Mechanism of recruiting Sec6/8 (exocyst) complex to the apical junctional complex during polarization of epithelial cells

Charles Yeaman*,‡, Kent K. Grindstaff and W. James Nelson

Department of Molecular and Cellular Physiology, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, CA 94305-5345, USA

*Present address: Department of Anatomy and Cell Biology, University of Iowa, Iowa City, IA 52242, USA
‡Author for correspondence (e-mail: charles-yeaman@uiowa.edu)

Accepted 18 September 2003
Journal of Cell Science 117, 559-570 Published by The Company of Biologists 2004
doi:10.1242/jcs.00893

Summary

Sec6/8 (exocyst) complex regulates vesicle delivery and polarized membrane growth in a variety of cells, but mechanisms regulating Sec6/8 localization are unknown. In epithelial cells, Sec6/8 complex is recruited to cell-cell contacts with a mixture of junctional proteins, but then sorts out to the apex of the lateral membrane with components of tight junction and nectin complexes. Sec6/8 complex fractionates in a high molecular mass complex with tight junction proteins and a portion of E-cadherin, and co-immunoprecipitates with cell surface-labeled E-cadherin and nectin-2α. Recruitment of Sec6/8 complex to cell-cell contacts can be achieved in fibroblasts when E-cadherin and nectin-2α are co-expressed. These results support a model in which localized recruitment of Sec6/8 complex to the plasma membrane by specific cell-cell adhesion complexes defines a site for vesicle delivery and polarized membrane growth during development of epithelial cell polarity.

Key words: Cell polarity, Cell membrane, Intercellular junctions, Intracellular membranes, Metabolism

Introduction

A diversity of cell types undergo polarized growth coupled to formation of structurally and functionally distinct membrane domains. For example, immediately following cell division yeast cells start to generate a new daughter cell bud by specifying delivery, docking and fusion of exocytic transport vesicles to a highly localized site on the plasma membrane (Finger and Novick, 1998); in neurons, one of many membrane extensions from the cell body continues to grow and differentiate into the axon while the remainder differentiate into dendrites (Dotti and Banker, 1987); in epithelial cells, the lateral membrane increases in surface area and length during cellularization of *Drosophila* blastoderm (Lecuit and Wieschaus, 2000), and following cell-cell adhesion in Madin-Darby canine kidney (MDCK) cells in culture (Vega-Salas et al., 1987). Defining mechanisms involved in directing anisotropic membrane growth is, therefore, a basic problem in understanding how cells generate polarity.

One important mechanism involves correct sorting and targeting of exocytic transport vesicles from the Golgi complex to the growth site on the plasma membrane. In neurons and epithelial cells (Craig et al., 1995; Keller and Simons, 1997; Winckler and Mellman, 1999; Mostov et al., 2000), and perhaps in yeast (Bagnat et al., 2001), classes of proteins are sorted in the *trans*-Golgi Network (TGN) into distinct transport vesicles which are then delivered efficiently to the correct membrane domain (axon or dendrite, and apical or basal-lateral, respectively); the actin and/or microtubule cytoskeleton are involved at this stage (Grindstaff et al., 1998a; Kreitzer et al., 2000; Stamnes, 2002). Final stages of transport vesicle docking and fusion require specific interactions between vesicle-associated v-SNAREs and plasma membrane-associated t-SNAREs (Rothman and Warren, 1994; Jahn and Sudhof, 1999). In polarized epithelial cells, different t-SNAREs are concentrated on apical (syntaxin 3) and basal-lateral (syntaxin 4) membrane domains (Low et al., 1996; Li et al., 2002), indicating that localization of t-SNAREs to specific membrane domains may control sites of polarized membrane growth. In yeast, however, t-SNAREs (Sso1p, Sso2p, Sec9p) are localized uniformly over the surface of the mother and daughter cell plasma membrane even though vesicle docking and fusion appears to occur, at least initially, only at the tip of the daughter cell bud (Brennwald et al., 1994; Finger and Novick, 1998). This observation indicates that additional cellular machinery is involved in directing vesicles to the correct growth site on the plasma membrane. Indeed, Novick and colleagues identified eight genes that acted very late in the secretory pathway, and showed that loss of function or deletion of any one of those genes resulted in accumulation of transport vesicles underneath the tip of the plasma membrane (Finger and Novick, 1998). The encoded proteins form a large complex, termed the exocyst, which is localized at the tip of the daughter bud during early membrane growth (TerBush et al., 1996).

The exocyst, also termed the Sec6/8 complex, has been found in other eukaryotic cells including neurons, polarized epithelial cells, exocrine cells and fibroblasts (Hsu et al., 1996; Grindstaff et al., 1998b; Shin et al., 2000; Yeaman et al., 2001). Biochemically, the Sec6/8 complex in neurons and epithelia

appears to be identical to the yeast exocyst in terms of protein components (Hsu et al., 1996; Kee et al., 1997; Grindstaff et al., 1998b). In these cells, as in yeast, the Sec6/8 complex is localized specifically at sites of polarized membrane growth. In neurons, the Sec6/8 complex localizes to the axon, at sites of synapse formation and at the growth cone (Hazuka et al., 1999; Vega and Hsu, 2001); in polarized MDCK epithelial cells, the Sec6/8 complex is localized to the lateral membrane near the apical junctional complex (Grindstaff et al., 1998b; Lipschutz et al., 2000) and this region was recently demonstrated to be an area of active exocytosis (Kreitzer et al., 2003); in exocrine cells, the Sec6/8 complex is localized to the apical surface, which undergoes rapid membrane growth upon release of zymogen granules (Shin et al., 2000).

Mammalian Sec6/8 complex, like its yeast counterpart, is important for specifying exocytic sites leading to localized membrane growth. Addition of function-blocking antibodies to Sec6/8 complex in polarized MDCK cells inhibited plasma membrane delivery of vesicles containing a basal-lateral membrane protein, but not those containing an apical membrane protein (Grindstaff et al., 1998b); functional mutants of the small GTPase RalA, which binds Sec5 (Moskalenko et al., 2002; Sugihara et al., 2002), disrupted Sec6/8 function in MDCK cells and caused mis-sorting of basal-lateral membrane proteins (Moskalenko et al., 2002). Expression of mutant Sec8 or Sec10 subunits blocked neurite outgrowth in PC12 cells (Vega and Hsu, 2001), and expression of mutant Exo70 blocked insulin-dependent GLUT-4 translocation to the plasma membrane of adipocytes (Inoue et al., 2003). That Sec6/8-dependent targeting of exocytic vesicles is an essential process is supported by the finding that a fortuitous knockout of the Sec8 gene in mouse was early embryonic lethal (Friedrich et al., 1997).

An important question is how the cytosolic Sec6/8 complex is recruited to specific sites on the plasma membrane that define where localized membrane growth occurs. In yeast, genetic epistasis identified Sec3p as the most membrane-proximal exocyst subunit, but the mechanisms by which this cytosolic protein becomes associated with plasma membrane have not been identified (Finger et al., 1998). More recently, it was suggested that recruitment of Sec3p to presumptive bud sites is regulated by Rho family GTPases (Guo et al., 2001; Zhang et al., 2001). A mammalian homologue of Sec3p has been described, but sequence analysis reveals that it lacks the domain responsible for interacting with Rho GTPases (Brymora et al., 2001; Matern et al., 2001). In contrast, mammalian Exo70 was recently shown to interact with a close relative of Cdc42, TC10, raising the possibility that this interaction is involved in recruiting Sec6/8 complex to plasma membrane (Inoue et al., 2003). Other clues are provided from the analysis of Sec6/8 complex distribution in MDCK epithelial cells. In the absence of cell-cell adhesion, the Sec6/8 complex comprises a cytosolic ~17S particle, but upon cadherin-mediated cell-cell adhesion it rapidly appears at contacting plasma membranes and eventually localizes near the apical junctional complex (Grindstaff et al., 1998b). Here, we define the membrane organization of the Sec6/8 complex in polarized epithelial cells, identify membrane proteins with which it associates, and establish that Sec6/8 is recruited to cell-cell contacts by complementation of Sec6/8 binding sites in non-adherent fibroblasts.

Materials and Methods

Antibodies, fluorescent probes and expression vectors

Mouse monoclonal antibodies against Sec6 (9H5 and 10C3) and Sec8 (2E9, 2E12, 5C3, 8F12 and 10C2) have been described previously (Hsu et al., 1996; Kee et al., 1997). Rabbit polyclonal antibody against Exo70 was provided by Dr Patrick Brennwald (University of North Carolina) (Yeaman et al., 2001). Rabbit polyclonal antibody against the conserved cytoplasmic domain of mouse E-cadherin (E2) was described previously (Hinck et al., 1994). Polyclonal antibodies to occludin, ZO-1, ZO-2, claudin-1 and claudin-2 were purchased from Zymed Laboratories, Inc. (So. San Francisco, CA). Polyclonal antibodies to afadin/AF-6 (Yamamoto et al., 1997) and ponsin (CAP) (Ribon et al., 1998) were purchased from Sigma (St. Louis, MO) and Upstate Biotechnology (Lake Placid, NY), respectively. Mouse anti-HA antibody was from Covance Research Products, Inc. (Denver, PA). Rabbit polyclonal and rat monoclonal antibodies to nectin-1α and nectin-2α (Takahashi et al., 1999) and the cDNA of mouse nectin-2α were generously provided by Dr Y. Takai (Osaka University, Japan). FITC-goat anti-mouse, FITC-goat anti-rat, Texas Red-donkey anti-mouse and Texas Red-donkey anti-rabbit IgG were purchased from Jackson Immunoresearch Labs (West Grove, PA).

The expression vector pcDNA3.1-IgK-2HA was constructed by subcloning an insert encoding the IgK signal peptide and 2XHA epitope into pcDNA3.1. To construct HA-tagged nectin-2α, cDNA encoding mouse nectin-2α was subcloned downstream of the IgK signal peptide and 2XHA tag.

Cell culture methodology

Madin-Darby canine kidney (MDCK) clone II cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1 g/l sodium bicarbonate (~12 mM, 'lo bicarb' medium), and supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and kanamycin as previously described (Nelson and Veshnock, 1987). In some experiments, cells were grown in 3.7 g/l sodium bicarbonate (~45 mM, 'hi bicarb' medium), although under these culture conditions cells appear unhealthy, contain numerous internal vacuoles, grow as tightly focused clusters of cells that fail to form confluent monolayers, and after several passages stop dividing. We note that the concentration of bicarbonate in blood is ~20-30 mM, depending on whether it is venous or arterial.

