Molecular Cloning and Characterization of Human Podocalyxin-like Protein

ORTHOLOGOUS RELATIONSHIP TO RABBIT PCLP1 AND RAT PODOCALYXIN

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The glomerular epithelial cell (podocyte) is a highly differentiated cell with characteristic interdigitating foot processes covering the outer aspect of the glomerular basement membrane. The space between these foot processes is spanned by a modified tight junction (slit diaphragm) and provides the large surface area for filtration. The foot processes are covered on their non-soluble (apical) surface with an anionic glyocalyx. A major component of this glyocalyx is thought to be podocalyxin, a sialoglycoprotein described by Kerjaschki and colleagues (7). Both the lectin binding properties of this protein and its distribution on the surface of podocyte foot processes and the luminal surface of vascular endothelial cells are similar to rat podocalyxin. However, this sialoglycoprotein differs from rat podocalyxin in its apparent molecular mass on SDS-PAGE (165/170-kDa doublet in contrast to a 140-kDa band for rat podocalyxin) and in its peptide digest pattern. Antibodies to this molecule and those to rat podocalyxin have been reported not to react across species (7), and neither of these molecules has been cloned to date.

We reported recently the cloning and characterization of a rabbit sialoglycoprotein with a size, staining characteristics, and tissue distribution similar to those of rat podocalyxin. We named this protein rabbit podocalyxin-like protein 1 (PCLP1) (8). Using the rabbit PCLP1 cDNA as a probe we have now cloned a human podocalyxin-like protein (PCLP). In this report we characterize the molecular structure of human PCLP and define its relationship with rabbit PCLP1 and rat podocalyxin.

EXPERIMENTAL PROCEDURES

cDNA Library, Screening, and Sequence Analysis—Total RNA from was prepared from renal cortex by modification of the CsCl/guanidine isocyanate method of Chirgwin et al. (9) as described previously (10). The kidneys used for RNA preparation were from a cadaver organ donor whose kidney could not be used for transplantation and a patient with congenital nephrotic syndrome (Finnish type) undergoing a pretransplant nephrectomy. Libraries were produced from these preparations by the custom library services of Stratagene, Inc. (La Jolla, CA). In addition, a commercial human heart cDNA library was used (Stratagene). These libraries were initially screened using rabbit PCLP1 cDNA as probes (11). Sequencing was done by the method of Sanger et al. (12) using the Sequenase kit (U. S. Biochemical Corp.) with modifications described previously (8, 13). Additional automated sequencing was performed by the sequencing core at the University of Michigan on a fee-for-service basis. All clones shown were sequenced in both directions. 5’-Rapid amplification of cDNA ends (RACE) was performed using 1 μg of renal cortical RNA isolated from a normal kidney or a kidney of a patient with congenital nephrotic syndrome and a kit from

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCLP, podocalyxin-like protein; PCLP1, podocalyxin-like protein 1; RACE, 5’-rapid amplification of cDNA ends; PCR, polymerase chain reaction; mAb, monoclonal antibody; GST, glutathione S-transferase.

9 5’-Rapid amplification of cDNA ends (RACE) was performed using 1 μg of renal cortical RNA isolated from a normal kidney or a kidney of a patient with congenital nephrotic syndrome and a kit from

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U97519.

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**Fig. 1. Diagrammatic illustration of human PCLP cDNA and derived protein structure.** Panel A, diagrammatic representation of the cDNA clones used to assemble the human PCLP sequence. The sources of the clones were: human heart cDNA library (LH10), cadaver kidney cDNA library (HP11), congenital nephrotic kidney cDNA library (NP2 and NP3), and PCR products derived from cadaver kidney (RACE5) or congenital nephrotic kidney (RACE12) RNA using the RACE technique. Kb, kilobases. Panel B, Kyle-Doolittle amino acid hydrophilicity plot and diagrammatic representation of PCLP protein structure derived from the nucleotide sequence. A single putative 26-amino acid transmembrane region is shown (solid black box). The NH<sub>2</sub>-terminal domain contains a hydrophobic 21-residue putative signal peptide (horizontal striped box). In addition are shown cysteines for possible disulfide linkage (shaded boxes), and a highly acidic COOH-terminal region (darkly shaded box). Panel C, Kyle-Doolittle amino acid hydrophilicity plot and diagrammatic representation of rabbit PCLP1 protein structure.

