Fabrication of a dual substrate display to test roles of cell adhesion proteins in vesicle targeting to plasma membrane domains

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Abstract While much is known of the molecular machinery involved in protein sorting during exocytosis, less is known about the spatial regulation of exocytosis at the plasma membrane (PM). This study outlines a novel method, dual substrate display, used to formally test the hypothesis that E-cadherin-mediated adhesion directs basolateral vesicle exocytosis to specific sites at the PM. We show that vesicles containing the basolateral marker protein VSV-G preferentially target to sites of adhesion to E-cadherin rather than collagen VI or a control peptide. These results support the hypothesis that E-cadherin adhesion initiates signaling at the PM resulting in targeted sites for exocytosis.

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1. Introduction

Many cellular processes display evidence of plasma membrane polarization, for example, the orientation of membranes in migrating cells, or the sorting of cellular contents to daughter cells during asymmetric division [1]. Cell polarization requires mechanisms that sort, target and incorporate membrane proteins in different membrane domains. The molecular machinery responsible for targeted exocytosis in polarized epithelial cells has begun to be delineated [2]. Indirect evidence indicates that E-cadherin-mediated cell–cell adhesion plays a role in initiating a signaling cascade that results in basolateral vesicle targeting and fusion at sites of cell–cell contact [1,3,4]. There has been no direct test, however, of the hypothesis that E-cadherin-mediated adhesion directs basolateral vesicle targeting to specific sites in the plasma membrane. This optically limits the spatial resolution of imaging vesicle fusion events along the plasma membrane. Serial reconstructions of horizontal optical (confocal) sections can be used for imaging, but this severely limits temporal resolution of vesicle delivery and results in decreased spatial resolution as vesicles move in and out of different optical sections [5]. These caveats have restricted high-resolution, real-time imaging of vesicle trafficking and fusion at sites of E-cadherin-mediated cell–cell adhesion.

The plasma membrane involved in cell–cell adhesion can be spatially re-oriented by inducing cells to adhere to E-cadherin bound to a planar surface (Fc-E-cadherin); the N-terminal of E-cadherin can be correctly orientated on the planar surface to enable proper homophilic trans interactions with endogenous E-cadherin on the cell [6]. In this way, the cell is essentially “flipped” on its side for high spatio-temporal resolution by total internal reflection fluorescence (TIRF) microscopy (Fig. 1). We have adapted this method further to test the specificity of basolateral vesicle targeting and fusion between different adhesive surfaces.

To this end, we designed a micropatterned protein array containing two of the following: an extracellular matrix protein that induces non-cadherin-mediated adhesion (collagen VI), combined with either E-cadherin (Fc-E-Cadherin) or a non-adherent control (Fc protein). This newly-developed hybrid technique, called dual substrate display (DSD), combines micro-contact printing [7] and Fc-E-cadherin display technology [6]. Thus, DSD permits in the same cell a comparative analysis of vesicle targeting between areas of integrin-mediated adhesion to collagen VI and areas of E-cadherin-mediated adhesion. Here, we describe how to prepare DSDs, and a series of experiments using DSD in combination with TIRF-microscopy to formally test the hypothesis that E-cadherin-mediated adhesion directs basolateral vesicle targeting to the plasma membrane.

2. Materials and methods

2.1. Dual substrate display

The methodology for the DSD technique is outlined in Fig. 1; in brief, glass coverslips are thoroughly cleaned and then a reactive surface is generated by silanization, a labeled protein is stamped onto the reactive surface, and a second labeled protein attached to the remaining activated surface. The details are as follows. Borosilicate 22 x 22 mm glass coverslips (VWR Scientific) were cleaned sequentially in hot 2% (v/v) NP-40 detergent solution, 3% (v/v) Nochromix (Godax Labs)/concentrated sulfuric acid solution, and 1:1 methanol:saturated

Abbreviations: DSD, dual substrate display; TIRF, total internal reflectance fluorescence; PDMS, polydimethylsiloxane; PM, plasma membrane

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KOH solution. The cleaned coverslips were dipped in 100% methanol, and then silanized in a 4% (v/v) solution of N-(2-aminoethyl)-3-amino-propyl-trimethoxy-silane (TCI America) in 95% methanol under nitrogen gas. The silanized coverslips were cured for 24–72 h at 30 °C.

Collagen VI (Sigma) and purified Fc-E-cadherin fusion protein [6] were labeled with Cy 3.5 dye and Cy 5.5 dye (Amersham), respectively, according to manufacturer’s directions. Aliquots were flash frozen and stored at −20 °C at a final working concentration of 500 μg/ml (collagen VI) and 200 μg/ml (Fc-E-cadherin) in 0.01 M phosphate buffer.

