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Cell Adhesion Assays: Fabrication of an E-cadherin Substratum and Isolation of Lateral and Basal Membrane Patches

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Summary

Cell adhesion between cells and with the extracellular matrix (ECM) results in dramatic changes in cell organization and, in particular, the cytoskeleton and plasma membrane domains involved in adhesion. However, current methods to analyze these changes are limited because of the small areas of membrane involved in adhesion, compared to the areas of membrane not adhering (a signal to noise problem), and the difficulty in accessing native protein complexes directly for imaging or reconstitution with purified proteins. The methods described here overcome these problems. Using a mammalian expression system, a chimeric protein comprising the extracellular domain of E-cadherin fused at its C-terminus to the Fc domain of human IgG1 (E-cadherin:Fc) is expressed and purified. A chemical bridge of biotin-NeutrAvidin-biotinylated Protein G bound to a silanized glass cover slip is fabricated to which the E-cadherin:Fc chimera binds in the correct orientation for adhesion by cells. After cell attachment, the basal membrane (a contact formed between cellular E-cadherin and the E-cadherin:Fc substratum) is isolated by sonication; a similar method is described to isolate basal membranes of cells attached to ECM. These membrane patches provide direct access to protein complexes formed on the membrane following cell-cell or cell-ECM adhesion.

Keywords

Epithelial cells; polarity; plasma membrane; membrane domains; cell–cell adhesion; cell–extracellular matrix adhesion; cadherin; integrin; collagen; substrate; plasma membrane; cytoskeleton; actin; microtubules; membrane patches

1. Introduction

Polarized epithelial cells have a higher-order organization involving cell–cell and cell–extracellular matrix contacts that orient cells into a monolayer that separates different biological compartments in the body. The cell surface bounded by these contacts (basal–lateral domain) is structurally and functionally distinct from the unbounded surface (apical domain; **refs.** 1,2). Understanding how different membrane domains are organized in polarized epithelial cells requires knowledge of how cells adhere to one another and the extracellular matrix, and how the resulting cell surfaces are converted into specific membrane domains by localized assembly and targeted delivery of specific proteins.

Epithelial cell–cell adhesion is mediated by a variety of membrane proteins, including classical cadherins, claudins/occludin, nectin, and desmosomal cadherins (3–5). Classical cadherins are single membrane spanning proteins with a divergent extracellular domain of five repeats and a conserved cytoplasmic domain. Binding between extracellular domains, which requires Ca^{2+} for protein conformation, is thought to involve multiple *cis*-dimers of

cadherin, forming *trans*-oligomers between cadherins on opposing cell surfaces (4). Binding between cadherin extracellular domains is weak, but strong cell–cell adhesion develops during lateral clustering of cadherins by proteins that link cadherin to the actin cytoskeleton (5). However, little is known about how these protein complexes assemble in cells, how the cadherin complex binds and organizes the actin cytoskeleton, or how other proteins identified at cell-cell contacts modify cadherin function and actin organization.

The analysis of mechanisms involved in cell adhesion has many limitations. Current methods that are available to investigate mechanisms of assembly of cadherin complexes include analysis of protein distributions in fixed or live cells, isolation of protein complexes with antibodies following extraction from cells, and in vitro dissection of protein-protein binding with bacterial expressed proteins or yeast two-hybrid analysis. None of these methods, however, allow direct access to native protein complexes on the membrane of cells. Another significant problem is that the initial interactions between cells, when protein complexes of interest are assembled and modified, occur on a very small area of plasma membrane relative to area of the membrane involved in cell attachment to extracellular matrix and the free cell surface. The “signal” from cell-cell adhesion complexes is correspondingly small compared to that from cell-ECM protein complexes, and hence difficult to isolate and analyze.

A step to overcome problems of the relatively small “signal” of initial cell–cell adhesion is to induce cells to adhere and spread on a substratum that promotes adhesion through cadherins rather than extracellular matrix. This requires purification of native E-cadherin and correct orientation on the substratum for cell adhesion. Recombinant deoxyribonucleic acid (DNA) technology offers a simple means for production of specific protein domains. Many eukaryotic and prokaryotic heterologous expression systems have been used to produce proteins in quantities suitable for biochemical analysis. However, a mammalian system is the best choice for production of the extracellular domain of mammalian transmembrane membrane proteins, especially when proper post-transitional modification of the protein is essential for function. To use E-cadherin for cell attachment, large amounts of protein that have been post-translationally modified are required: cleavage of presequence, and complex glycosylation are essential for E-cadherin function (3,4). To present the protein in the correct orientation for cell adhesion (N-terminus to N-terminus), E-cadherin is tagged at the C-terminus, thereby providing not only a way to correctly orientate the protein on artificial substratum for adhesion assays, but also an easy one-step method for protein purification (6).

To overcome the limitation of accessibility to native protein complexes on membranes, isolated substrate-bound membranes can be prepared simply and reproducibly from cells plated on cover slips or filters to provide a cell-free system for analyzing and reconstituting cytoskeleton-membrane interactions (7). Isolated membrane patches retain plasma membrane and the associated cytoskeleton, including actin filaments and in many cases microtubules. Nuclei are not retained and most internal organelles are removed. A good preparation yields thousands of patches per cover slip or filter. Isolated membrane patches provide an improved visualization of the cytoskeleton at the membrane, since the cytoskeleton not associated with the basal membrane is removed. Moreover, proteins on the basal membrane are more accessible to fixative, which is particularly advantageous in the case of tall cells such as MDCK cells on filters.

2. Materials

2.1. Expression and Purification of E-cadherin:Fc

1. pCEP4 Plasmid (Invitrogen, San Diego, CA).

2. CDM8 Plasmid (Invitrogen).
3. 293 Cells expressing EBV nuclear antigen-1 (Invitrogen).
4. Lipofectamine (Gibco-BRL, Gaithersburg, MD).
5. Hygromycin B (Calbiochem, La Jolla, CA).
6. High glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL).
7. Fetal bovine serum (FBS), cell culture grade (Sigma, St. Louis, MO).
8. Dimethyl sulfoxide (DMSO), cell culture grade (Sigma).
9. HiTrap Protein G sepharose 4B column (capacity 24 mg human IgG/mL drained gel; Pierce, Rockford, IL).
10. Centricon YM-10 filter (Amicon).
11. BCA Protein Assay Kit (Pierce).

