Siah-1 Interacts with the Intracellular Region of Polycystin-1 and Affects Its Stability via the Ubiquitin-Proteasome Pathway

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Abstract. Autosomal dominant polycystic kidney disease, characterized by extensive formation of renal cysts and progressive renal failure, is a genetic disorder caused by mutations in the PKD1 and PKD2 genes. The PKD1 gene product, polycystin-1, is a transmembrane protein with its N-terminus facing the extracellular region and C-terminus facing the cytoplasm. Polycystin-1 seems to be involved in regulating cell growth and maturation, but the precise mechanisms are not yet well defined. For investigating the function of the intracellular region of polycystin-1, the C-terminal cytoplasmic fragment of polycystin-1, PKD1-C, was used as bait in two-hybrid screening, and a polycystin-1-binding protein, the human homologue of Drosophila Seven in Absentia (Siah-1), which has a RING domain and promotes the ubiquitin-dependent proteasome pathway, was identified. It was shown that PKD1-C interacts with Siah-1 in vivo. In addition, interaction with Siah-1 induces the degradation of PKD1-C, shortening its half-life. PKD1-C and CD4 chimeric proteins, which are attached to the plasma membrane, also show similar results. Furthermore, ubiquitination and degradation of PKD1-C are increased in the presence of Siah-1, and overexpression of Siah-1 protein promotes the degradation of polycystin-1 via the ubiquitin-proteasome pathway. These results suggest that polycystin-1 is regulated by Siah-1 through the ubiquitin-dependent proteasome pathway.

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited human diseases and is estimated to occur in the population with a frequency of 1 in 1000. The presence of renal cysts is the major clinical feature of the disease, and cysts seem to increase in size and number throughout the lifetime of an affected individual. Although the renal lesion is the most prominent feature, ADPKD is a systemic disorder with a variety of other manifestations, including liver cysts, cerebral aneurysms, and cardiac valvular abnormalities (1–3). Most cases of ADPKD are caused by mutations of PKD1, the gene that encodes polycystin-1.

Polycystin-1 is a large integral membrane protein with several domains that suggest a role in cell–cell or cell–matrix interactions (4,5). It contains several transmembrane domains and a short intracellular C-terminal tail, suggesting that polycystin-1 might act as a receptor and that its C-terminal region might activate the signal pathway (4). Actually, several proteins have been reported to interact with the C-terminal cytoplasmic region of polycystin-1 (6–10). Recently, the C-terminal polycystin-1 region was found to activate protein kinase Cα (PKCα) and c-Jun N-terminal protein kinase (11) and trigger the accumulation of β-catenin (12), indicating that this domain might play a crucial role in the signaling of polycystin-1.

Drosophila Seven in Absentia (Sina) protein is a RING finger–containing protein that is critically involved in the neuronal development of the R7 photoreceptor cell in Drosophila (13–15). Sina functions downstream of the tyrosine kinase receptor Sevenless and Ras/Raf mitogen-activated protein kinase pathway (16–18). Recent evidence indicates that Sina promotes the ubiquitin-proteasome pathway–dependent degradation of tramtrack, a negative regulator of neuronal differentiation (19,20). In mammals, highly conserved Sina homologues (Siah-1 and Siah-2) are abundantly expressed in the brain as well as other tissues (21,22). Siah proteins reportedly interact with DCC (deleted in colorectal cancer) and regulate the degradation of DCC via the ubiquitin-proteasome pathway (23). These studies suggest that Siah proteins might function by mediating the ubiquitin-dependent degradation of target proteins (24).

In this study, we used yeast two-hybrid screening to identify proteins that regulate the function of polycystin-1 through their interactions with the C-terminal cytoplasmic region of polycystin-1 and isolated a polycystin-1–binding protein, Siah-1. We demonstrate that Siah-1 regulates the ubiquitination and degradation of polycystin-1 C-terminal fragments by the proteasome pathway. Thus, Siah-1 might play an important role in the turnover of polycystin-1 as well as in the function of polycystin-1.
Materials and Methods

Yeast Two-Hybrid Screen

The bait plasmid, PKD1-C, was constructed by subcloning the cytoplasmic C-terminal tail of human polycystin-1, the PKD1 gene product, into the pLexA vector. pLexA-PKD1-C was transformed into the yeast strain EGY48, a host strain that had been transformed with the reporter plasmid p8op-lacZ. For library selection, a single colony of EGY48 cells transformed with pLexA-PKD1-C was grown at 30°C overnight in minimal medium lacking uracil and histidine and was then transformed with a human fetal brain cDNA library constructed into pB42AD (Matchmaker; Clontech). A total of 2.5 × 10^6 independent transformants were plated on minimal medium lacking uracil, histidine, tryptophan, and leucine but containing 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal). After incubation at 30°C for 4 to 6 d, positive colonies were picked (25). The inserts of selected positive clones were sequenced and aligned in the NCBI BLAST database.

