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The polycystic kidney disease-related proteins Bicc1 and SamCystin interact

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ABSTRACT

Mutations in either the *Bicaudal-C* or the *Anks6* gene which encode the Bicc1 and SamCystin proteins respectively cause formation of renal cysts in rodent models of polycystic kidney disease, however their role in the mammalian kidney is unknown. Immunolocalization studies demonstrated that, unlike many other PKD-related proteins, SamCystin and Bicc1 do not localize to the primary cilia of cultured kidney cells. Epitope-tagged recombinant SamCystin and Bicc1 proteins were transiently transfected into inner medullary collecting duct (IMCD) cells and co-immunoprecipitated. The results showed that SamCystin self-associates, Bicc1 and SamCystin interact, the mutation responsible for PKD in the Han:SPRD-*Cy* rat disrupts the self-association of SamCystin but not the Bicc1–SamCystin interaction, and RNA may be an important component of the Bicc1–SamCystin complex. These studies provide the first evidence that Bicc1 and SamCystin interact at the protein level suggesting that they function in a common molecular pathway that when perturbed, is involved in cystogenesis.

Introduction

Renal tubular cysts are a feature of a number of inherited human disorders including polycystic kidney disease (PKD). Many rodent models for PKD have been characterized, including the *jcpk* mouse model and the *Cy* rat model which both carry mutations that lead to the formation of renal cysts in affected animals. A mutation in *Bicc1*, the mammalian orthologue of the *Drosophila Bicaudal-C* gene is responsible for disease in the *jcpk* mouse model [1]. The Bicc1 protein contains two types of functional domains: three tandem K homology (KH) domains near the N-terminus and a sterile alpha motif (SAM) domain near the C-terminus. The disease allele carried by the *jcpk* mouse (*Bicc1*^{jcpk}) contains a mutation that results in a frameshift leading to a premature stop which produces a severely truncated protein. This truncated protein is missing most of KH1, all of KH2 and 3 and all of the SAM domain [1]. The Bicc1^{jcpk} protein is predicted to be nonfunctional. SamCystin, the protein encoded by *Anks6* (formerly *Pkdr1*), has 10 tandem ankyrin repeats at its N-terminus and a SAM domain at its C-terminus [2]. In the Han:SPRD-*Cy* rat model, a single nucleotide base pair mutation in *Anks6* results in a replacement of a highly conserved arginine residue with a tryptophan residue in the SAM domain. The effect of this change on protein function is unknown.

The sterile alpha motif (SAM) is a conserved domain of approximately 70 residues that is found in a large number of bacterial and eukaryotic proteins [3,4]. Many of these proteins participate in protein–protein interactions, signal transduction pathways or various developmental processes [5]. In SAM domain-containing proteins that have been shown to participate in protein–protein interactions, these interactions can include homo-SAM [6–10] and hetero-SAM [10,11] domain interactions as well as heterotypic interactions with non-SAM domain-containing proteins [12–14].

The finding that two PKD-related proteins, Bicc1 and SamCystin contain SAM domains led us to speculate that perhaps both the Bicc1 and SamCystin proteins physically interact via their SAM domains. The data presented here provides evidence that both proteins co-localize to the same region of the cell and that SamCystin is involved in protein–protein interactions with both itself and Bicc1.

Materials and methods

Constructs. Anks6 cDNA (GenBank Accession No. NM001015028) was PCR amplified from IMAGE clone 7108955 (Open Biosystems, Huntsville, AL) using primers 5′-GAAATGGGCGAGGGCGCGCTG GCC-3′ and 5′-CCTGCTCGACACTGTTTCTTCTGGCCTTA-3′ (full-length *Anks6*); 5′-GAAATGGGCGAGGGCGCGCTGGCC-3′ and 5′-

