Proteolytic Cleavage and Nuclear Translocation of Fibrocystin Is Regulated by Intracellular Ca\(^{2+}\) and Activation of Protein Kinase C

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Fibrocystin, a type I membrane protein of unknown function, is the protein affected in the autosomal recessive form of polycystic kidney disease. Here we show that fibrocystin undergoes regulated proteolysis. Several proteolytic cleavages occur within the predicted ectodomain, whereas at least one cleavage occurs within the cytoplasmic portion. The latter generates a C-terminal intracellular fragment that harbors the nuclear localization signal KRKVSRLAVGERTAPKIPRIT and translocates to the nucleus. Proteolytic cleavage of fibrocystin occurs constitutively in long-term cultures of polarized inner medullary collecting duct cells (mIMCD-3). Activation of protein kinase C and release of intracellular Ca\(^{2+}\) are required for proteolysis under these conditions. In short-term cultures of human embryonic kidney 293 cells (HEK-293), proteolytic cleavage of fibrocystin can be elicited by stimulation of intracellular Ca\(^{2+}\) release or activation of protein kinase C. These results identify a novel Ca\(^{2+}\)-dependent pathway that signals from fibrocystin located in the cell membrane to the nucleus.

Autosomal recessive polycystic kidney disease (ARPKD)\(^{2}\) is a hereditary cause of kidney failure in infants and children. ARPKD affects 1 in 20,000 individuals and is characterized by aberrant epithelial cell proliferation, which causes cystic dilation of the renal collecting ducts, and abnormal development of intrahepatic bile ducts (1). Affected individuals present with bilateral kidney enlargement, intrauterine kidney failure, and oligohydramnios; the latter causes pulmonary hypoplasia and limb and facial abnormalities. Children who survive the perinatal period or develop ARPKD later in life develop chronic kidney disease and portal hypertension due to congenital hepatic fibrosis.

ARPKD is caused by mutations of the polycystic kidney and hepatic disease gene 1 (PKHD1) located on chromosome 6 (2). The protein encoded by PKHD1 is termed fibrocystin (also called polyductin, or tigmin (3–5)). Fibrocystin is an ~500,000 dalton type I membrane protein comprised of a large N-terminal ectodomain, a single transmembrane segment, and a short C-terminal cytoplasmic domain. The ectodomain contains arrays of IPT (Ig-like, plexins, transcription factors) domains and PbH1 (parallel β-helix repeats) domains. The structure of fibrocystin suggests that it may function as a receptor for an as yet unidentified ligand. However, the authentic function of fibrocystin and the mechanism of signal transduction remain unknown. Recent studies suggest that the molecular pathogenesis of PKD may involve primary cilia and associated Ca\(^{2+}\)-dependent signaling (6). Primary cilia are present on the apical membrane of renal tubular epithelial cells, and bending of the cilia in response to fluid flow shear stress elicits an increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)). Polycystin-1 and polycystin-2, which are affected in the autosomal dominant form of PKD, are located in primary cilia and are required for an initial Ca\(^{2+}\) influx that is triggered by fluid flow shear stress (6). Subsequently, the increase in [Ca\(^{2+}\)]\(_{i}\), is amplified by Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Inhibitors of intracellular Ca\(^{2+}\) release abolish the primary cilia-dependent increase in [Ca\(^{2+}\)]\(_{i}\), which suggests that Ca\(^{2+}\)-induced Ca\(^{2+}\) release is an obligatory step in the primary cilia signaling cascade (6).

Fibrocystin is located in the primary cilium as well as the basal body, which anchors the primary cilium in the cell body (7–10). Together with some similarities in disease manifestations, the overlapping subcellular localization of fibrocystin and polycystins suggests that they may be involved in a common pathway. However, so far, no such pathway has been described. Recent studies suggest that ciliary signaling may involve the regulated proteolysis of polycystin-1. Polycystin-1 undergoes regulated intramembrane proteolysis (RIP), which releases a C-terminal fragment that translocates to the nucleus. Disruption of primary cilia formation causes the accumulation of the fragment in the nucleus, where it affects AP-1 activity. The
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C-terminal fragment of polycystin-1 has also been shown to interact with STAT6 and the coactivator p100 in the nucleus.

