Adducin Promotes Micrometer-Scale Organization of β 2-Spectrin in Lateral Membranes of Bronchial Epithelial Cells

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Adducin promotes assembly of spectrin–actin complexes, and is a target for regulation by calmodulin, protein kinase C, and rho kinase. We demonstrate here that adducin is required to stabilize preformed lateral membranes of human bronchial epithelial (HBE) cells through interaction with β 2-spectrin. We use a Tet-on regulated inducible small interfering RNA (siRNA) system to deplete α -adducin from confluent HBE cells. Depletion of α -adducin resulted in increased detergent solubility of spectrin after normal membrane biogenesis during mitosis. Conversely, depletion of β 2-spectrin resulted in loss of adducin from the lateral membrane. siRNA-resistant α -adducin prevented loss of lateral membrane, but only if α -adducin retained the MARCKS domain that mediates spectrin–actin interactions. Phosphomimetic versions of adducin with S/D substitutions at protein kinase C phosphorylation sites in the MARCKS domain were not active in rescue. We find that adducin modulates long-range organization of the lateral membrane based on several criteria. First, the lateral membrane of adducin-depleted cells exhibited reduced height, increased curvature, and expansion into the basal surface. Moreover, E-cadherin-GFP, which normally is restricted in lateral mobility, rapidly diffuses over distances up to 10 μ m. We conclude that adducin acting through spectrin provides a novel mechanism to regulate global properties of the lateral membrane of bronchial epithelial cells.

INTRODUCTION

Lateral membranes of epithelial cells are one of the most abundant specialized membrane domains in metazoans, and are of major physiological importance as sites of cell adhesion and homeostasis of salt and water. The possibility that lateral membranes of epithelial cells have a spectrin-based membrane skeleton similar to erythrocytes has been suggested by localization of isoforms of spectrin and ankyrin at sites of cell–cell contact (Drenckhahn *et al.*, 1985; Nelson and Veshnock, 1986; Drenckhahn and Bennett, 1987; Peters *et al.*, 1995). Moreover, resident lateral membrane proteins including the Na/K ATPase and E-cadherin either bind directly or coimmunoprecipitate with ankyrin and spectrin (Nelson and Veshnock, 1987; Nelson and Hammerton, 1989; Nelson *et al.*, 1990; Morrow *et al.*, 1989; Davis and Bennett, 1990). Recently, β 2-spectrin and ankyrin have been demonstrated to be required for both stability and biogenesis of the lateral membrane of cultured epithelial cells (Kizhatil and Bennett, 2004; Kizhatil *et al.*, 2007a). Furthermore, E-cadherin has been shown to bind directly to ankyrin-G and to require ankyrin-G and β 2-spectrin for transport to the lateral mem-

brane (Kizhatil *et al.*, 2007b). Combined, these early and recent findings highlight the need for a closer examination of additional components of the spectrin-based membrane skeleton within epithelial lateral membranes.

The precise organization of spectrin in lateral membranes is not known. However, epithelial spectrin presumably participates in multiprotein complexes because it is insoluble in nonionic detergents that preserve protein interactions (Nelson and Veshnock, 1986). Spectrins do not assemble beyond a tetramer and associate only weakly with actin (Bennett and Baines, 2001). Formation of the spectrin network of erythrocytes thus requires accessory proteins that stabilize spectrin–actin complexes (Bennett and Baines, 2001). The detergent-resistant spectrin assembly in epithelial cells similarly is likely to involve additional proteins. Adducin is a key assembly protein that recruits both erythroid and nonerythroid spectrins to the fast growing ends of actin filaments (Gardner and Bennett, 1986, 1987; Bennett *et al.*, 1988; Kuhlman *et al.*, 1996; Li and Bennett, 1996; Li *et al.*, 1998). Loss of β -adducin results in increased fragility of erythrocyte membranes (Gilligan *et al.*, 1999; Chen *et al.*, 2007). Adducin is widely expressed in most cell types, including epithelial cells where it is localized at the lateral membrane (Kaiser *et al.*, 1989). Adducin is a target for protein kinase C (PKC) and Rho-kinase and can also be regulated with calcium and calmodulin (Ling *et al.*, 1986; Kuhlman *et al.*, 1996; Matsuoka *et al.*, 1996; Fukata *et al.*, 1999). Thus adducin represents an interesting candidate to analyze with respect to assembly of spectrin in epithelial cells.

We describe the use of a lentiviral-delivered inducible short hairpin RNA (shRNA) system to deplete α -adducin from human bronchial epithelial (HBE) cells. In contrast to β 2-spectrin and ankyrin-G, which are required for formation

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Abbreviations used: HBE, human bronchial epithelial cell; PKC, protein kinase C; CTL, control; ADD1, adducin siRNA 1; FRAP, fluorescence recovery after photobleaching; FLIP, fluorescence loss in photobleaching.

of the lateral membrane, we find that adducin functions to stabilize and promote long-range organization of the lateral membrane.

MATERIALS AND METHODS

Cell Culture and Immunofluorescence

HBE cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). For shRNA induction or transfection cells were seeded on the 1.4-mm coverslip inserts of MatTek plates at 200,000 cells/plates for 100% confluence, 100,000 cells/plate for 50% confluence, and 50,000 cells/plate for 25% confluence. Doxycycline (Sigma) was used at final concentration of 100 ng/ml. For immunofluorescence cells were fixed with 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) for 10 min at room temperature and then permeabilized with 0.2% Triton X-100 for 10 min at room temperature. To stain microtubules, cells were fixed in -20°C methanol for 5 min followed by 20 min incubation in PBS. Cells were blocked in PBS (pH 7.4) containing 3% fish oil gelatin (Sigma) and 4% bovine serum albumin (BSA). Primary antibodies were diluted in blocking buffer and incubated onto cells overnight at 4°C . Alexa Fluor secondary antibodies (488, 568, 633) were purchased from Molecular Probes (Eugene, OR), diluted in blocking buffer, and incubated for 3 h at 4°C . After washing of secondary antibodies, cells were covered with vectashield (Pierce, Rockford, IL) and processed for imaging using an LSM 510 Meta laser scanning confocal system.

Triton X-100 Extractions

During Triton X-100 extraction experiments for immunofluorescence, the growth media was washed away from cells with PBS, pH 7.4 (1.06 mM KH_2PO_4 , 155 mM NaCl, 2.97 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; PBS, Invitrogen) and then cells were incubated with PBS buffer supplemented with a protease inhibitor cocktail (10 $\mu\text{g}/\text{ml}$ AEBSF, 30 $\mu\text{g}/\text{ml}$ benzamide, 10 $\mu\text{g}/\text{ml}$ pepstatin, and 10 $\mu\text{g}/\text{ml}$ leupeptin) and containing 0.1% Triton X-100 for 7 min at room temperature. Extraction buffer was removed and cells were directly fixed in 3% PFA for 10 min at room temperature. PFA was washed away and cells were blocked and stained as stated above. During Triton X-100 extraction experiments for Western blots, six-well plates were used and the growth medium was washed away with PBS buffer. Cells were then incubated in 150 μl of PBS supplemented with the same protease inhibitor cocktail containing and 0.1% Triton X-100 for 7 min. The Triton X-100 solution was carefully suctioned away and mixed with 100 μl of protein gel loading buffer to bring to a final volume of 250 μl . The remaining nonsoluble material on the plate was rinsed once with PBS and solubilized with 250 μl of protein gel loading buffer. Both soluble and insoluble fractions were loaded onto SDS-PAGE gels and processed for immunoblotting. Total samples were generated from extra wells where no Triton X-100 was added and cells were directly lysed in 250 μl of 5 $\times$ PAGE.

