Characterization of cis-Autoproteolysis of Polycystin-1, the Product of Human Polycystic Kidney Disease 1 Gene*

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Polycystin-1 (PC1), the PKD1 gene product, plays a critical role in renal tubule diameter control and disruption of its function causes cyst formation in human autosomal dominant polycystic kidney disease. Recent evidence shows that PC1 undergoes cleavage at the juxtamembrane G protein-coupled receptor proteolytic site (GPS), a process likely to be essential for its biological activity. Here we further characterized the proteolytic cleavage of PC1 at the GPS domain. We determined the actual cleavage site to be between leucine and threonine of the tripeptide HLT3049 of human PC1. Cleavage occurs in the early intra-cellular secretory pathway and requires initial N-glycan attachment but not its subsequent trimming. We provide evidence that the cleavage occurs via a cis-autoproteolytic mechanism involving an ester intermediate as shown for Ntn hydrolases and EMR2.

Polycystin-1 (PC1) is encoded by the PKD1 gene, which is mutated in 85–90% of human autosomal dominant polycystic kidney disease (ADPKD), which affects 1 in 1000 people worldwide (1). ADPKD is characterized by the progressive development of numerous fluid-filled cysts derived from tubular epithelial cells in both kidneys, which leads to end-stage kidney disease in ~50% of affected individuals by the age of 60. ADPKD is a systemic disease with many extrarenal manifestations, including cyst formation in the liver and spleen and cardiovascular abnormalities (1).

PC1 is thought to function as a cell surface signaling receptor at cell-cell/cell-matrix junctions and as a mechano-sensor in renal primary cilia that activates signaling pathways involved in renal tubular differentiation (2–4). It is a 4302-amino acid (aa) 11-transmembrane glycoprotein with a large N-terminal extracellular region (ectodomain) of 3072 aa and a short cytoplasmic C-terminal tail of ~200 aa (5) (Fig. 1A). The ectodomain contains a novel combination of motifs. One of them is the ~1000-aa long receptor for egg jelly (REJ) domain, a structure of unknown function, which was originally described in sea urchin receptor for egg jelly protein (6).

Situated between the REJ domain and the first transmembrane domain is the GPS (G protein-coupled receptor proteolytic site) domain of ~50 aa (7, 8). It was first demonstrated to be the internal cleavage site for the neuronal G protein-coupled receptor, latrophilin/CIRL, with the actual cleavage site at HL ↓ T (where ↓ identifies the position of cleavage) (9). It was later identified in many proteins of the LNB-TM7 family, a group of seven-transmembrane receptors related to family-B G protein-coupled receptors (10). These proteins, like PC1, are extraordinary for having unusually large and complex N-terminal extracellular regions; they include EMR2, ETL, and Drosophila Flamingo.

We have previously shown indirectly that PC1 undergoes cleavage at the GPS domain (8). This reaction results in the N-terminal fragment (NTF) and C-terminal fragment (CTF), which remain tethered noncovalently. One unique aspect of PC1 cleavage is that it is incomplete when expressed in various cell types. The degree of cleavage (DOC; defined as the fraction of CTF over the sum of CTF and uncleaved full-length (uFL)) is typically ~50% (i.e. an equal amount of CTF and uFL). Partial cleavage has also been found for endogenous PC1 (8).

PKD1-associated missense mutations in the GPS domain and the neighboring REJ domain, as well as synthetic mutations at the predicted cleavage site, were found to disrupt the cleavage. They also resulted in loss of the functional properties of PC1 to activate the JAK2-STAT pathway and induce in vitro tubulogenesis of MDCK cells (8). These findings indicate that GPS cleavage likely plays a critical role for the biological function of PC1.

The GPS or GPS-like sequence is present at a similar juxtamembrane position of all PC1 family members (11, 12) (Fig. 1A). The cleavage property, however, varies among the few PC1 family members in which cleavage has been studied. Cleavage has also been demonstrated for suREJ3 (13), and PKDREJ and suREJ2 have recently been shown to be uncleaved (14, 15). The functional significance of cleavage or noncleavage for the PC1 family is currently unclear.

Proteolytic cleavage at the extracellular juxtamembrane position occurs in many membrane proteins including receptors and channel proteins (16). It serves a variety of functions ranging from precursor activation (e.g. ENaC) (17) to receptor inactivation (e.g. V2 vasopressin receptor) (18) to creation of a high-affinity binding pocket for ligands (19). It occurs generally