2.2. Fabrication of E-cadherin:Fc Substratum

1. 12-mm Glass cover slips, number 1.5, ceramic cover slip holders (Electron Microscope Sciences, Fort Washington, PA).
2. Nonidet P-40 (Sigma).
3. Nochromix (Pierce).
4. Branson Sonifier 250 (Pierce).
5. *N*-(2-aminethyl)-3-amino-propyltrimethoxysilane (Pierce).
6. Anhydrous methanol (Sigma).
7. 'Atmosbag' (Aldrich).
8. Tris-saline (20 m*M* Tris-HCl, pH 7.4; 137 m*M* NaCl).
9. Ringer's buffer (10 m*M* HEPES, pH 7.4; 154 m*M* NaCl, 7.2 mM KCl).
10. Sulfo-NHS-biotin (Pierce).
11. Biotin (Pierce).
12. NeutrAvidin (Pierce).
13. Biotinylated Protein G (Pierce).

2.3. Plating Cells and Cross-Linking Procedures for E-cadherin:Fc Substratum

1. Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection).
2. Twenty-four-well tissue culture plates.
3. 12-mm 0.45-μm Transwell polycarbonate filter membranes (Corning).
4. Type I collagen solution (*see* Note 1).
5. BS^3, DTSSP (Pierce).

2.4. Preparation of Basal Membranes From Cells Bound to Substrata by Sonication

1. Hypotonic buffer (15 m*M* HEPES, 15 m*M* KCl, pH 7.2).
2. Branson Sonifier 250 with 1/8-inch diameter microprobe.

3. Adjustable platform (support jack).
4. Ringers buffer (10 m*M* HEPES, pH 7.4; 154 m*M* NaCl; 7.2 m*M* KCl).

2.5. Fixing Basal Membranes for Microscopy

1. Methanol.
2. Formaldehyde.
3. Glutaraldehyde, $NaBH_4$.

3. Methods

3.1. Expression and Purification of E-cadherin:Fc

The methods described below outline 1) construction of the E-cadherin:Fc expression plasmid, 2) transfection and expression of E-cadherin:Fc in 293 cells, and 3) the purification of E-cadherin:Fc from tissue culture cells (Fig. 1).

3.1.1. E-cadherin:Fc Expression Plasmid—The expression vector CDM8FT (8) is used to express a chimeric protein comprising the complete extracellular domain of E-cadherin fused at the C-terminus to human immunoglobulin Fc domain (Fig. 1A; **ref.** 6). CDM8FT is derived from CDM8, an expression vector with the human cytomegalovirus early promoter and SV40 origin of replication.

1. CH2 and CH3 domains of human immunoglobulin G1, with or without the hinge region, are generated by polymerase chain reaction (PCR) using as templates either plasmid SIgpoly7 or a DNA fragment containing the Fc domain.
2. A *Xho*1 site (reading phase = CTC[Leu]-GAG[Glu]) is added to the 5′ end, and a *Not*1 site is added to the 3′ end downstream to a stop codon (TAA-A-GCG GCCGC).
3. The resulting PCR product (CH2-CH3) is subcloned into the *Xho*1/*Not*1 sites of CDM8FT, which results in an in-frame fusion with the hemagglutinin epitope tag on CDM8FT through the *Xho*1 restriction site.
4. The extracellular domain of canine E-cadherin is generated by PCR using cloned cDNA of canine E-cadherin as a template, with a Hind3 site and an *Xho*1 site (phase = Leu-Glu) at the 3′ and 5′ ends, respectively.
5. The PCR product, containing the start codon and the signal peptide of E-cadherin, is subcloned into the *Hind*3/*Xho*1 sites of the vector containing the CH2 and CH3 domains to replace the hemagglutinin tag; this results in an in-frame fusion of the E-cadherin domain with the CH2 and CH3 domain through the *Xho*1 site.

[1]Rat tails are used to prepare type I collagen. Briefly, place 5–10 tails in 95% ethanol. Prepare a 0.1% (v/v) acetic acid solution using sterile water, a sterile beaker and a sterile stir bar. Have the dilute acetic acid solution stirring at room temperature. Keep solution covered. Starting at the cut end of the tail, clamp two hemostats about 2–3 cm apart on a tail. While holding the hemostat in your left hand, twist or rotate the right hemostat 360 degrees and then pull. Keep pulling until it breaks off. You should have white collagen fibers at the end of the broken (2–3 cm) piece of the tail. Cut the white fibers off the broken piece of the tail using a sharp razor blade and place them on a glass gel plate. Continue breaking and pulling 2–3 cm pieces of the tail. You get less material the closer you get to the tip of the tail. Tease the collagen fibers by holding one end of the fibers stationary with one razor blade and then use a scraping motion with a razor blade at a 45° angle. You want to flatten the fibers and open them up. Put the teased fibers in the stirring acetic acid solution. They should turn translucent. When finished, put the beaker of collagen at 4°C and stir overnight. Next day, centrifuge three quarters full 50-mL Nalgene plastic tubes for 2 h at 15,000 rpm. Remove supernatant and save. Discard pellets. (Portion of the pellet will be gelatinous). Store supernatant as the stock solution at 4°C.

6. The complete coding region of the chimeric protein is excised from the vector plasmid with *Hind*3 and *Not*1 and subcloned into the *Hind*3/*Not*1 sites of the EBV Ori P-based expression vector, pCEP4.

The scheme can be modified to accommodate other types of affinity or epitope tags, and the E-cadherin extracellular domain can be replaced with the extracellular domain of different type I transmembrane proteins (6).

3.1.2. Transfection and Expression of E-cadherin:Fc in 293 Cells—The E-cadherin:Fc expression plasmid is transfected into 293 EBNA cells (293 cells expressing EBV nuclear antigen-1) maintained in high glucose DMEM/10% FBS. Transcription of the chimeric construct is driven by human cytomegalovirus early promoter in mammalian cells, and secretion into the growth medium is driven by the endogenous signal peptide of canine E-cadherin (Fig. 1).