Antibodies

Antibodies were generally used at either 1:1000 dilution for polyclonal and 1:500 dilution for monoclonal antibodies; polyclonal rabbit antibodies described earlier (Matsuoka *et al.*, 1998; Kizhatil *et al.*, 2007a) include anti- β 2-spectrin, anti-ankyrin-G, anti- α -adducin, anti-MARCKS; monoclonal mouse antibodies include anti-E-cadherin (Invitrogen), anti-Na/K ATPase antibody (Affinity BioReagents, Golden, CO), anti- α -tubulin antibody (Sigma); and polyclonal goat anti-B-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), chicken anti-green fluorescent protein (GFP; Aves Labs, Tigard, OR).

Immunoblots

Proteins were separated by electrophoresis on 3.5–17% gradient gels, transferred to nitrocellulose membrane, and subjected to immunoblotting. Membranes were blocked for 20 min with 4% BSA in blot buffer (10 mM phosphate buffer, 150 mM NaCl, 0.2% Triton-X 100, pH 7.4). Primary antibodies were generally used at 1:1000 dilution. Mouse primary antibodies were sandwiched with rabbit anti-mouse secondary (Pierce). Immunoblots were incubated with ^{125}I -conjugated protein A/G for 1 h, washed extensively in blot buffer, and developed using phosphor imager (Typhoon 9200; GE Life-sciences, Pittsburgh, PA).

cDNA Constructs

Lentiviral plasmids were obtained from addgene (www.addgene.com). They include pLVPT-tTR-KRAB, pMD2.G, psPAX2, pLVTHM. α -adducin shRNA-targeted sequence was GTGACTGCATCCAGTTTGG. Control shRNA targeted sequence was GAGGATCTGACTCCATCGC. Both adducin and control shRNA were first cloned into the pSuper-GFP plasmid using BglII/HindIII restriction sites. The shRNA cassette including the H1-promoter was transferred into the pLVTHM plasmid using EcoRI/ClaI sites. The final inducible shRNA plasmid was generated by transferring the Tet-O H1-shRNA cassette from pLVTHM into pLVPT-tTR-KRAB using MscI/FspI restriction sites. Hem-

agglutinin (HA)-adducin wild type (WT) is described in Matsouka *et al.* (1998) and was made shRNA resistant by mutation of base pairs 581–585 using the primer: CTTTACAGTGAAGTGAAGTCCAGTAGTTGGTTAAGATCAATC. To make HA-adducin 1–711, amino acid 712 was converted a stop codon by site directed mutagenesis (Stratagene, La Jolla, CA) using the forward primer: GAGGAGGGGGCCCGCGCTAACCTGGCAGCGATGGGTCTC. To make HA-adducin S716D/S726D two rounds of site-directed mutagenesis was performed using primers GTCTCCAGGCAAGTCCCGGATAAAAAGA-AGAAGAAGTTC for S716D and GAAGAAGTTCCTACCCCGGATTTT-TCTGAAGAAGAGCAAG for S726D. To make HA- α -adducin KK718AA, site-directed mutagenesis was used with the primer GCAAGTCCCGGTC-CAAAGCGCGCGGAAGTTCCTGATCCCGGCC.

E-cadherin-GFP was described previously (Kizhatil *et al.*, 2007a).

Lentivirus Production and Transduction of HBE Cells

Lentivirus was produced in human embryonic kidney 293T17 cells. 293T17 cells were seeded at 2.6×10^6 cells/10-cm dish, cultured for 24 h, and then transfected with 2 μg pLVPT-tTR-KRAB, 1 μg psPAX2, 0.5 μg pMDG.2 using Effectene (Invitrogen) transfection reagent. Viruses were collected from cell culture media at 48 and 72 h after transfection and concentrated using SW-28 swinging bucket rotor at 25,000 rpm for 2 h. Viral pellets were resuspended in 1 ml of DMEM and used immediately or frozen at -80°C for storage. HBE cells were transduced with concentrated lentiviral stock at $\sim 2 \times 10^6$ transducing units (TU)/ml for 8 h and then allowed to expand for 5 d. Cells were subsequently sorted by FACS analysis based on GFP levels where cells at the high or low end of GFP expression were eliminated.

Figure 1. HBE cells express α and γ forms of adducin. (A) Affinity-purified anti-MARCKS and anti- α -adducin antibodies were used to blot lysates from red blood cell ghosts (RBC) and HBE cells. Anti-MARCKS antibody recognizes α (105 kDa) and β (97 kDa) in RBCs and recognizes α (105 kDa) and γ (90 kDa) in HBE cells. The affinity-purified rabbit anti- α -adducin antibody recognizes a single polypeptide with M_r 105 kDa in both RBCs and HBE cells. (B) Immunofluorescence staining of HBE cells reveals α -adducin and β 2-spectrin both colocalize with E-cadherin in HBE cells. Scale bars, 20 μm . (C) Confocal slices taken of the XZ axis reveal extensive colocalization of α -adducin and β 2-spectrin, with E-cadherin along the lateral membrane. Scale bars, 10 μm .

Fluorescence Recovery after Photobleaching

HBE^{CTL} and HBE^{ADD1} cells were seeded at 60,000 cells/MatTek plate and were transfected with 250 ng of E-cadherin-GFP using Effectene. After 6 h of transfection cells were incubated in growth media supplemented with 100 ng/ml doxycycline for 72 h. Fluorescence recovery after photobleaching (FRAP) was performed on a laser scanning microscope (LSM5) live duoscan confocal system using an Apochromat 100× NA 1.4 objective. All experiments were performed at 37°C with 5% CO₂ using an Incubator XL-3-LSM humidified chamber (Zeiss, Germany). E-cadherin-GFP signal was bleached using a 488-nm laser at 100% transmission with a bleach scan speed of 25.61 μ s. Images were collected every second for 2 min at 3% laser transmission with scan speeds between 66.67 to 150 ms. Fluorescence intensities for background, whole cell, and the bleach region were collected using LSM 4.0 software. Phair's double normalization method was used to generate a FRAP recovery curve over time in seconds. The curve was fitted using the single exponential equation $y = y_0 + Ae^{-bx}$. The mobile fraction was calculated using the equation $Mob = -A/[1 - (y_0 + A)]$. Halftime of recovery was calculated using $-\ln 0.5/b$.

Fluorescence Lost in Photobleaching

Cells were transfected and prepared similar to FRAP experiments. Fluorescence lost in photobleaching (FLIP) was performed using the same LSM Live duoscan system for FRAP. Bleaching was repeated every 10 s with a 488-nm laser at 100% transmission and for 10 iterations with a bleach scan speed of 25 μ s. Images were collected every 5 s for 10 min with a scan speed of 170 ms at 1% laser transmission. Loss of fluorescence was recorded at the described region of interest with corrections for background fluorescence and acquisition bleaching to generate a normalized fluorescence curve over time in seconds.

