Polycystin-1: immunoaffinity isolation and characterisation by mass spectrometry

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Abstract Polycystin-1 is a putative 460 kDa membrane protein with a unique structure and is possibly representative of a new family of proteins. Its structure suggests an involvement in cell signalling and cell–matrix interactions. The amino acid sequence of polycystin-1 has to date been predicted from its gene sequence. This, to our knowledge, is the first report of the isolation and analysis of polycystin-1 at the protein level using mass spectrometry to confirm its predicted structure. The availability of purified polycystin-1 will allow a new approach to unravelling the complexity of the cell–cell and cell–matrix interactions of this large molecule in normal cells and its perturbation in disease. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Autosomal dominant polycystic kidney disease; Polycystin-1; Immunoaffinity purification; Anti-peptide antibody; Mass spectrometry; Protein identification

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common hereditary diseases in humans, with an incidence rate of about 1:1000 [1]. The predominantly affected organ in ADPKD is the kidney, where tubule sections enlarge to form epithelial-lined fluid-filled cysts [2]. There are different forms of ADPKD caused by mutations in at least three different alleles. Approximately 85% of ADPKD cases are caused by mutations in the *PKD1* locus. The rest of the ADPKD cases are accounted for by mutations in the *PKD2* and possibly the *PKD3* loci. PKD2 has a late onset and is less severe so some cases may be undiagnosed [3]. The *PKD1* protein product, polycystin-1, is predicted to be a plasma membrane-associated glycoprotein that contains 4302 amino acids and has a molecular weight of about 460 kDa [4,5]. Polycystin-1 is predicted to have a unique structure unlike that of any known protein and may be representative of a new family of proteins. The extracellular part of polycystin-1 has two putative leucine-rich repeats flanked by cysteine-rich domains, a cell-wall and stress-response component domain, a C-type lectin domain, a low-density lipoprotein domain, 16 'Ig-like' PKD repeats and a domain showing high homology to the sea urchin receptor for egg jelly. The intracellular part contains several potential phosphorylation sites, a proline-rich sequence, a heterotrimeric G protein activation sequence and a coiled-coil domain (reviewed in [6]). It is therefore thought that polycystin-1 may be involved in cell–cell and cell–matrix interactions, which are important in renal development but the nature of its involvement in the pathogenesis of ADPKD is not currently understood.

Previous studies in our laboratory have established that the extracellular component laminin has the ability to enhance the proliferation of tubular epithelial cells in vitro [7] and more recent studies have demonstrated using a fusion protein that the polycystin-1 C-type lectin domain binds carbohydrate structures present in type IV collagen [8]. Polycystin-1 expression in embryonic development of human tissues was measured using antibodies prepared against peptide sequences of the C-terminal region of the molecule and demonstrated that the staining pattern in the kidney varied with the stage of development [9].

Polycystin-1 interactions with potential intracellular and extracellular ligands and their roles in cell signalling have to date been studied using synthetic peptides and fusion proteins of particular sections of the molecule rather than the whole protein [8,10–14]. Furthermore, there are still discrepancies between the reported distributions of polycystin-1 in the literature [6]. Some of these discrepancies would be resolved by the availability of antibodies against the whole molecule. We therefore set out to isolate polycystin-1 from embryonic kidney fibroblast cells.

To our knowledge, there have not been any analytical studies confirming the structure or glycosylation of polycystin-1 as predicted from its gene sequence. Here we describe a method for the purification of intact whole polycystin-1 and confirm its identity and predicted structure using mass spectrometry.

2. Materials and methods

2.1. Extraction of soluble proteins from cells

Embryonic kidney epithelial cell monolayers (293) were cultured in Dulbecco's modified essential Eagle's medium supplemented with 10% foetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine (Sigma, UK). Confluent fibroblast cells (approximately 1.5×10^6 cells) were washed with phosphate-buffered saline (PBS), drained and placed on ice. Cells were lysed for 30 min using lysis buffer (100 μl, 50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.5% DOC, 0.1% SDS, 50 mM NaF, 5 μg ml^{-1} pepstatin, 25 μg ml^{-1} aprotinin, 25 μg ml^{-1} leupeptin, 5 mM EGTA and 1 mM PMSF,

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Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; MALDI-TOF MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

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Sigma, UK) followed by centrifugation at 10 000 × *g* for 10 min at 4°C. The supernatant was dialysed against 100 mM $NaHCO_3$ buffer, pH 8.6, prior to mixing with the antibody matrix.

2.2. Generation of polyclonal anti-peptide antiserum

Polyclonal antibodies were generated in rabbits against an antigenic polypeptide corresponding to amino acid residues 3147–3163 of polycystin-1 [9]. The peptide was coupled to keyhole limpet haemocyanin for immunisations and to ovalbumin for ELISA analyses, using *m*-maleimidobenzoyl-*N*-dihydroxysuccinimide ester (Sigma, UK) [15].

