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The exocyst localizes to the primary cilium in MDCK cells

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Abstract

Primary cilia play a role in the maintenance of tubular epithelial differentiation and ciliary dysfunction can result in abnormal cyst formation, such as occurs in autosomal dominant polycystic kidney disease (ADPKD). We previously showed that the exocyst, an eight-protein complex involved in the biogenesis of polarity from yeast to mammals, is centrally involved in cyst formation [Mol. Biol. Cell. 11 (2000) 4259]. Here we show that the exocyst complex localizes to the primary cilium in Madin–Darby canine kidney (MDCK) tubular epithelial cells. We further show that the exocyst is overexpressed in both cell lines and primary cell cultures of ADPKD origin, suggesting that the exocyst may be involved in the pathogenesis of ADPKD.

Keywords: Exocyst; Cilia; ADPKD

Cilia are thin rod-like organelles found on the surface of many eukaryotic cell types. Cilia extend outward from the basal body, a cellular organelle related to the centriole. Cilia are classified as primary (nonmotile) and motile. Structurally, cilia are covered by a membrane that is continuous with the plasma membrane and contain a central axenome composed of microtubules [2]. In the mammalian kidney, primary cilia have been observed on cells in the parietal layer of Bowman's capsule, the proximal tubule, the distal tubule, and in the principal, but not intercalated, cells, of the collecting duct [3]. In contrast to epithelia with numerous motile cilia, that are thought to have a propulsive function, the cells of these nephron segments contain a single nonmotile cilium, or, less commonly, two cilia [2].

Protein products of several genes which when mutated result in polycystic kidney disease, including PKD1 and PKD2 [4], have recently been localized to and/or are important for the function of the primary cilium of kidney epithelial cells [5–10]. It was proposed that renal primary cilia function as flow sensors based on the observation that flow rates in renal tubules result in a deflection in the cilia shaft [11]. The Madin–Darby canine kidney (MDCK) cell line, derived from the kidney tubules of a normal cocker spaniel in 1958 [12], is one of the best-characterized and most widely used epithelial cell lines [13]. Bending of the cilium of MDCK cells grown on coverslips caused intracellular calcium to substantially increase [14]. Initially extracellular calcium enters through mechanically sensitive channels, followed by calcium-induced calcium release from intracellular stores. The increase of intracellular calcium then spreads to adjacent cells, probably by a process that is dependent on gap junctions. Polycystins-1 and -2, the protein products of PKD1 and PKD2, are likely important functional elements for mechanosensation by the primary cilium of kidney epithelial cells. Polycystin-2 is a calcium channel [2]. Nauli and co-workers confirmed localization of polycystin-1 and polycystin-2 to the primary cilia of kidney tubular epithelium and went on to demonstrate that renal tubular epithelial cells

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from knockout mice that lack functional polycystin-1 form cilia, but do not demonstrate increases of intracellular calcium levels with mechanical stimulation of the cilia. Moreover, function-perturbing antibodies directed against polycystin-2 abrogated the increases in intracellular calcium following mechanical stimulation of the cilia [15].

The exocyst is a conserved eight-protein complex first identified as being involved in the exocytosis of vesicles in yeast [16] and is composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 [17,18]. The exocyst is involved in the biogenesis of polarity in species as diverse as yeast and mammals, and has been postulated to be the docking and targeting complex for vesicular traffic to the plasma membrane [19]. We and others have recently shown that the exocyst complex, in addition to its docking and targeting function, is also involved in protein synthesis, by interacting with the translational machinery of the endoplasmic reticulum (ER) [20,21]. The exocyst had previously been partially localized to the ER where it was shown to interact with and regulate the activity of calcium signaling complexes [22].

We previously demonstrated, using an in vitro model system in which MDCK cells are grown in a collagen matrix, that the exocyst complex is centrally involved in cystogenesis and tubulogenesis [1]. In addition, it was shown that the exocyst, which is significantly localized to the tight junction in kidney epithelial cells as part of its targeting and docking role, was mislocalized to the cytoplasm in primary cultures of ADPKD cells [23]. Here we examine the subcellular localization of the exocyst complex in MDCK cells with confocal laser microscopy and evaluate exocyst expression levels in ADPKD primary cell cultures and cell lines.