1. Transfect cells using lipofectamine with the following modifications of the protocol supplied with the reagent: 3×10^5 cells in 3-cm dishes are transfected with 0.5 μg of cesium chloride purified E-cadherin:Fc DNA/5 μL of lipofectamine in 1 mL of serum-free DMEM for 6 h. Using this protocol, >70% of cells are routinely transfected.
2. Two days after transfection, 200 μg/mL of hygromycin B is added to the growth medium.
3. After about 10 d, a hygromycin B resistant cell population emerges, which is expanded further in the presence of a reduced concentration of hygromycin B (100 μg/mL; *see* Note 2).
4. Store replicate cultures in liquid nitrogen at a density of 1×10^6 cells/mL in 1-mL aliquots in a solution of 90% FBS/10% DMSO.
5. Thaw cells rapidly in a 37°C water bath, dilute into 30 mL of high glucose DMEM/10% FBS in a 10-cm diameter plastic Petri dish, and incubate at 37°C in a humidified, 5% CO_2 in air atmosphere. After 2 h, change the medium.
6. Grow cells to confluency, and then divide between 10 15-cm Petri dishes, grow cells to confluency, and then divide once more between 50 15-cm Petri dishes. The cells do not adhere strongly to the plastic and they can be removed simply by pipetting a stream of warm medium. Grow cells to approx 75% confluency.
7. To collect medium containing secreted Fc-E-cadherin all traces of FBS are first removed from the culture: the growth medium is discarded and the cells are washed in three changes of high glucose DMEM (without FBS); cells are then incubated for 1–2 h in 20 mL of high-glucose DMEM (without FBS) at 37°C in a humidified, 5% CO_2 in air atmosphere; the growth medium is discarded and 15–20 mL of fresh high-glucose DMEM (without FBS) is added, and the cells are incubated for 2 d at 37°C in a humidified, 5% CO_2 in air atmosphere.
8. Collect media and combine (Fig. 1B). Floating cells and large debris are removed by centrifugation. The supernatant is passed through a 0.45-μm filter and then

[2]A major advantage of this approach is that stable producer cells can be generated in less than two wk once recombinant cDNA constructs are made. Production of chimeric E-cadherin:Fc from a pooled drug-resistant cell population is stable for at least 10 cell passages, and generally, a newly thawed replicate culture is used for a new round of protein purification. There is, however, some heterogeneity of expression levels in the population, and it may be possible to achieve a higher yield of secreted proteins by clonal isolation of cells and screening for higher expression. However, in light of the quantity of soluble chimeric protein produced, and the ease of generating stable cells, labor intensive cloning and screening of higher expressing cells can be avoided.

stored in sterile containers at 4°C, or frozen and stored at −80°C depending on the time interval between collection and protein purification.

9. To continue collecting conditioned medium, the cells need to be subdivided and grown for 2 d in the presence of FBS: divide approximately half of the cells into 50 15-cm Petri dishes and incubate in high glucose DMEM/10% FBS for 2 d or until they are approx 75% confluent; wash cells and incubate in high glucose DMEM (without FBS), and collect conditioned medium.

10. This procedure can be repeated for three collection cycles, and then the cells are discarded. A new vial of frozen cells is thawed and expanded for more protein collection.

3.1.3. Purification of E-cadherin:Fc—E-cadherin:Fc is purified from conditioned medium in one step using affinity chromatography on Protein G sepharose which binds the Fc domain of the chimeric protein (Fig. 1B). The methods outlined below describe the purification, concentration, and storage of the protein.

1. Equilibrate a 1 mL HiTrap protein A sepharose 4B column in 0.1 *M* Na phosphate, buffer, pH 7.0.

2. Apply conditioned medium containing E-cadherin:Fc to the column (flow rate, approx 60 mL/h) and discard the flow through.

3. Wash the column in 10 volumes of 0.1 *M* Na phosphate buffer, pH 7.0.

4. Elute E-cadherin:Fc with 0.1 *M* glycine-HCl, pH 2.6, at a flow rate of 60 mL/h and collect 400- to 500-μL fractions; neutralize eluant immediately with 1 *M* Tris-HCl, pH 9 (approx 400 μL of 0.1 *M* glycine-HCl, pH 2.6, is neutralized to pH approx 7 with 10 μL of 1 *M* Tris-HCl, pH 9).

5. Measure OD_{280} and combine fractions containing eluted protein.

6. Concentrate combined protein fractions using a Centricon YM-10 filter.

7. Exchange buffer to Ringer's buffer using the Centricon filter or dialysis.

8. Determine protein concentration with the BCA protein assay using BSA as a protein standard, and assess protein purity by sodium dodecyl sulfate-polyacrylamide page electrophoresis (SDS-PAGE) and Coomassie Blue staining.

9. Adjust concentration of purified E-cadherin:Fc to 200 μg/mL with Ringer's buffer, flash freeze aliquots (50 μL) in liquid nitrogen, and store at −80°C (protein is stable for several months at −80°C).

10. Approximately 0.2–0.5 mg E-cadherin:Fc is purified per liter of conditioned medium (Fig. 1B). The purified E-cadherin:Fc migrates as a monomer when treated with reducing agents (e.g., DTT) owing to disulfide bonds in the Fc domains (Fig. 1A), or a dimer in the absence of reducing agent (Fig. 1C).

3.2. Fabrication of E-cadherin:Fc Substratum

A glass surface is used for attachment of cells to fabricated E-cadherin:Fc substrates so that cells and membrane patches can be analyzed by immunofluorescence microscopy as well as used for biochemical reconstitution of protein complexes. The methods described below outline 1) the preparation of glass surfaces to form chemical binding sites by silanizing the glass surface, and 2) fabrication of the E-cadherin:Fc substratum (Fig. 2).