2.3. Purification of anti-peptide antibodies

The antigen-affinity gel was prepared by coupling the antigenic polypeptide to ω-aminohexyl-Sepharose 4B using *N*-γ-maleimidobutyryloxysuccinimide ester (Sigma, UK) [16]. The immunoglobulin fraction of the antiserum (10 ml) was first precipitated with 50% ammonium sulphate, redissolved in 100 mM sodium phosphate buffer, pH 7.5 (10 ml), dialysed against 40 mM phosphate buffer, pH 8, and mixed with the antigen-affinity gel (5 ml) for 2 h at room temperature. The gel was transferred to a column, washed with cold TBS, pH 7.5, and 1 ml fractions were collected on ice until the absorbance at 280 nm fell to 0.05. After washing with 20 ml of cold distilled water, bound antibodies were eluted with 100 mM glycine–HCl, pH 2.5. The pH of the eluted fractions was adjusted with 20 μl of 1 M Tris–HCl, pH 8. The column fractions were analysed for antibody activity by direct ELISA using the peptide ovalbumin as the solid phase antigen. Fractions showing antibody activity were pooled and dialysed against PBS. Several purifications were performed as described and the resulting purified antibody fractions pooled.

2.4. Preparation of the antibody-affinity gel

The purified antibodies (20 mg) were coupled to 2 g of CNBr-activated Sepharose 4B (Sigma, UK) as described in [17]. The antibody-affinity gel (5 ml) was first washed with 500 ml of 100 mM $NaHCO_3$ containing 200 mM NaCl followed by a wash with glycine–HCl, pH 2.5, and finally washed with PBS, before being stored in PBS containing 0.1% sodium azide.

2.5. Immunoaffinity purification of polycystin-1

The 293 cell extract was mixed with the antibody-affinity gel overnight at room temperature. The gel was transferred to a column, washed and the bound antigen eluted with glycine–HCl, pH 2.5, as described above for the affinity purification of the antiserum. Samples of the column fractions (20 μl) were diluted in 100 mM $NaHCO_3$ (100 μl), allowed to adsorb to microtitre plate wells (Griener) and analysed for polycystin-1 content by direct ELISA. Eluted fractions showing high polycystin-1 content were pooled, dialysed against PBS and lyophilised.

2.6. Analysis of the purified polycystin-1 using SDS–PAGE and Western blotting

The lyophilised sample was then analysed by SDS–PAGE and Western blotting. SDS–PAGE in 8% polyacrylamide gels, transfer to nitrocellulose membranes and silver staining were performed using standard procedures [18–20]. The membranes were blocked in TBS-T (0.1% Tween 20) containing 10% milk, incubated with rabbit anti-polycystin-1 antibodies (1:1000) and goat anti-rabbit horseradish peroxidase conjugate (1:20 000), followed by detection using enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

2.7. Analysis of the purified polycystin-1 using mass spectrometry

Following SDS–PAGE, silver staining and Western blotting, the band corresponding to polycystin-1 was excised from the stained gel, destained using 30 mM potassium ferricyanide and 100 mM sodium thiosulphate and then digested using trypsin as described in [21]. The digested peptide fragments were extracted from the gel using 50% acetonitrile/0.1% trifluoroacetic acid, lyophilised, resuspended in 5 μl of water and analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using a LASERMAT 2000 mass spectrometer (Thermo Bioanalysis, Hemel Hempstead, UK). The sample was prepared by mixing 0.5 μl of the tryptic digest with 0.5 μl of α-cyano-4-hydroxycinnamic acid directly on the MALDI target. The monoisotopic mass values of the tryptic digest fragments were used to search the NCBI non-redundant (nr) database using the program ProFound and analysis of the tryptic cleavage of polycystin-1 was performed using the program ProteinInfo (http://www.proteometrics.com).