Confluent monolayers of 'contact-naive' MDCK cells were generated by passaging cells at low density (2×10^6 cells/150 mm diameter dish) on two consecutive days. Following trypsinization, cells were centrifuged at 1000 ***g*** for 5 minutes and resuspended in DMEM containing 5 μM Ca^{2+} (LCM), supplemented with 10% dialyzed FBS. Cells were plated in LCM at low density (~5×10^4 cells/35 mm dish) on collagen-coated coverslips or high density (~8×10^5 cells/well) on 12 mm Transwell™ 0.45 μm polycarbonate filters (Costar Corp., Cambridge, MA) for immunofluorescent staining, or at confluent density (~2×10^7 cells/150 mm dish) for biochemical studies. Cell-cell contacts were synchronously induced by replacing LCM with DMEM (1.8 mM Ca^{2+}) for the times indicated in the figures. For latrunculin B treatment, fully polarized monolayers were treated with 2 μM latrunculin B for 1 hour at 37°C prior to immunofluorescent staining.

MDCK cells were transfected with pcDNA3.1-IgK-2HA-nectin-2α, using the calcium phosphate method (Ausubel et al., 1987). G418 resistant (500 μg/ml) colonies were isolated using cloning rings, and individual clones were expanded and screened by immunofluorescent staining and western blotting using anti-HA antibody. Mouse L cells stably transfected with a full-length mouse E-cadherin cDNA under the control of a dexamethasone inducible promoter (LE cells) were cultured in DMEM supplemented with 5% fetal bovine serum, 300 μg/ml G418, penicillin, streptomycin and kanamycin as described previously (Angres et al., 1996). E-cadherin expression was induced

in LE cells by culturing the cells in the presence of 1 μM dexamethasone (LE+Dex) for ~18 hours; E-cadherin expression is induced more than 100-fold under these conditions (Angres et al., 1996). Transient transfection of LE cells was achieved using the calcium phosphate precipitation method (Ausubel et al., 1987), with 15 μg of pcDNA3.1-IgK-2HA-nectin-2α, 48 hours prior to dexamethasone induction of E-cadherin expression.

Immunofluorescent staining

Cells were fixed in 4% paraformaldehyde for 30 minutes, before or after extraction at 0°C for 10 minutes with 1% Triton X-100 in buffer containing 10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.1 mg/ml RNase, 0.1 mg/ml DNase and protease inhibitors (1 mM pefabloc, and 10 μg/ml each of aprotinin, antipain, leupeptin, pepstatin A) (CSK buffer). Monoclonal Sec6 (mAb 9H5) or Sec8 (mAb 2E9) antibodies (as hybridoma supernatants diluted 1:5), monoclonal HA (mAb 16B12) antibody (as ascites fluid diluted 1:1000), polyclonal antibodies to E-cadherin (1:25), ZO-1 (1:300), afadin (1:250), or rat monoclonal anti-nectin-2α (1:100) were diluted in blocking buffer [Ringer's saline (154 mM NaCl, 1.8 mM Ca^{2+}, 7.2 mM KCl, 10 mM Hepes, pH 7.4)] containing 0.2% BSA, 0.5% normal goat serum and 0.5% normal donkey serum) and applied to cells for 2 hours at 4°C. After five washes in blocking buffer, fluorescein (FITC)- and Texas Red-conjugated secondary antibodies, diluted 1:200, were applied for 1 hour at 4°C. Coverslips and filters were washed five times and mounted in VectaShield (Vector Laboratories, Burlingame, CA). Samples were viewed with either a Nikon Microphot-FX microscope (63× or 100× objectives) or a Molecular Dynamics MultiProbe 2010 confocal laser-scanning microscope (63× objective) using a krypton/argon laser with 488 nm (FITC, GFP) and 568 nm (Texas Red) laser lines, as noted in figure legends. Digital images were obtained using a Kodak DCS 760 digital camera with a Nikon Microphot-FX microscope. In order to quantify distributions of Sec6, E-cadherin and ZO-1 on the lateral plasma membrane, serial 0.4 μm optical sections were collected from five different fields, and pixel intensities in red (E-cadherin or ZO-1) and green (Sec6) channels were quantified using Image Space software (version 3.01) from Molecular Dynamics.

Cell fractionation in iodixanol gradients

Cells were homogenized in isotonic sucrose buffer (0.25 M sucrose in 20 mM Hepes-KOH, pH 7.2, 90 mM potassium acetate, 2 mM magnesium acetate, and protease inhibitors) by repeated passage through a ball-bearing homogenizer (Varian Physics, Stanford University). Separation of different membrane compartments was achieved by centrifugation in three-step 10-20-30% (wt/vol) iodixanol gradients (Yeaman et al., 2001; Yeaman, 2003). Briefly, one-third of the post-nuclear supernatant was mixed with Opti-Prep (60% (wt/vol) iodixanol, Nycomed, Oslo, Norway) and homogenization buffer to generate solutions containing 10, 20 or 30% iodixanol. Equal volumes of these three solutions were layered in centrifuge tubes and samples were centrifuged at 353,000 ***g*** for 3 hours at 4°C in a Beckman Vti65 rotor. Fractions (0.5 ml) were collected, refractive indices were read, and proteins were separated by SDS-PAGE. Proteins were transferred from gels to Immobilon P membranes for immunoblotting as described below.

Superose 6 FPLC analysis

Confluent monolayers of MDCK cells were extracted for 10 minutes at 4°C in Tris-saline buffer containing 0.5% (v/v) NP-40 and protease inhibitors (1 mM PMSF and 10 μg/ml each of pepstatin A, leupeptin and antipain). Cell lysates were centrifuged at 15,000 ***g*** for 10 minutes. The supernatant fraction was centrifuged at 100,000 ***g*** for 30 minutes and passed through a 0.22 μm syringe filter (Millipore). 200 μl of this lysate was applied to a Superose 6 HR 10/30 column and fractionated as described previously (Stewart and Nelson, 1997). Fractions 6-28 were separated by SDS-PAGE and proteins were electrophoretically transferred to Immobilon P membranes for immunoblotting with specific antibodies.

Cell extraction and immunoprecipitation

Cells were rinsed 3× in Ringer's saline and solubilized in CSK buffer for 30 minutes at 4°C on a rocking platform. For analysis of Triton-soluble vs. Triton-insoluble Sec8 (Fig. 3), cells were scraped from the filter with a rubber policeman and sedimented in a microfuge for 10 minutes. The soluble supernatant was collected. The cell pellet was triturated in 100 μl SDS-PAGE sample buffer and incubated at 100°C for 10 minutes. For immunoprecipitation analysis, CSK extracts were precleared with 5 μl of preimmune serum and 50 μl *Staphylococcus aureus* cells (Pansorbin; Calbiochem Novabiochem, La Jolla, CA) for 1 hour at 4°C. For Sec8 immunoprecipitation, mAbs 2E12, 5C3 and 10C2 were covalently cross-linked to protein A Sepharose beads (Pharmacia LKB Nuclear, Gaithersburg, MD) with dimethyl pimelimidate (DMP), and 20 μl of immunoadsorbant was used per immunoprecipitation. Immunoprecipitation of E-cadherin, Exo70, ZO-1, ZO-2, occludin, claudin-1 and claudin-2 was performed with specific rabbit polyclonal antibodies (5 μg per sample) pre-bound to protein A Sepharose beads. Immunoadsorbants were incubated with pre-cleared cell extracts for 2 hours at 4°C, then washed under stringent conditions and prepared for SDS-PAGE as described previously (Pasdar and Nelson, 1988).

Cell surface biotinylation and chemical cross-linking

MDCK cells were biotinylated as previously described (Le Bivic et al., 1990). Briefly, cells cultured on 24 mm Transwell™ 0.45 μm polycarbonate filters were rinsed three times with Ringer's saline. For experiments involving avidin precipitation, Sulfo-NHS-SS-Biotin (Pierce, Rockford IL) (0.5 mg/ml in Ringer's saline) was applied to both apical and basal-lateral surfaces (0.5 ml apical/1 ml basal-lateral) and the cells were incubated twice for 20 minutes each at 4°C with gentle rocking. For experiments involving Sec8 immunoprecipitation and avidin western blotting (Fig. 6B), Sulfo-NHS-LC-LC-Biotin (Pierce) was used. The biotinylation reaction was quenched by washing cells in five changes of TBS (120 mM NaCl, 10 mM Tris, pH 7.4) containing 50 mM NH_4Cl and 0.2% BSA (quenching buffer) at 4°C.

Cells were chemically cross-linked as previously described (Hinck et al., 1994). Following biotinylation, cells were washed three times with Ringer's saline. 200 μg/ml dithiobis(succinimidylpropionate) (DSP) (Pierce Chemical Co., Rockford, IL), diluted in Ringer's saline, was added to the apical (1 ml) and basal-lateral (1 ml) compartment of the filter, and the cells were incubated for 20 minutes at room temperature on a rocking platform. DSP was prepared as a 100× stock in DMSO and diluted immediately before use. Cultures were washed five times with quenching buffer at 4°C. Cells were lysed for 1 hour in 1 ml/filter CSK buffer. Lysates were centrifuged at 15,000 ***g*** for 10 minutes, and supernatant fractions were transferred to clean tubes. 50 μl of lysate was removed and mixed with SDS-PAGE sample buffer for quantitation of total protein expression. The remaining lysate (950 μl) was combined with 50 μl avidin-agarose (Pierce) or 20 μl Sec8 immunoadsorbant, incubated for 2 hours at 4°C on a tube rotator, then washed under stringent conditions and prepared for SDS-PAGE as described previously (Pasdar and Nelson, 1988).

Gel electrophoresis and immunoblotting

Protein samples were incubated in SDS sample buffer for 10 minutes at 100°C before separation in 7.5% or 14% SDS polyacrylamide gels (Laemmli, 1970). Proteins were electrophoretically transferred from

gels to Immobilon PVDF membrane (Millipore Corp., Bedford, MA). Blots were blocked in Blotto [5% nonfat dry milk, 0.1% sodium azide in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 (TBS)] overnight at 4°C. Primary antibodies were incubated with blots at room temperature for 1 hour. After five washes, of 10 minutes each, in TBS containing 0.1% Tween 20, the blots were incubated with ^{125}I-labeled goat anti-mouse or goat anti-rabbit secondary antibody (ICN, Costa Mesa, CA) for 1 hour at room temperature. Blots were washed as above and exposed for autoradiography. The amount of labeled protein was determined directly using a Phosphorimager (Model 820, Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (version 1.2, Molecular Dynamics, Sunnyvale, CA).