3.2.1. Etching and Silanizing Glass Cover Slips

1. Load glass cover slips in a ceramic holder (used for all subsequent operations).
2. Submerge cover slips in warm (50–60°C) 2% (v/v) Nonidet P-40 in dH_2O and stir for 15–30 min.
3. Sonicate cover slips in warm 2% NP-40 in dH_2O solution for 5 min using a Branson Sonifer (1/8 in. microtip, setting no. 5).
4. Rinse three times for 5 min in dH_2O and allow to drain.
5. In a fume hood, slowly submerge coverslips in concentrated H_2SO_4 containing 3% Nochromix for 10 min, agitating occasionally by carefully dunking the ceramic holder up-and-down.
6. Slowly submerge cover slips in three changes of 100 mL of dH_2O for 3–6 min and agitate by dunking the ceramic holder up-and-down; allow to drain.
7. In a fume hood, slowly submerge coverslips in a solution of one part anhydrous methanol to one part dH_2O-saturated KOH for 10 min, and agitate occasionally by dunking the ceramic holder up-and-down.
8. Slowly submerge cover slips in three changes of 100 mL of dH_2O for 3–6 min and agitate by dunking the ceramic holder up-and-down; allow to drain.
9. Briefly submerge coverslips in 100% methanol.
10. Quick dry cover slips with a hot air dryer (a commercial hair dryer works well).
11. Prepare a solution of 95% methanol in dH_2O and adjust to pH 4.5–5.5 with glacial acetic acid (approx 40 mL 95% methanol + 1 μL of CH_3COOH).
12. Under nitrogen atmosphere, add *N*-(2-aminethyl)-3-amino-propyltrimeth-oxysilane to a final concentration of 4% (v/v) in the pH-adjusted 95% methanol solution, allow 5 min for hydrolysis and silanol formation.
13. Under nitrogen atmosphere, dip cover slips into silanol solution for 1–2 min and agitate occasionally by dunking the ceramic holder up-and-down.
14. Briefly submerge cover slips in anhydrous methanol.
15. Cure silanized cover slips at 40–50°C for 24 h.
16. Use silanized cover slips immediately.

3.2.2. Fabricating the E-cadherin:Fc Substratum—Silanizing glass cover slips with a long chain silane containing a free amine provide the base for chemical assembly of the E-cadherin:Fc substratum using a biotin-neutrAvidin-biotinylated protein G-E-cadherin:Fc sandwich (Fig. 2; *see* Note 3).

1. Incubate cover slips in a freshly prepared solution of 50 m*M* sulfo-NHS-LC-biotin (a long chain (LC), nonreversible biotinylating reagent that cross-links to free amines) in DMSO in the dark at 37°C for 2–3 h (*see* Note 4).
2. Wash cover slips in three changes of Tris-saline.

[3]This chemical assembly serves two purposes: 1) the E-cadherin:Fc chimera is attached at a distance from the glass surface with the purpose to allow some degree of lateral movement during binding to cell surface E-cadherin; and 2) binding of the E-cadherin:Fc chimera through the Fc domain to the Protein G correctly orients the N-terminus of the E-cadherin extracellular domain towards attaching cells. This chemical assembly results in a saturated density of approx 50,000 E-cadherin:Fc molecules/_m2.

3. Wash cover slips in three changes of 150 mL of Ringer's buffer.
4. Incubate cover slips in a fresh solution of 84 μ*M* NeutrAvidin in Ringer's buffer in the dark at room temperature for 2–3 h.
5. Wash cover slips in three changes of 150 mL of Ringer's buffer.
6. Incubate cover slips in a fresh solution of 17 μ*M* biotinylated protein G in Ringer's buffer in the dark at room temperature for 2–3 h.
7. Wash cover slips in three changes of 150 mL of Ringer's buffer.
8. Block excess free sites with a solution of 50 μ*M* biotin, 1 mg/mL BSA in Ringer's solution in the dark at room temperature for 2–3 h.
9. Wash cover slips in three changes of 150 mL of Ringer's buffer, and drain by touching the edge of the cover slip to a piece of filter paper.
10. Incubate cover slips in approx 4 μg of purified E-cadherin:Fc in 50 μL Ringer's buffer at room temperature for 1 h, or overnight at 4°C.
11. Wash cover slips in three changes of 150 mL of Ringer's buffer.
12. Use immediately for attachment of cells.

3.3. Plating Cells and Cross-Linking Procedures for E-cadherin:Fc or Collagen Substrata

Any cell type that expresses E-cadherin on the cell surface can be used for adhesion to the fabricated E-cadherin:Fc substratum (*see* Note 5); adhesion is specific as cells do not bind to substratum containing IgG of Fc instead of E-cadherin:Fc, or to E-cadherin:Fc when extracellular Ca^{2+} is left out of the medium. Before plating cells on E-cadherin:Fc, we passage cells each day for two days at very low cell density in order to render cells "contact naïve," during which time the cells have had little or no cell-cell adhesion and hence induction of cell-cell adhesion on an E-cadherin:Fc substratum will elicit formation of de novo adhesion complexes. Methods are also described for plating cells on type I collagen surfaces, as the methods used to prepare 'lateral' membranes attached to E-cadherin:Fc (**Subheading 3.4.**) can be also used to prepare basal membranes attached to extracellular matrix. Cells plated on E-cadherin:Fc accumulate E-cadherin and associated proteins on the attached "basal" membrane, and proteins characteristic of focal adhesion complexes involved in cell-ECM adhesion are not present. In contrast, cells attached to a type I collagen substratum accumulate integrin adhesion complexes on attached basal membranes, and not E-cadherin.

3.3.1. Attachment of Cells to an E-cadherin:Fc Substratum

1. Trypsinize a approx 50% confluent culture of MDCK cells, replate cells on 15-cm Petri dishes at a density of 1.5×10^6 cells/dish in DMEM/10% FBS, and incubate at 37°C in a humidified, 5% CO_2 in air atmosphere (the number of plates and cells

[4]In the methods outlined here, cover slips are placed with the silanized surface facing up on a flat surface (the caps of small polystyrene tubes can be used), approx 50 μL of solution is added (sufficient volume to cover a 12-mm cover slip), and incubated at room temperature in a humidified atmosphere. For washing, the cover slips are transferred to a ceramic cover slip holder (make sure the orientation of the silanized surface is noted) and placed in beaker containing the wash buffer.