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The abbreviations used are: PC1, polycystin-1; PKD, polycystic kidney disease; ADPKD, autosomal dominant PKD; HEK cells, human embryonic kidney cells; MDCK cells, Madin-Darby canine kidney cells; WT, wild type; aa, amino acid(s); REJ, receptor for egg jelly; BFA, brefeldin A; GPS, G-protein-coupled receptor proteolytic site; uFL, uncleaved full-length; NTF, N-terminal fragment; CTF, C-terminal fragment; DOC, degree of cleavage; ETL, EGF-TM7-latrophilin-related protein.
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by proteases along the secretary pathway (e.g. furin) (20) or at the plasma membrane (e.g. TACE sheddase) (21). However, several types of self-catalyzed protein modifications that do not require the intervention of other enzymes have been identified (22). The best characterized mechanism among these is the cis-autoproteolysis, a self-catalyzed protein rearrangement that results in cleavage at the HX-Y (T/S/C) (22, 23). This process is essential for the biological function of a diverse group of proteins that include Hedgehog, glycosylasparaginase, nucleoporin, and intein-containing proteins (24). It is initiated by a nucleophilic attack of the side chain hydroxyl group (T, S) or thiol group (C) of HX-Y (T/S/C) on the penultimate α-carboxyl group. The process is followed by the reversible N–O bond to a more reactive (thio)ester intermediate. The subsequent attack of the intermediate by a second nucleophile results in the irreversible cleavage of the scissile bond. Lin et al. (25) have recently described that the cleavage of EMR2, a LNB-TM7 member, occurs at its GPS domain through a similar cis-autoproteolytic mechanism. This result suggested that PC1 might be cleaved through the same mechanism.

In this study, we confirmed the cleavage site within the GPS domain of PC1 as proposed previously and identified the intracellular location at which PC1 cleavage takes place. We have provided evidence that PC1 is cleaved at the GPS domain through a similar cis-autoproteolytic mechanism involving an ester intermediate.

EXPERIMENTAL PROCEDURES

Cell Culture—The HEK293 cell line was obtained from the American Type Culture Collection (Manassas, VA). MDCK(hPKD1FLAG) with stable expression of hPKD1FLAG, human PKD1 cDNA with C-terminal FLAG epitope (8), was established as described by Boletta et al. (26). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37 °C under 5% CO2. Transfection of the cells was performed using Lipofectamine 2000 (Invitrogen). The HEK293 stable cell lines, HEK(hPKD1FLAG) and HEK(ED-Fc), were established using the Flp-InTM T-RExTM system (Invitrogen).

Reagents and Antibodies—All chemicals and reagents were obtained from Sigma unless otherwise specified. Complete protease inhibitor mixture was from Roche Applied Science. Agarose-conjugated anti-FLAG M2 antibody was from Sigma. Anti-Fc was from Jackson ImmunoResearch (West Grove, PA). Anti-CT, the polyclonal antibody against human PC1 cytoplasmic C-terminal tail is described elsewhere (27). Protein G-agarose beads were from GE Healthcare.

Generation of PC1 and ETL Cleavage Mutant Constructs—The construct wild-type (WT) hPKD1FLAG was the base plasmid for the generation of the cleavage mutants (8). The cleavage mutants were generated in a two-step procedure by PCR using pfu DNA polymerase (Stratagene, La Jolla, CA), as described in (8). To generate expression vector for the soluble human PC1 or ETL ectodomain-human immunoglobulin Fc fusion proteins, the Fc region of human IgG1, was PCR-amplified from a human immunoglobulin G1, Fc fragment cDNA (GenBankTM accession number AF150959) and inserted in-frame at the 3'-end of the ectodomain of human PC1 (residues 1–3072) or of ETL, respectively, at AvrII site using primers Fe-C-F(AvrII) and Fe-C(R-AvrII). The sequences of the primers used are as follows (AvrII site is underlined): Fe-C(AvrII), 5’-AAAAACCTAG-GCAAATCTTGTGACAAAACCTCA-3’; Fe-C(R-AvrII), 5’-TTTT-TCTTAGTCTATTACCCGAGACGGG-3’. The AvrII site used for cloning was introduced by site-specific mutagenesis. All constructs were confirmed by sequencing. EMR2 constructs were kindly provided by Dr. Hsi-Hsien Lin (Oxford, UK).

Purification of PC1 ED-Fc and N-terminal Sequencing—HEK cells with stable expression of ED-Fc, HEK(ED-Fc) were harvested and lysed in lysis buffer (20 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100) containing Complete protease inhibitor cocktails for 1 h on ice. The lysate was centrifuged at 9000 × g for 15 min at 4 °C. The cleared supernatant was subjected to immunoprecipitation using protein G-agarose beads overnight at 4 °C under constant and gentle rotation. The beads were washed three times with 10 ml of phosphate-buffered saline and eluted with 4 ml of 100 mM glycine (pH 2.5). The eluate was neutralized by adding 800 µl of 0.5 M sodium phosphate (pH 7.2) and concentrated using Centriprep-10 concentrators. The concentrated sample was loaded onto 10% SDS gel for electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane. The membrane was stained with Coomassie Brilliant Blue R-250, and the protein band was excised for N-terminal sequencing. N-terminal sequence was determined by automated Edman degradation using a protein sequencer from Applied Biosystems (Midwest Analytical, Inc).