Life Technologies Inc. according to the manufacturer's protocol with dimethyl sulfoxide 10% (v/v) added to the PCRs. The PCR product was ligated into the pCR 2.1 vector (Invitrogen, San Diego) and used to transform INV<sup>F</sup> competent cells. Data base management, sequence analysis, and comparison were done with version 8.0 of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI). Data base searches were performed using the Blast Network Service from the National Center for Biotechnology Information on the “non-redundant” data base from the Brookhaven Protein Data Bank, GenBank, EMBL, PIR, and SwissProt data bases (14).

**Construction and Purification of Fusion Proteins**—The following primers were used to PCR amplify regions of rabbit PCLP1 and human PCLP. To make the fusion protein used to raise antibodies, a portion of the human extracellular, transmembrane, and intracellular domain (base pairs 1004–1835) was PCR amplified using the primers TTTGAATTCAGATGCCAGCCAGCTCTACG and TTTGAATTCTTAGAGGTGCGTGTCTTCCTC. A region of the rabbit PCLP1 extracellular domain (base pairs 1726–2912) was PCR amplified using the primers TTTGGATCCTGCTGCTGC-TACACCCATGACGACG. A region of the rabbit PCLP1 intracellular domain (bases 1726–2912) was PCR amplified using the primers TTTGGATCCTGCTGCTGCTACACCCATGACGACG and TTTGAATTCCTTCATGGTGGCGTGTCTTCCTC.

**Preparation of Monoclonal Antibodies**—Monoclonal antibodies (mAbs) 3D3, 4F10 and 2A4 were produced from BALB/C mice immunized with purified human podocalyxin-glutathione S-transferase (GST) fusion protein containing the intracellular, transmembrane, and a part of the extracellular domain of human PCLP by standard methods as described previously (17). The resulting hybridomas grown out in 96-well plates were selected and subcloned based on immunofluorescence pattern assayed on cryostat sections of human renal cortex. The anti-β-galactosidase (clone Gal-40) used as a control IgM antibody was obtained from Sigma. The VWM control IgM antibody was provided by the Hybridosome Core Facility at the University of Michigan. The monoclonal antibody 5A (anti-rat podocalyxin) was kindly provided by Robert Orlando of the University of California, San Diego. Other antibodies used were as described previously (8, 18, 19). All mAbs were IgG except antibodies 2A4, 4F10, VWM, and anti-β-galactosidase, which were IgM.

**Northern and Southern blot Analysis**—A human multiple tissue Northern blot (CLONTECH Laboratories, Palo Alto, CA) containing 2 μg of poly(A)<sup>+</sup> RNA/lane was probed with <sup>32</sup>PdCTP-labeled human podocalyxin cDNA or β-actin cDNA. Prehybridization, hybridization, and washings were carried out per the ExpressHyb protocol (CLONTECH). The probes for this analysis were the human PCLP cDNA from base pair 1004 to 2029 which was PCR amplified with <sup>32</sup>PdCTP using the primers TTTGGATCCAGATGCCAGCCAGCTCTACG and ACAA-GAGAAATCCTGAGCA and a random <sup>32</sup>PdCTP-labeled β-actin cDNA as a RNA loading control. Conditions for the final wash were 0.1 × SSC and 0.1% SDS at 50 °C.

For Southern blot a portion of the human PCLP cDNA (base pairs 1603–1835) was PCR amplified using the primers TTTGAATTCAGATGCCAGCCAGCTCTACG and TTTGAATTCTTAGAGGTGCGTGTCTTCCTC with <sup>32</sup>PdCTP. A commercial Interspecies Zoo-Blot (CLONTECH Laboratories) containing 5 μg of genomic DNA/lane was probed with the human PCLP cDNA probe as described for Northern blot. Conditions for the final wash were 0.1 × SSC and 0.1% SDS at 50 °C.