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TGGGACGAGGAGGAAGAC-3′ (SamCystinΔSAM); or 5′-ATCACGCC GGTGTCTGTGTGCATGCAG-3′ and 5′-AAGGTACCGGGCCCCCCCTC GAGGTCGAC-3′ (SamCystinΔANK). For c-Myc-SamCystin (R823W), RT-PCR was performed using total kidney RNA from a *Cy*/*Cy* rat and primers 5′-GAAATGGGCGAGGGCGCGCTGGCC-3′ and 5′-CCTGCTCGACACTGTTTCTTCTGGCCTTA-3′. Amplicons were cloned into pCR8/GW/TOPO (Invitrogen, Carlsbad, CA), and subcloned into the EcoRI site of pCMV-3Tag-2A (Stratagene, La Jolla, CA). For V5-SamCystin, the full-length *Anks6* coding region was recombined from pCR8/GW/TOPO into pcDNA3.1/nV5-DEST (Invitrogen) using LR Clonase II enzyme mix (Invitrogen). cDNA encoding Bicc1 (GenBank Accession No. NM031397) was PCR amplified from IMAGE clone 2655954 (ATCC, Manassas, VA) using primers 5′-ATGGCCTCGCAGAGCGAG-3′ and 5′-ctaccagcggccactgacgct-3′ (full-length Bicc1); 5′-ATGGGGTGTCTTCCTCTGGTGTT-3′ and 5′-GTCAGTGGCCGCTGGTAG-3′ (Bicc1ΔKH); or 5′-TCCGAATTCGCCT TATG-3′ and 5′-TTATCCGGTCTCTCCAGTTGTCT-3′ (Bicc1ΔSAM). Amplicons were cloned into pCR8/GW/TOPO (Invitrogen). Bicc1, Bicc1(ΔKH), and Bicc1(ΔSAM) were recombined into pcDNA3.1/nV5-DEST (Invitrogen). For GFP-Bicc1, a full-length Bicc1 PCR product was cloned into pcDNA3.1/NT-GFP-TOPO (Invitrogen). All sequences were confirmed by nucleotide sequence analysis.

Cell culture and transfection. Mouse inner medullary collecting duct (IMCD) cells (American Type Culture Collection, Manassas, VA) were transiently transfected using Lipofectamine 2000 (Invitrogen) and harvested 48 h after transfection using M-PER (Pierce, Rockford, IL) containing Complete protease inhibitor (Roche Applied Science, Indianapolis, IN). RNase treated transfected lysates were incubated with 10 mg/μl RNase A (USB, Cleveland, Ohio) at 37 °C for 40 min, followed by co-immunoprecipitation as described below.

Immunofluorescence microscopy. IMCD cells were grown on collagen-coated coverslips (BD Biosciences, San Jose, CA). To assess protein localization within cilia, cells were transfected two days post-confluence with 4 μg of c-Myc-SamCystin or GFP-Bicc1 DNA. Cells were fixed 48 h post-transfection in a 1:1 acetone:methanol for 3 min, washed briefly in phosphate-buffered saline (PBS) and permeabilized for 10 min in PBS; 0.1% Triton-X 100. Cells were incubated for 30 min in 0.1% BSA diluted in PBS containing 0.2% Tween (PBS-T), rinsed briefly in PBS-T, and incubated for 1 h in PBS-T; 2.5% BSA; 2.5% normal goat serum (blocking buffer). All incubations were performed at room temperature. Cells were washed with PBS-T then incubated with primary antibodies for 1 hour. Antibodies were diluted 1:200 in blocking buffer. c-Myc-SamCystin was visualized using anti-c-Myc rabbit polyclonal antibody (Novus Biologicals, Littleton, CO); GFP-Bicc1 localization was determined by directly observing GFP fluorescence; acetylated α-tubulin mouse monoclonal antibody (Sigma) was used to visualize cilia, and anti-γ-tubulin mouse monoclonal antibody (Santa Cruz Biotech.) was used to visualize basal bodies. After primary antibody incubation, cells were washed with PBS-T and incubated for 1 h with fluorochrome-conjugated secondary antibodies (Molecular Probes, Eugene, OR) diluted 1:200 in blocking buffer: Alexa Fluor 568 goat anti-mouse for α-tubulin or γ-tubulin (red) and Alexa Fluor 488 goat anti-rabbit for c-Myc-SamCystin labeling (green). Nuclei were stained with DAPI (Roche). Cells were washed again in PBS-T and mounted using MOWIOL (Calbiochem, San Diego, CA). For SamCystin and Bicc1 co-localization, cells were co-transfected with 4 μg each of c-Myc-SamCystin and V5-Bicc1 DNA. V5-Bicc1 was visualized with anti-V5 mouse monoclonal antibody (Invitrogen) and Alexa Fluor 568 (red); c-Myc-SamCystin was visualized described previously.