Results

Spatial sorting of Sec6/8 complex during assembly of epithelial cell-cell junctions

The subcellular distribution of Sec6/8 complex was compared by immunofluorescence microscopy to those of tight junction (ZO-1), nectin complex (nectin, afadin), and cadherin complex (E-cadherin) proteins during assembly of epithelial junctional complexes at different times after synchronous induction of cell-cell adhesion. Previous studies have shown that different adhesion complexes are initially mixed upon cell contact, but then gradually sort out into the correct spatial order (Van Itallie et al., 1995; Ando-Akatsuka et al., 1999; Suzuki et al., 2002). Immediately after initiation of cell-cell adhesion (t=0 hour), Sec6 was co-distributed with ZO-1 and E-cadherin within newly formed intercellular contacts and, close to contacts, in detergent-insoluble cytosolic puncta that contained ZO-1 but not E-cadherin. After 1 hour, Sec6 was concentrated within the apical portion of the lateral membrane, similar to the distribution of ZO-1, but only partially overlapped that of E-cadherin, which also localized to the rest of the forming lateral membrane (Fig. 1A). Differences between Sec6 and E-cadherin distributions were even more evident at 3 hours, and at all other times, as Sec6 and ZO-1 distributions were confined to a region near the top of the lateral membrane. Quantification of pixel intensities at 24 hours show that the bulk of Sec6 is confined to an apical region of the lateral plasma membrane (sections 4-9), corresponding roughly to the upper ~1/3 of the E-cadherin-positive plasma membrane (Fig. 1C). At this time point, a subtle difference in the distributions of Sec6 and ZO-1 could be discerned (Fig. 1B,C), but higher resolution analysis by immuno-EM were thwarted as available Sec6 and Sec8 monoclonal antibodies were not reactive following glutaraldehyde fixation. Nectin-2α and afadin also co-localized with Sec6 at sites of early cell-cell adhesion (Fig. 2). In polarized cells, Sec6 co-localized with afadin within the apical junctional complex at the apex of the lateral membrane (Fig. 2).

Our observation that Sec6/8 complex is confined to an apical region of the lateral membrane (Figs 1, 2) (Grindstaff et al., 1998b) is different from the diffusely lateral membrane distribution reported recently by others (Kreitzer et al., 2003). Likely sources for this discrepancy are different cell culture conditions. In cells grown in medium containing 3.7 g/l sodium bicarbonate (‘hi bicarb’), as in the work of Kreitzer et al., Sec6/8 complex has a diffusely lateral distribution and is largely extracted in buffer containing non-ionic detergent (Fig. 3). In contrast, in cells cultured in medium containing 1 g/l sodium bicarbonate (‘lo bicarb’) Sec6/8 complex is tightly focused near the apical junctions (Figs 1, 2) and is much more resistant to detergent extraction (Fig. 3). Although the mechanism underlying this effect is not known, it is interesting to note that changes in cytoplasmic pH have been shown to affect the localization of other organelles (Heuser, 1989). In conclusion, while Sec6/8 complex localizes rapidly to E-cadherin–mediated cell-cell contacts with many other junctional components, it is spatially sorted with proteins of the apical junctional complex into an apical aspect of the lateral membrane.

Co-fractionation of membrane junctional adhesion complexes and Sec6/8 complex after cell-cell adhesion

Association of Sec6/8 complex with, and sorting from different junctional complexes following cell-cell adhesion was examined by separating different plasma membrane domains through linear 10-30% Opti-Prep gradients. These gradients resolve distinct membrane fractions containing intercellular junctions (δ ~1.16 g/ml), non-junction-associated basal-lateral membranes (δ ~1.10 g/ml) and apical membranes (δ ~1.07 g/ml); cytosolic proteins are recovered in higher density fractions (δ ~1.22 g/ml) (Yeaman, 2003). MDCK cells were homogenized 6 or 48 hours after induction of cell-cell adhesion, and homogenates were fractionated in Opti-Prep gradients. Distributions of Sec8 and junction-associated proteins (ZO-1, ZO-2, afadin, claudin-1, claudin-2, occludin, E-cadherin, nectin-1α and nectin-2α) were determined by SDS-PAGE and western blotting (Fig. 4).

After 6 hours contact, approximately half of Sec8 had been recruited from the cytosol to membranes, in agreement with previous results (Grindstaff et al., 1998b); note that other subunits of the Sec6/8 complex (Sec5, Sec6, Exo70) co-fractionate with Sec8 and, therefore, the distribution of Sec8 probably reflects the fractionation behavior of the holocomplex in these gradients (data not shown). At this early stage of polarity development, as spatial sorting of Sec6/8 complex is occurring (see Figs 1, 2), 80% of membrane-bound Sec8 was recovered in a peak at 1.16 g/ml and 20% in a peak at 1.10 g/ml. Three proteins that are associated with the cytoplasmic face of junctions (ZO-1, ZO-2 and afadin) were also enriched within the Sec8 peak at 1.16 g/ml (Fig. 4). E-cadherin was partially recovered in this fraction, but was also distributed throughout regions of lower density that contained only minor amounts of Sec8. In contrast, nectin-2α, an adhesion molecule enriched at zonula adherens (Takahashi et al., 1999), was recovered exclusively in a membrane peak at 1.16 g/ml, and thus co-fractionated with its binding protein, afadin, and with Sec8 (Fig. 4). Most of the occludin, and claudins-1 and -2 were recovered in two membrane fractions with peak densities of ~1.12 g/ml, which contained only minor amounts of ZO-1, ZO-2 and Sec8, and ~1.07 g/ml, which did not contain these proteins; at this time, neither occludin nor claudins were recovered in the 1.16 g/ml peak that contained most of the membrane-bound Sec6/8 complex, ZO-1 and ZO-2. Therefore, it is unlikely that either membrane recruitment or spatial sorting of the Sec6/8 complex to intercellular junctions involves interactions with transmembrane proteins of epithelial tight junctions.

Forty-eight hours after induction of cell-cell adhesion, ~80% of Sec8 co-fractionated with ZO-1, ZO-2 and nectin-1α in the

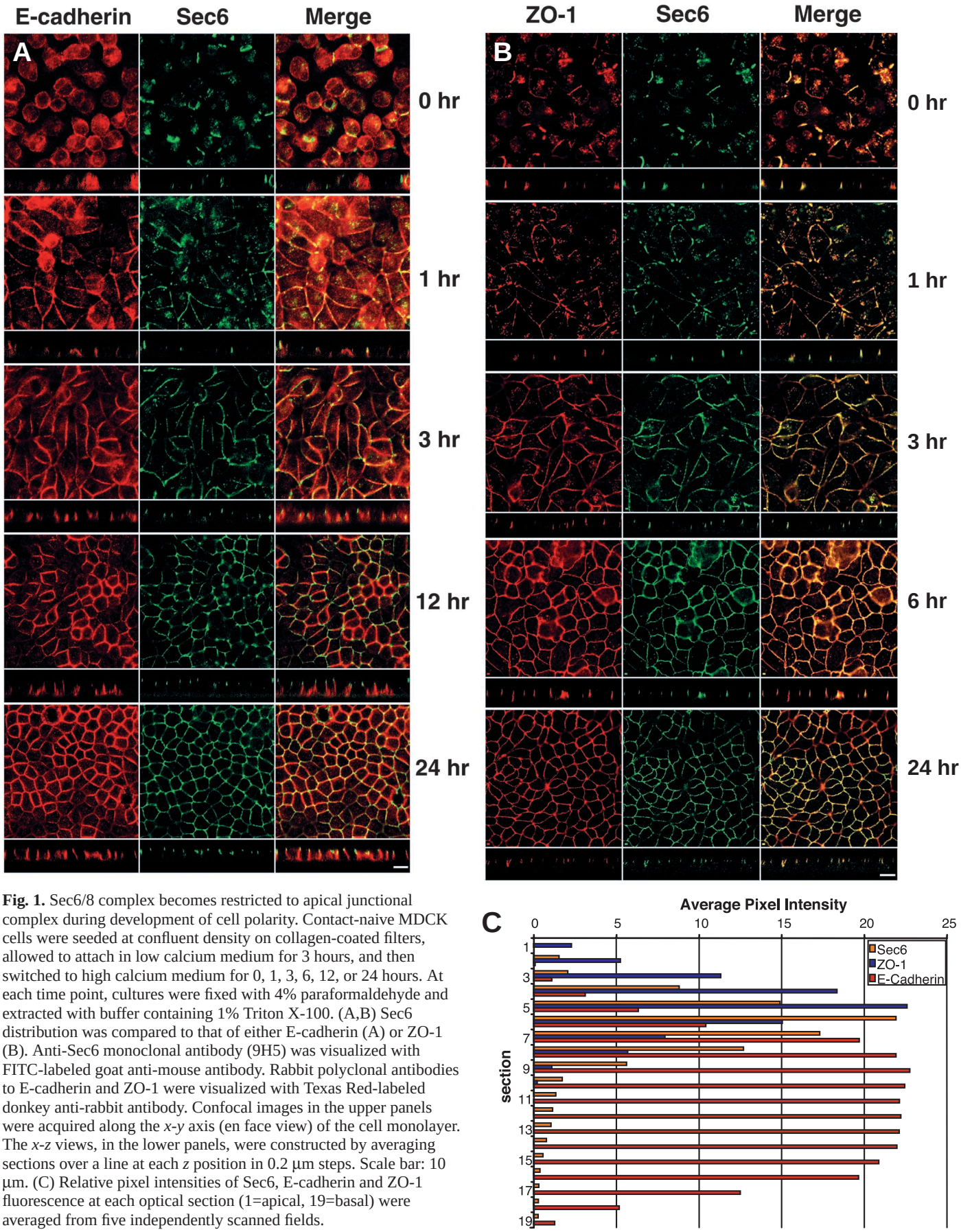


Fig. 1. Sec6/8 complex becomes restricted to apical junctional complex during development of cell polarity. Contact-naive MDCK cells were seeded at confluent density on collagen-coated filters, allowed to attach in low calcium medium for 3 hours, and then switched to high calcium medium for 0, 1, 3, 6, 12, or 24 hours. At each time point, cultures were fixed with 4% paraformaldehyde and extracted with buffer containing 1% Triton X-100. (A,B) Sec6 distribution was compared to that of either E-cadherin (A) or ZO-1 (B). Anti-Sec6 monoclonal antibody (9H5) was visualized with FITC-labeled goat anti-mouse antibody. Rabbit polyclonal antibodies to E-cadherin and ZO-1 were visualized with Texas Red-labeled donkey anti-rabbit antibody. Confocal images in the upper panels were acquired along the *x-y* axis (en face view) of the cell monolayer. The *x-z* views, in the lower panels, were constructed by averaging sections over a line at each *z* position in 0.2 μm steps. Scale bar: 10 μm. (C) Relative pixel intensities of Sec6, E-cadherin and ZO-1 fluorescence at each optical section (1=apical, 19=basal) were averaged from five independently scanned fields.