**Glomerular Isolation, Protein Extraction, and Western Blots**—The human kidneys used were as described for RNA preparation. Glomerular isolation and extraction were performed at 4 °C by differential sieving as described previously (18). Rabbit glomeruli were isolated from New Zealand White rabbits (2.0–2.5 kg) by iron oxide magnetization as described previously (20). Rat glomeruli were isolated from Harlan Sprague Dawley rats by progressive sieving using 180-, 106-, and 75-μm sieves as described by Salant et al. (21). For glomerular
extraction, $5 \times 10^4$ glomeruli were suspended in 1 ml of phosphate-buffered saline containing 1% Triton X-100, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, 2 mM EDTA, and 8 M urea and sonicated in six short bursts of 10 s as described previously (19). Glomerular extracts were analyzed by SDS-PAGE. Blots (model SBD-1000 Polyblot, American Bionetics, Hayward, CA) were performed as described previously (19). Western blots were developed using the ECL reagent (Amersham Corp.).

Immunoprecipitation Studies—Immunoprecipitations were carried out using a modification of published protocols (22). For immunoprecipitation experiments rat glomerular extract was preabsorbed with anti-mouse IgM (m chain-specific)-agarose beads (Sigma). The mAbs (2A4 and 4F10 or control IgMs anti-β-galactosidase and VWM) were incubated with anti-mouse IgM agarose beads, washed four times with Tris-buffered saline, and incubated with the preabsorbed rat glomerular extract for 20 min at room temperature and then overnight at 4 °C on a rotor. Beads were washed six times with Tris-buffered saline containing protease inhibitors (Complete™ protease inhibitor, Boehringer Mannheim). Samples were prepared as described for Western blot.

Immunofluorescence Studies—Kidney segments were cut on a cryostat for subsequent analysis by indirect immunofluorescence performed using the primary antibodies described for Western blot as described previously (20). For the blocking experiment shown the primary antibody was preincubated with 20 μg of the immunizing fusion protein.

RESULTS AND DISCUSSION

Cloning and Sequencing of Human PCLP—Initial human PCLP clones were obtained by screening with rabbit PCLP1 cDNA. These positive clones were used for subsequent screening of the three human cDNA libraries used (one heart and two renal cortical libraries). Thirty-four clones were obtained by this method, and two additional clones were produced by an anchored PCR strategy using RNA from a normal kidney (clone RACEN5) and the kidney from a patient undergoing a pretransplant nephrectomy for congenital nephrotic syndrome (clone RACEC12). Fig. 1A shows six clones that were used to assemble the nucleotide sequence. The cDNAs spanned 5869 base pairs (Figs. 1 and 2). An initiator methionine (base pairs 251–253) was identified using the following criteria. (a) The sequence was consistent with the putative signal peptide. The double underlined 26 hydrophobic amino acids represent a putative transmembrane region. Potential sites for N-linked glycosylation (black triangles), glycosaminoglycan attachment (dashed underlines), and disulfide linkages (black circles) are shown. Multiple serines and threonines are present in the extracellular domain for potential O-linked glycosylation. At positions 315–320 the boxed nucleotides and their corresponding amino acids were found in only one (RACEC12) of two clones in this region. Nucleotide 435 was G in one clone, making amino acid 62 an arginine. Nucleotide 837 was C in one clone, making amino acid 196 a serine. A region of 96 base pairs (tagagac agtgtttcac catgtcagcc aggctggtct tgaactcctg acctcgggtg atctgcccac cttggcctcc caaagtgctg ggattacag) at position 963 which codes for a 32-amino acid span with 78% identity to Ahu-derived amino acid sequences was found in clones NP2 and LH10 and was not included in this diagram.
Kozak's consensus sequence (first methionine in the open reading frame, purine in position −3) (24). (b) A likely site for signal peptidase cleavage at amino acid 21 is preceded by hydrophobic amino acids in 15 out of 20 positions (25). (c) The 250 base pairs upstream of the putative start methionine is highly GC-rich (78%) and contains numerous CpG “islands” compatible with this region being a 5′-untranslated region (26).