[5]We use MDCK cells, an established cell line derived from canine kidney, and mouse L cell fibroblasts in which canine E-cadherin expression is controlled by an inducible dexamethasone promoter (*see* **ref.** 9). To seed MDCK GII cells on E-cadherin:Fc substrate, we trypsinize the cells briefly. Cells can also be lifted from the tissue culture dishes using non-enzymatic methods, but we have found cells to adhere to the cadherin substrate better after light trypsinization. Cells are plated in DMEM with 2% instead of the usual 10% FBS to reduce the amount of ECM proteins introduced through the serum. However, certain growth factors in the serum are required for cell-cell-adhesion, which is why we recommend not omitting the serum completely. Cells will start to spread on the E-cadherin:Fc substratum after 1–2 h of incubation, which is slightly slower than the time for cells to spread on collagen-coated cover slips. MDCK G cells will secrete their own extracellular matrix over time, but no focal contact staining is observed until >12 h on the substratum.

will depend on the number of E-cadherin:Fc/cover slips that will be used in an experiment).

2. After 24 h, trypsinize the cells and re-plate them on 15 cm Petri dishes at a density of 1.5×10^6 cells/dish in DMEM/10% FBS and incubate at 37°C in a humidified, 5% CO_2 in air atmosphere for a further 24 h.
3. Trypsinize the cells, combine and re-suspend in DMEM/2% FBS at different cell densities ($1.5–2.0 \times 10^6$ cells will make an instantly confluent cell culture on a 12-mm cover slip).
4. Place 12-mm glass cover slips with an E-cadherin:Fc substratum (*see* **Subheading 3.2.**) in the well of a 24-well cluster dish, and aspirate excess Ringer's buffer.
5. Add 0.5 mL of a single cell suspension (3×10^6 cells/mL) to the well.
6. Cells attach to the E-cadherin:Fc substratum at 1*g* for 1 h, or by centrifugation at 500*g* for 5 min in a swinging bucket rotor at room temperature (do not slow the spin by breaking as this will swirl cells into the center of the cover slip).
7. Incubate cells at 37°C in a humidified, 5% CO_2 in air atmosphere for up to 6 h (this period is sufficient for cells to fully spread on the E-cadherin:Fc substratum, and to assemble protein complexes specific to cadherin-mediated cell-cell contacts; *see* Note 5).

3.3.2. Attachment of Cells to Fabricated Collagen Substratum

1. Dilute a stock solution of rat tail type I collagen (*see* Note 1) 1/10 in 0.1% (v/v) acetic acid.
2. Add diluted collagen solution to cover slips for 2 min or to 12-mm 0.45-μm Transwell polycarbonate filter membranes, and then remove excess by aspiration.
3. Air-dry cover slips or filters under UV light for no more than 1–2 h (we use the clean bench UV light).
4. Plate cells on 12-mm collagen-coated glass cover slips in 24-well tissue-culture dishes, or 12-mm Transwells at a confluent cell density.
5. Cells are cultured at least 2 d to allow for secretion of endogenous ECM and more stable attachment to the cover slip (*see* Note 6).

3.3.3. Cross-Linking Cells to E-cadherin:Fc Substratum

1. Rinse cells free of DMEM/FBS with Ringer's buffer containing 1.8 m*M* $CaCl_2$.
2. Incubate cells for 20 min at room temperature in Ringer's buffer containing 1 mM solution of the membrane-impermeable cross-linkers BS^3 or DTSSP to stabilize the interaction between cellular cadherin and E-cadherin:Fc.
3. Quench excess cross-linker by adding glycine to a final concentration of 20 m*M* and incubate for an additional 5 min at room temperature.

[6]In the absence of chemical cross-linking to the substratum, cells should be confluent and plated for at least 24 h, although longer is better because cells will have a more stable attachment to the substratum. Nonconfluent cells or cells plated less than 24 h are not well retained after sonication without previous crosslinking. The 24-well plates or the 12-mm filters work best for the sonication procedure. Because the interactions to the substrate are more mature and more stable, membrane patches can be generated even at early time points after plating without cross-linking, but cross-linking of cells to the substrate greatly increases the number of membrane patches obtained.

3.4. Preparation of Basal Membranes From Cells Bound to Substrata by Sonication

The method described below outlines how to generate cover slips covered in basal membranes of cells attached to either an E-cadherin:Fc (Fig. 3) or extracellular matrix (Figs. 4 and 5). The method is simple: hypotonic swelling of cells followed by brief sonication, which results in the removal of the dorsal (apical) cell surface, nucleus and intracellular membranes and organelles, leaving behind on the cover slip the basal membrane attached to the substratum (*see* Figs. 3–5). The method described below is adapted for E-cadherin:Fc substratum and the epithelial cells used in our laboratory (*see* **ref.** 7 for background information and variations on the methodology).

1. Rinse cells three times in hypotonic buffer containing 1.8 m*M* $CaCl_2$, and incubate in the same buffer for 5 to 10 min at room temperature (*see* Note 7).
2. Fill the tissue-culture well nearly to the brim with hypotonic buffer.
3. Place the tissue culture dish on an adjustable platform/support jack.
4. Raise the dish until the tip of the 1/8-in. diameter microprobe of the Branson Sonifier 250 is 4–8 mm above the cells (*see* Note 8).
5. Wearing earphones to protect your ears, sonicate cells with a brief (less than 1 s) pulse, duty cycle 20, and output 19–22% (*see* Note 9).
6. Rinse membranes briefly in Ringer's buffer before fixation (*see* **Subheading 3.5.**). When buffer is aspirated from the cover slip or filter, the area that has been rendered into basal patches is visible as a hole in the cell layer. The hole will vary in size from preparation to preparation (*see* Note 10).

3.5. Fixing Basal Membranes for Microscopy

Isolated membrane patches expose the cytoplasmic face of the membrane, and thereby the protein complexes that form the cellular attachments to E-cadherin or extracellular matrix. These membrane patches can be used for direct analysis of protein distributions by immunofluorescence microscopy or electron microscopy. However, of greater significance in terms of understanding the regulation of protein complex interactions and assembly, these membrane patches can be used to selectively remove, or add back proteins to the complexes. The methods described below outline fixation of membrane patches in 1) cold methanol, 2) glutaraldehyde, or 3) formaldehyde for immunofluorescence microscopy (*see* Note 11).

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[7] Any hypotonic buffer should work. Both 5-min and 10-min swelling times give nice patches on cover slips. Filters may need a longer swelling time, so we swell cells for 10 min to be safe.