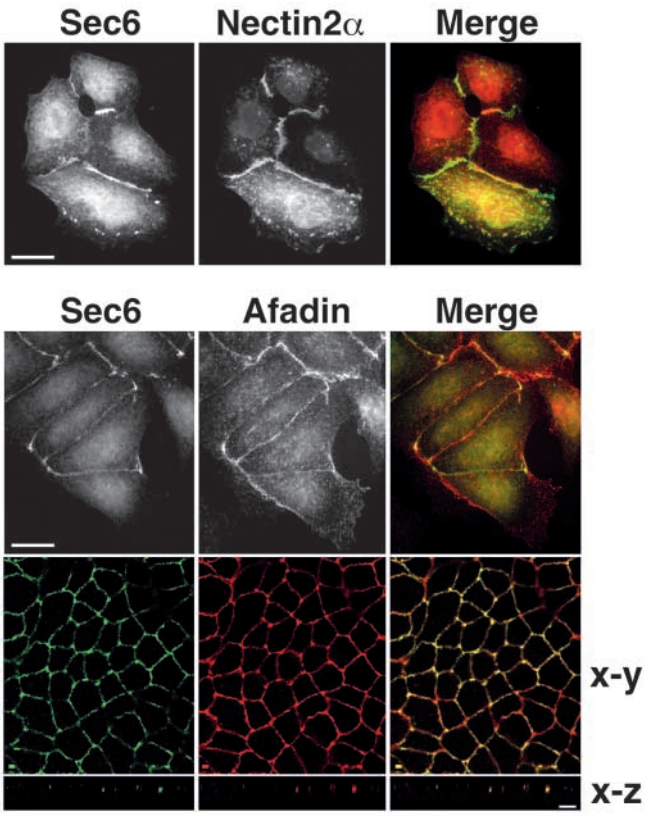


Fig. 2. Sec6 co-localizes with components of the nectin complex in early cell-cell contacts and polarized MDCK cells. (Top panels) Low-density cultures of MDCK cells were allowed to form calcium-dependent cell-cell contacts for 1 hour, and then fixed with 2% paraformaldehyde before extraction with 1% Triton X-100. Sec6 distribution was compared to that of afadin or nectin-2α. (Bottom panel) Confluent MDCK cultures on polycarbonate filters were fixed and extracted 24 hours after induction of calcium-dependent cell-cell adhesion. Sec6 distribution was compared to that of afadin. Anti-Sec6 monoclonal antibody (9H5) was visualized with FITC-labeled goat anti-mouse antibody (in the afadin panels) or with Texas Red-labeled donkey anti-mouse antibody (in the nectin panel). Rabbit polyclonal antibody to afadin was visualized with Texas Red-labeled donkey anti-rabbit antibody, and rat monoclonal antibody to nectin-2α was visualized with FITC-labeled goat anti-rat antibody. Confocal images were obtained as described in Fig. 1 legend. Scale bar: 10 μm.

1.16 g/ml membrane peak (Fig. 4). Forty percent of E-cadherin was also present in this membrane fraction, but the majority (60%) was still associated with membranes of lower density. Significantly, occludin was now largely recovered in the membrane peak at 1.16 g/ml together with ZO-1/-2. Therefore, assembly of tight junctions, as defined by isopycnic gradient centrifugation, occurs after the recruitment and spatial sorting of Sec6/8 complex.

Fractionation of junctional proteins associated with Sec6/8 complex

We sought evidence for direct association between Sec6/8 complex and different junctional membrane complexes using a cell fractionation and co-immunoprecipitation strategy. Detergent extracts of MDCK cells were fractionated by Superose 6 FPLC and elution patterns of different proteins determined by western blotting. E-cadherin was eluted as a single peak at fraction 14, corresponding to a protein complex of apparent molecular size ~1.4×10^6 Da based on the elution of globular protein standards (Fig. 5) (see also Stewart and Nelson, 1997). The elution of Sec8 overlapped that of E-cadherin with a peak at fraction 13. This corresponds to a (globular) protein complex of >2000 kDa, which is much larger than the isolated Sec6/8 complex, which has an apparent native molecular mass of 650-700 kDa (Hsu et al., 1996). Afadin, ZO-1, ZO-2 and ponsin were also in this region, although their peaks were offset by a fraction or two from that of Sec8, indicating that the bulk of these proteins were in smaller complexes than the bulk of Sec8 (Fig. 5A). Nectin-2α was eluted predominantly in fractions 18 and 22 in peaks that were different from those of ponsin and afadin, probably because it dissociated from afadin/ponsin complexes under our extraction and/or fractionation conditions.

To determine whether a complex containing E-cadherin and Sec8 was present in MDCK cells, each Superose 6 column fraction in this region was immunoprecipitated with anti-E-cadherin antibodies followed by western blotting for Sec8. In cell extracts prepared either 3.5 hours or 3 days following induction of cadherin-mediated adhesion, Sec8 co-precipitated with E-cadherin, and the peak of the Sec8/E-cadherin complex

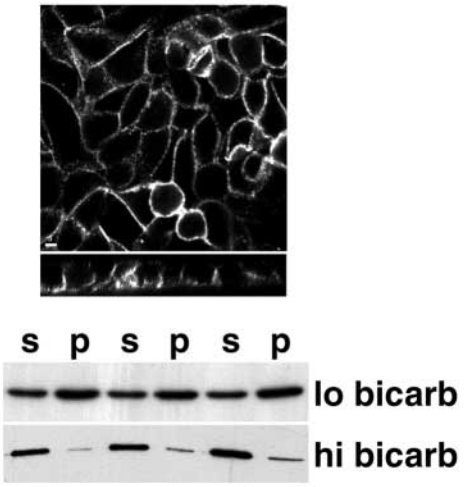


Fig. 3. Effect of sodium bicarbonate concentration on Sec6/8 complex distribution. Confluent MDCK cultures on polycarbonate filters were grown in DMEM containing either 1 g/l ('lo bicarb') or 3.7 g/l ('hi bicarb') sodium bicarbonate for 48 hours. (Top) Cultures were fixed with 4% paraformaldehyde before extraction with buffer containing 1% Triton X-100. Anti-Sec6 monoclonal antibody (9H5) was visualized with FITC-labeled goat anti-mouse antibody. Confocal images were obtained as described in Fig. 1 legend. Scale bar: 5 μm. (Bottom) Triplicate filters of cells grown in hi or lo bicarbonate were extracted successively in Triton X-100 and SDS, as described in Materials and Methods. Sec8 in Triton-soluble ('s') and Triton-insoluble ('p') fractions was quantified by SDS-PAGE and western blotting. Protein levels were quantified using a Molecular Dynamics Phosphorimager. In 1 g/l bicarbonate, Sec8 is enriched at the apical junction (Fig. 1) and is only partially (~30%) soluble in Triton X-100. In 3.7 g/l bicarbonate, Sec8 is diffusely distributed along the lateral and basal membranes and is almost entirely (~90%) soluble in Triton X-100.

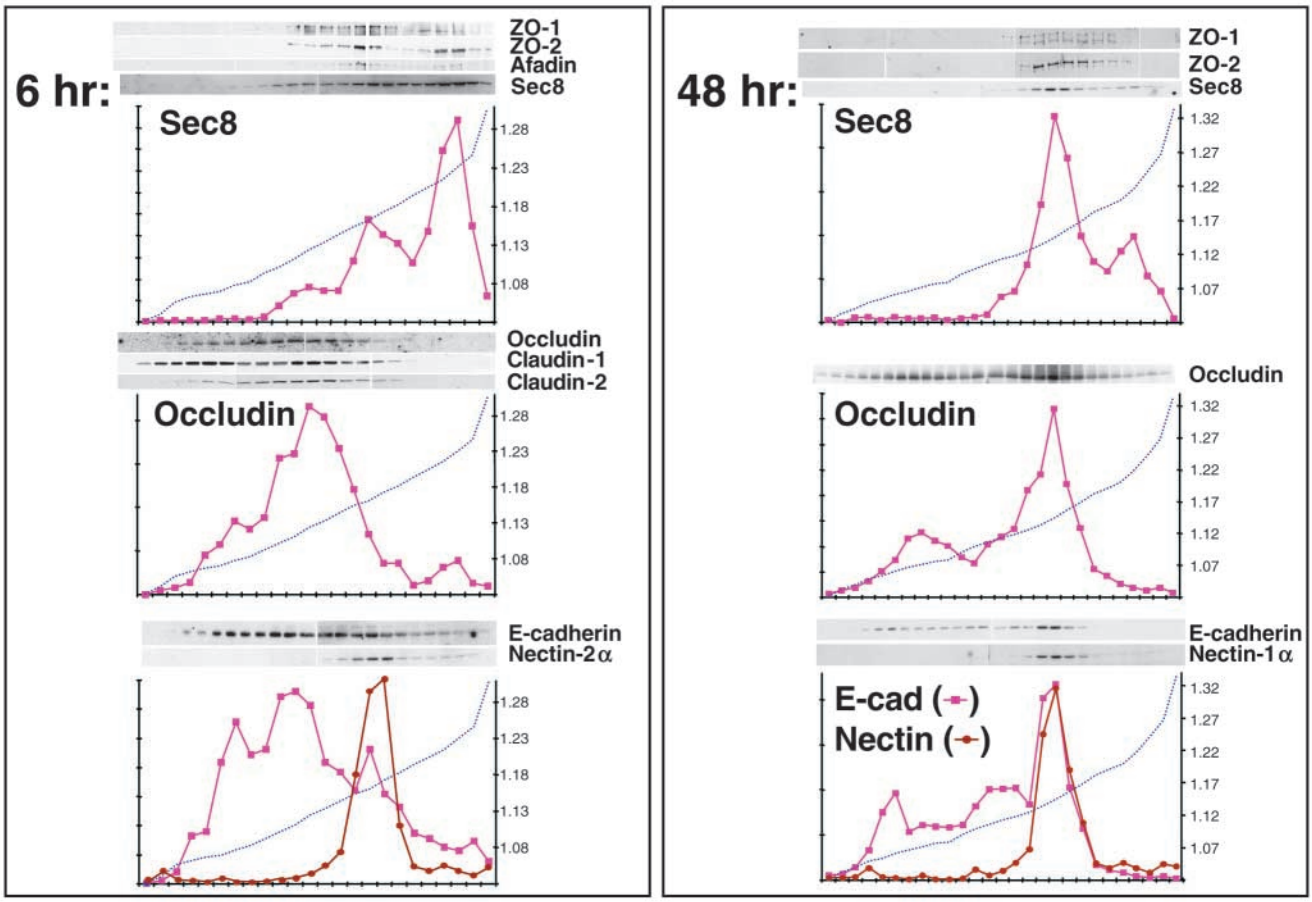


Fig. 4. Fractionation of MDCK cells in iodixanol gradients. MDCK cells were homogenized either 6 hours or 48 hours after induction of calcium-dependent cell-cell adhesion. Post-nuclear supernatants were mixed with 10%, 20% and 30% (w/v) iodixanol, layered step-wise in centrifuge tubes and centrifuged at 350,000 ***g*** for 3 hours. The presence of Sec8, ZO-1, ZO-2, afadin, occludin, claudin-1, claudin-2, E-cadherin, nectin-1α and nectin-2α in gradient fractions was assayed by SDS-PAGE followed by immunoblotting with specific antibodies. Protein levels were quantified using a Molecular Dynamics Phosphorimager. Densities of each fraction were calculated after measuring refractive indices with a refractometer, and are plotted as dotted lines on each graph with values (in g/ml) indicated on the *y*-axis.

