into functional tissues. Understanding how these cells function offers new approaches for the design of bioactive materials. Beyond the biomolecular aspects of these proteins, a clearer understanding of how the biophysics of their presentation modulates cell signaling will provide new strategies for developing such devices. In this report, we provide a new demonstration that lateral mobility of E-cadherin proteins enhances recognition, allowing functional activation of the downstream signaling processes at lower surface concentrations than their immobile counterparts.

To better understand the role of surface concentration in this response, we imaged the distribution of Cy5-labeled, membrane-tethered Ecad/Fc. The distribution of Ecad/Fc tethered to bilayer-bearing beads that missed landing on the cell (illustrated by the one in region 1 of Figure 3B) was compared to those that landed on a cell (illustrated by region 2) from z-series stacks taken at 0.5 μm intervals starting at the cell-coverslip interface. For beads that did not interact with the cell, fluorescence intensity was uniform; Ecad/Fc was evenly distributed around the bead in any given slice. However, the intensity of fluorescence decreased with distance from the surface, likely owing to the mismatch in refractive index between the bead and media as well as photobleaching of the Ecad/Fc protein. At the cell-bead interface, limited to the first 1–2 μm of cell-bead contact, Ecad/Fc recruitment was compared using the average intensity of Ecad/Fc fluorescence across 2 μm -wide side-projections of beads that included the bead diameter (and thus the bottom of the cell-bead interface), as illustrated in Figure 3b. Recognizing the decrease in fluorescence associated with distance in the stacks, and that overall intensity varied from bead to bead, the fluorescence intensity at the bottom of each bead (the cell-bead interface) was normalized to that measured at the bead diameter, half-way up the bead. This measure of concentration was higher for beads contacting cells (1.4 ± 0.23 , mean $\pm$ s.d., $n = 6$ beads) than those off the cells (1.08 ± 0.18 , $n = 6$ beads), corresponding to a 33% increase in concentration in the cell-bead interface, much less than the four-fold difference associated with the immobilized protein beads used in this report. However, additional structure was observed at smaller spatial scales. Top-down, average projections of the cell-bead interface (Figure 3b) showed that Ecad/Fc organized into small clusters, the dimensions of which we could not determine finer than the diffraction limit of our microscope (roughly half a micrometer at the Cy5 wavelength). The possible role of sub-resolution clustering is of particular interest given the development of the hop diffusion hypothesis^{19; 27}, which proposes that the cell cytoskeleton selectively modulates long-range diffusion of membrane proteins. Emerging methods, such as the use of nanopatterned surfaces to capture this non-Brownian motion³² in conjunction with high-resolution microscopy, will provide new insight towards identifying the role of spatial effects and lateral mobility in cell-cell signaling.

To the design of biomaterials, this emerging field will provide new engineering guidelines. Control over the micro-/nano-scale topology and dynamics of biomolecules presented on a material surface, through careful polymer design or patterning, may provide improved replication of cell-cell communication, improving the performance of these systems.

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Figure 1. The influence of ligand mobility on cell response was tested by presenting to cells Ecad/Fc either tethered to a lipid bilayer (mobile) or directly adsorbed (immobile) onto silica beads.

Figure 2.

Lateral mobility of tethered Ecad/Fc protein. **(a)** FRAP of Ecad/Fc tethered to a supported lipid bilayer formed on a planar coverslip. **(b)** Corresponding experiment carried out on a series of silicate beads bearing bilayer-tethered (mobile) or directly adsorbed (immobile) Ecad/Fc. For the series illustrating a bead with mobile Ecad/Fc, background-adsorbed protein allows visualization of the edge of the FRAP region. The intensity profiles presented in the last column show the intensity of Ecad/Fc along the yellow dotted lines indicated in the prebleach image for each type of bead.

Figure 3.

Laterally mobile E-cadherin is more effective at inducing Rac1 recruitment than its immobile counterpart. **(a)** Cell interaction with Ecad/Fc-presenting beads 15 minutes after engagement. The method of presentation and estimated surface concentration of Ecad/Fc dimers is indicated at the top of each column. Accumulation of Rac1 around the site of interaction was interpreted as functional E-cadherin engagement. The bottom row highlights the regions delineated by yellow squares in the top row; yellow arrowheads help to further highlight individual interactions of beads with the cells. **(b)** Local clustering of laterally-mobile E-cadherin. The top image illustrates the interaction of beads presenting bilayer-tethered, Cy5-labeled Ecad/Fc to MDCK epithelial cells; the red signal (DsRed-Ecad) is shown to indicate cell position. The lower images illustrate the distribution of bilayer-tethered, Cy5-labeled Ecad/Fc in both top and side projections; region 1 shows a bead not encountering a cell, while region 2 illustrates a pair of beads that engaged the cell surface. The yellow lines indicate the center of a 2 μm -wide region containing the side projection.

Figure 4.

Recruitment of GFP-Rac1 as a function of mobility and surface area concentration. **(a)** The average fluorescence intensity of Rac1-GFP within a narrow band surrounding a bead was normalized by the average fluorescence in a nearby reference region on the cell surface. **(b)** Comparison of Rac1 recruitment as a function of bead preparation. Approximate concentrations of Ecad/Fc (in molecules per square micrometer) are shown in parentheses. * $P < 0.05$ compared to beads containing a high concentration of Ecad/Fc; two-way ANOVA. Data are mean $\pm$ s.e.m., representing > 25 beads per independent experiment, collected from > 4 cells in each experiment. Data collected over 4 independent experiments for beads presenting Ecad/Fc and two independent experiments for DOPC.