Immunoprecipitation and Western Blot Analysis—Transfected HEK293 or MDCK cells were washed twice with phosphate-buffered saline and lysed in lysis buffer containing Complete protease inhibitor mixture for 1 h on ice. The lysate was centrifuged at 9000 × g for 15 min at 4 °C. The cleared supernatant was subjected to immunoprecipitation using agarose-conjugated anti-FLAG M2 antibody at 4 °C under constant rotation. The beads were washed three times with lysis buffer. The immunoprecipitated proteins were eluted in SDS loading buffer by incubation at 95 °C for 3 min. The eluted immunoprecipitation product was resolved on a 4% or 3–8% SDS gel and electrophoresed onto a polyvinylidene difluoride membrane. The membrane was probed with primary antibodies and then with the secondary horseradish peroxidase-conjugated antibody (GE Healthcare). The signal was detected with SuperSignal West Pico Chemiluminescent detection system (Pierce).

In Vitro Cleavage Assay—HEK cells transfected with expression constructs of full-length WT or mutant PC1 were harvested for lysis as described above. The PC1 proteins were bound to agarose-coujugated anti-FLAG M2 antibody. For in vitro cleavage reaction, the beads were evenly distributed to the tubes for different time points. For example, the beads were evenly distributed to nine tubes for four time points. Tube 1 was a negative control at time zero. 250 µM hydroxylamine was added to four of the remaining eight tubes, and the other four
FIGURE 1. Determination of human PC1 cleavage site within the GPS domain. A, schematic diagram of the structure of full-length PC1. LRR, leucine-rich repeat; R1 and R2–R16, PKD repeats; CLD, C-type lectin domain; LDL-A, low density lipoprotein class A module; TM, transmembrane domain; C-ter, cytoplasmic C-terminal tail. The recombinant PC1 used in the study has a C-terminal FLAG tag as indicated. The cleavage products, NTF and CTF, and the ectodomain are indicated. Underneath the diagram is the multiple sequence alignment of GPS sequences of PC1 family proteins (upper part) and LNB-TM7 proteins (lower part) using ClustalW. The predicted cleavage site of human PC1 is marked by an arrow. The position of the three residues in respect to the cleavage site (−2, −1, and +1) is indicated at the top. The two pairs of cysteine are indicated. Note that only PC1, PKD1-L1, and cLOV1 contain the GPS domain with one pair of cysteines. h, human; m, mouse; c, Caenorhabditis elegans; su, sea urchin; b, bovine. LPH/CL, latrophilin. The black box indicates a 17-amino acids insertion in PKDREJ proteins. B, schematic diagram of chimeric proteins of the PC1 ectodomain (residues 1–3072) fused to the Fc fragment of human immunoglobulin of WT (ED-Fc) and of mutant (T/R(−1)-Fc) with a point mutation of Thr to Arg at the −1 position of the predicted cleavage site (B). ED-Fc(R) is same as ED-Fc except that Fc cDNA is inserted in the reverse orientation. The expected GPS cleavage products of ED-Fc, NTF and Stalk-Fc (34 kDa) fragment, are marked. C, ED-Fc, ED-Fc(R), and T/R(−1)-Fc proteins were immunoprecipitated (IP) from the HEK293 cells expressing the respective proteins with protein G-agarose beads and detected on Western blot (IB; 4–20% SDS gel) by anti-Fc. The positions of uFL ED-Fc and the Stalk-Fc are indicated on the right. D, ED-Fc from the HEK293 cells with stable expression was immunopurified using protein G-agarose beads and stained with Coomassie Blue after SDS-PAGE (10%) and electrotransfer to a polyvinylidene difluoride membrane. The protein band corresponding to Stalk-Fc was excised for N-terminal sequencing. E, amino acid sequences surrounding the cleavage site contained in ED-Fc, with an arrow indicating the point of cleavage. The N-terminal five amino acids of Stalk-Fc fragment determined are indicated by a gray box. The Fc fragment is indicated by a white box.
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**A.** Subcellular localization of PC1 cleavage and role of N-glycosylation in cleavage. A, site of action of glycoprotein trafficking along the major secretory pathway and processing inhibitors used in the study, shown here only from the ER-to-Golgi medial cisternae. BFA inhibits protein transport from the ER to Golgi, whereas monensin inhibits protein intra-Golgi transport. ERGIC, ER-to-Golgi intermediate compartment. The N-linked oligosaccharide is indicated within boxes. Enzymes of the major N-glycan processing steps are: OST, oligosaccharyl transferase; GlcI, α-glucosidase I; GlcII, α-glucosidase II; ER ManI and -II, ER α1,2-mannosidase I and II; GnT1, N-acetylglucosaminyltransferase I; Golgi ManI and -II, α-mannosidase I and II. The inhibitors are indicated above the respective enzymes: CAS, castanospermine; DNJ, 1-deoxynojirimycin; ManDNJ, 1-deoxyxamnnojirimycin; SW, swainsonine. B, Western blots demonstrating the cleavage pattern of FLAG-tagged PC1 transiently expressed in HEK293 (lanes 1–8) or stably expressed in MDCK cells (lanes 9–12) after treatment with the inhibitors of protein trafficking and glycosidases as indicated. The inhibitors were added to the cells for 24 h prior to cell lysis. The final concentration used for each chemical was: tunicamycin (TM), 0.2 μg/ml; BFA, 10 μg/ml; monensin, 10 μM; castanospermine, 50 μM; 1-deoxynojirimycin, 1 μM; 1-deoxyxamnnojirimycin, 0.25 mM; swainsonin, 100 μM. Dimethyl sulfoxide (DMSO) was used as a negative control and had no effect on PC1 cleavage. The protein lysates were immunoprecipitated with anti-FLAG M2 antibody and probed with anti-CT. The positions of uFL (as a negative control) and had no effect on PC1 cleavage. The protein lysates were immunoprecipitated with anti-FLAG M2 antibody and probed with anti-CT. The positions of uFL (as a negative control) and had no effect on PC1 cleavage. The protein lysates were immunoprecipitated with anti-FLAG M2 antibody and probed with anti-CT. The positions of uFL (as a negative control) and had no effect on PC1 cleavage. The protein lysates were immunoprecipitated with anti-FLAG M2 antibody and probed with anti-CT. The positions of uFL (as a negative control) and had no effect on PC1 cleavage. The protein lysates were immunoprecipitated with anti-FLAG M2 antibody and probed with anti-CT. The positions of uFL (as a negative control) and had no effect on PC1 cleavage. The protein lysates were immunoprecipitated with anti-FLAG M2 antibody and probed with anti-CT. The positions of uFL (as a negative control) and had no effect on PC1 cleavage. The protein lysates were immunoprecipitated with anti-FLAG M2 antibody and probed with anti-CT.