Here we show that fibrocystin undergoes regulated proteolytic cleavage releasing a cytoplasmic fragment that translocates to the nucleus. We identify factors that are necessary and sufficient to trigger proteolytic cleavage, and we define a nuclear translocation signal located within the cytoplasmic portion of fibrocystin.

MATERIALS AND METHODS

Reagents and Antibodies—Dantrolene, caffeine, ruthenium red, calphostin, thapsigargin, carbachol, and phorbol 12-myristate 13-acetate (PMA) were from Sigma. Mifepristone and anti-V5 antibody were from Invitrogen. Monoclonal mouse anti-fibrocystin antibody was described previously (10). To generate rabbit anti-fibrocystin serum (IgG8739), rabbits were immunized with a polyacrylamide gel-purified glutathione S-transferase fusion protein containing the cytoplasmic domain of mouse fibrocystin (amino acids 3869–4060).

Plasmids—cDNA encoding full-length (12,225 bp) human fibrocystin was assembled from 12 PCR fragments that were synthesized using human kidney cDNA as template and verified by sequencing. A V5 epitope tag was attached in-frame to the C terminus by removing the stop codon and inserting the DNA fragment into pcDNA3.1-V5/His (Invitrogen). The resultant plasmid was termed pFC-V5. The plasmid pGeneFC was created by inserting the fibrocystin coding region into pT7-7V5 (Clontech). The plasmid pGene/V5 (Invitrogen). Inserting a cDNA encoding EGFP into pGeneFC created plasmid pGeneEGFP. The plasmid pGeneEGFP was generated essentially as described previously (15). Successful fractionation was confirmed by the distribution of the membrane protein polycystin-2 and the nuclear protein proliferating cell nuclear antigen. To concentrate nuclear proteins, nuclear extract was precipitated with trichloroacetic acid.

Immunofluorescence and Microscopy—MDCK cells were grown in 6-well dishes and transfected with plasmids using Effectene. Twenty-four or 48 h later, cells were fixed with acetic/methanol (1:1) and subjected to fluorescence microscopy. Paraffin-embedded kidney sections were deparaffinized and rehydrated. Immunofluorescence was performed using anti-fibrocystin (2B) and Cy3-conjugated anti-mouse IgG (Molecular Probes). Images were obtained by deconvolution microscopy (Zeiss Axioplan-2, Openlab).

RESULTS

Proteolysis of Fibrocystin Produces a C-terminal Nuclear Fragment—A 12,225-bp DNA fragment encoding human fibrocystin was assembled from 12 PCR fragments, cloned into the mammalian expression plasmid pcDNA3.1, and verified by sequencing. To facilitate detection of the recombinant protein, a C-terminal V5 epitope tag was added, and the resultant plasmid was termed pFC-V5. Mouse inner medullary collecting duct cells (mIMCD-3), which endogenously express native fibrocystin (14), were transfected with pFC-V5 or empty
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pcDNA3.1, and cellular proteins were analyzed after 3 days. Immunoblotting utilizing an antibody directed against the C-terminal V5 epitope tag revealed a high molecular weight band corresponding to full-length fibrocystin as well as a series of proteolytic fragments (Fig. 1A).

To further investigate the proteolytic cleavage of fibrocystin, we generated mIMCD3/FC cells in which the expression of recombinant human fibrocystin can be induced by treatment with mifepristone. Immunoblot analysis of cells expressing recombinant fibrocystin revealed the presence of proteolytic fragments similar to those seen in transient transfection experiments (Fig. 1B).

The approximate sites of proteolytic cleavage of fibrocystin were estimated from the molecular weights of the proteolytic fragments. Since the cytoplasmic domain of human fibrocystin including the epitope tag has a calculated molecular mass of 25 kDa, the 21-kDa fragment (Fig. 1B) is likely produced by cleavage in the cytoplasmic domain close to the transmembrane region. Proteolytic fragments of fibrocystin that retain the membrane-spanning segment as well as the C-terminal V5 epitope tag have a minimal molecular mass of 27.5 kDa, suggesting that fragments named FCC to FCF were generated by proteolytic cleavage in the ectodomain of fibrocystin. The site of cleavage producing the fragment named FCB may be within the transmembrane segment.