Experimental procedures

Cell lines and primary cultures. Human renal epithelia of normal collecting tubule [24] and ADPKD cyst origin [25] were microdissected and grown in serum-free defined media and conditionally immortalized by retroviral delivery of an immortalizing vector containing the temperature-sensitive SV40 T-antigen allele tsA58U19 and neomycin resistance [26,27]. After 6 weeks of selection in geneticin (G418, 500 μg/ml) cells were grown to 70% confluence in T25 flasks coated with type 1 collagen at the permissive temperature of 33 °C and then subjected to 7 days at 37 °C, at which temperature T antigen was degraded and the cells were highly differentiated as assessed by antigen and marker analysis [27].

Primary cultures of epithelial cells derived from kidneys from patients with endstage ADPKD and from age-matched normal human kidneys were prepared as described previously [24,25]. Briefly, ADPKD cyst epithelia or normal collecting tubules were microdissected from nephrectomy specimens perfused in situ with Collins salts solution, and allowed to attach overnight to collagen-coated tissue culture plastic and then outgrowth monolayers were cultured in defined, growth-factor-supplemented media until confluence was reached. Cells were collected for analysis after three washes in cold PBS containing a comprehensive protease-inhibitor cocktail.

Cell culture. MDCK type II cells were grown on Transwell filters for 6 days and maintained in MEM-containing Earle's balanced salt solution, supplemented with 5% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO_2/95% air.

Antibodies/reagents. Monoclonal mouse anti-rSec6 and -8 was used at 1:500 for Western blot and 1:100 for immunofluorescence (StressGen). Sec10 polyclonal antibody was used at 1:100 for immunofluorescence [1]. Monoclonal antibody against β-actin (Sigma) was used at 1:1000 for Western blot. Monoclonal antibody to β-tubulin IV (BioGenex) and polyclonal sheep antibody to β-tubulin (Cytoskeleton) were used at 1:100 for immunofluorescence. Goat anti-mouse HRP (Jackson Labs) was the secondary antibody used for Western blots (1:15,000) (Jackson ImmunoResearch). Texas Red-conjugated goat anti-mouse and FITC-conjugated goat anti-sheep and anti-rabbit secondary antibody, 1:200 dilution (Jackson), were used as the secondary antibody for immunofluorescence.

Western blot/immunoprecipitation. Cells were lysed in 0.5% SDS lysis buffer and prepared in standard fashion [1]. Immunoprecipitation was performed using 4–8 μg of antibody against Sec6 and Sec8. The immunoprecipitate was then run on an SDS–PAGE gel. The protein bands were detected by incubations with the same antibody followed by goat anti-mouse HRP (Jackson Labs) as the secondary antibody (1:15,000), and ECL (Amersham–Pharmacia).

Immunofluorescence and confocal microscopy. Cells were rinsed in PBS and fixed for 30 min with 4% paraformaldehyde as previously described [1,28]. Nonspecific binding sites were blocked and the cells were permeabilized using 0.7% fish skin gelatin and 0.025% saponin. Samples were placed in medium containing either monoclonal antibody to β-tubulin IV (BioGenex) and polyclonal antibody to Sec10 [1] or polyclonal sheep antibody to β-tubulin (Cytoskeleton) and monoclonal antibody to Sec6 or Sec8 (StressGen). After extensive washing, the samples were incubated in blocking buffer containing either goat anti-mouse Texas Red-conjugated and goat anti-rabbit FITC-conjugated secondary antibody or goat anti-mouse Texas Red-conjugated and goat anti-sheep FITC-conjugated secondary antibody, 1:200 dilution (Jackson). Cells were postfixed with 4% paraformaldehyde and mounted. Confocal images were collected using a krypton–argon laser (BioRad 1024). Ciliary/centriolar staining was not seen when secondary antibody alone was used.

Results

We chose to perform the detailed subcellular localization of the exocyst using MDCK cells, as this renal tubular epithelial cell line is well studied, well characterized, and forms a confluent monolayer in cell culture. Furthermore, we grew the MDCK cells on Transwell filters for 6 days prior to fixation and staining, as growth on semi-permeable filters, in comparison to growth on plastic dishes, more rigorously simulates the polarized state [28]. For example, MDCK cells are typically 3–5 μm tall when grown on plastic, but 10–15 μm tall when grown on filters [29]. Excellent monoclonal antibodies, verified by multiple groups including ours [1,22,30], are commercially available against exocyst complex proteins Sec6 and Sec8. Because other subunits of the exocyst cofractionate and co-localize with Sec6 and Sec8 [1,30], the localization of Sec6 and Sec8 likely reflects the localization of the holocomplex. Following fixation and staining, we used confocal immunofluorescence microscopy to examine the localization of Sec6 and Sec8.