Plasmids

The full-length human Siah-1 was subcloned into a pcDNA3.1/His to obtain an Xpress fusion protein. The C-terminal cytoplasmic region of polycystin-1 and deletion series were constructed. PKD1-CT were generated by digestion of pcDNA 3.0/HA with EcoRI and XhoI, and PKD1-CD4CT were generated by digestion of pUHD10.1 containing the full-length human CD4 with AgeI and BamHI (26). pEGFP-N3—expressing green fluorescent protein (GFP) was used as the transfection control. The human ubiquitin was subcloned into a pcDNA3.1/His to obtain an Xpress fusion protein.

Protein Half-Life Experiments

293T cells were co-transfected with pcDNA3.0/HA-PKD1-CT1 or pUHD10.1-PKD1-CD4CT1 and either the pcDNA3.1/His-Siah-1 or pcDNA3.1/His vector by using LipofectAMINE reagent (Invitrogen). After incubation for 36 h at 37°C, cells were washed with PBS and then incubated with DMEM containing 40 μg/ml cycloheximide for the indicated durations (12). Cells were lysed after an appropriate chase time. The level of protein was analyzed by anti-HA antibody (sc-805; Santa Cruz Biotechnology) and quantified with TINA2.0 (27).

[^35S]Metionine Pulse-Chase Experiments

293T cells were co-transfected with pcDNA3.0/HA-PKD1-CT1 or pUHD10.1-PKD1-CD4CT1 and either the pcDNA3.1/His-Siah-1 or pcDNA3.1/His vector. Forty-eight hours later, the cells were washed and incubated for 2 h with Met-free DMEM containing 100 μCi of [^35S]Met. After incubation for 2 h, the radioactive medium was removed by extensive washes with nonradioactive DMEM. Cells were then incubated for chase intervals of 0, 2, 4, 6, 8, and 10 h in nonradioactive DMEM supplemented with 8% FBS. Cells were lysed after appropriate chase time. An equal amount of protein from each lysate was immunoprecipitated using anti-HA antibody (sc-805; Santa Cruz Biotechnology). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. The level of protein was quantified with TINA2.0.

Proteolysis Inhibitor Treatment of Cells

293T cells expressing Siah-1 and either PKD1-CT1 or PKD1-CD4CT1 were incubated for 8 h at 37°C with the proteasome inhibitors MG132 (10 μM; Calbiochem) and ALLN (20 μM; Calbiochem), the cysteine protease inhibitor ALLM (20 μM; Calbiochem), or the minus-inhibitor control DMSO. Cells were then lysed and analyzed by SDS-PAGE and immunoblotting.

Western Blot Analysis

Proteins that were purified from transfected cells were separated by 12% SDS-PAGE and transferred to nitrocellulose filters (28). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and 0.5% Tween20 (TBST) for 1 h and then incubated for 3 h at room temperature with primary antibodies in TBST. After three 10-min washes in TBST, membranes were incubated for 1 h at room temperature with the peroxidase-labeled secondary antibody in TBST. After three additional 10-min washes in TBST, the membrane-bound antibodies were visualized by the enhanced chemiluminescence detection system (Amersham Bioscience).

Results

Identification of Siah-1 as a PKD1-Binding Protein

Polycystin-1, the PKD1 gene product, is an integral membrane protein with an N-terminal extracellular region and a C-terminal cytoplasmic region that contains a coiled-coil motif. To identify cellular proteins that interact with the cytoplasmic region of polycystin-1, we used PKD1-C, the C-terminal 153 amino acids of polycystin-1, as bait in yeast two-hybrid screens of cDNA libraries. Of positive clones in yeast transformants, one clone encoded human Siah-1. The Siah-1 protein contains an N-terminal RING finger motif (C(H)C) followed by a conserved cysteine-rich region, which might represent a novel class of Zn2+-binding motifs (Figure 1A). The interaction was further confirmed by the growth of the transformed colonies in medium lacking uracil, histidine, tryptophan, and leucine and development of blue color as seen for the positive control, whereas the negative controls, transformed with pLexA-PKD1-C alone (clone 1) or empty vectors without inserts (clone 3), did not grow in minimal medium or develop color (Figure 1B).