**B.** Determination of the Cleavage Site within the GPS Domain of PC1—
The exact site of cleavage within the GPS domain of human PC1 was proposed to be at HL\(^{T3049}\) (7, 8). We sought to confirm the cleavage site by N-terminal amino acid sequencing of the \(~150\)-kDa CTF fragment from HEK(hPKD1\(^{FLAG}\), which stably expresses human FLAG-tagged PC1 at the C terminus, but were unable to obtain sufficient quantity of the product. We therefore resorted to using ED-Fc, the human PC1 ectodomain (aa 1–3072) fused to human IgG Fc fragment (28) (Fig. 1B). ED-Fc was cleaved when expressed in cells, as evident by detection of a \(~34\)-kDa Stalk-Fc band by anti-Fc (Fig. 1C). The T/R(+1)-Fc, which contains the T to R mutation at the critical +1 position of HL\(^{T3049}\), was not cleaved. This result confirmed that ED-Fc was cleaved at the same site as the full-length PC1. We isolated the \(~34\)-kDa Stalk-Fc for N-terminal sequencing following SDS-PAGE (Fig. 1D) and determined the sequence of five N-terminal residues as TAFGA (Fig. 1E). We therefore concluded that the cleavage site of PC1 is indeed at the predicted HL\(^{T3049}\), a site 23 aa N-terminal to the first transmembrane domain.

**Subcellular Localization of PC1 Cleavage—** We sought to define the intracellular location where PC1 cleavage occurs by
using inhibitors affecting protein trafficking (Fig. 2A). Brefeldin A (BFA) blocks membrane protein export out of ER by disassembling the Golgi complex and fusion of Golgi cisternae with ER, whereas monensin blocks intra-Golgi protein trafficking (29). In HEK293 cells, PC1 cleavage was considerably reduced by BFA, as evident by reduced DOC (~15%) (Fig. 2B, lane 2), compared with the untreated control (~50%) (lane 1). However, it was not affected by monensin (lane 3). This result indicated that a modest amount of PC1 was cleaved in ER and a considerable amount was cleaved post-ER, probably in the ER-Golgi intermediate compartment (30). This result differs from that of EMR2, which is cleaved exclusively in ER (25). To assay whether the location of PC1 cleavage may differ between cell types, we performed the same analyses in MDCK cells with stable PC1 expression. We found that both BFA (up to 500 μg/ml) and monensin only minimally affected PC1 cleavage (lanes 9, 11 and 12), indicating that cleavage occurred predominantly in the ER in MDCK cells. Our results indicate that PC1 cleavage takes place in the early secretory pathway but the location may differ between cell types.