The first stop codon in the open reading frame occurred at base pairs 1835–1837 (Fig. 2). This would correspond to a 1584-base pair or 528-amino acid open reading frame. After removal of the 21-amino acid signal peptide the peptide is calculated to have a molecular mass of 54 kDa. A Blast search of the non-redundant data bases showed significant similarities only to rabbit PCLP1 (8).

Analysis of the derived amino acid sequence shows a single 26-amino acid hydrophobic region compatible with a single membrane spanning domain (Fig. 1B and Fig. 2) similar to rabbit PCLP1 (Fig. 1C) (8). The region COOH-terminal to the hydrophobic putative transmembrane domain contains positively charged amino acids as is typically described for the cytoplasmic side of transmembrane proteins (27). We have shown previously that rabbit PCLP1 is a transmembrane protein with an NH2-terminal extracellular domain, and we have aligned human PCLP in this fashion.

**Analysis of the Intracellular Domain**—Similar to rabbit PCLP1 the intracellular domain contains 75 amino acids, which included one potential protein kinase C site (amino acid 457) and two potential casein kinase II phosphorylation sites (amino acids 488 and 516). Overall this region was highly acidic (pI 4.2) with the final 10 amino acids containing 4 aspartic acid and 3 glutamic acid residues (Fig. 1, B and C and Fig. 2) as described previously for rabbit PCLP1 (8).

Analysis of the Extracellular Domain—The 406 amino acids of the extracellular domain (after signal peptide cleavage) were analyzed for potential structural features and sequence motifs using the Peptidestructure and Motifs programs. There were five potential sites for N-linked glycosylation. In the extracellular region from amino acids 22 to 295 the high serine and threonine content (39%) provides numerous sites for potential O-linked glycosylation some with proline in the −1 and +3 positions, which is seen in many O-glycosylation sites (28). There are three serine-glycine sites and one serine-glycine-glycine site for potential glycosaminoglycan attachment (Fig. 3), but it should be noted that these sites lack acidic residues 2–3 amino acids amino-terminal to the serine, which has been shown to increase the acceptor activity for glycosaminoglycans (29, 30).

Four cysteines for potential disulfide linkage were present in the extracellular domain (Fig. 1B and Fig. 2) as described previously for rabbit PCLP1 (8).

In the extracellular domain there were three differences in nucleotide sequence of the clones which resulted in amino acid changes. The clone RACEN5 derived from RNA from a patient with congenital nephrotic syndrome had as nucleotide 435 a G, making amino acid 62 an arginine instead of a threonine, and nucleotides 315–320 were missing deleting amino acids 23 and 24. Both clones in this region were obtained by the RACE methodology so cloning artifacts cannot be excluded. Nucleotide 837 was a C in clone NP3, making amino acid 196 a serine instead of a leucine.

**Northern Blot Analysis**—Northern blot analysis was performed on RNA from multiple tissues to determine transcript size and the tissue distribution of human PCLP mRNA expression (Fig. 3). A 32P-labeled PCR product from base 1004 to base 2029 was used as probe. A major band was seen at approximately 5.9 kilobases with minor bands at 9.6 and 4.4 kilobases. The mRNA transcript expression was highest in the kidney, pancreas, and heart. Lesser amounts were present in the placenta, lung, and skeletal muscle; a low but detectable signal was present in brain and liver. This tissue distribution is similar to that seen with rabbit PCLP1 on Northern blot (8).

**Characterization of Antibodies to Human PCLP Fusion Proteins**—The cDNA coding for base pairs 1004–1836 of the hu-

**FIG. 3. Northern blot analysis.** A human multiple tissue Northern blot containing 2 μg of poly(A)+ RNA/lane was probed with a 32P-dCTP-labeled human PCLP cDNA (base pairs 1004–2029) (top) or random 32P-dCTP-labeled control human β-actin (bottom). Conditions for the final wash were: 0.1 × SSC, 0.1% SDS at 50 °C. The lanes are: A, heart; B, brain; C, placenta; D, lung; E, liver; F, skeletal muscle; G, kidney; and H, pancreas. The major transcript is at 5.9 kilobases (kb) with minor transcripts seen at 9.5 and 4.4 kilobases. Transcripts were present at high levels in kidney, heart, and pancreas; lesser levels were present in placenta, lung, and skeletal muscle; and low but detectable levels were in liver and brain.