Co-immunoprecipitation and western blotting. Co-immunoprecipitation was performed using the ProFound c-Myc-Tag Co-IP Kit (Pierce). Briefly, 400 μg of protein were applied to spin columns containing 10 μl of anti-c-Myc antibody-coupled agarose (0.5 μg/μl) and incubated overnight at 4 °C with gentle mixing. Columns were washed three times with Tris-buffered saline containing 0.05% Tween-20, and proteins were eluted by boiling with ImmunoPure Lane Marker Non-Reducing Sample Buffer (Pierce). Twenty micrograms of protein from transfected cell lysates and 12.5 μl of co-immunoprecipitation eluates were separated on 10% polyacrylamide–SDS gels under denaturing conditions. Proteins were transferred electrophoretically to 0.45 μM nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in PBS containing 0.2% Tween-20 and 5% dry milk. Blots were incubated overnight in PBS + 0.2% Tween-20 and 0.5% dry milk (PBST-M) containing primary antibodies diluted as follows: anti-c-Myc mouse monoclonal antibody (Clontech, Mountain View, CA) 1:5000; anti-V5-HRP (Invitrogen) 1:5000. After washing, anti-c-Myc blots were incubated for 1 hour in goat anti-mouse-HRP (Novagen, San Diego, CA) diluted 1:100,000 in PBST-M. The HRP signals were detected using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA).

Results

Localization of SamCystin and Bicc1 in IMCD cells

To analyze the subcellular distribution of SamCystin and Bicc1, IMCD cells were transiently transfected with constructs encoding c-Myc-SamCystin and GFP-Bicc1 recombinant proteins. Schematic diagrams of all constructs used in transfection experiments are shown in Fig. 1. Visualization by immunofluorescent microscopy revealed that c-Myc-SamCystin and GFP-Bicc1 localized to the cytoplasm. Occasional punctate expression of GFP-Bicc1 was observed in the nucleus (data not shown).

To determine whether SamCystin and Bicc1 co-localize in the cytoplasm, IMCD cells were co-transfected with c-Myc-SamCystin and V5-Bicc1 constructs. Double-labeling with anti-c-Myc antibody and anti-V5 antibody showed that SamCystin (Fig. 2E, green) and Bicc1 (Fig. 2F, red) have overlapping expression patterns throughout the cytoplasm (Fig. 2G, yellow-merge).

As many PKD-related proteins localize to primary cilia or basal bodies, [15–18] antibodies to acetylated α-tubulin (cilia marker) and γ-tubulin (basal body marker) were used to determine if either SamCystin or Bicc1 localize in these structures. IMCD cells were grown post-confluence to allow growth of primary cilia prior to transfection. In transfected cells, neither c-Myc-SamCystin nor GFP-Bicc1 localize in primary cilia (Fig. 2A and C) or basal bodies (Fig. 2B and D).

SamCystin and Bicc1 interact

To determine if SamCystin and Bicc1 interact, IMCD cells were transfected with c-Myc-SamCystin, V5-Bicc1, or co-transfected with both constructs and immunoprecipitation was performed. Expression of the recombinant proteins was verified by western blot analysis using anti-c-Myc antibody and anti-V5 antibody (Fig. 3A–C, Input). Immunoprecipitations were performed by incubating protein from transfected cell lysates with immobilized anti-c-Myc antibody and analyzed as described for the input lysates. (Fig. 3A–C, IP). In Fig. 3A, the presence of V5-Bicc1 in the co-immunoprecipitation eluates supported the hypothesis that SamCystin and Bicc1 physically interact.

To determine whether the mutation found in the *Cy* PKD rats disrupts the interaction, protein from cell lysates expressing c-Myc-SamCystin(R823W) and V5-Bicc1 were immunoprecipitated. The c-Myc-SamCystin(R823W) construct encodes the altered form of SamCystin with an arginine-to-tryptophan substitution at residue 823. Bicc1 was co-immunoprecipitated, indicating that this amino acid change does not abolish the interaction (Fig. 3B).