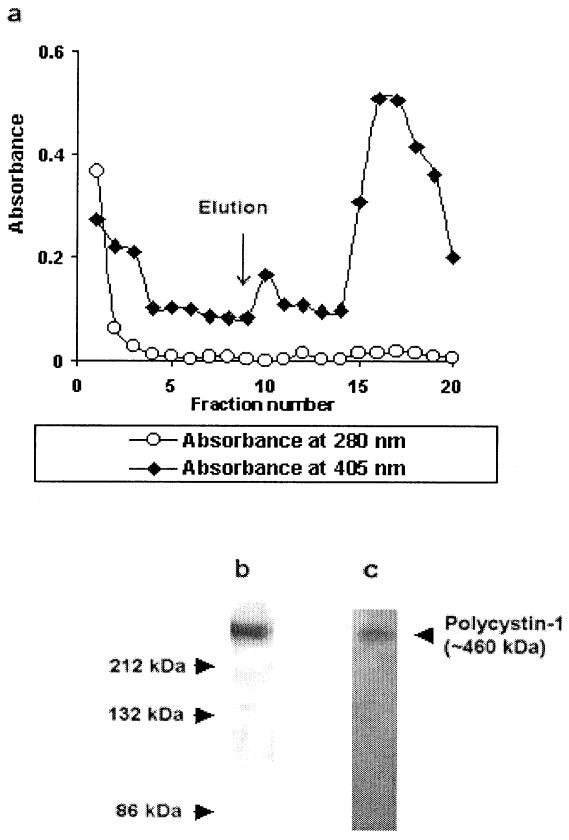

Fig. 1. The affinity purification of polycystin-1. a: Analysis of fractions obtained from the anti-polycystin-1 antibody matrix mixed with cell extract. The arrow indicates the start of elution with glycine–HCl, pH 2.5. Protein content was measured by absorbance at 280 nm and polycystin-1 content by absorbance at 405 nm following ELISA. Eluted fractions with high 405 nm readings following ELISA were pooled, dialysed and lyophilised as described. The lyophilised material was analysed by (b) SDS–PAGE followed by (c) Western blotting as described in the text.

3. Results and discussion

Following the extraction of proteins from 293 cells, an antibody-affinity matrix was used to prepare a purified sample of polycystin-1. Anti-polycystin-1 antibodies were affinity-purified prior to the preparation of the antibody matrix. Comparison of antibody activity in the antiserum before and after coupling (data not shown) indicated a high coupling efficiency. The extracted proteins were mixed with the antibody-affinity gel and the bound polycystin-1 was eluted using low pH (Fig. 1a). Eluted fractions containing high levels of polycystin-1 were pooled and treated as described above. The lyophilised sample was analysed by SDS–PAGE and Western blotting (Fig. 1b,c). A single band of approximately 460 kDa, corresponding to the predicted molecular weight of polycystin-1, was visualised. Although no other proteins copurified with polycystin-1 were detected by SDS–PAGE, the possibility of the occurrence of undetectable levels of copurified materials was not ruled out because of the limitations of SDS–PAGE and silver staining. Therefore, the visualised band was analysed by mass spectrometry instead of directly analysing the lyophilised isolated sample.

Mass spectrometry was used to further characterise the isolated protein because of the inaccurate estimation of the mo-

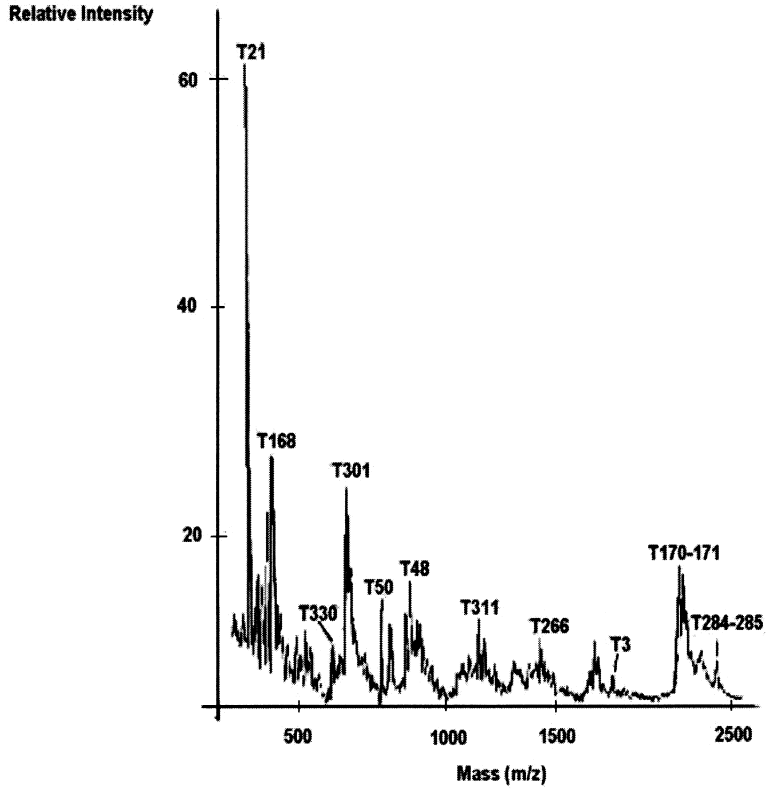

Fig. 2. Mass spectrum of the tryptic digest of polycystin-1 obtained by MALDI-TOF MS. The positions of selected tryptic fragments (T) numbered from the amino-terminal (obtained by the program ProteinInfo) are shown above the corresponding prominent peaks.