RESULTS

α -Adducin Expression and Localization in HBE Cells

As a first step in evaluating the function of adducin in human bronchial epithelial (HBE) cells, we determined the major isoforms expressed in these cells and their localization. Adducins are a family of three closely related polypeptides encoded by distinct genes (α -, β -, and γ -adducins; Joshi *et al.*, 1991; Dong *et al.*, 1995). α -Adducin forms heterodimers and tetramers with β -adducin and most likely γ -adducin (Dong *et al.*, 1995; Hughes and Bennett, 1995). α -Adducin is expressed in nearly all cell types, whereas β - and γ -adducins have specialized patterns of expression (Bennett and Baines, 2001). We immunoblotted HBE and red blood cell (RBC) lysates with a polyclonal antibody directed against the C-terminal MARCKS-domain, which is shared by the major polypeptides of α -, β -, and γ -adducin. The MARCKS-domain antibody recognizes α -(M_r 105 kDa) and β -(M_r 97 kDa) adducin in RBC membranes (Figure 1A, lane 1). However, the MARCKS antibody recognizes α -(M_r 105 kDa) and γ -adducin (M_r 90 kDa), but not β -adducin in HBE cells (Figure 1A, lane 2). To specifically detect α -adducin and not γ -adducin in HBE cells, we generated an antibody directed against the tail domain of α -adducin, amino acids 536-737. This antibody was affinity-purified and recognizes a single polypeptide band with an apparent molecular weight of ~105 kDa in both HBE cells and RBCs (Figure 1A, lanes 3 and 4).

Immunofluorescent staining of α -adducin in HBE cells shows localization at sites of cell-cell contact and colocalization with E-cadherin in XY and XZ sections (Figure 1Bc). On the basis of the colocalization of β 2-spectrin with E-cadherin (Figure 1Bc), we conclude that endogenous α -adducin colocalizes with β 2-spectrin along the lateral membrane and is excluded from the apical and basal membranes in HBE cells.

An Epithelial Cell Line for Inducible Depletion of α -Adducin

Considering adducin forms α - β and likely α - γ heterotetramers (Hughes and Bennett, 1995; Dong *et al.*, 1995), we chose to target α -adducin for shRNA depletion. This strategy elim-

inates the possibility of compensation through up-regulation of β -adducin in HBE cells, which is a concern based on the finding that γ -adducin is up-regulated in RBCs of the β -adducin knockout mouse (Gilligan *et al.*, 1999). We first identified a shRNA that was effective in knocking down α -adducin after screening six potential candidates through cotransfection of human GFP- α -adducin cDNA and pSuper shRNA plasmids into mouse NIH3T3 fibroblasts. We next introduced this shRNA into epithelial cells using an inducible all-in-one bicistronic lentiviral transfer plasmid encoding a Tetracycline (tet)-on-regulated shRNA cassette, a Tet repressor protein (KRAB), and GFP (Szulc *et al.*, 2006). After lentiviral infection, two cell lines were generated with HBE^{ADD1} containing the α -adducin-specific shRNA and HBE^{CTL} containing a shRNA targeted toward α -adducin but harboring 3 mis-sense changes rendering it nonfunctional toward α -adducin. Each cell line was sorted by FACS to eliminate cells containing either very low or very high levels of GFP, resulting in a homogeneous population more evenly regulated through doxycycline.

HBE^{CTL} and HBE^{ADD1} cells were grown to confluence and allowed to establish lateral membranes before the addition of doxycycline and induction of siRNA expression. Cells were then monitored for adducin knockdown at 24-h intervals by immunoblots as a measure of total protein in the culture and by immunofluorescence to estimate levels in individual cells. As determined from immunoblots, α -adducin is depleted by ~80% after 72 h of doxycycline treatment

Figure 2. Adducin is required to maintain lateral membrane height. (A) Confluent monolayers of HBE^{ADD1} were allowed to form lateral membranes then induced to express the ADD1 shRNA using 100 ng/ml doxycycline for the indicated lengths of time. Cell extracts were collected from each time point and blotted using rabbit antibodies: anti- α -adducin, anti- β 2-spectrin; mouse antibodies: anti-E-cadherin. (B) HBE^{ADD1} cells were fixed at 0-, 24-, 48-, and 72-h time points after doxycycline treatment and costained using anti- α -adducin (red) and anti-E-cadherin (green), revealing changes in cross-sectional surface area. (C) Lateral membrane height and surface area were measured using LSM510 software on images collected from 25 fields for each time point and then correlated with α -adducin levels. Differences in cell height and surface area were confirmed to be statistically significant using Student's *t* tests; $n > 50$ for control and knockdown cells. (D) The single time point of 72 h was used to collect XZ confocal slices from HBE^{ADD1} or HBE^{CTL} cells. HBE^{CTL} were stained with mouse gp135 antibody (green), and tight junctions were stained through mouse anti-ZO-1 (green). DNA is stained in blue, whereas adducin is stained in red. HBE^{ADD1} cells also maintain tight junctions and apical polarity. Scale bars, 20 μ m.

(Figure 2A). Moreover, we did not observe any compensatory increase in β - or γ -adducin using the MARCKS antibody (not shown). Total protein levels of β 2-spectrin and E-cadherin were equal at all time points measured (Figure 2A). Immunofluorescence staining of confluent monolayers revealed the greatest reduction of α -adducin at 72 h, where staining was reduced 85–95% (Figure 2B). However, ~5% of cells retain normal α -adducin expression, contributing to the 20% α -adducin detected in immunoblots of HBE^{ADD1} cells at the 72-h time point.

We next determined if loss of adducin effects viability and polarity of epithelial cells. Less than 1% of HBE^{CTL} and HBE^{ADD1} cells cultured with doxycycline are permeable to trypan-blue after 72 h under conditions where α -adducin was depleted by 80% (Supplementary Figure 1). Thus adducin depletion does not lead to reduction in cell viability, at least in the time frame of these experiments. We next evaluated the effects of α -adducin depletion on apical polarity of epithelial cells. Knockdown of α -adducin does not alter localization of gp135, a marker for the apical membrane (Figure 2D). In addition, the tight junction marker ZO-1 is positioned normally at the boundary between apical and lateral membranes in α -adducin-depleted cells (Figure 2D). These results show that adducin depletion alters neither cell viability nor general apical-lateral polarity of epithelial cells.

Adducin Is Necessary to Stabilize Preformed Lateral Membranes

The inducible shRNA delivery system allows depletion of α -adducin from epithelial cells with fully formed lateral membranes. Depletion of α -adducin from columnar HBE cells results in a substantial reduction of lateral membrane height from 7.5 μ m down to 3.5 μ m. Loss of cell height is accompanied by an increase in cross sectional area of apical/basal surfaces from 169 to 462 μ m² (Figure 2C). α -Adducin levels decrease at a faster rate than the loss of the lateral membrane or the expansion of apical surface area (Figure 2C). This lag between loss of adducin and loss of membrane suggests that either some time is required for cells to adjust to the absence of adducin or that nearly complete loss of adducin must occur before membranes are depleted.