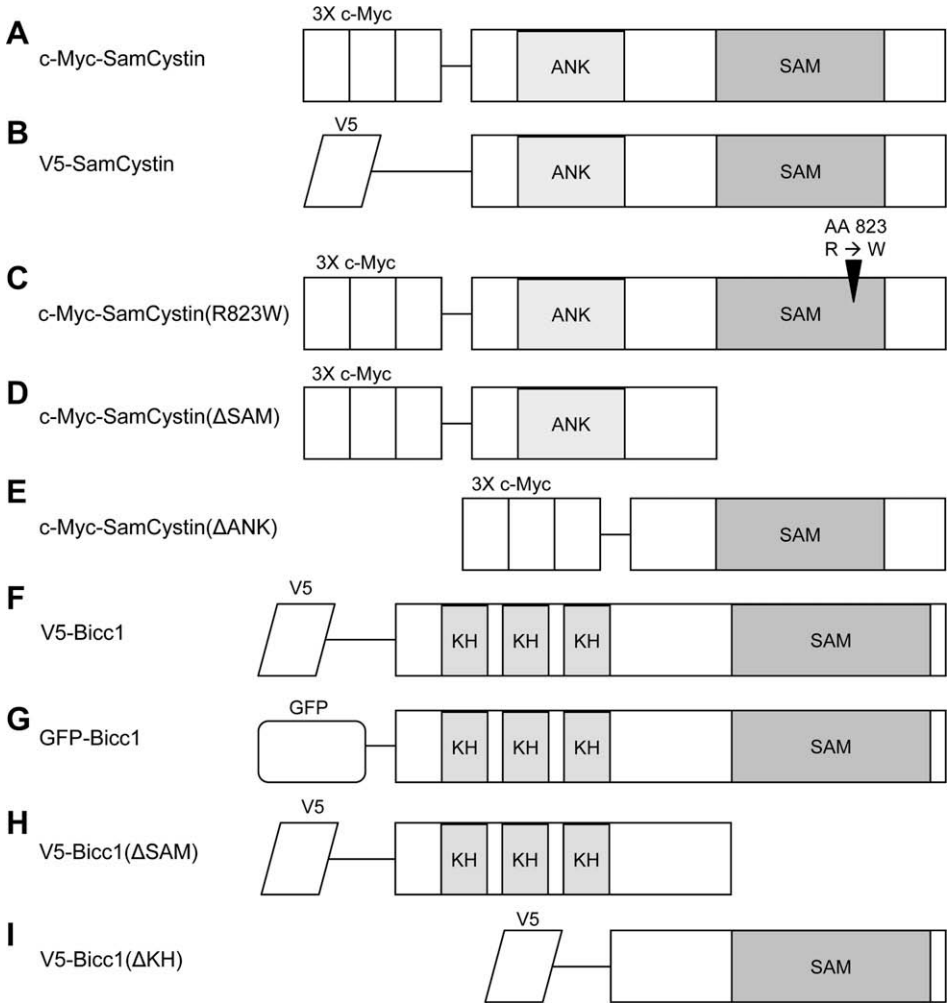

Fig. 1. Schematic of recombinant proteins. All recombinant proteins have a N-terminal epitope tag (3X c-Myc, V5, or GFP) as indicated. (A and B) Full-length SamCystin. (C) Full-length SamCystin protein with a single amino acid substitution at position 823 within the SAM domain (*Anks6*^{Cy} allele) [2]. (D) SamCystin, SAM domain deleted. (E) SamCystin, ankyrin repeat region deleted. (F and G) Full-length Bicc1. (H) Bicc1, SAM domain deleted. (I) Bicc1, KH domains deleted.

To further define which functional domains of each protein are involved in the interaction, a series of deletion constructs were used in co-immunoprecipitation experiments. Initially, the ability of SamCystin recombinant proteins with deletions of either the ankyrin repeats or the SAM domain to interact with Bicc1 was tested. As shown in Fig. 3B, c-Myc-SamCystin(ΔANK) is able to pull-down V5-Bicc1, while c-Myc-SamCystin(ΔSAM) is not. Collectively, these data suggest that SamCystin interacts with Bicc1 via its SAM domain, but elimination of the ankyrin repeats does not interfere with the SamCystin–Bicc1 interaction.

To determine which domain of Bicc1 is involved in the interaction with SamCystin, constructs with deletions of either the KH or SAM domains of Bicc1 were used (Fig. 3C). Interaction was detected between c-Myc-SamCystin and V5-Bicc1(ΔSAM), but not with V5-Bicc1(ΔKH). This indicates that Bicc1 KH domains are important in mediating the interaction with SamCystin.