lecular weight due to the limitations of SDS–PAGE especially for such a high molecular weight protein. The protein band visualised in the stained gel was excised and subjected to in-gel tryptic digestion. The tryptic digest was extracted and its components detected by MALDI-TOF MS (Table 1 and Fig. 2). The molecular weights obtained for the fragments were used to search the NCBInr database using the program ProFound. The search parameters were as follows: database, NCBInr (4/17/2001); taxonomy category, *Homo sapiens*; protein mass range, 300–500 kDa; protein p*I* range, 0–14; search for, single protein; digest chemistry, trypsin; maximum missed cuts, 1; modification, by acrylamide (Cys); charge state, MH^+ and peptide mass tolerance (MON), ± 0.1 Da. The result of the search is a single hit which is the human polycystic kidney disease 1 protein, polycystin-1 (accession numbers AAC37576, A38971 and NP_000287) with an estimated Z score of 2.43 and sequence coverage of 18% (about 36% if the glycosylation sites of polycystin-1 are taken into consideration), i.e. the observed peptides cover 18% of the entire polycystin-1 amino acid sequence and 36% of the non-glycosylated parts of the protein. The result obtained is in the 99th percentile, i.e. there are 1% of random matches that can yield a higher Z score than this search. Using the program ProteinInfo, the theoretical tryptic cleavage of polycystin-1 as predicted from its gene

Table 1
Identities of selected tryptic fragments of polycystin-1 observed by MALDI-TOF MS

Tryptic fragment	Peptide sequence (residue position in polycystin-1)	Observed mass (Da)	Calculated mass (Da)	Δ (Da)
T3	GCGPCEPPCLCGPAPGAACR (29–48)	1858.6	1858.7	−0.1
T49	YSPVVEAGSDMVFR (953–966)	1556.6	1556.7	−0.1
T50	WTINDK (967–972)	776.32	776.39	−0.07
T64	LTAYVTGNPAHYLFDWTFGDGSSNTTVR (1313–1340)	3090.3	3090.4	−0.1
T102	NHVSWAQAQVR (1954–1964)	1295.7	1295.6	+0.1
T110	AFNALGSENR (2042–2051)	1078.4	1078.5	−0.1
T127	VALPGVDVSR (2204–2213)	1012.6	1012.5	+0.1
T137	LAAGVEYTFSLTVWK (2330–2344)	1684.7	1684.8	−0.1
T212	HLDGDR (3153–3158)	712.31	712.33	−0.02
T241	TETLALQR (3507–3514)	931.59	931.52	+0.07
T263	SEELWPWMAHVLLPYVHGNQSSPELGPPR (3719–3747)	3326.5	3326.6	−0.1
T266	LQEALYPDPPGPR (3753–3765)	1452.6	1452.7	−0.1
T306	LWMGLSK (4138–4144)	834.36	834.45	−0.09
T330	VHPSST (4297–4302)	627.29	627.31	−0.02

Peptide matches were obtained using the programs ProteinInfo and ProteinFind (www.proteometrics.com). Δ (Da) is the difference between calculated and observed masses in Da.

sequence results in 330 tryptic fragments. The fragments we obtained by the experimental tryptic cleavage range from tryptic fragment numbers 3 to 330 (Fig. 2), which cover residues 29–4302 of polycystin-1.

Native polycystin-1 is predicted to have about 60 glycosylation sites (SwissProt accession number P98161). Some of the products of the tryptic digestion did not match the predicted masses which may possibly be due to the fact that they contain glycosylation sites and hence differ from the computer predicted masses. Glycosylation residues are thought to be found within the tryptic fragments T7–T13, T15–T20, T22–T47, T51–T63 and T68–T99 which are all predicted by the program ProteinInfo but not observed using the MALDI-TOF MS. Our results, therefore, do not rule out glycosylation of the native protein, but rather support glycosylation at the predicted positions. Furthermore, when the glycosylation sites are taken into consideration, the observed peptides cover 36% of the entire amino acid sequence of polycystin-1. The identity of the purified product as intact polycystin-1 is therefore confirmed by three independent pieces of evidence, namely molecular weight, antigenicity and MALDI-TOF MS.

This, to our knowledge, is the first report of an analytical study of polycystin-1 at the protein level, which confirms the molecular weight and structure as predicted from the gene sequence. The isolation of purified polycystin-1 will allow new approaches for the investigation of its cell–cell and cell–matrix interactions as well as its distribution during development and in ADPKD.

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