Synthesis of the silicon wafer caste was carried out at the Stanford Nanofabrication Facility as outlined previously [8]. Patterns were generated with an Autocad computer program, which were subsequently etched onto a chrome-on-quartz mask using electron beam lithography. Four-inch silicon wafers were vapor-primed with hexamethyl disylazane (HMDS), an adhesion promoter, before being spin-coated with Shipley 3612 Photoresist at a depth of 1 μm. Coated wafers were exposed to UV radiation through the chrome-on-quartz mask, and then developed for 90 s in tetramethyl ammonium hydroxide, and chemically etched. Upon rinsing the remaining Photoresist away, the pattern of interest remains on the wafer. The wafer was subject to a final vapor primed coat with HMDS to allow ease of removal of the elastomer in the following step. From this wafer caste a poly(dimethylsiloxane) (PDMS) elastomer mold was formed by baking liquid Sylgard 184 (Dow Corning) in a 60-cm cell culture dish in which the wafer has been taped face up for 30 min at 60 °C. One-centimeter square “stamps” were cut out of the PDMS elastomer mold, oxidized for 30 s using a plasma sterilization unit (Harrick Scientific), and then coated with a 15 μl droplet of Cy3.5-collagen VI for 20 min. Each stamp was dried with a nitrogen gas stream, and placed in contact with a previously cleaned and silanized quartz coverslip (Fig. 1). The coverslip and stamp were inverted, and a 40 g weight was used to apply pressure to the coverslip for 30 min, to facilitate transfer of the collagen to the silanized surface.

The remaining preparation of the Fc substrate was identical to that reported previously [6]. Micropatterned silanized coverslips were incubated with fresh sulfo-NHS-biotin (50 mM in DMSO; Pierce) for 2 h in the dark at room temperature. This results in the covalent reaction between the free amines on the exposed areas of silane (i.e., those areas not masked by ECM) and the sulfo-NHS-biotin. Coverslips were sequentially incubated with Neutravidin (5 mg/ml in PBS; Pierce), biotinylated protein A (0.3 mg/ml in PBS; Pierce), dd-Biotin (15 mM in DMSO; Pierce), and Cy5.5-labeled purified Fc-E-cadherin fusion protein (200 μg/ml; [6]) for 1 h each at room temperature in the dark, with 3 × PBS washes between each incubation. Where appropriate, purified IgG heavy chain (Fc; 200 μg/ml; Sigma) was substituted in the final step in place of Fc-E-cadherin protein as a negative (non-adhesive) control.

2.2. Cell culture

A Madin Darby canine kidney (MDCK) IIG cell line stably expressing VSVG-YFP, a marker for basolateral exocytosis, was generated using Lipofectamine 2000 (Invitrogen) transfection. Fluorescent Activated Cell Sorting flow cytometry, followed by standard cell cloning selection procedures. The cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum, 1% PSK, and 400 μM G418 at a very low density to
maintain a “contact-naïve” phenotype and prevent cell polarization. Cells were trypsinized briefly, rinsed in PBS, and allowed to spread for 3 h on the patterned DSD surface before imaging. Cells were imaged in DMEM without phenol red supplemented with 25 mM HEPES-buffered-saline.

2.3. Total internal reflectance fluorescence imaging

Images were acquired at 37 °C on an Olympus microscope (IX-70, Olympus America Inc.) using a high numerical aperture lens (Apo-chromat 60×, NA 1.45, Olympus) with an evanescent field with a decay length of less than 100 nm. The cells were first photobleached for 60 s.
to remove existing plasma membrane fluorescence. Time-lapse sequences of the 488-nm wavelength channel were acquired at 8.5–11 frames/s. A single reference image was collected from the DIC and Cy3.5 channels for subsequent correlation later. Images were acquired with ≥2 × 2 binning, using a 12-bit cooled CCD camera (Hamamatsu); the camera was controlled using a script written in OpenLab software. Inverse monochrome images were exported as 8-bit TIFF files, and imported into ImageJ (NIH) for analysis. Image stacks were subjected to Kalman filtering if necessary prior to scoring for targeting events to reduce the shot noise from the camera.