was present in fraction 13, as was Sec8 but it was higher than that of the major cadherin-containing complex (fraction 14) (Fig. 5B). Note that our previous studies showed that cadherin is in a stoichiometric complex with α- and β-catenin in these fractions (Stewart and Nelson, 1997). Our results show that there is more than one cadherin complex within the single peak of E-cadherin, and that Sec8 is associated with a large, cadherin-containing protein complex. We hypothesize that a subset of junctional adhesion complexes (e.g., those found in fractions 12-13) contains the Sec6/8 complex.

ZO-1 has an elution profile that overlaps that of Sec8, but we failed to co-immunoprecipitate Sec8 with ZO-1 from FPLC fractions (data not shown) or whole cell extracts (Fig. 6A). In contrast, Sec8 co-precipitated with ZO-2 from lysates of polarized MDCK cells but much less was associated with ZO-2 in lysates from contact-naive cells (Fig. 6A). The amount of Sec8 co-precipitating with ZO-2 and the Exo70 subunit, which exists in a stoichiometric complex with Sec8, was similar (Kee et al., 1997; Hsu et al., 1998). Immunoprecipitation of claudin-1, claudin-2 or occludin from MDCK cell lysates showed that some ZO-2 was recovered in association with each of these proteins, but Sec8 was not detected in these complexes (Fig. 6B), indicating that the fraction of ZO-2 that is associated with Sec6/8 complex is distinct from that associated with these tight junction membrane proteins. Immunofluorescent staining of cells after latrunculin B treatment supports this interpretation. This treatment disrupts the actin cytoskeleton and causes junctional complexes to dissociate. For a short period of time after latrunculin treatment, remnants of broken junctions remained at the plasma membrane. Immunofluorescent staining shows that ZO-2 localized to punctate structures, and that Sec6 co-localized with ZO-2 within a subset of these structures (Fig. 6C). Therefore, we conclude that ZO-2 is associated with more than one type of structure on the lateral plasma membrane, and that a subset of these structures contains the Sec6/8 complex.

Isolation of junctional membrane proteins bound to Sec6/8 complex

As an alternative method to determine whether Sec6/8 complex is bound to transmembrane proteins, cells were biotinylated and extracted, either directly or after cross-linking with DSP, and precipitated with avidin-agarose beads. Approximately

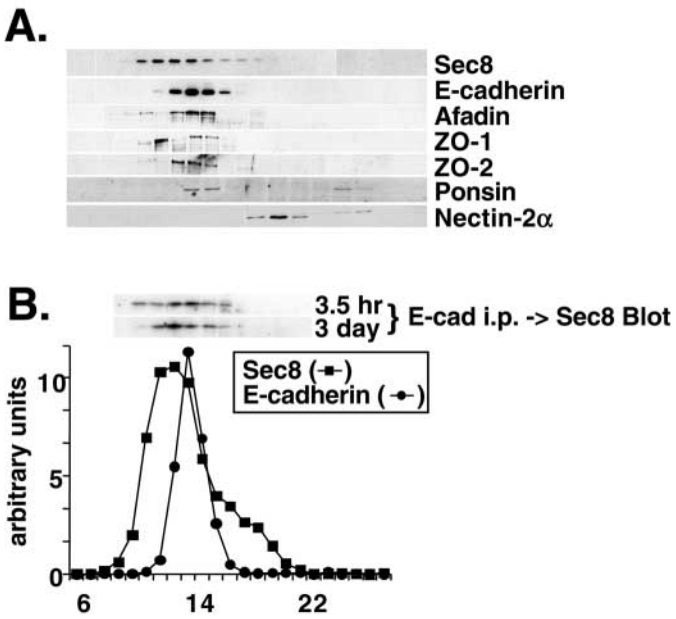
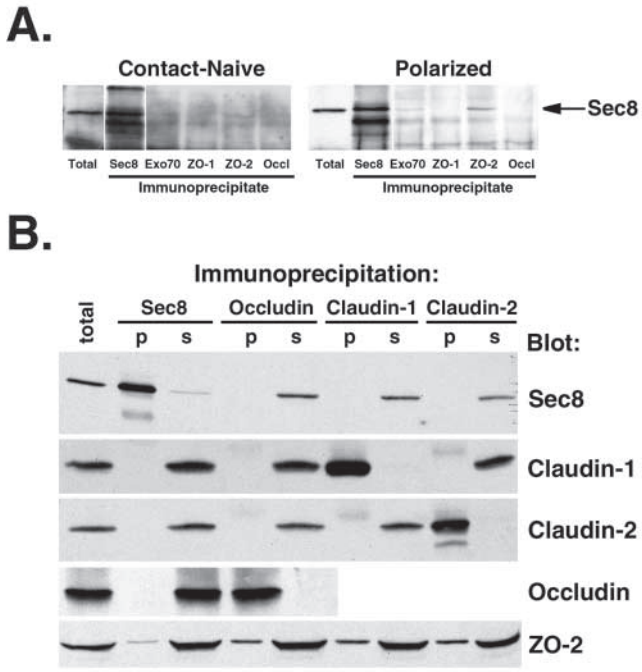


Fig. 5. Fractionation of junctional proteins associated with Sec6/8 complex. (A) Detergent extracts of polarized MDCK cells were fractionated by Superose 6 FPLC as described in Materials and Methods. Fractions 6-28 were divided into equal aliquots, separated by SDS-PAGE, and transferred to Immobilon P membranes. Membranes were probed with antibodies to Sec8, E-cadherin, afadin, ZO-1, ZO-2, ponsin or nectin-2α. Protein levels were quantified using a Molecular Dynamics Phosphorimager. The elution profiles of Sec6 and E-cadherin are shown in B. The elution peaks of globular protein standards with known relative molecular masses were also determined: thyroglobulin, M_r=669,000 (fraction 16); apoferritin, M_r=443,000 (fraction 19); catalase, M_r=232,000 (fraction 22); bovine serum albumin, M_r=66,000 (fraction 24). (B) Coimmunoprecipitation of Sec8 with E-cadherin adhesion complex. MDCK cells were extracted either 3.5 hours or 3 days after induction of calcium-dependent cell-cell adhesion and extracts were fractionated by Superose 6 FPLC. Each fraction (10-19) was subjected to immunoprecipitation with anti-E-cadherin E2 antiserum. Immunoprecipitated material was eluted in SDS-PAGE sample buffer and the presence of Sec8 in each fraction was assayed by SDS-PAGE followed by immunoblotting. Protein levels were quantified using a Molecular Dynamics Phosphorimager.



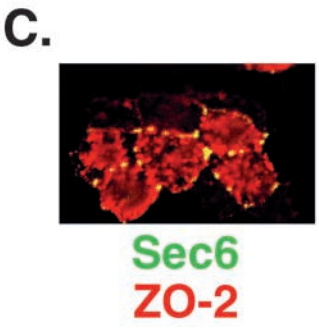


Fig. 6. Sec8 associates with a fraction of ZO-2. (A) MDCK cells were extracted in 1% Triton X-100 either 0 hours (contact-naive) or 48 hours (polarized) after inducing calcium-dependent cell-cell adhesion. Extracts were subjected to immunoprecipitation with specific antibodies to Sec8, Exo70, ZO-1, ZO-2 or occludin. The presence of Sec8 in precipitated immune complexes was assessed by SDS-PAGE followed by immunoblotting with Sec8 antibodies. (B) MDCK cells were homogenized 48 hours after induction of calcium-dependent cell-cell adhesion and junction-enriched membrane fractions were isolated by isopycnic density gradient centrifugation as described in Fig. 2. Membranes were extracted in 1% Triton X-100 and subjected to immunoprecipitation with antibodies specific for Sec8, occludin, claudin-1 or claudin-2. The presence of each of these proteins and of ZO-2 in precipitated immune complexes was assessed by SDS-PAGE followed by immunoblotting with specific antibodies. (C) Polarized MDCK cultures on polycarbonate filters were treated with 2 μM latrunculin B for 1 hour, then fixed with 2% paraformaldehyde before extraction with buffer containing 1% Triton X-100. Anti-Sec6 monoclonal antibody (9H5) was visualized with FITC-labeled goat anti-mouse antibody and anti-ZO-2 polyclonal antibody was visualized with Texas Red-labeled donkey anti-rabbit antibody.

20% of total Sec8 was covalently cross-linked to transmembrane protein(s) and recovered in avidin-agarose precipitates (Fig. 7A). If the biotinylation step was omitted none of the Sec8 bound to avidin-agarose beads, and if the DSP cross-linking step was omitted <5% of Sec8 was co-precipitated with transmembrane proteins.

To identify biotinylated, membrane-spanning protein(s) to which Sec6/8 complex was bound, extracts of biotinylated cells were immunoprecipitated with anti-Sec8 antibodies and probed by western blotting with HRP-avidin. As an additional control for specificity, Sec8 was immunoprecipitated from MDCK cells cultured in low calcium medium containing EGTA, which causes the internalization of Sec6/8 complex from the plasma membrane (Grindstaff et al., 1998b) and, therefore, transmembrane proteins associated with the Sec6/8 complex are either no longer bound or not accessible to cell surface biotinylation. Two prominent biotinylated proteins of 7 and 12×10^4 Da, and several less prominent ones, co-precipitated with Sec8 (Fig. 7B). These proteins were not detected when DSP cross-linking was omitted, nor were they observed in extracts of cells that had been cultured in EGTA. The 120 kDa protein was identified as E-cadherin, and the 70 kDa protein was identified as nectin-2α (Fig. 7C); note that the nectin-2α blot contains several additional bands, all of which were not detected in the Sec8 immunoprecipitate of biotinylated membrane proteins from whole cells (Fig. 7B).