Because adducin is required to maintain lateral membrane height, we next wanted to address its role during the initial formation of the lateral membrane. De novo formation of the lateral membrane can be followed in cells undergoing cell division. Epithelial cells rapidly build their lateral membrane from the base upward during cytokinesis. α -Tubulin labeling was used to identify the midbody of cells transitioning between late anaphase and telophase, whereas β -catenin provided a lateral membrane marker to follow membrane biogenesis. To determine if α -adducin is required for lateral membrane biogenesis, we followed the formation of the lateral membrane between anaphase and telophase in HBE^{CTL} and HBE^{ADD1} cells using α -tubulin and β -catenin labeling. In HBE^{CTL} cells, the lateral membrane begins to form at the base during the end of anaphase and continues upward until the entire membrane has been established by the end of cytokinesis (Figure 3). In HBE^{ADD1} cells lateral membranes reach a length of 3.5 μ m in the absence of α -adducin. Adducin-depleted cells do not achieve the height of control cells, suggesting a different set point for the extent of the lateral membrane. However, at least the initial phase of lateral membrane biogenesis proceeds normally in the absence of adducin.

Loss of adducin from HBE cells presents an interesting contrast to depletion of either ankyrin-G or β 2-spectrin. Loss of ankyrin-G or β 2-spectrin abolishes de novo formation of

Figure 3. Biogenesis of the lateral membrane after cytokinesis proceeds in the absence of α -adducin. Cells were treated with 100 ng/ml doxycycline for 72 h then fixed and stained with α -tubulin (green) and β -catenin (red). Left panel represents collapsed Z-series collected at 0.3- μ m intervals, whereas right panel shows XZ section taken at 0.05- μ m intervals with the addition of staining for α -adducin to show level of depletion. Arrow depicts the forming lateral membrane as marked by β -catenin. Scale bars, (XY) 20 μ m; (XZ) 5 μ m.

lateral membranes during mitosis (Kizhatil and Bennett, 2004; Kizhatil *et al.*, 2007a), whereas loss of adducin has no effect (Figure 3B). Moreover, cells depleted of either ankyrin-G or β 2-spectrin accumulate E-cadherin in the *trans*-Golgi (Kizhatil *et al.*, 2007b). In contrast, E-cadherin is not reduced at the lateral membrane and does not appear in an intracellular compartment in adducin-depleted cells (Figure 2). Adducin thus is not required for either formation of new membrane during mitosis or for transport of E-cadherin to the lateral membrane.

Figure 4. β 2-spectrin is required for accumulation of adducin at the lateral membrane. (Top row) Staining of β 2-spectrin after transfection with control (pSuper plasmid) or β 2-spectrin shRNA plasmids. (Bottom row) Staining of α -adducin after transfection with control or β 2-spectrin shRNA plasmids. Asterisks show sites of cell-cell contact where α -adducin staining is eliminated. Scale bars, 20 μ m.

Figure 5. The MARCKS domain of α -adducin is required for lateral membrane targeting and rescue of lateral membranes upon endogenous α -adducin depletion. (A) Diagram depicting domain organization for α -adducin, with the core domain at the N-terminal half (a.a. 1-365) followed by the neck (a.a. 351-500) and tail domain (a.a. 500-711) and ending with the MARCKS-domain (a.a. 711-737). Two lysines that are predicted to be required for β 2-spectrin interactions are underlined, and the PKC sites are highlighted in red. (B) HBE cells were transfected with shRNA resistant HA- α -adducin constructs with the displayed amino acid substitutions and stained using a monoclonal HA antibody. Scale bars equal 20 μ m. (C) HBE^{CTL} or HBE^{ADD1} cells were transfected at 70% confluence with HA- α -adducin WT cDNA, containing nucleotide substitutions at nucleotides 581-584 conferring shRNA resistance. After 24 h after transfection cells were treated with 100 ng/ml doxycycline for 72 h, then fixed and stained with monoclonal HA antibody (green) and rabbit ankyrin-G antibody (red). Images were taken along the XZ axis to measure lateral membrane height. Arrow head points to lateral membrane of an untransfected cell; arrow points to an HA- α -adducin WT transfected cell. HA- α -adducin 1-711 cDNA lacks the MARCKS domain sequence, whereas HA- α -adducin KK718AA contains amino acids substitution of two lysines at positions 718 and 719 to alanines. HA- α -adducin S716D/S726D contains substitution of serines 716 and 726, representing the major PKC sites, to aspartic

α -Adducin and β 2-Spectrin Are Functionally Coupled in Epithelial Cells

Biochemical studies using both erythrocyte spectrin and brain spectrin have shown that adducin enhances binding of β -2 spectrin to the fast-growing ends of F-actin filaments (Gardner and Bennett, 1987; Bennett *et al.*, 1988; Joshi and Bennett, 1990; Li and Bennett, 1996; Li *et al.*, 1998). Association of adducin with lateral membrane is likely to occur through interaction with spectrin, which associates with ankyrin-G190 (Kizhatil *et al.*, 2007a). To determine the effects of β 2-spectrin depletion on adducin, we turned to the system used by Kizhatil *et al.* (2007a) using cotransfection of the shRNA plasmid with the marker peGFP-N1 plasmid (Figure 4, top row). Cells depleted of β 2-spectrin show drastically reduced levels of α -adducin on the lateral membrane (Figure 4, bottom row). Levels of α -adducin are reduced to nearly background levels in some cells (asterisk), whereas other areas show very low levels of adducin expression, likely representing sites where β 2-spectrin depletion is not complete. These results reveal that accumulation of adducin at lateral membranes is strongly dependent on the presence of β 2-spectrin on the lateral membrane.

To determine if adducin's interaction with spectrin is required for its function and localization, we turned to rescue experiments using mutants of α -adducin and adducin domains (Figure 5A). We first performed rescue experiments using a cDNA encoding a hemagglutinin (HA)-tagged human α -adducin containing four silent mutations conferring resistance to the ADD1 shRNA. HA- α -adducin WT is targeted to the lateral membrane in HBE cells (Figure 5B). HBE^{ADD1} cells transfected with HA- α -adducin WT and treated for 72 h with doxycycline retain their lateral membranes (Figure 5C). In the first panel of Figure 5C we can see both rescued cells and untransfected cells within the same field showing their respective differences in lateral membrane height. These results confirm that phenotypes observed are due to shRNA knockdown of α -adducin.

The MARCKS domain is required for interaction of α -adducin with β 2-spectrin and actin (Li *et al.*, 1998). Moreover, phosphorylation at serines 716 and 726 within the MARCKS domain abolishes adducin activity in vitro and leads to the dissociation of spectrin-actin structures in cultured cells and platelets (Matsuoka *et al.*, 1996; Matsuoka *et al.*, 1998; Barkalow *et al.*, 2003). We therefore determined the role of the MARCKS domain in α -adducin activity in preserving HBE lateral membranes. Transfection of HA- α -adducin 1-711, lacking the MARCKS domain, into the HBE^{ADD1} cell line failed to rescue the lateral membrane height and led to an average lateral membrane height of 3.7 ± 0.4 μ m (Figure 5Cd, top right). This construct also did not target to the lateral membrane (Figure 5B). Lysines 718–722 lie between serines 716 and 726 and may interact directly with β 2-spectrin. We converted lysines 718–719 to alanines to make HA- α -adducin KK718AA. Interestingly, this construct does not prevent reduced height of the lateral membrane, resulting in transfected HBE^{ADD1} cells with lateral membrane heights of $\sim 4 \pm 0.8$ μ m (Figure 5Cd, bottom left). Similar to the MARCKS deletion, HA- α -adducin KK718AA does not assemble at the lateral membrane (Figure 5B). Finally, we converted serines 716 and 726 to aspartic acid to mimic the phosphorylated state of α -adducin. HA- α -adducin S716D/

acid. Scale bars, 10 μ m. (D) Only full-length WT HA- α -adducin cDNA can rescue the lateral membrane from ~ 3.5 to ~ 7 μ m upon depletion of endogenous α -adducin. Error bars, SEM; $n \geq 12$ for each construct.