2.4. Event scoring and statistics

Time-lapse image stacks were scored for “targeting events”, which were defined as punctate structures that appeared and persisted for longer than 1 s (8–11 frames) before vanishing (see Fig. 2a). Events were scored and their x-y coordinates recorded upon the disappearance of the vesicle fluorescence. After scoring all of the events over a given time frame (45–70 s), the x-y coordinates for all events were imported into Microsoft Excel for analysis and graphical display. Next, the coordinates for six 16 μm² areas each of the collagen IV, and either Fc or Fc-E-Cadherin were measured and imported into Microsoft Excel. A script was written into Microsoft Excel for automatically binning events on areas of collagen VI, Fc, or Fc-E-cadherin (Fig. 2b). The number of events was then divided into the area (96 μm²) and time (45–70 s) and converted into units of events/μm² h. A mean and standard error were calculated by pooling the results of three different cells. For analysis of significance, a two-tailed student’s t-test was performed to determine the likelihood of the differences in the event distributions occurring by chance; a cutoff of P < 0.05 was used to indicate significance. The ratio of events occurring over areas of collagen versus Fc or Fc-E-cadherin was calculated for each cell and a mean and standard error were generated from three cells on each substrate. For the dwell time analysis, each targeting event was followed through serial time frame images to calculate its time to disappearance. Dwell times were then binned into fast (<5 s), medium fast (5–10 s), medium (10–15 s), medium slow (15–20 s) and slow (>20 s) events. A mean and standard error were generated from counting 20–30 events in a given area in three cells on each substrate. For analysis of significance, a two-tailed Student’s t-test with a cutoff of P < 0.05 was performed to determine the likelihood of the differences in the event distributions occurring by chance.

4. Discussion

Examples of spatial regulation of exocytosis include membrane delivery during filopodial extension, and basolateral protein trafficking during polarization in epithelial cells where exocytic traffic is directed to sites of E-cadherin-mediated cell–cell adhesion during lateral membrane growth [2,4]. Exocytic delivery of post-Golgi vesicles does not appear to be uniformly spread over the entire cell surface, but is instead often restricted to sub-domains. It was first noted over a quarter of century ago that the apico-lateral membrane junction is a “hotspot” for integral membrane protein recycling in polarized epithelial cells [9]. Indeed, recent imaging demonstrates that basolateral vesicles are targeted preferentially to this portion of the lateral membrane [5]. E-cadherin is likely to play a role in defining sites for exocytosis at the plasma membrane, perhaps by recruiting the exocyst tethering complex to the membrane [2,4].

To isolate effects of E-cadherin from other cell adhesion proteins, our laboratory has previously characterized both whole cell and ventral membrane patches adherent to a Fc-E-cadherin substrate [6]. Of note, MDCKII epithelial cells exhibit calcium-dependent adhesion and spreading on the Fc-E-cadherin substrate even in the absence of additional serum factors [6]. Immunofluorescence of fixed cells has demonstrated localization and clustering of cellular E-cadherin, catenins, and other protein complexes characteristic of basolateral membrane domains [6,10]. Competition experiments using 35S-labeled Fc-E-cadherin indicate that the surface concentration of Fc-E-cadherin is approximately 50,000 molecules per μm² [6]. The biotin-Neutravidin-biotin lattice both assures the correct orientation of Fc-E-cadherin for homotypic binding interactions with endogenous E-cadherin on cell surfaces (Fig. 1B), and provides some molecular flexibility to the substrate to allow for clustering of E-cadherin molecules within the plasma membrane of contacting cells, as is normally observed for E-cadherin-mediated cell–cell adhesion [4,11].
Here we demonstrate the methodology for arraying two bioactive proteins in close apposition on a surface (termed dual substrate display). This method makes use of micro-contact printing to apply the first protein in a pattern on a micron to submicron scale. By functionalizing the coverslip surface prior to stamping the initial protein, a second protein can be arranged in a complementary pattern on the surface. By forming subcellular-sized patterns, this method allows for the establishment of signaling micro-domains at the surface of attached cells to contrast effects of two different adhesion ligands that are sampled by a single cell.

As a specific test of DSD, we show that vesicles containing a basolateral membrane protein (VSV-G protein) are targeted preferentially to sites of E-cadherin-mediated adhesion, compared to sites of collagen IV adhesion. Due to the method of preparation, we could not compare directly vesicle targeting on a DSD containing Fc-E-cadherin and Fc; however, since there was no difference in delivery of VSVG to areas of collagen IV and Fc (Fig. 2d), we assume that vesicle delivery on the Fc/Fc-E-cadherin DSD would be similar to that on the collagenIV/Fc-E-cadherin DSD. These results support the hypothesis that E-cadherin-mediated adhesion specifies sites for basolateral vesicle targeting at the plasma membrane. The DSD assay provides a platform for future experiments to dissect molecular pathways responsible for converting E-cadherin-mediated adhesion into changes in basolateral vesicle targeting.

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Appendix A. Supplementary data

TIRF imaging of vesicle targeting events. This video demonstrates a representative 60 second sequence of an inverse TIRF time capture series of VSV-G-YFP cells plated on DSD. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.08.037.

References