Since there have been no reports of KH domain involvement in protein–protein interactions yet it is well known that proteins containing KH domains bind and regulate RNA, we suspected that the Bicc1–SamCystin interaction might be indirect and possibly involve a RNA intermediate. Bicc1 and SamCystin co-transfected cell lysates were shown to contain RNA as detected by RT-PCR assays (data not shown). When these lysates were incubated with RNase A, the amount of Bicc1 recovered by co-IP was notably reduced (Fig. 3F), supporting the hypothesis that the presence of RNA is important for the interaction.

SamCystin self-associates

Because other proteins containing SAM domains form either homodimers or homo-oligomers, we tested the hypothesis that SamCystin proteins self-associate. Protein from cell lysates that were co-transfected with c-Myc-SamCystin and V5-SamCystin were immunoprecipitated with anti-c-Myc antibody. As shown in Fig. 3D, SamCystin proteins physically interact.

To characterize the interaction between SamCystin proteins in more detail, immunoprecipitations were carried out using protein from cells expressing V5-SamCystin and either c-Myc-SamCystin(ΔSAM), or c-Myc-SamCystin(ΔANK). Fig. 3E shows that deletion of the ankyrin repeats and deletion of the SAM domains abolishes self-interaction. This indicates that both functional domains of SamCystin are necessary in order for self-association to occur. Likewise, the altered version of SamCystin encoded by the *Cy* allele represented by c-Myc-SamCystin(R823W) was not able to maintain an interaction with V5-SamCystin (Fig. 3E).

Discussion

While mutations in *Bicc1* and *Anks6* have been shown to cause polycystic kidney disease in rodent models, [1,2] the role of these proteins in the mammalian kidney is unknown. In this study, we demonstrate that SamCystin self-associates and interacts with Bicc1.