Figure 6. Increased solubility of $\beta 2$ -spectrin in Triton X-100 in α -adducin-depleted HBE cells. (A) HBE^{CTL} and HBE^{ADD1} cells were seeded to confluence, treated with 100 ng/ml doxycycline for 72 h and then extracted with 0.2% Triton X-100 for 10 min at room temperature. Cells were fixed and stained for $\beta 2$ -spectrin; untreated (left panel), Triton X-100 treated (right panel). Confocal XY sections were taken from different fields and revealed similar reduction of $\beta 2$ -spectrin levels after extraction in HBE^{ADD1} cells. Diagonal white line represents regions of interests used to measure fluorescence intensities. (B) Line graph of representative level of fluorescence intensity between control (blue, top) and adducin depleted (red, bottom) cells taken from LSM imager software. F.I., fluorescence intensity, a.u., arbitrary units. (C) Cells were extracted as above but in the presence of protease inhibitors separating soluble and insoluble fractions. Samples were loaded in 5 $\times$ PAGE buffer and blotted for $\beta 2$ -spectrin. (D) Fluorescence was measured using LSM imaging software. Quantitation of immunofluorescence was performed by measuring lateral membrane intensities of 10 regions of interests from each field of cells, number fields = 12; total number of lateral membranes = 120, (n = 120). (E) Quantitation for blots were performed in triplicates over three separate experiments each in triplicates (n = 6). Protein levels were normalized using GAPDH for total, soluble, and insoluble fractions. Numbers are the mean from those experiments with SDs calculated for each data set (error bars). Scale bars, 20 μ m.

S726D does not prevent reduction of the lateral membrane in HBE^{ADD1} cells leading to lengths of $3.9 \pm 0.6 \mu\text{m}$ (Figure 5Cd, bottom right). Moreover, HA- α -adducin S716D/S726D is not assembled at the lateral membrane in HBE cells (Figure 5B). The lack of rescue in these three mutant constructs is consistent with the possibility that α -adducin interactions with $\beta 2$ -spectrin are required for the maintenance of epithelial lateral membranes. It also is conceivable that the MARCKS domain interacts with proteins and/or phospholipids in addition to spectrin. Although we cannot exclude this possibility, we have observed that adducin constructs lacking the head domain but retaining the MARCKS domain do not target to the lateral membrane and do not rescue (data not shown). Thus the MARCKS domain alone, which would be expected to interact equivalently with phospholipids as intact adducin, is not sufficient for membrane targeting or rescue.

To evaluate the *in vivo* effect of α -adducin depletion on $\beta 2$ -spectrin localization and detergent solubility in HBE cells, we extracted HBE^{CTL} and HBE^{ADD1} cells with Triton X-100 and stained with the polyclonal $\beta 2$ -spectrin antibody. In HBE^{CTL} cells, 90% of lateral membrane-associated $\beta 2$ -spectrin is resistant to extraction by Triton X-100 (Figure 6Ad, top row), whereas the majority of intracellular staining is depleted. In contrast, in HBE^{ADD1} cells we observe a 43% reduction of $\beta 2$ -spectrin levels on the lateral membrane after Triton X-100 extraction, and intracellular $\beta 2$ -spectrin is reduced to background levels (Figure 6Ac, bottom row). Immunoblots reveal a 30% increase in the detergent solubility of $\beta 2$ -spectrin in the HBE^{ADD1} cell line when compared with the level of $\beta 2$ -spectrin solubility in HBE^{CTL} cells (Figure

6Ce). Ankyrin-G is nearly completely insoluble in Triton X-100 in polarized epithelial cells. Staining of extracted cells from an adducin-depleted population reveals that ankyrin-G increased to 26% soluble from $\sim 7\%$ in control cells, based on immunofluorescence analysis of lateral membranes (Supplementary Figure 2). Western blotting shows an increase in detergent solubility of ankyrin-G from ~ 5 to $\sim 16\%$ in adducin-depleted cells. We conclude that loss of α -adducin expression in HBE cells leads to conversion of a significant population of $\beta 2$ -spectrin and ankyrin-G from a detergent-insoluble to a detergent-soluble pool. Together with the critical role of the MARCKS domain in adducin function described above, these results suggest a direct interaction of adducin with spectrin.

Adducin Is Necessary to Minimize Lateral Membrane Deformations

HBE cells within a confluent and polarized monolayer typically are hexagonally packed with straight and noncurved lateral membranes of uniform cross-sectional area from top to bottom. We wondered if adducin depletion and weakening of the spectrin network would affect the curvature of the lateral membrane in confluent monolayers. X-Z sections of adducin-depleted cells labeled for three different lateral membrane proteins (ankyrin-G, Na/K ATPase, and E-cadherin) revealed marked irregularities and a gain of membrane curvature (Figure 7B). All three markers extended into the basal surface of adducin-depleted cells, indicating either increased diffusion or transposition of the entire lateral membrane.

We next evaluated mixed cultures where cells from HBE^{CTL} and HBE^{ADD1} cell lines (identified by GFP expres-

Figure 7. Adducin is necessary to maintain cell shape and restrict basal accumulation of lateral membrane proteins. (A) View from top of a monolayer stained with E-cadherin reveals changes to cross-sectional surface area. View from XZ plane in both HBE^{CTL} and HBE^{ADD1} cells stained through markers for the lateral membrane including; E-cadherin, Na/K ATPase, and Ankyrin-G. Resident lateral membrane proteins can often be seen accumulating at the basal surface of adducin depleted cells (B). Arrows points to effected HBE cells. (C) Normal HBE cells were seeded with HBE^{CTL} cells at a 10:1 ratio and induced with doxycycline for 72 h. HBE^{CTL} cells are identified through GFP expression and display normal lateral membrane shape and epithelial morphology. HBE^{ADD1} cells are also identified through GFP expression and can be seen to display curvature of the lateral membrane combined with increased number of cell-cell contacts. (D) Thirty-six percent of adducin-depleted cells display loss of normal epithelial morphology, whereas only 3% of control cells show similar changes to cell shape; $n = 31$ HBE^{CTL}, $n = 33$ HBE^{ADD1}. (E) Measurement of cell-cell contact numbers seen in both GFP-positive cell populations; 30 cells were imaged and counted for each group and were displayed as function of the number of cell-cell contacts. Scale bars, (XY) 20 μm ; (XZ) 10 μm .