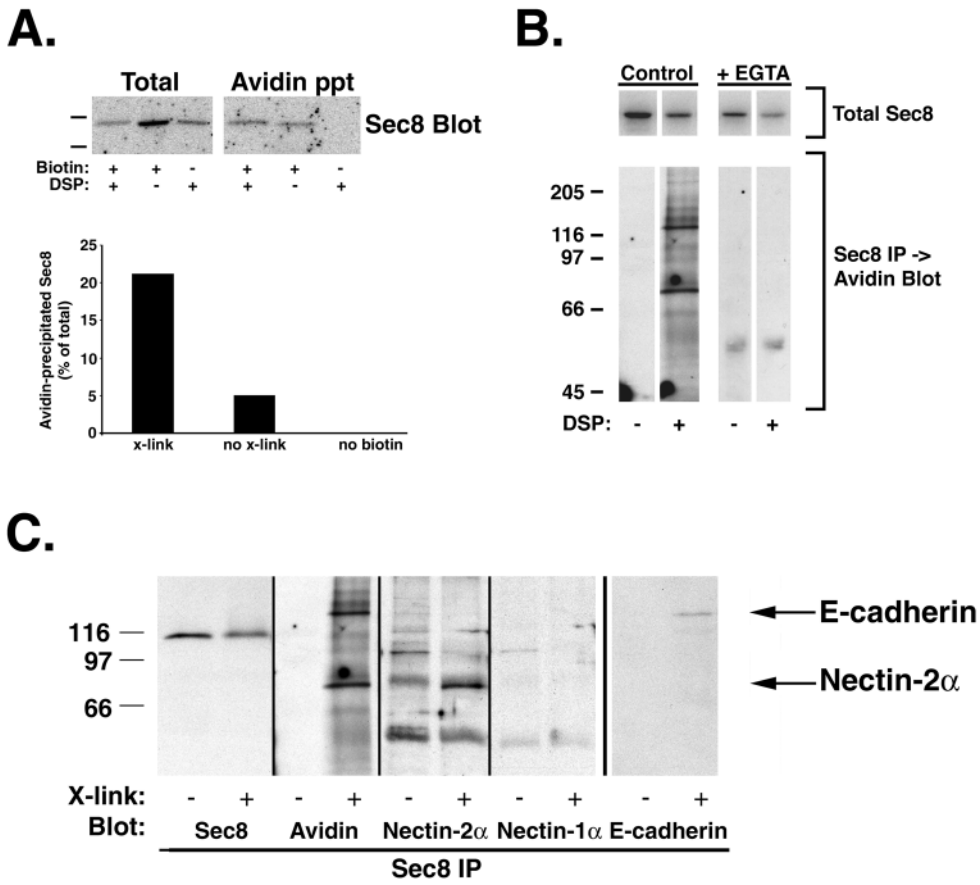


Fig. 7. Sec6/8 complex is associated with E-cadherin and nectin-2α. Polarized MDCK cells cultured on polycarbonate filters were surface labeled with either Sulfo-NHS-SS-Biotin (A) or Sulfo-NHS-LC-LC-Biotin (B,C) and extracted with 1% Triton X-100 either directly (no x-link or –DSP) or following (x-link or +DSP) chemical cross-linking with the membrane-permeable cross-linker DSP. Non-biotinylated controls (no biotin) were subjected to cross-linking prior to extraction. (A) Extracts were incubated with avidin-agarose, and the presence of Sec8 in avidin precipitates was assayed by SDS-PAGE followed by immunoblotting with anti-Sec8 antibody. (B,C) Extracts were subjected to immunoprecipitation with anti-Sec8 antibodies, and the presence of biotinylated proteins, Sec8, nectin-2α, nectin-1α and E-cadherin in precipitated immune complexes was assessed by SDS-PAGE followed by immunoblotting with HRP-avidin or specific antibodies. '+EGTA' cultures were incubated in LCM + 2 mM EGTA for 6 hours prior to biotinylation.

Further evidence of the specificity of the Sec8/nectin-2α interaction is indicated by the fact that nectin-1α, which co-fractionates with Sec8 in isopycnic density gradients, was not detected in Sec8 immunoprecipitates (Fig. 7C).

Reconstitution of Sec6/8 recruitment to cell-cell contacts in fibroblasts requires E-cadherin and nectin-2α

We evaluated the requirement for E-cadherin and nectin-2α in recruitment of Sec6/8 complex to junctional complexes by reconstituting cell-cell adhesion in LE fibroblasts expressing E-cadherin under the control of the dexamathasone (Dex) promoter (Angres et al., 1996). LE cells were transiently transfected with nectin-2α cDNA, and 48 hours later Dex (1 μM) was added to half the cultures to induce E-cadherin expression. After a further 18 hours, cultures were fixed and stained for Sec8 and either E-cadherin or nectin-2α; triple staining was not possible because only mouse and rabbit antibodies to these proteins were available, but we note that all cells express endogenous Sec8, and Dex-induction of E-cadherin results in uniform expression of E-cadherin in all cells (see Angres et al., 1996). E-cadherin staining was very weak in untreated cells, but was strong in Dex-treated cultures and was found prominently at cell-cell contacts (Fig. 8). Sec6/8 complex was localized in the cytosol of LE cells cultured in the absence of Dex, whether cells expressed nectin-2α or not (Fig. 8, upper panels, arrows). Therefore, homotypic nectin-2α-mediated adhesion is not sufficient to drive plasma membrane recruitment of Sec6/8 complex in L cells. Sec6/8 complex was also cytosolic in Dex-treated L cells that had not been transfected with the nectin-2α expression vector. Because Sec6/8 complex is cytosolic in L cells expressing only E-cadherin, E-cadherin-mediated adhesion is also insufficient to drive plasma membrane recruitment of Sec6/8 complex in these cells (Fig. 8, lower panels, arrows). Sec6/8 was only observed at junctions between E-cadherin-expressing cells when the cells also expressed nectin-2α (Fig. 8, lower panels, arrowheads). Furthermore, Sec6/8 was localized to cell-cell contacts only when both E-cadherin and nectin-2α were expressed and co-localized at the contact. Thus, expression of either E-cadherin or nectin-2α is insufficient, but both are necessary to recruit Sec6/8 complex to membrane sites of cell-cell adhesion.

Discussion

Sec6/8 complex is essential for targeted exocytosis of post-Golgi transport vesicles to the plasma membrane in a variety of polarized cells. However, Sec6/8 complex is not uniformly distributed over the plasma membrane but is restricted to sites active in vesicle delivery at the yeast daughter cell bud, growing lateral membranes of polarized epithelial cells and growth cones of hippocampal neurons (TerBush et al., 1996; Grindstaff et al., 1998b; Hazuka et al., 1999). As the complex is assembled from eight cytosolic protein subunits (TerBush et al., 1996; Aoki et al., 1997; Kee et al., 1997; Grindstaff et al., 1998b), it must associate with plasma membrane proteins that have a restricted distribution in order to execute its function in

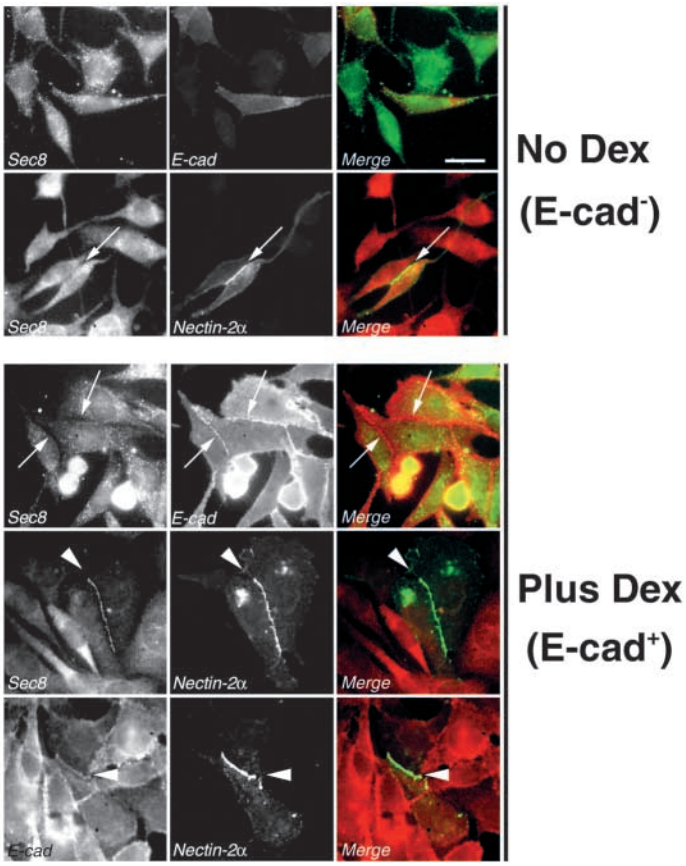


Fig. 8. E-cadherin and nectin-2α cooperate to recruit Sec6/8 complex to intercellular contacts in fibroblasts. LE cells, or LE cells that had been transiently transfected with pcDNA3.1-IgK-2HA-nectin-2α, were cultured without (no Dex) or with (plus Dex) 10^{-6} M dexamethasone for 18 hours to induce E-cadherin expression. Cells were fixed with 4% paraformaldehyde and then extracted with buffer containing 1% Triton X-100. Anti-Sec8 monoclonal antibody (2E9) was visualized either with FITC- or Texas Red-labeled secondary antibodies. Anti-E-cadherin polyclonal antibody (UVO) was visualized with Texas Red-labeled secondary antibody. Anti-nectin-2α rat monoclonal antibody was visualized with FITC-labeled secondary antibody. Arrows indicate homotypic cell-cell contacts formed between adjacent cells expressing either nectin-2α or E-cadherin only. Arrowheads indicate cell-cell contacts between E-cadherin-expressing cells in which nectin-2α was also expressed. Scale bar: 30 μm.

localized vesicle delivery. Therefore, identification of membrane binding site(s) for Sec6/8 complex is important for understanding the function(s) of this essential complex.