[8] The distance between microprobe and cover slip/filter is measured by eye. The distance does not have to be precise because after sonication there is a gradient of whole cells to patches, and since there are thousands of patches per cover slip or filter, there will usually be plenty even if many whole cells remain. Cells that are more resistant to rupture, for example polarized epithelial cells on Transwell filters, should be held closer to the microprobe (4 mm), whereas more delicate cells, for example fibroblasts on cover slips, should be held a few mm further from the microprobe (7 mm). Once one gets the feel for it, obtaining nice patches is very reproducible.

[9] The duty cycle on the sonifier is set at 20, and the output varies according to the substrate (coated-glass or filter), the type of cells, and how long the cells have been plated. The output setting ranges from around 19% maximum power for fibroblasts to around 23% maximum power for MDCK cells polarized on filters.

[10] Preparation-to-preparation variation (the size of the hole in the cell monolayer after sonication, the number of basal patches, the number of membranes with microtubules) depends on how long cells are plated, the distance of the micro-probe from the cover slip/filter, and the intensity of the sonication burst. There will usually be whole cells present towards the edge of the cover slip, but these cells are not suitable for microscopy as they are damaged. The presence of whole cells means that these preparations are not suitable for biochemical isolation of basal membranes. Do not attempt to deliver more than one sonication burst to a cover slip/filter to increase the area of basal patches, because the patches already present will be ruined.

[11] Although the isolated membranes are thin, approx 1 μm, the best high-resolution images are obtained by confocal or deconvolution microscopy. For the images in Fig. 5, image stacks of 0.20 μm in thickness were taken on an Olympus IX-70 inverted microscope with a 100X oil-immersion objective 1.35 N.A. (Olympus Corp.) and captured by a cooled CCD camera (Photometrics Ltd.). Images are collected and processed using Delta Vision de-convolution software (Applied Precision, Seattle, WA) on a Silicon Graphics workstation (Silicon Graphics Corp.).

3.5.1. Methanol Fixation

1. Precool 100% methanol to −20°C.
2. Fix membrane patches in −20°C precooled 100% methanol for 10 min.
3. Rinse briefly in 0.1% Triton in Ringer's buffer to break the surface tension.
4. Rinse briefly in two changes of Ringer's buffer.

3.5.2. Formaldehyde Fixation

1. Dilute 37% (v/v) formaldehyde solution to 4% (v/v) in Ringer's buffer immediately before use.
2. Fix membrane patches in 4% (v/v) formaldehyde solution for 10 min at room temperature.
3. Rinse in three changes of Ringer's buffer.

3.5.3. Glutaraldehyde Fixation (Suitable for Staining Microtubules)

1. Fix membrane patches in freshly prepared 0.3% (v/v) glutaraldehyde in Ringer's buffer.
2. Rinse briefly three times in Ringer's buffer.
3. Incubate three times for 5 min each in 1 mg/mL $NaBH_4$ in Ringer's buffer to quench unreacted aldehyde groups.
4. Rinse in three changes of Ringer's buffer.

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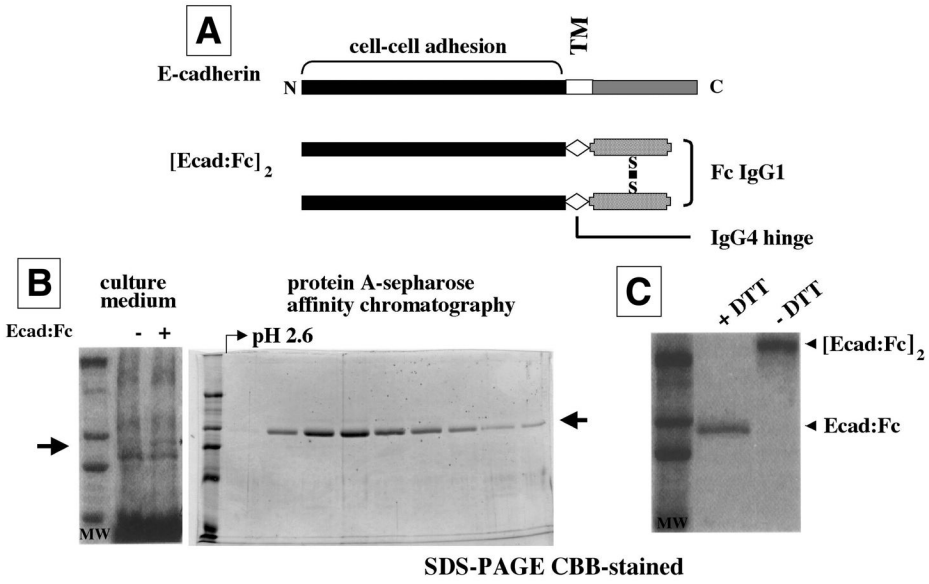

Fig. 1.

Construction, expression and purification of E-cadherin:Fc. **(A)** E-cadherin is a single transmembrane protein, and the extracellular domain is required for cell-cell adhesion. E-cadherin extracellular domain is fused to the hinge region of IgG4 and the Fc domain of human IgG1 to generate a dimeric E-cadherin:Fc chimeric protein. **(B)** Culture medium from control (−) cells and cells expressing E-cadherin:Fc (+) separated by SDS-PAGE and stained with Coomassie-Brilliant Blue (CBB) shows secretion of E-cadherin:Fc. E-cadherin:Fc is purified from culture medium in one-step by affinity chromatography on protein A sepaharose 4B and elution with buffer at pH 2.6, as shown by SDS-PAGE and CBB staining (arrow). **(C)** E-cadherin:Fc migrates as a dimer ([Ecad-Fc]$_2$) or monomer (Ecad-Fc) in SDS-PAGE in the absence or presence of reducing agent DTT, respectively (MW, molecular weight standard proteins from top: myosin [205 kDa], β-galactosidase [115 kDa], phosphorylase b [105 kDa], and bovine serum albumin [BSA; 68 kDa]).