sion) were grown with 10-fold excess normal HBE cells so that each GFP-positive cell was surrounded by GFP-negative HBE cells. When normal cells are mixed with HBE^{CTL} cells, GFP-positive cells maintain normal lateral membrane shape with little to no curvature along their lateral membranes (Figure 7C, top). However, when HBE^{ADD1} cells are mixed with normal HBE cells, we observe significant increases in lateral membrane curvature as well as number of neighboring epithelial cells in direct contact (Figure 7C, bottom). The curvature suggests that levels of membrane tension between normal and adducin-depleted cells are uneven. This phenotype is seen in 36% of GFP-positive cells from HBE^{ADD1} cells, whereas it is only observed in 3% of HBE^{CTL} cells (Figure 7D). This represents a 10-fold increase in susceptibility to shape changes at the lateral membrane after adducin depletion. In addition to increases to lateral membrane curvature, HBE^{ADD1} cells often are unable to

maintain the hexagonal arrangement found in normal cells and in HBE^{CTL} cells. Control cells average six cell-cell contacts per GFP-positive cell with only 1 of 30 cells displaying 8 or more cell-cell contacts (Figure 7E). However, adducin-depleted cells average between 7 and 8 cell-cell contacts per GFP-positive cell with 10 of 30 cells displaying 8 or 9 cell-cell contacts (Figure 7E). Thus, when mixed with normal HBE cells adducin-depleted cells are unable maintain the normal hexagonal arrangement found in compact polarized monolayers.

Enhanced Long-Range Mobility of E-Cadherin in α -Adducin-depleted HBE Cells

Spectrin deficiencies have been correlated with increased lateral mobility of integral membrane proteins both in erythrocytes and excitable membranes (Fowler and Bennett, 1978; Sheetz *et al.*, 1980; Nishimura *et al.*, 2007). Moreover, a mutant E-cadherin with reduced ankyrin-G-binding activity

Figure 8. The mobile fraction of E-cadherin-GFP is increased in cells lacking α -adducin. (A) HBE^{ADD1} or HBE^{CTL} cells were transfected with E-cadherin-GFP. After 24 h cells were treated with 100 ng/ml doxycycline for 72 h. Photobleaching was carried out at sites of cell–cell contact using a single FRAP configuration that consistently resulted in 60–70% reduction of immunofluorescence. Shown here is a representative bleach experiment from E-cadherin-GFP expressed in either HBE^{CTL} or HBE^{ADD1} cells with the bleached area marked with a red rectangle. The typical increase in recovery can be seen for the E-cadherin-GFP transfected into the HBE^{ADD1} cell line. (B) E-cadherin-GFP was bleached and allowed to recover for 2 min, with fluorescence recovery captured at 1-s intervals. Normalized fluorescence was calculated using corrections for background signal and acquisition bleaching and plotted versus time in seconds. (C) Mobile fraction of E-cadherin-GFP was calculated using the Phair method. Median mobile fractions are 0.31 for HBE^{CTL} and 0.55 for HBE^{ADD1}. A scatter plot of each calculated mobile fraction is presented to show variations from the median; $n = 29$ HBE^{CTL}, $n = 28$ HBE^{ADD1}. Scale bars, 10 μm .

exhibits increased lateral diffusion in HBE cells (Kizhatil *et al.*, 2007b). We therefore evaluated the lateral mobility of E-cadherin in adducin-depleted cells by measuring the FRAP of E-cadherin-GFP.

E-cadherin-GFP is recruited to the lateral membrane at sites of cell–cell contact in both HBE^{CTL} and HBE^{ADD1} cell lines (Figure 8A). Bleaching parameters were set to deplete the level of the E-cadherin-GFP signal on the lateral membrane by ~60–70% (Figure 8A, top row). E-cadherin-GFP shows an enhanced recovery from bleaching in the HBE^{ADD1} cell line, suggesting that loss of α -adducin has altered the mobility of E-cadherin-GFP (Figure 8A, bottom row). To generate a statistically relevant change in mobile fraction we bleached 28 examples each from the HBE^{CTL} and HBE^{ADD1} cell lines. Our results show a clear trend of increased mobility of E-cadherin-GFP in the HBE^{ADD1} cell line (Figure 8B). The median mobile fraction for E-cadherin-GFP in the HBE^{CTL} cell line is 0.31, with a recovery half-time of 27.5 s, whereas the median mobile fraction for E-cadherin-GFP increased to 0.55 in the HBE^{ADD1} cell line and the recovery half-time decreased to 21.5 s (Figure 8C). This represents a 77% increase in the mobile fraction of E-cadherin-GFP, which is a significant change in E-cadherin mobility in adducin-depleted cells.

We next wanted to determine the distance that E-cadherin-GFP traversed during FRAP and in particular whether effects of adducin-depletion reflected loss of local or long-range restraints. We addressed this issue by measuring FLIP at sites separated at least 10 μm from the bleach site (Figure 9Ab). By continuously bleaching a single region of the lateral membrane and collecting successive images from a distant site, we could determine the extent of lateral mobility of E-cadherin-GFP. Region of interest (ROI) 1 (red square) was bleached every 10 s, and images were collected every 5 s for 10 min from ROI2 (Figure 9A). In HBE^{CTL} cells levels of E-cadherin-GFP outside the photobleached region are remarkably resistant to photobleaching. ROI2 (white square) in HBE^{CTL} is ~10 μm from ROI1 and shows an 18% reduction in fluorescence intensity during the 10-min time course

(Figure 9B). Using the same parameters, we see that ROI2 in the HBE^{ADD1} example is 12 μm from ROI1 but displays a 57% reduction in fluorescence intensity (Figure 9B). We performed additional FLIP experiments on 15 examples from HBE^{CTL} and HBE^{ADD1} cell lines to calculate an average change in FLIP over the 10-min interval. In HBE^{CTL} cells the median change in fluorescence intensity of ROI2 is ~19%, whereas in HBE^{ADD1} cells we observe a significant loss in fluorescence of ~50% (Figure 9C). These results demonstrate that adducin restricts micrometer-scale diffusion of E-cadherin in the lateral membrane.

Levels of E-cadherin-GFP fluorescence are depleted as a function of distance from the site of photobleaching. E-cadherin-GFP is quickly and more efficiently depleted within 1 to 4 μm of the bleached area in both control and adducin-depleted cells (Figure 9De). In control cells at sites over 5 μm from the site of photobleaching we observe stability of the E-cadherin-GFP signal, which maintains 80% of original fluorescence even after 10 min of bleaching (Figure 9D). This level of fluorescence stability is not observed in α -adducin-depleted cells. In these cells, only 40% of E-cadherin's original fluorescence signal remained after 10 min of photobleaching at distances greater than 4 μm from ROI1 (Figure 9E). Additionally, even at 16 μm from the site of photobleaching α -adducin-depleted cells retain only 50% of their original E-cadherin-GFP signal after 10 min of photobleaching. These results confirm a gain of long-range diffusion of E-cadherin-GFP upon depletion of α -adducin from epithelial cells.