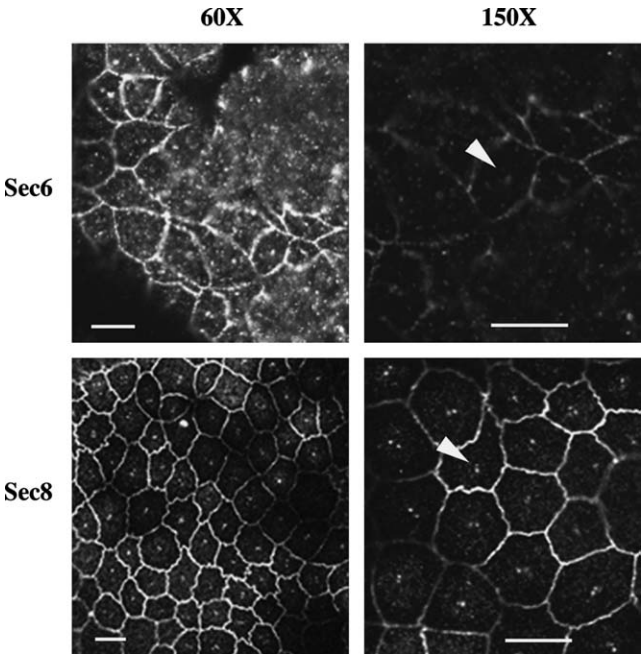

Fig. 1. Antibodies against exocyst component Sec6 and Sec8 show staining in a ciliary/centriolar pattern. Madin–Darby canine kidney (MDCK) cells were grown to confluency on a 12-mm (0.4-μm pore size) filter (Costar) for 6 days. The cells were then stained with primary antibody against the Sec6 and Sec8 components of the exocyst followed by Texas Red-conjugated goat anti-mouse secondary antibody. A BioRad 1024 confocal microscope was used to image the cells. Using a 60× oil objective, typical tight junction exocyst staining is seen along with intracellular staining consistent with the endoplasmic reticulum. Prominent exocyst staining in the center of the cell is also seen. Using the 60× oil objective with a 2.5× "zoom" (150×), a ciliary/centriolar staining pattern, in addition to the tight junction and intracellular staining, can be appreciated (arrowheads). Note that not all cells are in the same plane of focus, which typically occurs when filters are mounted during immunofluorescence staining. Bar = 10 μm.

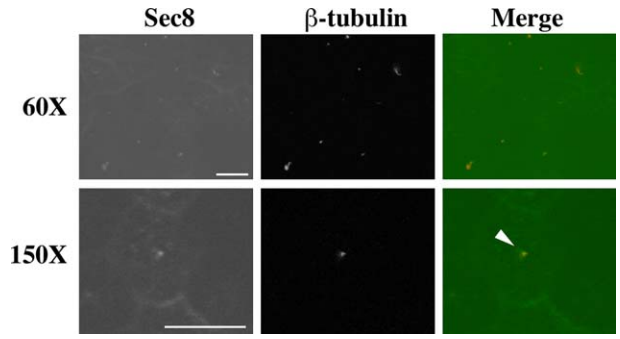

Fig. 2. The exocyst partially localizes to the primary cilium/centriole. MDCK cells were grown to confluency on a 12-mm (0.4-μm pore size) filter (Costar) for 6 days. The cells were then stained with primary antibody against the Sec8 component of the exocyst and the centriole/ciliary component β-tubulin, followed by goat anti-mouse Texas Red-conjugated and goat anti-sheep FITC-conjugated secondary antibody. Co-localization of exocyst component Sec8 and the centriole/ciliary component β-tubulin was seen using confocal microscopy with 60× oil objective. The co-localization, seen as a yellow color in the merged section, is better appreciated using the 60× oil objective with a 2.5× "zoom" (arrowhead). Note that Sec8 and β-tubulin staining are shown in black and white in the first two panels, with only the merged third panel in color.

We found exocyst staining localized largely to the tight junction area as has been described previously by us and others [1,30]. There was also intracellular staining consistent with localization to the endoplasmic reticulum, which we and others recently reported [20,21,31]. Finally, there was prominent staining near the center of the cell at the level of the centriole/cilia. This was more evident with the Sec8 antibody, but could also be appreciated with the Sec6 antibody (Fig. 1). Polyclonal antibodies against Sec10, that we previously generated [1], also demonstrated prominent staining in a centriole/cilia pattern; however, Sec10 and Sec15 have been shown to form the only separate subcomplex within the greater exocyst subcomplex [32], precluding generalization of exocyst localization from these data.