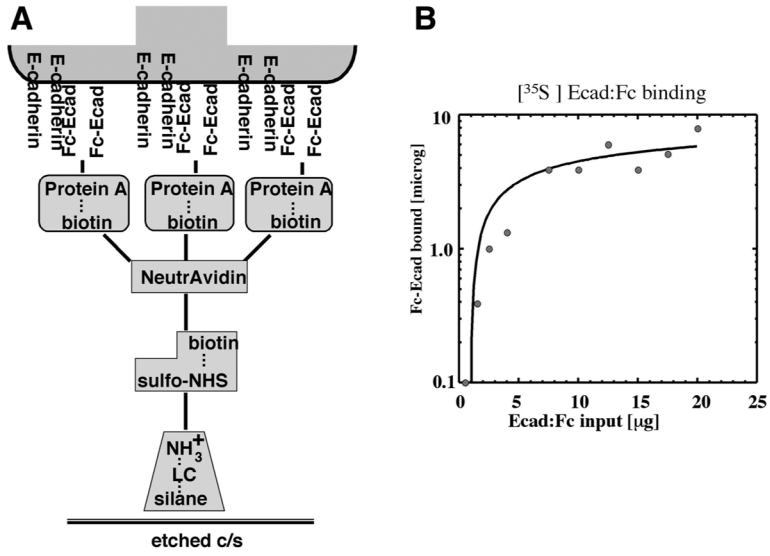

Fig. 2.

Fabrication of E-cadherin:Fc substratum. **(A)** Scheme of chemical assembly of E-cadherin:Fc on a cover slip. A glass cover slip is silianized with a long-chain silane containing an amine, to which sulfo-NHS-biotin is linked. NeutrAvidin and then biotinylated protein A are added sequentially, followed by purified E-cadherin:Fc. E-cadherin on the surface of cells binds to the correctly oriented E-cadherin:Fc substratum. **(B)** ^{35}S-methionine/cysteine-labeled E-cadherin:Fc was purified from cells incubated with ^{35}S-methionine/cysteine as described in **Subheading 3.1.3.** and used in a dilution series to determine the saturation density of E-cadherin:Fc on the substratum as approx 50,000 molecules/μm^2.

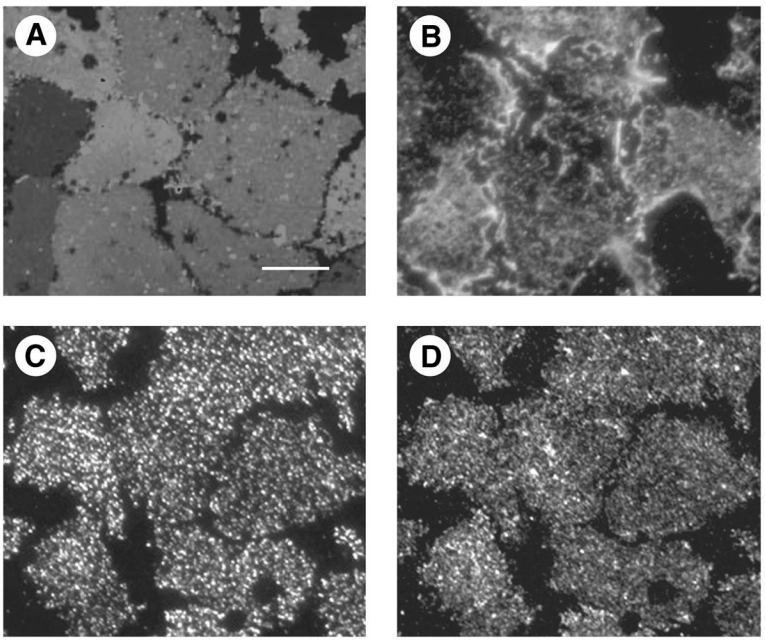

Fig. 3.

Characterization of basal membrane patches from MDCK cells on E-cadherin:Fc substratum. **(A)** Staining with membrane dye, DiIC18(3), of isolated basal membrane patches on E-cadherin:Fc substratum. **(B)** Staining with monoclonal actin antibody (Chemicon). **(C)** Basal membranes on E-cadherin:Fc substratum stained with antibody directed against the intracellular domain of E-cadherin. **(D)** Staining with β-catenin antibody. All fixations were with 4% formaldehyde in Ringer's buffer. Bar, 10 μm.

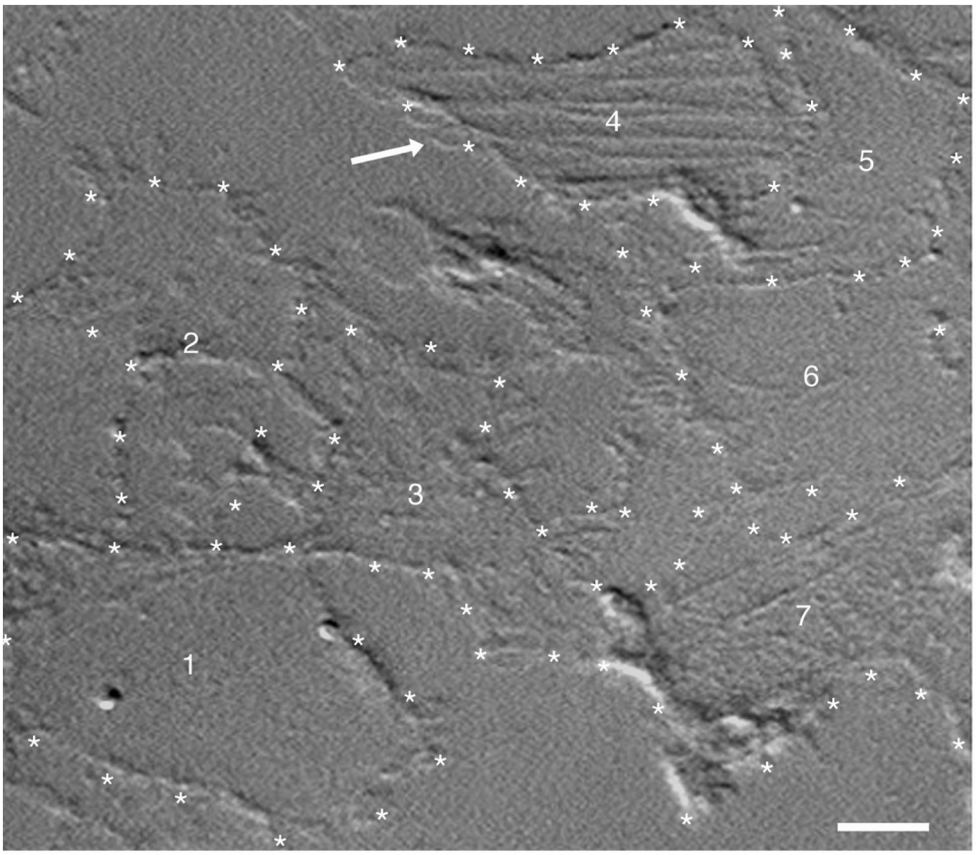

Fig. 4.