**FIG. 4. Indirect immunofluorescence of human kidney cortex.** Indirect immunofluorescence of cryostat sections of human kidney with mAb 4F10 (panels A and B), mAb 3D3 (panel C), and mAb 3D3 preincubated with the immunizing fusion protein (panel D). Panels C and D were photographed at the same exposure time. Note that the endothelial cells in the capillaries and blood vessels are labeled (arrowheads) in panels A and C. At high magnification (panel B) the unstained glomerular basement membrane can be appreciated between the intense fluorescence of the glomerular epithelial cell foot processes and the capillary endothelial cells (arrows). The bars represent 10 μm (panels A, C, and D) or 2.5 μm (panel B).
human PCLP protein was PCR amplified and ligated into the expression vector PGEX-KT. The fusion protein was purified with glutathione-agarose affinity chromatography and used to immunize mice. Three monoclonal antibodies 3D3, 4F10, and 2A4 appeared to recognize a protein in human renal cortical sections on immunofluorescence (Fig. 4, A–C, antibodies 4F10 and 3D3 shown) with a glomerular epithelial cell and vascular endothelial cell distribution similar to rabbit PCLP1 (8) and rat podocalyxin (1). The signal was abolished when the fusion protein used to raise the antibodies (Fig. 4D, mAb 3D3 shown).

To define further the epitopes recognized by these antibodies the immunizing fusion protein containing the intracellular, transmembrane and a portion of the extracellular domain of human PCLP (base pairs 1004–1836), a fusion protein containing the extracellular domain of human PCLP (base pairs 1004–1492), and a fusion protein containing the intracellular, N-terminus transmembrane domains both rabbit PCLP1 and human PCLP had 2 potential casein kinase II and a protein kinase C phosphorylation sites.

Comparison of Human PCLP and Rabbit PCLP1—We have reported previously the cloning and molecular characterization of a rabbit protein with size, staining, and tissue distribution similar to those of rat podocalyxin (8), and we have called this protein rabbit podocalyxin-like protein 1. On immunofluorescence of kidney sections antibodies to rabbit PCLP1 and human PCLP showed a strong signal in the glomerulus and on the endothelium of the blood vessels in their respective species. Overall the cDNA nucleotide sequences for rabbit PCLP1 and human PCLP showed 72% identity. Both sequences were highly GC-rich in the 5′-untranslated region. Rabbit PCLP1 has an open reading frame of 531 amino acids excluding an alternative splice, and human PCLP had an open reading frame of 528 amino acids. The peptide cores of rabbit PCLP1 and human PCLP were calculated at 55 and 54 kDa, respectively. Both molecules had a 21-amino acid putative signal peptide, multiple sites for potential O-linked and N-linked glycosylation, along with 4 cysteines for potential disulfide interactions in their extracellular domains. The position of 3 of the potential N-linked glycosylation sites was well conserved and the position of the 4 cysteines was identical in both species relative to the transmembrane region (Figs. 1 and 2). In the intracellular domains both rabbit PCLP1 and human PCLP had 2 potential casein kinase II and a protein kinase C phosphorylation sites at identical positions.

Despite the similarities between rabbit PCLP1 and human PCLP the overall amino acid identity between rabbit PCLP1 and human PCLP was only 48%, whereas transmembrane and intracellular domains had 96% amino acid identity. The extracellular NH2-terminal regions showed a low degree of identity (36% identity) except for the putative signal peptide regions (75% identical). This is shown graphically in the similarity plot in Fig. 6. This degree of dissimilarity is reported for the murine/human homologs of CD28 (68% identical) (31) and the murine/human homologs of the CD28 ligand B7 (44% identical) (32). Similarly the human mucosal addressin cell adhesion molecule (MAdCAM-1) shows 39% identity to its murine homolog, but both bind specifically to their αβ-integrin ligand (33).