The β-tubulin isoform IV is centriole/cilia specific [4]. We confirmed that the prominent exocyst staining in the center of the cell was the centriole/cilia, by performing double staining using antibodies against exocyst components and this isoform of β-tubulin. Colocalization of Sec6, Sec8 (Fig. 2), and Sec10 with β-tubulin was seen.

Given that the cilia are likely involved in the pathogenesis of ADPKD [2] and the exocyst is involved in cyst formation [1], we examined protein levels of exocyst components Sec6 and Sec8 in primary cell cultures (Fig. 3A) from kidneys of patients with ADPKD and immortalized cell lines that we had previously generated (Fig. 3B) [26]. We compared these levels to primary cell cultures from normal human kidneys (Fig. 3A) and immortalized cell lines of normal human collecting tubules that we had also previously generated (Fig. 3B) [26]. Equal amounts of protein were used, either for immunoprecipitation (IP), or for loading in each lane, as determined by BCA protein analysis. We compared the protein expression levels by both IP-Western (Figs. 3A and B) and straight Western blotting (Fig. 3C). We found that there was a 3- to 5-fold increase in Sec6 (Figs. 3A and C) and Sec8 (Fig. 3B) protein expression in ADPKD cell lines and primary cultures compared to normal controls.

Our results in Fig. 3 were different than those of Charron and colleagues who had shown that while the exocyst complex was mislocalized in primary cell cultures of ADPKD tissue compared to normal renal tissue, the levels of exocyst proteins Sec6 and Sec8 were unchanged. In the Methods section, they described their studies as having been done using early primary cultures (passage four or less) [23]. Primary cell cultures are created by enzymatically digesting tissue, i.e., with trypsin, in order to separate cells before plating them in suspension on tissue culture dishes and might be expected to include several types of cells [33]. It has been shown that ADPKD cells have a growth advantage [34].

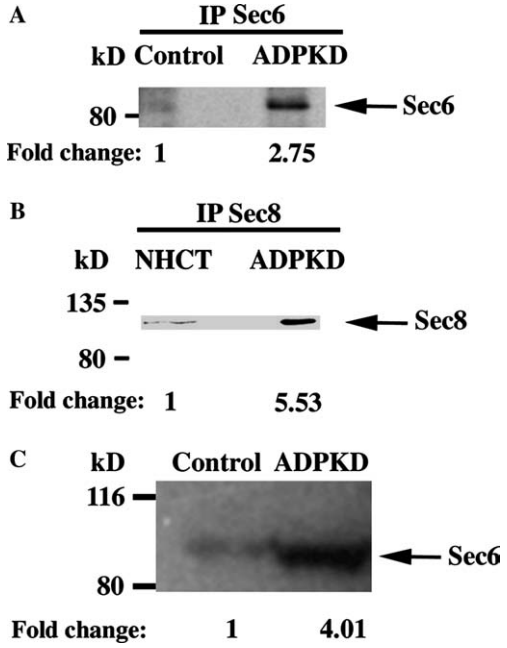

Fig. 3. Exocyst protein is enriched in ADPKD cells. (A) Increased amounts of Sec6 per microgram total protein were seen in primary cell cultures from ADPKD compared to normal kidney tissue (control). IP-Western. (B) Similar results for Sec8 were seen in cell lines created from ADPKD cells compared to cell lines from normal human collecting tubules (NHCT). Equal amounts of total protein were assayed as determined by the BCA Protein Assay (Pierce). IP-Western. (C) Western blot from primary cell cultures gave similar results. Fold change was determined using NIH Image quantification of the bands and was normalized to the control. This figure is representative of primary cell cultures from 13 different ADPKD patients/cysts and 9 normal human kidneys.

Therefore, assuming individual ADPKD cells contain greater levels of exocyst complex, then ADPKD primary cell cultures, after being repeatedly passaged, might become enriched in both ADPKD cells and exocyst protein. For the experiments demonstrating increased levels of exocyst protein in ADPKD primary cell cultures (Fig. 3), we used late passage primary cultures (passage ten or greater), and immortalized cell lines have also been repeatedly passaged. To test if this might explain the discrepancy between our data and those of Charron and colleagues, we examined exocyst expression in serial primary cell cultures from ADPKD tissue and from normal human kidney. We found that as the passage number increased, the levels of the exocyst proteins Sec6 (Fig. 4A) and Sec8 (Figs. 4B and C) also increased per μg of total protein. The levels of Sec6 and Sec8 (Fig. 4D), however, did not change with increasing passage number in the primary cell cultures from normal renal tissue. Equal amounts of protein loading in each lane were confirmed by both the BCA assay and by stripping the blots and reprobing for actin (Figs. 4C and D). IP-Western results were consistent with the results obtained by straight Western blot, and the enrichment of Sec6 (Fig. 4A) and Sec8 (Fig. 4B) continued through passage 7. Results for passage 10 and greater are shown in Fig. 3.