We have shown that in epithelial cells Sec6/8 complex is restricted to plasma membrane sites by cell-cell adhesion proteins specific to the apical junctional complex. This conclusion is based on results of four independent experimental approaches. First, Sec6/8 complex is rapidly recruited from the cytosol to cell-cell contacts of mixed junctional composition following the onset of E-cadherin-mediated adhesion and, subsequently, is spatially sorted away from the bulk of lateral membrane proteins and enriched within the apical junctional complex containing tight junction and nectin complexes. Second, consistent with this spatiotemporal redistribution during polarity development, Sec6/8 complex co-fractionates with membranes specifically enriched in apical junctional proteins. Third, Sec6/8 complex co-immunoprecipitates with specific components of apical junctions, and in a screen to uncover membrane-spanning proteins associated with Sec6/8 complex in MDCK cells the major binding partners were revealed to be E-cadherin and nectin-2α. Fourth, recruitment of Sec6/8 complex to intercellular contacts can be reconstituted in fibroblasts when E-cadherin and nectin-2α are co-expressed and functional on adjacent cells. Therefore, components of calcium-dependent (E-cadherin-based) and -independent (nectin-based) adhesion systems meet criteria expected of a binding site for the Sec6/8 complex: they co-localize, co-fractionate and co-immunoprecipitate with Sec6/8 complex and can functionally reconstitute the 'epithelial-like' recruitment of Sec6/8 complex to intercellular junctions when exogenously expressed in fibroblasts.

How components of E-cadherin and nectin-based adhesion complexes function to recruit Sec6/8 complex to intercellular junctions remains to be determined; considering that the Sec6/8 complex comprises eight subunits, the E-cadherin complex at least four proteins, and the nectin complex at least three proteins, it is likely to be complicated. However, the observation that both E-cadherin and nectin-2α are required for plasma membrane binding of Sec6/8 complex in fibroblasts suggests that the two adhesion systems cooperate to initially recruit Sec6/8 complex from the cytosol. Subsequently, Sec6/8 complex associates with only a fraction of the E-cadherin complex, as defined by immunofluorescence and protein complex fractionation, suggesting that interactions with the nectin complex and associated proteins (such as ZO-2) may further refine the localization of Sec6/8 complex to the apical junctional complex as polarity develops. Interestingly, the only cytosolic protein known to bind nectin is afadin, and afadin can be recruited to E-cadherin contacts in L cells in the absence of nectin through its interaction with α-catenin (Mandai et al., 1997). However, Sec6/8 complex is not assembled at intercellular contacts formed under these conditions, indicating that nectin itself is also required. It is unlikely that Sec6/8 complex binds directly to nectin-2α, though. Our data show that nectin-2α is associated with Sec8, but that preservation of this complex requires mild chemical cross-linking of cells prior to detergent solubilization. In the absence of cross-linking, nectin is not physically associated with Sec8 or with the E-cadherin complex and migrates as a smaller complex in Superose FPLC. Since in L cells nectin-2α is required to drive plasma membrane recruitment of Sec6/8 complex, we hypothesize that nectin-2α may be required to drive the initial association of Sec6/8 with nascent junctions, but then may dissociate.

In epithelial cells, identification of components of cadherin- and nectin-based adhesions as binding site(s) for Sec6/8 complex on the plasma membrane is functionally significant. Under low calcium culture conditions, cadherin-mediated adhesion is prevented but nectin-based adhesion, which is independent of extracellular calcium, should persist (Aoki et al., 1997; Takahashi et al., 1999). Sec6/8 complex is cytosolic under these conditions (Grindstaff et al., 1998b). Early morphological studies showed single MDCK cells have rudimentary apical and basal membrane domains, but relatively little lateral membrane surface area (Vega-Salas et al., 1987).

However, E-cadherin-mediated adhesion, through α-catenin-bound afadin, drives assembly of nectin complexes at cell-cell contacts (Tachibana et al., 2000; Pokutta et al., 2002). Sec6/8 complex can now be recruited specifically to cell-cell contacting membranes and participate in recruitment of basal-lateral transport vesicles to that domain of the plasma membrane. Consequently, the surface area of lateral plasma membranes increases more than six-fold following induction of cadherin-mediated adhesion and Sec6/8 recruitment (Vega-Salas et al., 1987). Interestingly, during *Drosophila* blastoderm cellularization membrane growth occurs by exocytic vesicle insertion from the apical membrane to form ~30 μm long lateral membranes (Lecuit and Wieschaus, 2000), although it is not known whether the Sec6/8 complex is involved.

In polarized epithelial cells, the Sec6/8 complex is localized to the apical junctional complex, and inhibition of Sec6/8 complex function greatly reduces exocytic basal-lateral vesicle delivery to the plasma membrane from the Golgi complex (Grindstaff et al., 1998b). Several other proteins involved in exocytosis are also localized to this site including, rab3b (Weber et al., 1994), rab8 (Huber et al., 1993), rab13 (Zahraoui et al., 1994), VAP-33 (Lapierre et al., 1999) and the Sec1p homolog Munc18c (our unpublished data). An early report identified the apical junctional domain as a site for exocytosis of proteins recycled from the apical plasma membrane (Louvard, 1980). More recently, exocytosis was shown to occur preferentially within the upper half of the lateral membrane (Kreitzer et al., 2003). However, studies of t-SNARE/syntaxin distributions in renal epithelial cells have revealed that syntaxin 4 (Low et al., 1996; Li et al., 2002) and the mammalian homolog of lethal giant larvae Mlgl (Müsch et al., 2002) are present over the entire surface of the lateral membrane domain, and not just at the apical junctional complex, and inhibition of t-SNARE function in MDCK cells decreases basal-lateral vesicle delivery (Ikonen et al., 1995). Remarkably, differences in spatial distributions of exocytic components in epithelial cells are similar to those in budding yeast, in which the exocyst and Sec1p are localized to the tip of the daughter bud (TerBush et al., 1996; Carr et al., 1999) and the t-SNAREs (Sso1/2p, Sec9p) and LGL homologues (Sro7/77) are distributed uniformly over the mother and daughter cell plasma membranes (Brennwald et al., 1994; Lehman et al., 1999). The functional significance of these differences in distributions of exocytic machinery on the plasma membrane is unclear. Perhaps, t-SNAREs are sufficient to promote vesicle delivery to the plasma membrane, but at specific stages of cell polarization, when rapid and localized membrane growth is required, Sec6/8 (exocyst) complex cooperates with the core exocytic machinery to provide a higher affinity destination for exocytic vesicles than t-SNAREs alone. This could account for the observation that apical trafficking in MDCK cells, while dependent on Syntaxin3 function, appears to be independent of the Sec6/8 complex (Grindstaff et al., 1998b).

Considering that many polarized cell types do not express E-cadherin or nectins, these adhesion molecules cannot be the only proteins involved in translating external spatial cues into intracellular signals that recruit the Sec6/8 complex to sites of membrane growth. Indeed, studies in other cell types have revealed interactions between Sec6/8 complex and septins (Hsu et al., 1998), a calcium signaling complex (Shin et al., 2000) and both Rho and Ral GTPases (Guo et al., 2001; Zhang et al., 2001; Moskalenko et al., 2002; Sugihara et al., 2002; Inoue et al., 2003). It is likely that this essential protein complex, which has retained an important function in exocytosis throughout evolution, has evolved more than one mechanism for recruitment to the plasma membrane in order to adapt to different environmental cues for polarized membrane growth.

This work was supported by a grant from the National Institutes of Health to W.J.N. (GM35227) and a Howard Hughes Medical Institute Biomedical Research Support Program Faculty Startup Package to C.Y.

References

Ando-Akatsuka, Y., Yonemura, S., Itoh, M., Furuse, M. and Tsukita, S. (1999). Differential behavior of E-cadherin and occludin in their colocalization with ZO-1 during the establishment of epithelial cell polarity. *J. Cell Physiol.* **179**, 115-125.

Angres, B., Barth, A. and Nelson, W. J. (1996). Mechanism for transition from initial to stable cell-cell adhesion: kinetic analysis of E-cadherin-mediated adhesion using a quantitative adhesion assay. *J. Cell Biol.* **134**, 549-557.

Aoki, J., Koike, S., Asou, H., Ise, I., Suwa, H., Tanaka, T., Miyasaka, M. and Nomoto, A. (1997). Mouse homolog of poliovirus receptor-related gene 2 product, mPRR2, mediates homophilic cell aggregation. *Exp. Cell Res.* **235**, 374-384.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1987). *Current Protocols in Molecular Biology.* New York: John Wiley and Sons.

Bagnat, M., Chang, A. and Simons, K. (2001). Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. *Mol. Biol. Cell* **12**, 4129-4138.

Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V. and Novick, P. (1994). Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. *Cell* **79**, 245-258.

Brymora, A., Valova, V. A., Larsen, M. R., Roufogalis, B. D. and Robinson, P. J. (2001). The brain exocyst complex interacts with RalA in a GTP-dependent manner: identification of a novel mammalian Sec3 gene and a second Sec15 gene. *J. Biol. Chem.* **276**, 29792-29797.

Carr, C. M., Grote, E., Munson, M., Hughson, F. M. and Novick, P. J. (1999). Sec1p binds to SNARE complexes and concentrates at sites of secretion. *J. Cell Biol.* **146**, 333-344.

Craig, A. M., Wyborski, R. J. and Banker, G. (1995). Preferential addition of newly synthesized membrane protein at axonal growth cones. *Nature* **375**, 592-594.

Dotti, C. G. and Banker, G. A. (1987). Experimentally induced alteration in the polarity of developing neurons. *Nature* **330**, 254-256.

Finger, F. P., Hughes, T. E. and Novick, P. (1998). Sec3p is a spatial landmark for polarized secretion in budding yeast. *Cell* **92**, 559-571.

Finger, F. P. and Novick, P. (1998). Spatial regulation of exocytosis: lessons from yeast. *J. Cell Biol.* **142**, 609-612.

Friedrich, G. A., Hildebrand, J. D. and Soriano, P. (1997). The secretory protein Sec8 is required for paraxial mesoderm formation in the mouse. *Dev. Biol.* **192**, 364-374.

Grindstaff, K. K., Bacallao, R. L. and Nelson, W. J. (1998a). Apiconuclear organization of microtubules does not specify protein delivery from the trans-Golgi network to different membrane domains in polarized epithelial cells. *Mol. Biol. Cell* **9**, 685-699.

Grindstaff, K. K., Yeaman, C., Anandasabapathy, N., Hsu, S.-C., Rodriguez-Boulan, E., Scheller, R. H. and Nelson, W. J. (1998b). Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in polarized epithelial cells. *Cell* **93**, 731-740.

Guo, W., Tamanoi, F. and Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat. Cell Biol.* **3**, 353-360.

Hazuka, C. D., Foletti, D. L., Hsu, S. C., Kee, Y., Hopf, F. W. and Scheller, R. H. (1999). The sec6/8 complex is located at neurite outgrowth and axonal synapse-assembly domains. *J. Neurosci.* **19**, 1324-1334.