DISCUSSION

Adducin has been hypothesized to direct assembly of β 2-spectrin–actin networks at specific sites in cells, based on its behavior in biochemical assays (Bennett *et al.*, 1988). In support of this proposal, we present evidence that adducin is required to stabilize a β 2-spectrin assembly at the lateral membranes of HBE cells. We use a Tet-on regulated lentiviral delivered siRNA system to deplete α -adducin from con-

Figure 9. Enhanced long range mobility of E-cadherin-GFP in adducin-depleted cells. (A) E-cadherin-GFP was bleached repeatedly every 10 s for 10 min, with images collected every 5 s. Region of interest (ROI) 1 (red square) represents the bleached region, whereas ROI2 (white square) is the site used to monitor loss of fluorescence. (B) Loss in fluorescence was plotted for E-cadherin-GFP in both HBE^{CTL} and HBE^{ADD1} cells after corrections for background signal and fluorescence lost during image acquisition. ROI2 for HBE^{CTL} is plotted in blue; ROI2 for HBE^{ADD1} is plotted in red. Curves represent the median fluorescence from 15 samples in both cell lines. (C) A scatter plot of fluorescence from all samples is shown to display variations from the median; $n = 15$ HBE^{CTL}, $n = 15$ HBE^{ADD1}. (D and E) Fluorescence intensity was measured as a function of distance from ROI1. Intensities were measured every 1.2 μm from ROI1 up to 16.8 μm away, representing 15 ROIs. Three time points were used to measure fluorescence intensities reflecting changes to signal levels from prebleach levels at time 0 min: blue, 1 min; maroon, 5 min; and red, 10 min. (D) Left graph, HBE^{CTL} cells; (E) right graph, HBE^{ADD1} cells. Scale bars, 10 μm .

fluent HBE cells. Inducible depletion of α -adducin in HBE cells resulted in increased detergent solubility of spectrin accompanied by reduced height of the lateral membrane after normal membrane biogenesis during mitosis. siRNA-resistant α -adducin cDNA prevented loss of lateral membrane, but only if α -adducin retained the MARCKS domain that mediates β 2-spectrin-actin interactions (Li *et al.*, 1998). Phospho-mimetic versions of adducin with S/D substitution at PKC phosphorylation sites in the MARCKS domain were not active in rescue. We show that adducin modulates long-range organization of the lateral membrane based on several criteria. First, the lateral membrane of adducin-depleted cells exhibited increased curvature and expansion into the basal surface. In addition, adducin-depleted cells exhibited loss of hexagonal packing. Moreover photobleaching experiments reveal that E-cadherin-GFP, which normally is restricted in lateral mobility, rapidly diffuses over distances up

to 10 μm in adducin-depleted cells. We conclude that adducin acting through spectrin provides a novel mechanism to regulate global properties of the lateral membrane of bronchial epithelial cells.

Spectrin is organized as a lattice in erythrocytes which preserves cell shape and promotes resistance to deformation during circulation (Bennett and Baines, 2001). Our findings support the working hypothesis that adducin promotes a spectrin network that has a similar stabilizing role for lateral membranes in epithelial cells. Depletion of adducin is likely to reduce assembly of spectrin tetramers into high-ordered structures and lead to disorder in the spectrin-actin network. This model can explain the phenotypes observed during α -adducin depletion including loss of lateral membrane height and enhanced lateral mobility of E-cadherin.

Phosphorylation of adducin by PKC within its MARKS domain inhibits activity in promoting spectrin-actin complexes in vitro and leads to the disassembly of spectrin-actin in activated platelets (Matsuoka *et al.*, 1998; Barkalow *et al.*, 2003). Given that conversion of serines 716 and 726 to aspartic acids inhibits targeting of α -adducin to the lateral membrane (Figure 5), we would predict that phosphorylation by PKC would also prevent association of adducin with β 2-spectrin in vivo. It is of interest in this regard that adducin is associated with the atypical PKC, PKC-lambda, in 3T3-L1 adipocytes, and may be constitutively phosphorylated, at least in these cells (Laustsen *et al.*, 2001). The par 3/par 6/atypical PKC complex localized at tight junctions (Izumi *et al.*, 1998) is a candidate to promote localized phosphorylation of adducin at tight junctions, which could contribute to limiting the lateral membrane.

The spectrin-actin junction in erythrocytes contains accessory proteins in addition to adducin including protein 4.1, dematin, tropomyosin, and tropomodulin that are all expressed in many types of cells (Bennett and Baines, 2001). Interestingly, depletion of tropomodulin 3 from lateral membranes of Caco-2 epithelial cells results in reduced cell height and increased cross-sectional area without a general loss of epithelial polarity (Weber *et al.*, 2007). These results were also attributed to dysfunction of spectrin-actin assembly and suggest a common mechanism to maintaining lateral membrane stability through activities of spectrin-junctional structures. It will be of interest to evaluate roles of additional proteins involved in promoting assembly of spectrin networks in epithelial cells. Adducin, protein 4.1 and tropomodulin are all substrates for PKC, suggesting the possibility that the entire spectrin-actin junction could be coordinately modulated by the same protein kinase(s) and phosphatase(s).

It will be important in future work to determine the relevance of adducin in bronchial epithelial cells and other epithelial tissues in vivo. Expectations from this study are that β 2-spectrin- and ankyrin-G-based pathways in initial formation of the lateral membrane will operate normally. However, we would predict that the ability of the lateral membrane to resist mechanical stress would be impaired in the absence of adducin. Thus, adducin-deficient bronchial epithelial cells may progress normally through development but subsequently be more susceptible to damage during adult life.