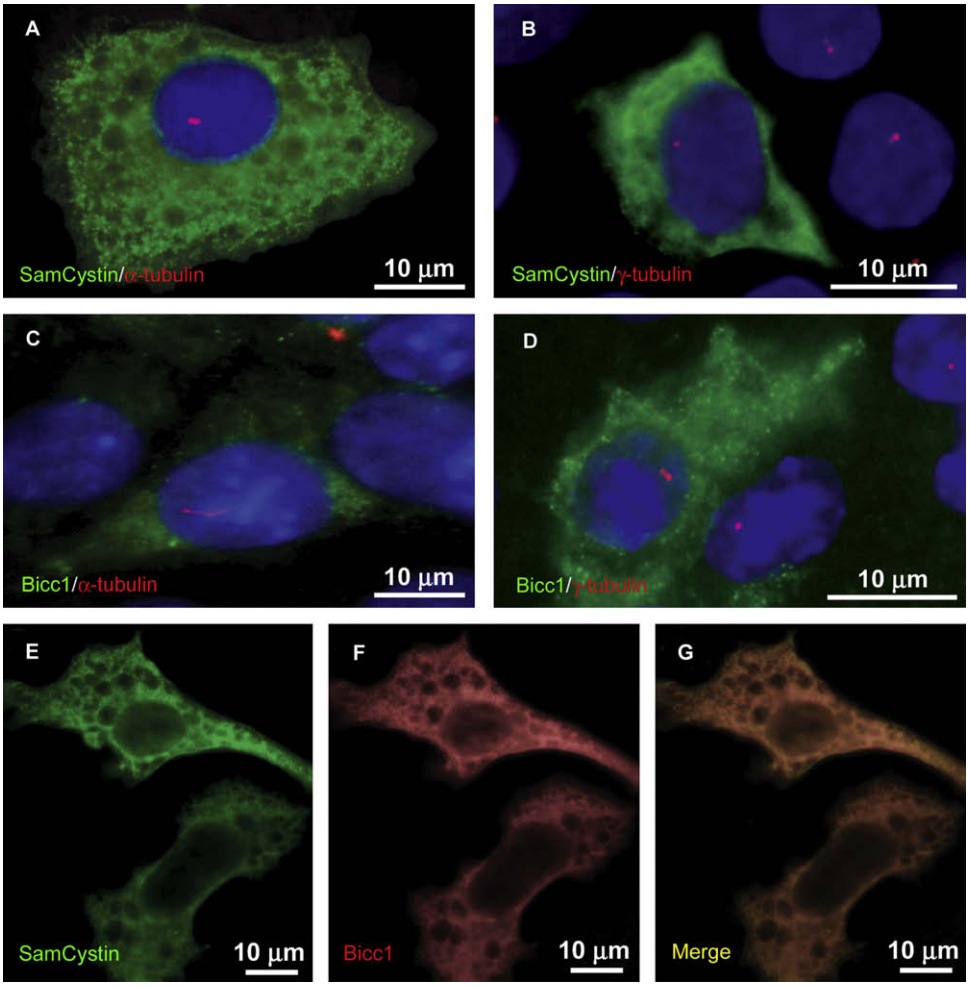

Fig. 2. Localization of SamCystin and Bicc1. Immunostaining of primary cilia with anti-acetylated α-tubulin antibody (A and C, red) or anti-γ-tubulin antibody (B and D, red). (A and B) Localization of c-Myc-SamCystin. Immunostaining with anti-c-Myc antibody (green). (C and D) Localization of GFP-Bicc1 was visualized directly (green). (E–G) Immunostaining of cells co-transfected with c-Myc-SamCystin and V5-Bicc1 with anti-c-Myc antibody (E, green) or anti-V5 antibody (F, red). Merged images (G) reveal co-localization (yellow) of SamCystin and Bicc1 proteins in the cytoplasm. Nuclei were stained with DAPI (blue) in all images.

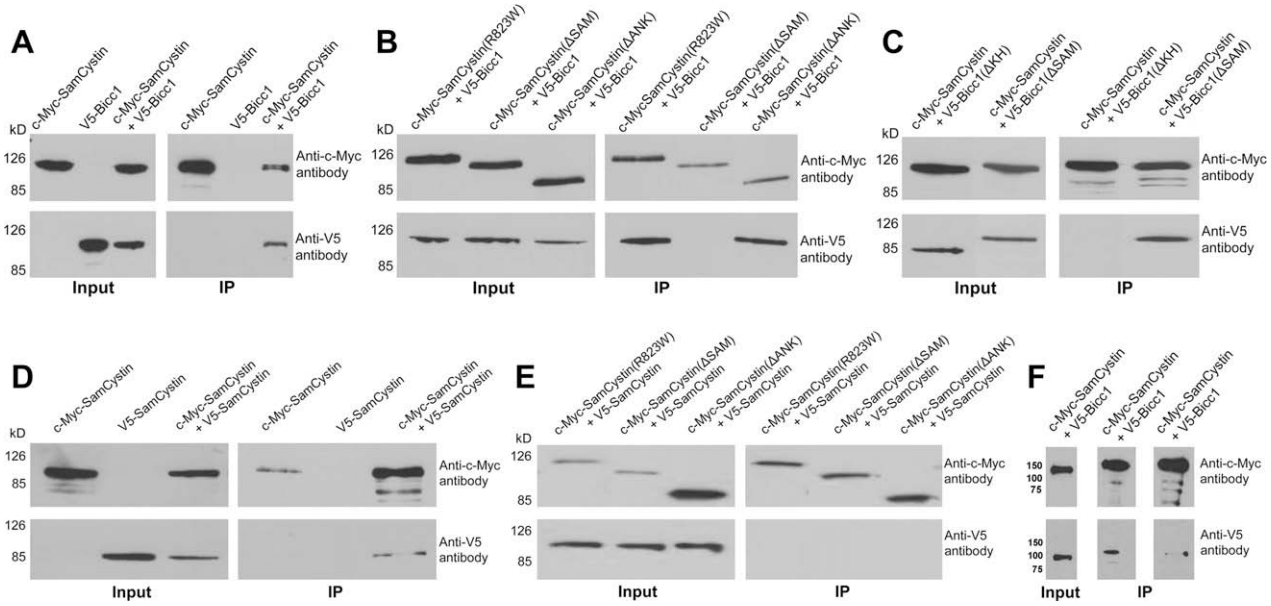

Fig. 3. SamCystin and Bicc1 Co-immunoprecipitation. IMCD cells were transfected with various constructs as indicated. Twenty micrograms of total protein extracted from transfected cell lysates (Input) or 12.5 μl of eluate from immunoprecipitation using immobilized anti-c-Myc antibody (IP) were analyzed by western blot analysis. Proteins were detected using either anti-c-Myc antibody or anti-V5 antibody. (A–C) Co-immunoprecipitation involving SamCystin and Bicc1. (D and E) Self-association between SamCystin proteins. (F) Effect of RNaseA on SamCystin–Bicc1 interaction.