To determine the subcellular localization of the proteolytic fragments, we prepared membrane, cytosolic, and nuclear extracts and analyzed the proteins by immunoblotting. Fragments that were calculated to contain the transmembrane domain (FCC to FCF) were found in the membrane fraction, as expected (Fig. 1B). In contrast, the 21-kDa fragment containing the C terminus of fibrocystin was detected in the nuclear fraction. This result suggests that the cytoplasmic domain of fibrocystin undergoes proteolytic cleavage, releasing a 21-kDa fragment that translocates to the nucleus. In some experiments, the C-terminal domain of fibrocystin (not shown). Minor, smaller peptides were also detected, raising the possibility that endogenous FCA is subjected to further endoproteolytic processing (Fig. 1C).

Cytoplasmic Domain of Fibrocystin Contains a Nuclear Localization Signal—To determine the localization of the cytoplasmic domain of fibrocystin in vivo, kidney sections of 21-day old mice were stained with an antibody specific for the cytoplasmic region of the protein. Antibody staining confirmed that the cytoplasmic domain of fibrocystin was located in the nuclei of renal tubular epithelial cells (Fig. 2A). The cytoplasmic domain was also found in the cytosol as well as the primary cilia when the cells were observed in a different focal plane (data not shown). Interestingly, the fibrocystin staining in the nucleus exhibited a speckled pattern.

To define the mechanism of nuclear localization, the cytoplasmic domain of fibrocystin was linked to EGFP or DsRed and expressed in MDCK cells. The subcellular localization of the fusion proteins was determined by fluorescence microscopy. A recombinant protein containing the cytoplasmic domain of fibrocystin (amino acids 3876–4059) and EGFP was located exclusively in the nucleus, whereas EGFP by itself was predominantly in the cytosol (Fig. 2B). Similarly, a fusion protein containing the cytoplasmic domain of fibrocystin and DsRed was located in the nucleus. Like endogenous fibrocystin, the fusion proteins were not diffusely distributed in the nucleus but were concentrated in structures that had a speckled subnuclear distribution. To identify the nuclear structures, cells were transfected with plasmids encoding the cytoplasmic domain fused to DsRed, and the nucleoli were counterstained with an RNA-specific stain. Co-staining demonstrated that the fusion proteins containing the cytoplasmic domain were located in nucleoli (Fig. 2C).

To identify the region within the cytoplasmic domain of fibrocystin that is required for nuclear localization, plasmids...
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encoding EGFP fused to the N-terminal portion (amino acids 3876–3970), the central portion (amino acids 3973–4031), and the C-terminal portion (amino acids 4043–4059) of the cytoplasmic domain were transfected into MDCK cells. Only an EGFP fusion protein containing the N-terminal portion was located in the nucleus, whereas fusion proteins containing the central or C-terminal portions remained in the cytosol (Fig. 3A). To verify these findings using a different cell type and method, plasmids encoding the V5 epitope-tagged N-terminal portion and central portion of the cytoplasmic domain were transfected into HEK-293 cells. Immunoblot analysis of nuclear and cytosolic fractions revealed that the N-terminal portion but not the central portion was present in the nuclear fraction (Fig. 3B). To further define the sequence mediating nuclear localization, plasmids encoding various regions of the cytoplasmic domain of fibrocystin fused to EGFP were generated, and the subcellular localizations of the fusion proteins were evaluated. These experiments defined the minimal nuclear localization signal (NLS) to be KRKVSRLAVTGERTAT.

Further deletions within this sequence abolished nuclear localization.