Discussion

We report two principal findings, both of which are quite interesting. First, we show that the exocyst

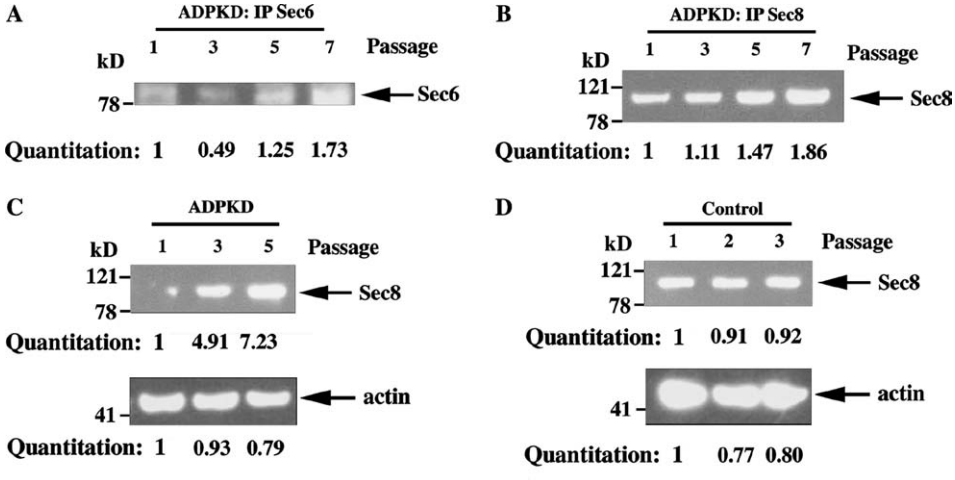

Fig. 4. Exocyst protein is enriched in primary cultures of ADPKD cells with increasing passage number. Increasing amounts of Sec6 (A) and Sec8 (B) per microgram total protein were seen as the passage number increased from one to seven in primary cell cultures from ADPKD kidney tissue. IP-Western. (C) A similar increase in the amount of Sec8 was seen in primary cultures from ADPKD kidney tissue compared to normal kidney tissue as the passage number increased from one to five using Western blot. (D) In contrast, there was no change in Sec8 levels with increasing passage number in primary cell cultures from normal human kidney tissue. Western blot. Equal amounts of total protein were assayed as determined by the BCA Protein Assay (Pierce) (A–D) and by staining for β-actin (C,D). Note that the amount of Sec8 protein was similar in passage three in both the ADPKD and control primary cell cultures, run on the same gel, but by passage five the Sec8 protein level was ~2-fold that of controls (C,D). NIH Image quantification of the bands was performed with normalization to passage 1. Each experiment was repeated three times.

complex localizes to the primary cilium in the MDCK renal tubular epithelial cell line. Second, we show that the exocyst complex is overexpressed in ADPKD primary cultures and immortalized cell lines in comparison to primary cultures of normal kidney and immortalized cell lines of normal human collecting tubules.

It is important to note that the exocyst complex has been shown to relocalize both throughout the yeast life cycle [35] and during cyst and tubule formation in mammalian cells [1]. There is precedent for proteins acting at multiple locations in the cell. For example, β-catenin is present in cell–cell junctions and binds to the cytoplasmic domain of a family of Ca^{2+}-dependent cell adhesion molecules, the cadherins [36]. β-Catenin also acts in the nucleus by binding to transcription factors of the T cell factor-lymphoid enhancer factor (Tcf-Lef) family [37–39].

The localization of the exocyst to the primary cilium of renal tubular epithelial cells, which we report here, as well as to the ER and tight junction, places the exocyst in a position where it could be involved in the initiation and propagation of the signals that ultimately lead to the polycystic kidney phenotype [5–10]. Previous reports demonstrate a functional association of the exocyst complex with microtubules [40], and microtubules form the axenome of the cilia [2].

Our data demonstrating that the exocyst complex localizes to the cilium in MDCK cells and is overexpressed in ADPKD cells, along with previous data showing that the exocyst is misexpressed in primary cell cultures of ADPKD tissue [23], suggest that the exocyst may be involved in the pathogenesis of ADPKD.

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