Heuser, J. (1989). Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J. Cell Biol.* **108**, 855-864.

Hinck, L., Nathke, I. S., Papkoff, J. and Nelson, W. J. (1994). Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J. Cell Biol.* **125**, 1327-1340.

Hsu, S. C., Ting, A. E., Hazuka, C. D., Davanger, S., Kenny, J. W., Kee, Y.

and Scheller, R. H. (1996). The mammalian brain rsec6/8 complex. *Neuron* **17**, 1209-1219.

Hsu, S. C., Hazuka, C. D., Roth, R., Foletti, D. L., Heuser, J. and Scheller, R. H. (1998). Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. *Neuron* **20**, 1111-1122.

Huber, L. A., Pimplikar, S., Parton, R. G., Virta, H., Zerial, M. and Simons, K. (1993). Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. *J. Cell Biol.* **123**, 35-45.

Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C. and Simons, K. (1995). Different requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells. *Cell* **81**, 571-580.

Inoue, M., Chang, L., Hwang, J., Chiang, S. H. and Saltiel, A. R. (2003). The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. *Nature* **422**, 629-633.

Jahn, R. and Sudhof, T. C. (1999). Membrane fusion and exocytosis. *Annu. Rev. Biochem.* **68**, 863-911.

Kee, Y., Yoo, J. S., Hazuka, C. D., Peterson, K. E., Hsu, S. C. and Scheller, R. H. (1997). Subunit structure of the mammalian exocyst complex. *Proc. Natl. Acad. Sci. USA* **94**, 14438-14443.

Keller, P. and Simons, K. (1997). Post-Golgi biosynthetic trafficking. *J. Cell Sci.* **110**, 3001-3009.

Kreitzer, G., Marmorstein, A., Okamoto, P., Vallee, R. and Rodriguez-Boulan, E. (2000). Kinesin and dynamin are required for post-Golgi transport of a plasma-membrane protein. *Nat. Cell Biol.* **2**, 125-127.

Kreitzer, G., Schmoranzer, J., Low, S. H., Li, X., Gan, Y., Weimbs, T., Simon, S. M. and Rodriguez-Boulan, E. (2003). Three-dimensional analysis of post-Golgi carrier exocytosis in epithelial cells. *Nat. Cell Biol.* **5**, 126-136.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lapierre, L. A., Tuma, P. L., Navarre, J., Goldenring, J. R. and Anderson, J. M. (1999). VAP-33 localizes to both an intracellular vesicle population and with occludin at the tight junction. *J. Cell Sci.* **112**, 3723-3732.

Le Bivic, A., Quaroni, A., Nichols, B. and Rodriguez-Boulan, E. (1990). Biogenetic pathways of plasma membrane proteins in Caco-2, a human intestinal epithelial cell line. *J. Cell Biol.* **111**, 1351-1361.

Lecuit, T. and Wieschaus, E. (2000). Polarized insertion of new membrane from a cytoplasmic reservoir during cleavage of the Drosophila embryo. *J. Cell Biol.* **150**, 849-860.

Lehman, K., Rossi, G., Adamo, J. E. and Brennwald, P. (1999). Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. *J. Cell Biol.* **146**, 125-140.

Li, X., Low, S. H., Miura, M. and Weimbs, T. (2002). SNARE expression and localization in renal epithelial cells suggest mechanism for variability of trafficking phenotypes. *Am. J. Physiol. Renal Physiol.* **283**, F1111-1122.

Lipschutz, J. H., Guo, W., O'Brien, L. E., Nguyen, Y. H., Novick, P. and Mostov, K. E. (2000). Exocyst is involved in cystogenesis and tubulogenesis and acts by modulating synthesis and delivery of basolateral plasma membrane and secretory proteins. *Mol. Biol. Cell* **11**, 4259-4275.

Louvard, D. (1980). Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA* **77**, 4132-4136.

Low, S. H., Chapin, S. J., Weimbs, T., Komuves, L. G., Bennett, M. K. and Mostov, K. E. (1996). Differential localization of syntaxin isoforms in polarized Madin-Darby canine kidney cells. *Mol. Biol. Cell* **7**, 2007-2018.

Mandai, K., Nakanishi, H., Satoh, A., Obaishi, H., Wada, M., Nishioka, H., Itoh, M., Mizoguchi, A., Aoki, T., Fujimoto, T. et al. (1997). Afadin: A novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction. *J. Cell Biol.* **139**, 517-528.

Matern, H. T., Yeaman, C., Nelson, W. J. and Scheller, R. H. (2001). The Sec6/8 complex in mammalian cells: characterization of mammalian Sec3, subunit interactions, and expression of subunits in polarized cells. *Proc. Natl. Acad. Sci. USA* **98**, 9648-9653.

Moskalenko, S., Henry, D. O., Rosse, C., Mirey, G., Camonis, J. H. and White, M. A. (2002). The exocyst is a Ral effector complex. *Nat. Cell Biol.* **4**, 66-72.

Mostov, K. E., Verges, M. and Altschuler, Y. (2000). Membrane traffic in polarized epithelial cells. *Curr. Opin. Cell Biol.* **12**, 483-490.

Müsch, A., Cohen, D., Yeaman, C., Nelson, W. J., Rodriguez-Boulan, E. and Brennwald, P. J. (2002). Mammalian homolog of Drosophila tumor suppressor lethal (2) giant larvae interacts with basolateral exocytic machinery in Madin-Darby canine kidney cells. *Mol. Biol. Cell* **13**, 158-168.

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**, 533-536.

Pasdar, M. and Nelson, W. J. (1988). Kinetics of desmosome assembly in Madin-Darby canine kidney epithelial cells: temporal and spatial regulation of desmoplakin organization and stabilization upon cell-cell contact. I. Biochemical analysis. *J. Cell Biol.* **106**, 677-685.

Pokutta, S., Drees, F., Takai, Y., Nelson, W. J. and Weis, W. I. (2002). Biochemical and structural definition of the l-afadin- and actin-binding sites of alpha-catenin. *J. Biol. Chem.* **277**, 18868-18874.

Ribon, V., Herrera, R., Kay, B. K. and Saltiel, A. R. (1998). A role for CAP, a novel, multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions. *J. Biol. Chem.* **273**, 4073-4080.

Rothman, J. E. and Warren, G. (1994). Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.* **4**, 220-233.

Shin, D. M., Zhao, X. S., Zeng, W., Mozhayeva, M. and Muallem, S. (2000). The mammalian Sec6/8 complex interacts with Ca(2+) signaling complexes and regulates their activity. *J. Cell Biol.* **150**, 1101-1112.

Stamnes, M. (2002). Regulating the actin cytoskeleton during vesicular transport. *Curr. Opin. Cell Biol.* **14**, 428.

Stewart, D. B. and Nelson, W. J. (1997). Identification of four distinct pools of catenins in mammalian cells and transformation-dependent changes in catenin distributions among these pools. *J. Biol. Chem.* **272**, 29652-29662.

Sugihara, K., Asano, S., Tanaka, K., Iwamatsu, A., Okawa, K. and Ohta, Y. (2002). The exocyst complex binds the small GTPase RalA to mediate filopodia formation. *Nat. Cell Biol.* **4**, 73-78.

Suzuki, A., Ishiyama, C., Hashiba, K., Shimizu, M., Ebnet, K. and Ohno, S. (2002). aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization. *J. Cell Sci.* **115**, 3565-3573.

Tachibana, K., Nakanishi, H., Mandai, K., Ozaki, K., Ikeda, W., Yamamoto, Y., Nagafuchi, A., Tsukita, S. and Takai, Y. (2000). Two cell adhesion molecules, nectin and cadherin, interact through their cytoplasmic domain-associated proteins. *J. Cell Biol.* **150**, 1161-1176.

Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A. et al. (1999). Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein. *J. Cell Biol.* **145**, 539-549.

TerBush, D. R., Maurice, T., Roth, D. and Novick, P. (1996). The Exocyst is a multiprotein complex required for exocytosis in Saccharomyces cerevisiae. *EMBO J.* **15**, 6483-6494.

Van Itallie, C. M., Balda, M. S. and Anderson, J. M. (1995). Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells. *J. Cell Sci.* **108**, 1735-1742.

Vega, I. E. and Hsu, S. C. (2001). The exocyst complex associates with microtubules to mediate vesicle targeting and neurite outgrowth. *J. Neurosci.* **21**, 3839-3848.

Vega-Salas, D. E., Salas, P. J., Gundersen, D. and Rodriguez-Boulan, E. (1987). Formation of the apical pole of epithelial (Madin-Darby canine kidney) cells: polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell-cell interactions. *J. Cell Biol.* **104**, 905-916.

Weber, E., Berta, G., Tousson, A., St John, P., Green, M. W., Gopalokrishnan, U., Jilling, T., Sorscher, E. J., Elton, T. S., Abrahamson, D. R. et al. (1994). Expression and polarized targeting of a rab3 isoform in epithelial cells. *J. Cell Biol.* **125**, 583-594.

Winckler, B. and Mellman, I. (1999). Neuronal polarity: controlling the sorting and diffusion of membrane components. *Neuron* **23**, 637-640.

Yamamoto, T., Harada, N., Kano, K., Taya, S., Canaani, E., Matsuura, Y., Mizoguchi, A., Ide, C. and Kaibuchi, K. (1997). The Ras target AF-6 interacts with ZO-1 and serves as a peripheral component of tight junctions in epithelial cells. *J, Cell Biol*, **139**, 785-795.

Yeaman, C. (2003). Ultracentrifugation-based approaches to study regulation of Sec6/8 (exocyst) complex function during development of epithelial cell polarity. *Methods* **30**, 198-206.

Yeaman, C., Grindstaff, K. K., Wright, J. R. and Nelson, W. J. (2001). Sec6/8 complexes on trans-Golgi network and plasma membrane regulate late stages of exocytosis in mammalian cells. *J. Cell Biol.* **155**, 593-604.

Zahraoui, A., Joberty, G., Arpin, M., Fontaine, J. J., Hellio, R., Tavitian, A. and Louvard, D. (1994). A small rab GTPase is distributed in cytoplasmic vesicles in non polarized cells but colocalizes with the tight junction marker ZO-1 in polarized epithelial cells. *J. Cell Biol.* **124**, 101-115.

Zhang, X., Bi, E., Novick, P., Du, L., Kozminski, K. G., Lipschutz, J. H. and Guo, W. (2001). Cdc42 interacts with the exocyst and regulates polarized secretion. *J. Biol. Chem.* **276**, 46745-46750.