Relationship of Rat Podocalyxin, Rabbit PCLP1, and Human PCLP—On Western blot of human glomerular extract both mAbs 2A4 and 3D3 recognized a 160/165-kDa doublet similar to that described previously by Kerjaschki et al. (7) for the
highly conserved, we have used this region of the human PCLP since the intracellular domain of PCLP appears to be recognized by mAbs 2A4 and 4F10.

epitopes present in human PCLP and rabbit PCLP1 which are recognized and immunoprecipitated rat podocalyxin. We conclude that rat podocalyxin contains the conserved intracellular domain of rabbit PCLP1 and human PCLP (Fig. 7, panel C). The anti-human PCLP mAbs in the rat. The anti-podocalyxin mAb 5A recognized a 140-kDa band on Western blot of rat glomerular extract (Fig. 7, panel C). In contrast the mAb 3D3 did not react across species.

Immunoprecipitations of rat glomerular extracts were performed with the anti-PCLP mAbs 2A4 and 4F10 or control IgM antibodies to define further the protein recognized by the anti-PCLP mAbs in the rat. The anti-podocalyxin mAb 5A recognized a 140-kDa band on Western blot of rat glomerular extract immunoprecipitated with mAbs 2A4 and 4F10 (Fig. 7E). The mAb 5A also recognized a 140-kDa band (rat podocalyxin) in the glomerular extract lane. The anti-human PCLP mAb 2A4 also recognized a 140-kDa band on Western blot of rat glomerular extract (Fig. 7, panel C). The anti-human PCLP mAb 2A4 also recognized a 140-kDa band on Western blot of rat glomerular extract immunoprecipitated with mAbs 2A4 and 4F10 (Fig. 7E). The mAb 5A also recognized a 140-kDa band (rat podocalyxin) in the glomerular extract lane. The anti-human PCLP mAb 2A4 also recognized a 140-kDa band on Western blot of rat glomerular extract (Fig. 7, panel C).

Conservation of the PCLP Intracellular Domain between Species—Since the intracellular domain of PCLP appears to be highly conserved, we have used this region of the human PCLP cDNA to probe for sequences homologous to this domain in the genomic DNAs of a wide range of species (Fig. 8). Under high stringency conditions one restriction fragment (two for monkey) of genomic DNA was found to hybridize to this probe for all eukaryotes tested except yeast. This observation supports the conclusion that the intracellular domain of PCLP is highly conserved in vertebrates and suggests that podocalyxin-like protein is a single copy gene.

Kerjaschki and colleagues have described previously a human protein similar to rat podocalyxin which has a size and tissue distribution similar to those of the protein characterized in this report (7). However this molecule recognized by mAb PHM5 differs from rat podocalyxin in apparent molecular mass on SDS-PAGE gels and the sizes of proteolytic fragments seen on peptide digests. Kerjaschki and colleagues therefore suggested that this human sialoglycoprotein may be evolutionary distinct but have a function similar to podocalyxin (rat). Our findings would support the contention that the podocalyxin-like protein from man would have a different pattern from rat podocalyxin on proteolytic peptide maps because of a poorly conserved amino acid sequence in the extracellular domain, and it would have a different apparent molecular mass on SDS-PAGE. We conclude that these proteins (rat podocalyxin, human PCLP, and rabbit PCLP1) are derived from a single related gene in rat, rabbit, and human which codes for a molecule with a highly conserved intracellular and transmembrane and a variable extracellular domain.

The calculated putative size of human PCLP and rabbit PCLP1 peptide is similar, but the apparent mass on SDS-PAGE differs considerably suggesting that post-translational modification differs considerably among species. We reported
previously for rabbit PCLP1 that the discrepancy between the calculated size of the peptide and the observed mass on SDS-PAGE is accounted for by post-translational modifications, most likely glycosylation. The interspecies differences in apparent molecular mass are likely to be due to differences in glycosylation as well. We speculate that the major function of the PCLP extracellular domain is to support large negatively charged carbohydrate residues which contribute to the podocyte’s anionic glyocalyx. The composition of the extracellular domain peptide framework shows considerable drift between species and even within species as reported here. If this is the case we speculate that this molecule might be a target for immune recognition on the endothelial surface of transplanted organs.

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