Intracellular Ca\textsuperscript{2+} Release Is Necessary and Sufficient to Trigger Fibrocystin Proteolysis—To determine whether the proteolytic cleavage of fibrocystin is affected by changes in intracellular Ca\textsuperscript{2+} concentration and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, mIMCD3/FC cells were pretreated with thapsigargin prior to induction of fibrocystin expression. Pretreatment with 0.1 \( \mu \text{M} \) thapsigargin to deplete intracellular Ca\textsuperscript{2+} stores (16) abolished the generation of the FCA fragment (Fig. 4A). Similarly, depletion of ryanodine-sensitive Ca\textsuperscript{2+} stores by pretreatment with 5 \( \mu \text{M} \) caffeine also prevented the generation of FCA (Fig. 4A). Pretreatment with 200 \( \mu \text{M} \) ruthenium red, a nonspecific Ca\textsuperscript{2+} channel inhibitor, or treatment with 75 \( \mu \text{M} \) dantrolene, which interferes with ryanodine receptor (RyR)-mediated intracellular Ca\textsuperscript{2+} release, abolished the formation of FCA (Fig. 4A).

To quantitatively measure fibrocystin cleavage, we developed a luciferase assay similar to the ones employed to quantify the proteolytic cleavage of amyloid precursor protein (APP) and low density lipoprotein receptor-related protein-1 (LRP1) (17, 18). A plasmid (FC-GV) encoding the synthetic transcription factor Gal4-VP16 fused to the C terminus of fibrocystin was generated. Proteolytic cleavage of the fibrocystin fusion protein releases Gal4-VP16, which translocates to the nucleus and activates a Gal4-responsive luciferase reporter gene (pG5-luc). Therefore, luciferase expression correlates with fibrocystin cleavage. Transfection of mIMCD3 cells with a plasmid encoding FC-GV stimulated luciferase activity 2.6-fold when compared with cells expressing LDLR-GV (low density lipoprotein receptor-related protein-1) (LRP1) (17, 18). A plasmid encoding the synthetic transcription factor Gal4-VP16

<FIGURE 2. The fibrocystin cytoplasmic domain is a nuclear protein. A, section of P21 mouse kidney stained with anti-fibrocystin antibody (red). Nuclei were counterstained with DAPI (blue). Arrows indicate nuclear staining. tu, tubules. Bars, 10 \( \mu \text{m} \). B, MDCK cells were transfected with plasmids encoding EGFP (green), EGFP fused to the cytoplasmic domain of fibrocystin (FCA-EGFP, green) or DsRed fused to the cytoplasmic domain of fibrocystin (FCA-DsRed, red). Nuclei were counterstained with DAPI (blue). C, MDCK cells were transfected with pFCA-DsRed (red), and nucleolar RNA was stained with RNASelect (green). Nuclei were counterstained with DAPI (blue).>
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Activation of PKC Is Necessary and Sufficient for the Generation of FCA—The inhibition of fibrocystin proteolysis in the presence of the PKC inhibitor calphostin C suggested an involvement of members of the group of conventional PKC (PKCa, PKCB1, PKCB2, or PKCγ). To test whether activation of PKC is sufficient to induce fibrocystin cleavage, HEK-293 cells were transfected with pFC-V5 and exposed to activators of protein kinases. Treatment with PMA, an activator of PKC, produced a marked increase of proteolytic cleavage, which was prevented by pretreatment with calphostin C (Fig. 5A). In contrast, no cleavage was observed when cells were left untreated or treated with the protein kinase A activator 8-bromo-cAMP. However, besides inducing proteolysis, PMA also increased the total expression of fibrocystin, controlled by the cytomegalovirus promoter, which made it difficult to unequivocally correlate PKC activation with fibrocystin cleavage.

To address this issue, we used two additional approaches: (i) replacement of the PKC-responsive cytomegalovirus promoter and (ii) genetic activation of PKC. mIMCD3/FC cells, in which fibrocystin is expressed under the control of a mifepristone-responsive promoter, were treated with calphostin C 24 h after induction, and cleavage was analyzed 30 h later. Treatment with calphostin C did not alter expression levels of fibrocystin but produced a dose-dependent inhibition of fibrocystin proteolysis (Fig. 5B). Next, we employed a constitutively active pseudosubstrate mutant of PKCa (PKCaA/E) (21). Cotransfection of a plasmid encoding PKCaA/E resulted in fibrocystin proteolysis and generation of FCA, whereas cotransfection of empty expression plasmid did not affect processing (Fig. 5C). The presence or absence of fetal calf serum did not markedly affect cleavage.