Role of N-Glycosylation of PC1 for Cleavage—PC1 is heavily N-glycosylated (31, 32). We examined the role of N-glycosylation for PC1 cleavage. We found that inhibition of N-glycosylation of PC1 by tunicamycin (TM), as evident by increased mobility of the full-length PC1, abolished cleavage in both HEK293 and MDCK cells (Fig. 2B, lanes 4 and 10), indicating that the N-glycan addition to PC1 is required for cleavage, likely for correct folding of the protein. We examined whether the subsequent processing of the N-glycan plays a role for its cleavage using glycosidase inhibitors in HEK293 cells (Fig. 2A). Hepatocystin is the regulatory subunit of glucosidase II that carries out the initial glucose trimming and thus the ER quality control of newly synthesized glycoproteins in concert with lectin chaperones (33–35). Mutations of hepatocystin cause autosomal dominant polycystic liver disease (36, 37). We found that castanospermine (CAS) and 1-deoxynojirimycin (DNJ), the inhibitors of both glucosidase I and II (38), did not affect PC1 cleavage (Fig. 2B, lanes 5 and 6), indicating that glucose trimming and thus hepatocystin are not required for PC1 cleavage. In agreement with this result, inhibitors of mannosidases of the Golgi, 1-deoxymannojirimycin (ManDNJ) and swainsonine (SW), did not affect PC1 cleavage (Fig. 2B, lanes 7 and 8). Our results show that PC1 cleavage occurs after the transfer of the oligosaccharide precursor but does not require its subsequent processing.

**Evidence for cis-Autoproteolytic Mechanism for PC1 Cleavage**—The sequence of the GPS domain in PC1 does not match the consensus cleavage site of any known intracellular processing proteases. We noticed that the cleavage tripeptide HL T of PC1 matches the cleavage site sequence HX T/S/C of the cis-autoproteolytic proteins, although no overall sequence similarity outside of it could be recognized between them (Fig. 3A). To determine whether PC1 cleavage might occur through a similar cis-autoproteolytic mechanism, we first examined the requirement of the +1 position residue for cleavage (Fig. 3, B and C). Such a mechanism requires that only Thr,
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Ser, and Cys, which contain a nucleophile side chain (–OH or -SH group), can support cleavage. We found that this was indeed the case. Substitution of Thr by Ser or Cys did not disrupt the cleavage, although the latter reduced the DOC (3–5%). Cleavage also occurred when the whole HL ↓ T was replaced by the cleavage tripeptide HF ↓ S of nucleoporin (39). In contrast, substitution of Thr to Val, Gly, or Arg blocked cleavage (Fig. 3C).

We next examined the formation of the postulated peptide ester, the hallmark of the cis-autoproteolysis, in the FLAG-purified WT PC1 expressed in HEK293 cells. The sample represents the steady-state condition of the cells and contains ~50% of uFL available for the study. Formation of an ester intermediate in the known cis-autoproteolytic proteins is usually inferred from the increased cleavage rate in vitro by strong nitrogen nucleophiles such as hydroxylamine (23, 25, 40). This effect is a result of the facilitated hydrolysis of the (thio)ester intermediate (the rate-limiting step in the cis-autoproteolysis) by virtue of the high reactivity of hydroxylamine against peptide (thio)ester.

We found no cleavage of the uFL PC1 occurring in vitro at various temperatures (4, 25, or 37 °C) regardless of the presence or absence of hydroxylamine as judged by the unchanged DOC (~50%) (Fig. 3D, shown for reactions at 4 °C).

While our kinetic study was under way, Lin et al. (25) reported that EMR2 is cleaved at its GPS domain via the cis-autoproteolysis mechanism. We reexamined PC1 cleavage using the condition as described in that study but still found no cleavage (not shown). Our result suggests that no detectable amount of stable ester intermediate was present in the sample during the incubation. Alternatively, the postulated oxygen ester intermediate might not be susceptible to hydroxylamine under our neutral conditions.

We therefore performed the same analysis with FLAG-purified T/C(+1) mutant, in which the presumed thioester intermediate is known to be much more reactive to hydroxylamine than the oxygen ester in WT, as found for protein splicing reaction (41). We found that T/C(+1) uFL underwent slow cleavage at 4 °C (to minimize activity of potential contaminating proteases) in the absence of hydroxylamine, with no cleavage apparent in the first 5 h of incubation (DOC ~3%; Fig. 3E).

However, cleavage consistently proceeded to a DOC of ~11% after 16 h of incubation, a ~4-fold of progression. In the presence of hydroxylamine, this DOC was reached after only 1 h of incubation and remained unchanged over time. Higher temperatures (25 or 37 °C) did not result in a higher DOC (not shown). Therefore, hydroxylamine shortened the t1/2 (defined as the time to reach half of the maximal DOC) from >5 h to <15 min, which is a >20-fold increase of the cleavage rate (Fig. 3F).

In the presence of iodoacetamide, a thiol-alkylation reagent, this T/C(+1) cleavage no longer occurred even with hydroxylamine (Fig. 3G).