Visualization of patches by differential interference contrast microscopy. MDCK clone II/G were plated on collagen-coated cover slips for 2 d, sonicated, and isolated membranes were imaged by differential interference contrast microscopy. Patches are outlined by asterisks. Note the actin fibers present on the membrane indicated by the arrow. Bar, 10 μm.

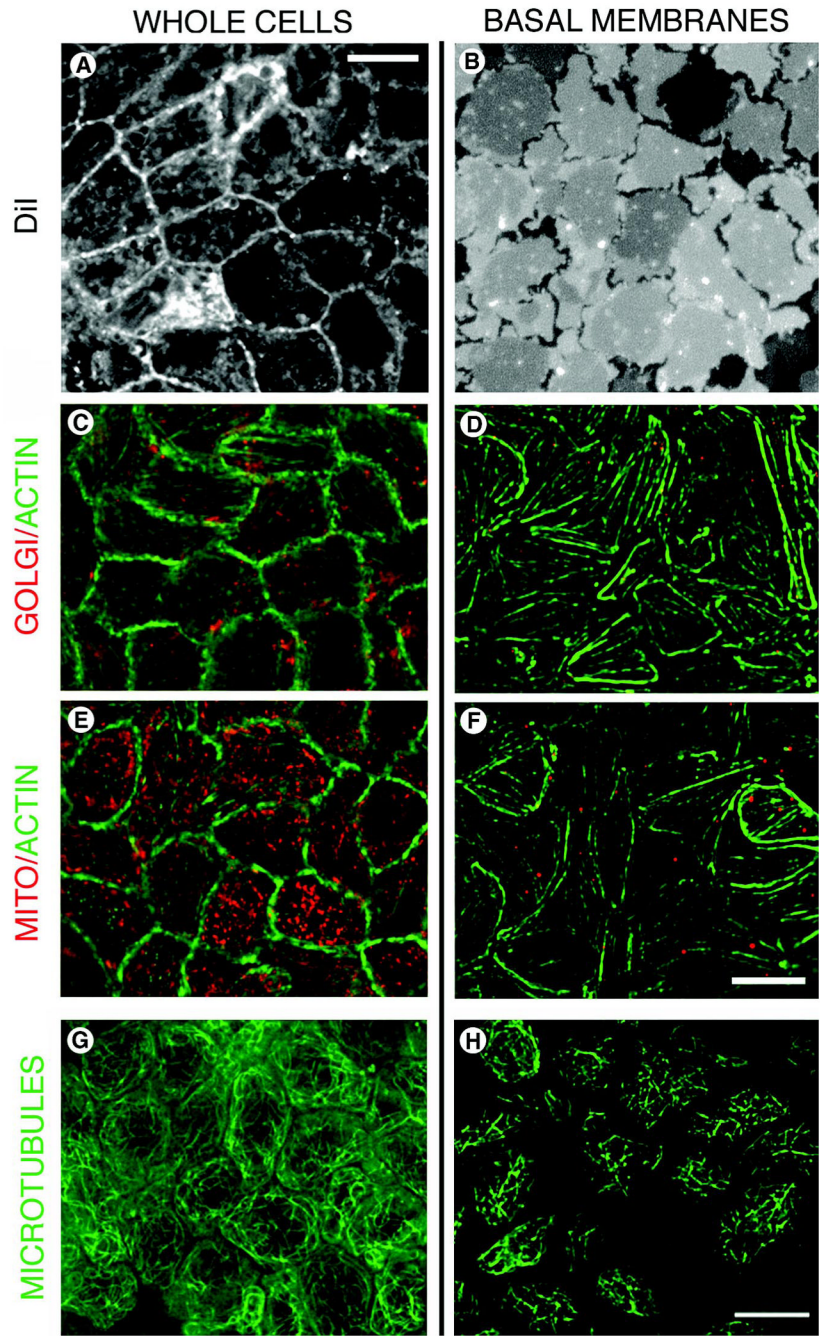

Fig. 5.

Characterization of basal membrane patches from MDCK cells on filters. **(A,B)** Staining with the membrane dye, DiIC18(3), of whole MDCK cells grown on filters **(A)**, and isolated membrane patches **(B)**, which show few internal organelles remaining after sonication. Bar, 10 μm. **(C,D)** Staining for the Golgi protein, p115 (red) and actin (green) on the basal membrane of intact MDCK cells grown on filters **(C)**, and isolated basal membranes which retain actin fibers whereas p115-containing Golgi membranes are largely removed **(D).** **(E,F)** Staining for mitochondria (red) and actin filaments (green) of the basal membrane of intact MDCK cells **(E)**, and isolated basal patches which show preservation of actin

filaments but absence of mitochondria **(F)**. Bar, 10 μm. Fixation of cells for Golgi and mitochondria staining was with 4% formaldehyde diluted in Ringers buffer. A polyclonal antibody to Golgi component p115 was a gift from Dr. Suzanne Pfeffer (Stanford University, Stanford, CA). A monoclonal antibody to mitochondrial Hsp70 was from Affinity Bioreagents. **(G,H)** GFP microtubules on the basal membrane of intact MDCK cells expressing GFP-tubulin **(G)**, and isolated basal membranes, which show improved visibility of the cytoskeleton **(H)**. Bar, 10 μm. green fluorescent protein -microtubules were fixed with 0.3% glutaraldehyde in BRB80 buffer, for 10 min for isolated membranes or 20 min for intact cells, with the addition of 0.1% Triton X-100 for intact cells.