In Vivo Interaction of Siah-1 with the Intracellular Region of Polycystin-1

To determine whether polycystin-1 associates with the Siah-1 protein in vivo, we used lysates of 293T cells that were co-transfected with Xpress-tagged Siah-1 and either truncated PKD1-CT or PKD1-CD4CT in co-immunoprecipitation experiments; PKD1-CT are proteins tagged with HA and PKD1-CD4CT are chimeric proteins that contain the human CD4, a plasma membrane-bound protein (Figure 2A). PKD1-CT1 and PKD1-CD4CT1 were co-immunoprecipitated with Xpress-tagged Siah-1, but PKD1-CT3 and PKD1-CD4CT3, a truncated form lacking the coiled-coil domain, did not co-immunoprecipitate (Figure 2B). These results confirm the specific association of polycystin-1 C-terminal fragments with the Siah-1 protein in mammalian cells and suggest that the coiled-coil domain might have a role in the interaction.

Siah-1 Protein Regulates PKD1-C Expression

To determine whether the Siah-1 protein regulates polycystin-1 expression by binding its cytoplasmic region, we assessed the stability of PKD1-C in the presence or absence of Siah-1 by
Western blot analysis. 293T cells were co-transfected with mammalian expression vectors that contained PKD1-CT1 or PKD1-CD4CT1 and either the pcDNA3.1/His control vector or pcDNA3.1/His-Siah-1. In 293T cells, in comparison with the control transfection, co-transfection of PKD1-C and Siah-1 revealed markedly reduced expression levels of both PKD1-CT1 and PKD1-CD4CT1 (Figure 3). To determine further whether the Siah-1 protein regulates the degradation of polycystin-1, we examined the effect of Siah-1 on the half-life of PKD1-C. 293T cells were co-transfected with PKD1-CT1 or PKD1-CD4CT1 and the pcDNA3.1/His control vector or pcDNA3.1/His-Siah-1 and then treated with the protein synthesis inhibitor cycloheximide (Figure 4A). In cells that were co-transfected with PKD1-C and the control vector, the half-life of PKD1-C protein was ~10 h. In contrast, when PKD1-C was co-expressed with Siah-1, the half-life of the PKD1-C protein was reduced to ~4 h (Figure 4A). It is interesting that the cytoplasmic PKD1-C (PKD1-CT1) showed similar stability as the plasma membrane-bound PKD1-C (PKD1-CD4CT1). [35S]Methionine pulse-chase experiments also confirmed that the half-life of PKD1-C is affected by the presence of Siah-1, as shown in Figure 4B. Taken together, these results indicate that the Siah-1 protein has the ability to regulate polycystin-1 turnover through interaction with the polycystin-1 cytoplasmic region.

**Siah-1 Regulates PKD1-C Degradation via the Ubiquitin-Proteasome Pathway**

The ubiquitin-proteasome pathway regulates the degradation of many proteins, and we sought to determine whether the enhanced degradation of the PKD1 C-terminal fragment by Siah-1 is mediated through this pathway. 293T cells that had...
been co-transfected with PKD1-CT1 or PKD1-CD4CT1 and either control vector or Siah-1 were treated for 8 h with various inhibitors of the proteolytic pathway, and the level of PKD1-C expression was then analyzed by Western blotting. As shown in Figure 5A, the enhanced degradation of PKD1-CT1 and PKD1-CD4CT1 by Siah-1 was blocked by MG132, a potent inhibitor of proteasome function. A similar effect was also observed when cells were treated with ALLN (Calpain inhibitor I), an inhibitor of protease and proteasome function. In contrast, ALLM (Calpain inhibitor II), a calcium-dependent cysteine protease inhibitor and not an inhibitor of proteasome function, had no effect.

Because proteasome-dependent proteolysis involves the ubiquitination of target proteins, we investigated whether Siah-1 accelerates the degradation of polycystin-1 by promoting the ubiquitination of polycystin-1. HA-tagged PKD1-CT1 was co-expressed in 293T cells along with Xpress-tagged ubiquitin in the absence or presence of exogenous Siah-1. Cell lysates were immunoprecipitated with the anti-HA antibody and immunoblotted with the anti-Xpress antibody to detect ubiquitin-conjugated PKD1-CT1 (Figure 5B). In the presence of Siah-1, the immunoprecipitate contained high-molecular-weight smears detected by the anti-Xpress antibody, indicating that PKD1-CT1 was ubiquitinated. This result was similarly shown in PKD1-CD4CT1 experiments (data not shown). Taken together, these results indicate that Siah-1 targets polycystin-1 for ubiquitin-dependent degradation via the proteasome.