These results suggest that no stable thioester was accumulated in the protein preparation, but a small detectable amount of thioester was generated during the cleavage reaction of T/C(+1) by the side chain thiol group of the C(+1), with hydrolysis of the thioester being the rate-limiting step. As a negative control, we tested the T/V(+1) mutant and showed that it was not cleaved in the presence of hydroxylamine (Fig. 3H), confirming that T/C(+1) cleavage occurred at the HL ↓ C tripeptide sequence rather than at a nearby site such as an Asn–Gly bond (42). Our results together provide strong evidence for the cis-autoproteolytic mechanism of PC1 cleavage. It is important to note that only ~8% of full-length T/C(+1) present in the sample was cleaved in vitro.

The observed resistance of PC1 cleavage to completion has not been described for other cis-autoproteolytic proteins. To examine whether our finding of limited cis-autoproteolysis of PC1 represents a unique property of PC1 or whether it is due to the altered conformation as a result of detergent extraction of the protein from the membrane, we generated several mutant ED-Fc, similar to the EMR2 Fc mutants used by Lin et al. (25). We found that the cleavage of T/C(+1)-Fc could be enhanced only minimally with hydroxylamine (Fig. 4A). H/R(−2)-Fc showed a trace of cleavage, whereas H/K(−2)-Fc was as resistant to cleavage as the negative control T/V(+1)-Fc (Fig. 4A).

The parallel control experiment with the equivalent slow cleavage EMR-Fc mutant showed the increased cleavage rate by hydroxylamine (Fig. 4C) as reported previously (25). The results of the soluble PC1 ectodomain proteins further confirmed that most of uFL at steady state were truly incapable of cis-autoproteolysis.
In Vitro Cleavage of ETL Molecule—To determine whether this property of PC1 also occurs in other GPS-containing proteins, we analyzed the cleavage of ETL (43), another LNB-TM7 member with a similar size to EMR2. We found that the secreted ETL-ED-Fc or its T/C(+/H11001) mutant was virtually resistant to further cleavage by hydroxylamine (Fig. 4B), in contrast to the slow cleavage EMR2 mutant (Fig. 4C).

DISCUSSION

In this study, we provide several lines of evidence that PC1 cleavage at the GPS domain occurs through a cis-autoproteolytic mechanism involving an ester-intermediate via N-O acyl rearrangement. First, PC1 was cleaved at the predicted tripeptide HL(2T3049) (Fig. 1), which conform to the cleavage site HX(2T/S/C) of cis-autoproteolytic proteins. Second, a nucleophile hydroxyl or thiol group at the +1 position is necessary for the cleavage (Fig. 2, A–C), as required by cis-autoproteolysis. Third, the in vitro cleavage rate of the slow cleavage T/C(+1) mutant was increased >20-fold by hydroxylamine, indicating the formation of a slowly hydrolyzed thiol-ester intermediate in the course of the cleavage reaction (Figs. 3, E–G, and 4A). Although a direct demonstration of a true autoproteolysis is difficult, the observed increase of the reaction rate by hydroxylamine at 4 °C argues against traces of contaminating proteases being responsible for the cleavage in vitro.

Our study provides independent confirmation that cleavage at the GPS domain is through cis-autoproteolysis (Fig. 5A), as shown previously by Lin et al. for EMR2 (25). Our data support the notion that cis-autoproteolysis is a general property of all proteins containing a functional GPS domain.

Our investigation, however, has revealed a unique cleavage property of PC1 that distinguishes it from other known cis-autoproteolytic proteins. We found that the WT full-length PC1 isolated from the

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**FIGURE 5. Proposed model for cis-autoproteolysis of PC1 at GPS. A**, proposed cis-autoproteolytic mechanism for PC1 cleavage. When newly synthesized PC1 polypeptide is correctly folded in the ER after N-glycosylation, a tight strain at HL(2T3049) is generated to confer the ability of Thr(3049) to initiate the cis-autoproteolysis. The active hydroxyl of Thr(3049) is deprotonated by His(3047) and launches a nucleophile attack on the α-carbonyl of Leu(3048) (step I). The attack results in the formation of a transitional tetrahedral intermediate (step II), which collapses by protonation of the amino group of Thr(3049) to form an ester intermediate via an N-O acyl shift (step III). The final step is hydrolysis of the ester that results in cleavage of the peptide bond between Leu(3048) and Thr(3049) (step IV). **B**, two-pathway model for PC1 cleavage in vivo. The nascent PC1 can follow one of two pathways. In the cleavage pathway, PC1 is cleaved via the cis-autoproteolytic mechanism as described in A. The non-cleavage pathway leads PC1 to be trapped in a blocked (uncleavable) state. It is not known whether the two pathways are convertible.
cells, which represents its steady-state condition, was not competent for cis-autoproteolysis. Only ~8% of the slow cleavage T/C(+1) mutant was able to undergo cis-autoproteolysis. The inefficiency of cis-autoproteolysis of PC1 in vitro could be confirmed by using the soluble ED-Fc proteins. This is in contrast to the equivalent EMR2 mutant, which could be efficiently cleaved by hydroxylamine. Slow cleavage mutants of other cis-autoproteolytic proteins typically showed complete cleavage in vitro with large increases in rates by strong nucleophiles (23, 39, 44).