To quantify the cleavage of fibrocystin stimulated by PKC activation, luciferase reporter assays were performed. HEK-293 cells were cotransfected with pFC-GV and pG5-luc, and PKC activity was stimulated by treatment with PMA or expression of the PKCaA/E mutant. Fibrocystin proteolysis was evaluated by measuring luciferase activity. Treatment with PMA increased luciferase activity 27-fold, whereas the addition of 8-bromo-cAMP did not significantly affect luciferase activity. Co-expression of PKCaA/E stimulated luciferase activity 19-fold but had no effect on cells expressing the negative control LDLR-GV (Fig. 5D). Taken together, these results demonstrate that fibrocystin-specific proteolysis is PKC-dependent.

DISCUSSION

The data presented here indicate that fibrocystin is subjected to site-specific proteolytic cleavage. At least six different fragments that contain the C terminus of fibrocystin are generated. Although the abundance of the larger proteolytic fragments varied under different experimental conditions, a 21-kDa fragment (FCA) was consistently observed. The 21-kDa fragment contains most of the cytoplasmic domain of fibrocystin and was primarily observed in the nucleus. In contrast, full-length fibrocystin has been localized primarily in the primary cilium, basal body region, and apical plasma membrane, and to a lesser degree, in the cytoplasm. These findings suggest that fibrocystin undergoes regulated proteolysis releasing a cytoplasmic C-terminal fragment that translocates from the cilium, apical...
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FIGURE 4. Intracellular Ca^{2+} release modulates fibrocystin cleavage. A, mIMCD3/FC cells were grown for 4 days and incubated with 0.1 mM thapsigargin (lane 2), 5 mM caffeine (lane 3), 75 μM dantrolene (lane 5), 200 μM ruthenium red (lane 7), or no additions (lanes 1, 4, and 6). After 2 h, media were replaced with media lacking serum but containing the same supplements, and in addition, 10 nM mifepristone. Cell lysates were prepared after 24 h and analyzed by immunoblotting using anti-V5 antibody. B, mIMCD-3 cells were transfected with 0.2 μg of pGS-luc and either 0.2 μg of pFC-GV or 0.2 μg of pLDLR-GV. After 24 h, cells were incubated in serum-free media in the presence or absence of 75 μM dantrolene. Relative luciferase activity was determined after 48 h. Data presented are mean ± S.E. of three separate transfections. The fibrocystin/Gal4-VP16 fusion protein is shown schematically below. The shaded box indicates the transmembrane segment; filled boxes indicate the N-terminal signal sequence and the C-terminal Gal4-VP16 fusion protein. N, N terminus. C, HEK-293 cells were transfected with 6 μg of pFC-VS, and 8 h later, incubated in serum-free media containing 1–8 mM caffeine, 0.5 μM calphostin, or no additions. Cytosines were lysed after 16 h, and extracts were analyzed by immunoblotting using anti-V5 antibody. D, HEK-293 cells stably expressing the m3 muscarinic receptor (lanes 1–4) and untransfected HEK-293 cells (lanes 5–8) were transfected with 6 μg of pFC-VS. Eight h later, the cells were incubated in serum-free media containing 5 mM caffeine (lanes 2, 4, 6, and 8), 100 μM carbamol (lanes 3, 4, 7, and 8), or no additions. Cytosines were lysed after 16 h, and extracts were analyzed by immunoblotting using anti-V5 antibody.