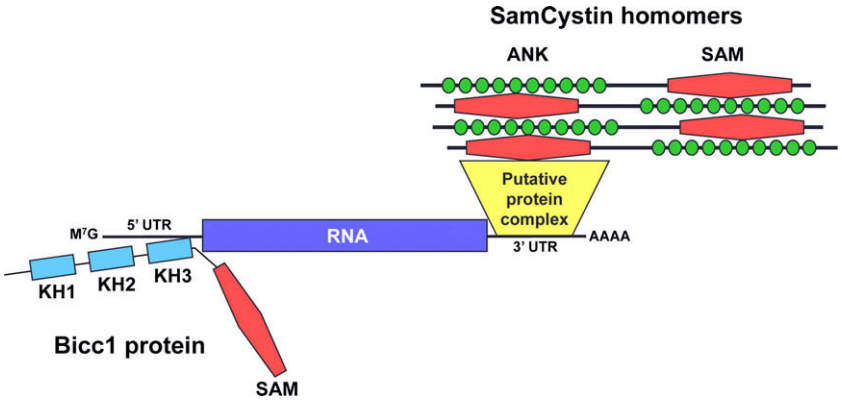

Fig. 4. Predicted model for SamCystin–Bicc1 interaction. In this model, SamCystin would form head-to-tail associations with itself. The Bicc1 KH domains would associate with an as yet unidentified RNA molecule. The SamCystin SAM domain would interact with an unidentified protein or protein complex that also associates with the RNA molecule bound by Bicc1.

Many proteins implicated in PKD are localized to the primary cilium or the basal body of kidney tubular epithelial cells. Defects in these proteins result in abnormal cilia structure or function that leads to cystogenesis [15–18]. In our studies, SamCystin and Bicc1 did not localize to the primary cilia or basal bodies of cultured kidney cells but instead, are primarily expressed throughout the cytoplasm.

Studies of *Drosophila* mutants demonstrate that Bic-C is important for localizing RNA and regulating translation in developing oocytes [19–22] and recent work in our lab has shown that mouse Bicc1 KH domains bind synthetic RNA in vitro [23]. Although specific mRNA targets of the mouse Bicc1 protein have not been identified, we speculate that it acts similar to its orthologues as a regulator of translation. Localization of Bicc1 primarily in the cytoplasm is consistent with this proposed function.

While Samcystin self-associates, the presence of the arginine-to-tryptophan change encoded by the mutant *Anks6*^{Cy} allele is sufficient to disrupt self-association. Using the DGK δ1 SAM domain as a structural template, [24] a molecular model of the SamCystin SAM domain predicts that the SAM domain folds into five distinct α-helices with both the N- and C-termini pointing outward and that the site of mutation in the *Cy* rat (arginine 823) is located on the largest helix on an exposed surface in a region that in DGK δ1 serves as an interaction interface for self-association [25–29] (Supplementary data). Analysis using MUpro, a program designed to predict changes in protein stability due to single residue mutations [30], indicates that this mutation decreases the stability of the protein to a level that could potentially affect protein-binding ability. We speculate that the altered protein encoded by the *Anks6*^{Cy} allele acts in a dominant negative fashion to disrupt SamCystin homodimer formation which leads to molecular changes in renal epithelial cells that promote cyst formation.

Based on our data, we propose a model to describe the SamCystin–Bicc1 interaction such that SamCystin, similar to other proteins such as Shank, tankyrase, and Sans which contain both ANK repeats and a SAM domain [6,31,32], acts as a molecular scaffold, forming high molecular mass complexes via self-association, while also binding multiple interaction partners (Fig. 4). Deletion of either the ANK or SAM domains abolished SamCystin self-association consistent with the idea that SamCystin proteins associate via head-to-tail interactions. SamCystin and Bicc1 interact but this interaction may be indirect, potentially through RNA and protein intermediates. In our model, SamCystin homomers associate in a head-to-tail manner, their SAM domains interacting with another unidentified protein or protein complex that concurrently binds to specific RNAs. Bicc1 would associate with the resulting protein complex by binding the specific RNAs via its KH domains. This model explains why the Bicc1 KH but not SAM domain is important for the interaction. In summary, our working model is that Bicc1 in association with SamCystin, a possible scaffolding protein act together in a complex to localize and regulate the translation of specific mRNAs that are important in the kidney. Mutations that disrupt this interaction lead to translation dysregulation which results in renal cystogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.113.

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