Our results suggest that although full-length PC1 molecules are cleaved by cis-autoproteolysis in the cells, the uncleaved molecules are in an uncleavable or blocked state. This notion is supported by the result of our previous pulse-and-chase experiment (8). The pulse-labeled WT PC1 became cleavage after 15 min of chase and gradually decreased the amount to a level equal to that of NTF within 2 h, a ratio that remained unchanged for at least another 3 h. Therefore, about half of the nascent PC1 molecules could accomplish cleavage within 2 h, whereas the other half remained uncleavable for a prolonged period of time before being degraded. At any given steady state, most of the uncleaved molecules are thus expected to be in this uncleavable or blocked state, with too few cis-autoproteolysis competent PC1 molecules to be detectable in vitro. The T/C(+1) mutation also causes significant slowing of cleavage in other cis-autoproteolytic proteins (39, 44). Decreased cleavage rate of PC1 T/C(+1) likely results in a small but sizable amount of the cis-autoproteolysis-competent molecule still present in steady state, which we could detect in ~8% of full-length proteins in vitro.

Based on these considerations, we propose a model in which newly synthesized PC1 can proceed through two competing pathways: the “cleavage” pathway, which leads to irreversible cis-autoproteolytic cleavage, and the “non-cleavage” pathway, which leads to trapping in the blocked state (Fig. 5B).

We have shown previously that endogenous PC1 in the kidney and in human endothelial cells is also partially cleaved, where both the uncleaved PC1 and the tethered cleavage products were detectable (8). This finding suggests that cleaved and non-cleaved PC1 molecules have different functions in vivo. We have recently generated a Pkd1 knock-in mouse expressing the non-cleavable T/V(1101) PC1 protein. The mutant mice overcome embryonic lethality, as found for the Pkd1 knock-out mice (45, 46), but develop severe polycystic kidney disease after birth (47). These data provide strong evidence that the non-cleaved PC1 plays a critical role in embryonic development, whereas the cleaved PC1 molecules are critical for kidney tubular integrity. The cleavage of PC1 may therefore be required to activate signaling pathways involved in renal tubular differentiation and maturation. It is tempting to speculate that the “two-pathway” cleavage is designed to produce both types of PC1 molecules, thus increasing the functional diversity of PC1. We found that ETL was similarly resistant to cleavage in vitro and may also have the two-pathway cleavage. We suggest that the mode of two-pathway cleavage might apply for a subset of GPS-containing LNB-TM7s and PC1 family members. Drosophila Flamingo, a molecule involved in regulating planar polarity, appears to be cleaved incompletely in embryos (48), and might belong in this category.

cis-Autoproteolysis is based on the ability of threonine, serine, or cysteine to initiate a proximal and reversible N–O or N–S acyl shift (22). However, proteins are not cleaved at every one of these residues. These amino acids acquire cis-autoproteolytic potential only when the conditions are met that favor the equilibrium toward a N–O or N–S shift and facilitate the (thio)ester formation (23): 1) a highly strained and energetically unfavorable tight turn at the scissile peptide bonds; 2) distant residues precisely arranged around the cleavage site to create oxyanion holes and provide proton transfer networks for the cleavage reaction.

The PKD1-associated missense mutations in the distant REJ domain previously found to disrupt the cleavage might affect those critical residues involved in the oxyanion hole formation and proton transfer. It is also conceivable that cellular factors (such as post-translational modifications or binding to chaperone and/or ligands) that affect any of these structural constraints would influence the course of PC1 cleavage in the two pathways and thus the relative ratio of uFL and cleavage products at the steady state. The initial attachment of Glc3Man2GlcNAc2 (probably required for correct folding) was required for cleavage, but the subsequent glucose trimming and quality control in ER, and therefore hepatocystin, are not necessary for cleavage. Further studies are required to identify the factors that regulate cleavage of PC1 and determine the relative abundance of the cleaved and uncleaved PC1 proteins in vivo.