membrane, or cytoplasm to the nucleus. The protease that mediates fibrocystin cleavage remains unidentified, but inhibitor studies suggest that it is not γ-secretase or calpain (data not shown). Similar to other proteases that are subject to regulated proteolysis, only a small fraction of fibrocystin appears to be cleaved. In particular, the pattern of proteolytic cleavage (close proximity to the transmembrane domain, nuclear translocation of the resulting cytoplasmatic fragment) resembles RIP of other type I membrane proteins. Nuclear signaling of cytoplasmatic fragments generated by RIP is most extensively described for Notch but also found in the case of APP, ErbB4, NRG-1, CD44, N-cadherin, and lipoprotein receptor-related protein (22). RIP controls vital signaling mechanisms that are crucial for regulatory processes and disease pathogenesis. Often, the cleavage releasing the nuclear fragment is preceded by an obligatory proteolytic event in the ectodomain of the protein. It is conceivable that the proteolytic cleavage occurring in the ectodomain of fibrocystin is also required for a subsequent step that releases the cytoplasmatic fragment.

Nucleoli are the site of rDNA transcription, assembly of ribosomes, and biogenesis of small ribonucleoprotein particles and influence cell cycle progression and transcription by sequestering specific trans-acting factors (24). The nucleolar localization of FCA suggests that fibrocystin may play an important role in regulating these processes. Several lines of evidence indicate that the release of intracellular Ca^{2+} is a prerequisite for fibrocystin proteolysis. Constitutive cleavage of fibrocystin observed in a long term cell culture system could be prevented by pharmacological inhibition of Ca^{2+}-induced Ca^{2+} release. No cleavage of fibrocystin was observed under basal conditions in a short term cell culture system, but cleavage and nuclear translocation could be induced by pharmacological or receptor-mediated stimulation of intracellular Ca^{2+} release. These findings suggest that signaling cascades that affect [Ca^{2+}], might act as central regulators or mediators of fibrocystin cleavage.

One Ca^{2+}-dependent signaling cascade that is considered to be central in the development of polycystic kidney disease
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involves primary cilia. Primary cilia located on the apical surface of renal tubular epithelial cells are involved in sensing luminal fluid flow. Shear stress, caused by the flow of fluid across the epithelial cell layer, bends the cilium and triggers an increase in [Ca^{2+}]. Proteolytic cleavage of polycystin-1 appears to involve primary cilia. The loss of primary cilia or obstruction of fluid flow results in the accumulation of the polycystin-1 C-terminal cytoplasmic fragment in the nucleus. It is possible that primary cilia are also involved in the regulation of fibrocystin proteolysis. Fibrocystin proteolysis was observed in long term cultures of mIMCD-3 cells, which form primary cilia, and no cleavage was observed in short term HEK-293 cell cultures, which lack primary cilia. However, no effect of fluid flow on fibrocystin cleavage has been detected (data not shown). Therefore, further experimentation will be required to clarify the role of primary cilia and the fluid-dependent increase in [Ca^{2+}], on fibrocystin cleavage.

Cleavage of fibrocystin is stimulated by activation of PKC. Studies using PKC activators, the PKC-specific inhibitor calphostin C, and a constitutively active PKC mutant indicate that activation of PKC is necessary and sufficient to cause fibrocystin proteolysis. Activation of PKC can circumvent the requirement for release of intracellular Ca^{2+}, indicating that PKC activation is likely to be downstream to Ca^{2+} release. Conventional PKCs are activated by intracellular Ca^{2+} release and represent potential downstream effectors of Ca^{2+}-induced Ca^{2+} release. Although it is not clear how activation of PKC triggers fibrocystin cleavage, one possibility is that PKC regulates the trafficking of intracellular vesicles, allowing fibrocystin to interact with the protease. Interestingly, PKC activation is also involved in the processing of APP. Modulators of PKC activity have been shown to alter APP processing and reduce the levels of β-amyloid in animal models (25). Similarly, alteration of fibrocystin proteolysis achieved by pharmacological modulation of PKC activation or intracellular Ca^{2+} release might represent a possible therapeutic strategy to treat PKD.

In summary, we propose a model in which cytosolic Ca^{2+} concentration and activation of PKC control a proteolytic process that leads to the release of the cytoplasmic C-terminal fragment of fibrocystin. Because of the presence of a NLS, the C-terminal fragment (FCA) is translocated to the nucleus. It is possible that primary cilia in a signaling cascade is suggested by mechanistic similarities with other proteins subjected to RIP.

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