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REFERENCES

- Barkalow, K. L., Italiano, J. E. Jr., Chou, D. E., Matsuoka, Y., Bennett, V., and Hartwig, J. H. (2003). Alpha-adducin dissociates from F-actin and spectrin during platelet activation. *J. Cell Biol.* 161(3), 557–570.
- Bennett, V., Gardner, K., and Steiner, J. P. (1988). Brain adducin: a protein kinase C substrate that may mediate site-directed assembly at the spectrin-actin junction. *J. Biol. Chem.* 263(12), 5860–5869.
- Bennett, V., and Baines, A. J. (2001). Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. *Physiol. Rev.* 81(3), 1353–1392.
- Chen, H. *et al.* (2007). Combined deletion of mouse dematin-headpiece and beta-adducin exerts a novel effect on the spectrin-actin junctions leading to erythrocyte fragility and hemolytic anemia. *J. Biol. Chem.* 282(6), 4124–4135.
- Davis, J. Q., and Bennett, V. (1990). The anion exchanger and Na⁺/K⁺-ATPase interact with distinct sites on ankyrin in in-vitro assays. *J. Biol. Chem.* 265(28), 17252–17256.
- Dong, L., Chapline, C., Mousseau, B., Fowler, L., Ramsay, K., Stevens, J. L., and Jaken, S. (1995). 35H, a sequence isolated as a protein kinase C binding protein, is a novel member of the adducin family. *J. Biol. Chem.* 270(43), 25534–25540.
- Drenckhahn, D., Schluter, K., Allen, D. P., and Bennett, V. (1985). Colocalization of band 3 with ankyrin and spectrin at the basal membrane of intercalated cells in the rat kidney. *Science* 230(4731), 1287–1289.
- Drenckhahn, D., and Bennett, V. (1987). Polarized distribution of Mr 210,000 and 190,000 analogs of erythrocyte ankyrin along the plasma membrane of transporting epithelia, neurons and photoreceptors. *Eur. J. Cell Biol.* 43(3), 479–486.
- Fukata, Y., Oshiro, N., Kinoshita, N., Kawano, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., and Kaibuchi, K. (1999). Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. *J. Cell Biol.* 145(2), 347–361.
- Fowler, V., and Bennett, V. (1978). Association of spectrin with its membrane attachment site restricts lateral mobility of human erythrocyte integral membrane proteins. *J. Supramol. Struct.* 8, 215–221.
- Gardner, K., and Bennett, V. (1986). A new erythrocyte membrane-associated protein with calmodulin binding activity. Identification and purification. *J. Biol. Chem.* 261(3), 1339–1348.
- Gardner, K., and Bennett, V. (1987). Modulation of spectrin-actin assembly by erythrocyte adducin. *Nature* 328(6128), 359–362.
- Gilligan, D. M., Lozovatsky, L., Gwynn, B., Brugnara, C., Mohandas, N., and Peters, L. L. (1999). Targeted disruption of the beta adducin gene (Add2) causes red blood cell spherocytosis in mice. *Proc. Natl. Acad. Sci. USA* 96(19), 10717–10722.
- Hughes, C. A., and Bennett, V. 1995. Adducin: a physical model with implications for function in assembly of spectrin-actin complexes. *J. Biol. Chem.* 270(32), 18990–18996.
- Izumi, Y., Hirose, T., Tamai, Y., Hirai, S., Nagashima, Y., Fujimoto, T., Tabuse, Y., Kempfues, J., and Ohno, S. (1998). An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of *Caenorhabditis elegans* polarity protein PAR-3. *J. Cell Biol.* 143(1), 95–106.
- Joshi, R., and Bennett, V. (1990). Mapping the domain structure of human erythrocyte adducin. *J. Biol. Chem.* 265(22), 13130–13136.
- Joshi, R., Gilligan, D. M., Otto, E., McLaughlin, T., and Bennett, V. (1991). Primary structure and domain organization of human alpha and beta adducin. *J. Cell Biol.* 115(3), 665–675.
- Kaiser, H. W., O'Keefe, E., and Bennett, V. (1989). Adducin: Ca⁺⁺-dependent association with sites of cell-cell contact. *J. Cell Biol.* 109(2), 557–569.
- Kizhatil, K., and Bennett, V. (2004). Lateral membrane biogenesis in human bronchial epithelial cells requires 190-kDa ankyrin-G. *J. Biol. Chem.* 279(16), 16706–16714.
- Kizhatil, K., Yoon, W., Mohler, P. J., Davis, L. H., Hoffman, J. A., and Bennett, V. (2007a). Ankyrin-G and beta2-spectrin collaborate in biogenesis of lateral membrane of human bronchial epithelial cells. *J. Biol. Chem.* 282(3), 2029–2037.
- Kizhatil, K., Davis, J., Davis, L., Hoffman, J., Hogan, B., and Bennett, V. (2007b). Ankyrin-G is a molecular partner of E-cadherin in epithelial cells and early embryos. *J. Biol. Chem.* July 9 [Epub ahead of print].
- Kuhlman, P. A., Hughes, C. A., Bennett, V., and Fowler, V. M. (1996). A new function for adducin. Calcium/calmodulin-regulated capping of the barbed ends of actin filaments. *J. Biol. Chem.* 271(14), 7986–7991.
- Laustsen, P. G., Lane, W. S., Bennett, V., and Lienhard, G. E. (2001). Association of protein kinase C(lambda) with adducin in 3T3-L1 adipocytes. *Biochim. Biophys. Acta* 1539(1–2), 163–172.
- Li, X., and Bennett, V. (1996). Identification of the spectrin subunit and domains required for formation of spectrin/adducin/actin complexes. *J. Biol. Chem.* 271, 15695–15702.
- Li, X., Matsuoka, Y., and Bennett, V. (1998). Adducin preferentially recruits spectrin to the fast growing ends of actin filaments in a complex requiring the MARCKS-related domain and a newly defined oligomerization domain. *J. Biol. Chem.* 273, 19329–19338.
- Ling, E., Gardner, K., and Bennett, V. (1986). Protein kinase C phosphorylates a recently identified membrane skeleton-associated calmodulin-binding protein in human erythrocytes. *J. Biol. Chem.* 261(30), 13875–13878.
- Matsuoka, Y., Hughes, C. A., and Bennett, V. (1996). Adducin regulation. Definition of the calmodulin-binding domain and sites of phosphorylation by protein kinases A and C. *J. Biol. Chem.* 271(41), 25157–25166.
- Matsuoka, Y., Li, X., and Bennett, V. (1998). Adducin is an in vivo substrate for protein kinase C: phosphorylation in the MARCKS-related domain inhibits activity in promoting spectrin-actin complexes and occurs in many cells, including dendritic spines of neurons. *J. Cell Biol.* 142(2), 485–497.
- Morrow, J. S., Cianci, C. D., Ardito, T., Mann, A. S., and Kashgarian, M. (1989). Ankyrin links fodrin to the alpha subunit of Na,K-ATPase in Madin-Darby canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108(2), 455–465.
- Nelson, W. J., and Veshnock, P. J. (1986). Dynamics of membrane-skeleton (fodrin) organization during development of polarity in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 103(5), 1751–1765.
- Nelson, W. J., and Hammerton, R. W. (1989). A membrane-cytoskeletal complex containing Na⁺/K⁺-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* 108(3), 893–902.
- Nelson, W. J., and Veshnock, P. J. (1987). Ankyrin binding to (Na⁺ + K⁺)-ATPase and implications for the organization of membrane domains in polarized cells. *Nature* 328(6130), 533–536.
- Nelson, W. J., Shore, E. M., Wang, A. Z., and Hammerton, R. W. (1990). Identification of a membrane-cytoskeletal complex containing the cell adhesion molecule uvomorulin (E-cadherin), ankyrin, and fodrin in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 110(2), 349–357.
- Nishimura, K., Akiyama, H., Komada, M., and Kamiguchi, H. (2007). Beta-IV spectrin forms a diffusion barrier against L1CAM at the axon initial segment. *Mol. Cell Neurosci.* 34(3), 422–430.
- Peters, L. L., John, K. M., Lu, F. M., Eicher, E. M., Higgins, A., Yialamas, M., Turtzo, L. C., Otsuka, A. J., and Lux, S. E. (1995). Ank3 (epithelial ankyrin), a widely distributed new member of the ankyrin gene family and the major ankyrin in kidney, is expressed in alternatively spliced forms, including forms that lack the repeat domain. *J. Cell Biol.* 130, 313–330.
- Sheetz, M. P., Schindler, M., and Koppel, D. E. 1980. Lateral mobility of integral membrane proteins is increased in spherocytic erythrocyte. *Nature* 285, (5765):510–1.
- Szulc, J., Wiznerowicz, M., Sauvain, M. O., Trono, D., and Aebischer, P. (2006). A versatile tool for conditional gene expression and knockdown. *Nat. Methods* 3(2), 109–116.
- Weber, K. L., Fisher, R. S., and Fowler, V. N. (2007). Tmod3 regulates polarized epithelial cell morphology. *J. Cell Sci.* 120, 3625–3632.