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REFERENCES
1. Gabow, P. A. (1993) N. Engl. J. Med. 329, 332–342
2. Boletta, A., and Germino, G. G. (2003) Trends Cell Biol. 13, 484–492
3. Harris, P. C., and Torres, V. E. (2006) Curr. Opin. Nephrol. Hypertens. 15, 456–463
4. Nauli, S. M., Alenghat, F. J., Luo, Y., Williams, E., Vassilev, P., Li, X., Elia, A. E., Lu, W., Brown, E. M., Quinn, S. J., Ingber, D. E., and Zhou, J. (2003) Nat. Genet. 33, 129–137
5. Hughes, J., Ward, C. J., Peral, B., Aspinwall, R., Clark, K., San Millan, J. L., Gamble, V., and Harris, P. (1995) Nat. Genet. 10, 151–160
6. Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) J. Cell Biol. 133, 809–817
7. Ponting, C. P., Hofmann, K., and Bork, P. (1999) Curr. Biol. 9, R585–R588
8. Qian, F., Boletta, A., Bhunia, A. K., Xu, H., Liu, L., Abrahi, A. K., Watnick, T. J., Zhou, F., and Germino, G. G. (2003) Proc. Natl. Acad. Sci. U. S. A. 99, 16981–16986
9. Krasnoperov, G. V., Bittner, M. A., Beavis, R., Kuang, Y., Salnikov, K. V., Chepurny, O. G., Little, A. R., Plotnikov, A. N., Wu, D., Holz, R. W., and Petrenko, A. G. (1997) Neuron 18, 925–937
10. Stacey, M., Lin, H. H., Gordon, S., and McKnight, A. J. (2000) Trends Biochem. Sci. 25, 284–289
11. Li, A., Tian, X., Sung, S. W., and Somlo, S. (2003) Genomics 81, 596–608
12. Yuasa, T., Takakura, A., Denker, B. M., Venugopal, B., and Zhou, J. (2004) Genomics 84, 126–138
13. Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2002) J. Biol. Chem. 277, 943–948
14. Butscheid, Y., Chubanov, V., Steger, K., Meyer, D., Dietrich, A., and Gud
15. Galindo, B. E., Moy, G. W., and Vacquier, V. D. (2004) Dev. Growth Differ. 46, 53–60
16. Hooper, N. M., Karran, E. H., and Turner, A. J. (1997) Biochem. J. 321, 265–279
17. Hughey, R. P., Bruns, J. B., Kinlough, C. L., Harkleroad, K. L., Tong, Q., Carattino, M. D., Johnson, J. P., Stockand, J. D., and Kleyman, T. R. (2004) J. Biol. Chem. 279, 18111–18114
18. Kojro, E., and Fahrenholz, F. (1995) J. Biol. Chem. 270, 6476–6481
19. Williams, J. F., McClain, D. A., Dull, T. J., Ullrich, A., and Olefsky, J. M. (1990) J. Biol. Chem. 265, 8463–8469
20. Taylor, N. A., Van De Ven, W. J., and Creemers, J. W. (2003) FASEB J. 17, 1215–1227
21. Malemud, C. J. (2006) Front. Biosci. 11, 1696–1701
22. Perler, F. B. (1998) Nat. Struct. Biol. 5, 249–252
23. Xu, Q., Buckley, D., Guan, C., and Guo, H. C. (1999) Cell 98, 651–661
24. Lin, H. H., Chang, G. W., Davies, J. Q., Stacey, M., Harris, J., and Gordon, S. (2004) J. Biol. Chem. 279, 31823–31832
25. Boletta, A., Qian, F., Bhunia, A. K., Phakdeekitcharoen, B., Hanaoka, K., Guggino, W., Monaco, L., and Germino, G. G. (2000) Mol. Cell 6, 1267–1273
26. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A. K., Piontek, K., Tsiokas, L., Sukhatme, V. P., Guggino, W. B., and Germino, G. G. (2000) Nature 408, 990–994
27. Guan, C., Cui, T., Tao, V., Liao, W., Ewel, A., Whitaker, R., and Paulus, H. (1998) J. Biol. Chem. 273, 9695–9702
28. Bornstein, P., and Balian, G. (1977) Methods Enzymol. 47, 132–145
29. Nechiporuk, T., Urness, L. D., and Keating, M. T. (2001) J. Biol. Chem. 276, 4150–4157
30. Guan, C., Cui, T., Tao, V., Liao, W., Benner, J., Lin, C. L., and Combs, D. (1996) J. Biol. Chem. 271, 1732–1737
31. Lu, W., Peisel, B., Babakhanlou, H., Pavlova, A., Geng, L., Fan, X., Larson, C., Brent, G., and Zhou, J. (1997) Nat. Genet. 17, 179–181
32. Piontek, K. B., Huso, D. L., Ginberg, A., Liu, L., Bedja, D., Zhao, H., Gabrielson, K., Qian, F., Mei, C., Westphal, H., and Germino, G. G. (2004) J. Am. Soc. Nephrol. 15, 3035–3043
33. Yu, S. Q., He, X. B., Gao, J. G., Zuo, J., Hackmann, K., Xu, H. X., Germino, G., and Qian, F. (2006) J. Am. Soc. Nephrol. 17, 89A (abstr.)
34. Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R. W., Schwarz, T. L., Takeichi, M., and Uemura, T. (1999) Cell 98, 585–595