**Siah-1 Regulates the Degradation of Endogenous Polycystin-1 via the Ubiquitin-Proteasome Pathway**

To address the question of whether Siah-1 protein could regulate the degradation of endogenous polycystin-1, the PKD1 gene product, we examined the effect of overexpression of Siah-1 on the protein level of endogenous polycystin-1. Cell lysates were prepared from 293 cells that were transfected with pcDNA3.1/His-Siah-1 or pcDNA3.1/His control vector, and the levels of exogenous Siah-1 and endogenous polycystin-1 in these lysates were determined by Western blot analysis (Figure 6). Comparison with the control transfection reveals that the expression of exogenous Siah-1 significantly downregulated the protein level of endogenous polycystin-1. For determining further whether the downregulation effect of Siah-1 overexpression is mediated via the ubiquitin-proteasome degradation pathway, cells that expressed Siah-1 were treated with various inhibitors of protein degradation. Comparison of the polycystin-1 levels in treated versus untreated cells showed that the downregulation effect of Siah-1 overexpression was blocked....
by the proteasome inhibitor MG132 but not by the cysteine protease inhibitor ALLM (Figure 6). These results suggest that Siah-1 protein regulates the degradation of endogenous polycystin-1 via the ubiquitin-proteasome pathway. As previously reported for Siah-1 (15,23), the expression levels of Siah-1 were increased upon inhibition of the proteasome activity by MG132, suggesting that the stability of Siah-1 itself is controlled by the proteasome pathway.

**Discussion**

The functional characterization of polycystin-1 and the genetic manipulation of full-length polycystin-1 have been hin-
Polycystin-1 contains several transmembrane domains and a short intracellular C-terminal tail that might activate a signal pathway (4). To understand the function of PKD1, we used the C-terminal cytoplasmic fragment of polycystin-1, PKD1-C, as bait in yeast two-hybrid screens and identified one polycystin-1-binding protein, Siah-1, which is the human homologue of the Drosophila Seven in Absentia (Sina) gene. Furthermore, we have demonstrated that the Siah-1 protein interacts with the C-terminal cytoplasmic region of DCC, a transmembrane protein such as polycystin-1, and regulates the degradation of the C-terminal cytoplasmic region of DCC and the full-length DCC (23). We think that the C-terminal region of polycystin-1 likely plays a central role in polycystin-1 functions and that polycystin-1 is regulated by Siah-1 via the ubiquitin-proteasome pathway in a similar manner to DCC. As we expected, our results suggest that in addition to these proteins, polycystin-1, a membrane protein, is another substrate of Siah-1 ubiquitin ligase.

Overexpression of polycystin-1 was reported to induce cell cycle arrest by upregulating p21, indicating that polycystin-1 might be involved in the cell cycle (31,32). It is well known that cell cycle proteins, including cyclins and p53, are regulated by the ubiquitin-proteasome pathway (33,34). If polycystin-1 is involved in the cell cycle, polycystin-1 also might be regulated by the ubiquitination pathway. From these findings, we assume that when a signal inducing cell arrest is transmitted to polycystin-1 to act as a receptor in the membrane, the C-terminal region of polycystin-1 might activate the JAK-STAT pathway and then induce p21, resulting in cell arrest. In the case of cell cycle progression, however, polycystin-1 would be quickly

Figure 5. Siah-1 regulates PKD1-C via the ubiquitin-proteasome pathway. (A) The effect of Siah-1 on PKD1-CT1 and PKD1-CD4CT1 degradation is blocked by proteasome inhibitors. 293T cells were co-transfected with PKD1-CT1 or PKD1-CD4CT1 and either pcDNA3.1/His vector or pcDNA3.1/His-Siah-1. After 36 h, cells were incubated for 8 h at 37°C with DMSO (−, inhibitor lane), MG132, ALLN, and ALLM. Lysates were prepared, and the levels of PKD1-C expression were detected by Western blot analysis. The data represent the means of three experiments. (B) Siah-1 promotes ubiquitination of PKD1-CT1. 293T cells were co-transfected with the indicated plasmids, incubated for 36 h, and treated with MG132 for 8 h. HA-tagged PKD1-CT1 proteins were then immunoprecipitated from the cell lysates with the anti-HA antibody, separated by SDS-PAGE, and transferred to Western blots. The immunoprecipitates were probed with the anti-Xpress antibody to identify PKD1-CT1 to which the Xpress-ubiquitin polypeptide had been attached.
C-terminal cytoplasmic region of polycystin-1. the ubiquitin-proteasome pathway through interaction with the degraded. Siah-1 might act as the rapid regulator of polycystin-1 via the ubiquitin-proteasome pathway through interaction with the C-terminal cytoplasmic region of polycystin-1.

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References


