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Identification of a human homologue of the sea urchin receptor for egg jelly: a polycystic kidney disease-like protein

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Previous studies have shown sequence similarity between a region of the autosomal dominant polycystic kidney disease (ADPKD) protein, polycystin-1 and a sea urchin sperm glycoprotein involved in fertilization, the receptor for egg jelly (suREJ). We have analysed sequence databases for novel genes encoding PKD/REJ-like proteins and found a significant region of homology to a large open reading frame in genomic sequence from human chromosome 22. Northern analysis showed that this is a functional gene [termed the polycystic kidney disease and receptor for egg jelly related gene (*PKDREJ*)], but unlike polycystin-1, has a very restricted expression pattern; the ~8 kb transcript was found exclusively in testis, coincident with the timing of sperm maturation. The *PKDREJ* transcript was cloned by screening a testis cDNA library and RT–PCR which revealed a 7660 bp mRNA terminating with a 900 bp 3′UTR and a polyA tail. Comparison with genomic sequence showed that *PKDREJ* is intronless; sequencing the mouse orthologue revealed a similar structure. The predicted human PKDREJ protein has 2253 amino acids (calculated molecular mass 255 kDa) and sequence similarity over ~2000 amino acids with polycystin-1, corresponding to the predicted membrane associated region and the area of homology (~1000 amino acids) with the suREJ protein (the REJ module). The suREJ protein binds the glycoprotein coat of the egg (egg jelly), triggering the acrosome reaction, which transforms the sperm into a fusogenic cell. The sequence similarity and expression pattern suggests that PKDREJ is a mammalian equivalent of the suREJ protein and therefore may have a central role in human fertilization.

INTRODUCTION

Fertilization is initiated by binding of the intact sperm to the glycoprotein coat of the egg. This event triggers an influx of extracellular Ca^{2+} (1) which activates the acrosome reaction; an exocytic remodelling of the sperm outer membrane which releases the acrosomal contents. Acrosome reacted sperm are then able to penetrate the glycoprotein coat and fuse with the egg plasma membrane. In mammals, the precise steps involved in the initial recognition event are not fully determined (2). The mammalian egg glycoprotein coat is termed the zona pellucida (ZP) and in mice, the ZP protein, ZP3, is thought to be involved in the initial binding to sperm and to act as the inducer of the acrosome reaction (3). In a quest for the sperm ZP adhesion molecule several different proteins that bind ZP3 have been identified, including β-1,4 galactosyl transferase (GalTase) (4,5), a 56 kDa protein (sp56) (6) and a tyrosine-phosphorylated protein (p95) (7). However, in each case some doubt about the protein's precise role has come from sequence analysis (8,9), localization data (10), or the fertility of null mice (11).

In sea urchin, similar events are required for fertilization; binding of the sperm to the egg glycoprotein coat, egg jelly (EJ) and triggering the acrosome reaction by Ca^{2+} influx. However, in sea urchin, events are better characterized with a 210 kDa sperm membrane glycoprotein being the receptor for egg jelly (suREJ) which triggers the acrosome reaction (12). This assertion is supported by evidence that purified suREJ binds to EJ (13) and that a suREJ mAb induces the acrosome reaction, induction that is blocked by suREJ protein (14). Cloning and sequence analysis indicated that the suREJ has a single transmembrane domain and a large extracellular region consisting of an EGF motif, two C-type lectins and a region of homology of ~1000 amino acids with polycystin-1 (14,15).

Polycystin-1 is the product of the major gene, *PKD1*, for the common genetic disease, autosomal dominant polycystic kidney disease (ADPKD). Polycystin-1 is a large protein (4302 amino acids) with an extensive extracellular region that contains several functional motifs (16) including, the REJ homologous region (REJ module) (14,15) and a C-type lectin, plus an area predicted to contain multiple transmembrane domains. However, it is not closely related to any known protein family and its precise function remains unclear. A second ADPKD gene, *PKD2*, has also been identified. This encodes a 968 amino acid protein, polycystin-2, which is similar to part of the membrane associated region of polycystin-1 and shows homology to the α subunit of

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voltage gated Ca^{2+} and Na^{+} channels (17). Recently, a third polycystin-like molecule has been identified (polycystin-L) that is similar in structure to polycystin-2, but which has not been associated with renal cystic disease (18). To better understand the role(s) of PKD/REJ-like proteins we have searched cDNA and genomic databases for related molecules. This search identified a gene on chromosome 22 predicted to encode a 2253 amino acid PKD/REJ-like protein, PKDREJ. Expression studies showed that the polycystic kidney disease and receptor for egg jelly related gene (*PKDREJ*) is testis specific suggesting that it is a human homologue of the suREJ.

RESULTS

Identification of the gene *PKDREJ* in genomic DNA

BLAST and FASTA analysis of the EMBL database with polycystin-1 and -2 sequence revealed significant hits in two cosmids located in chromosome region 22q13 that had been sequenced as part of the Human Chromosome 22 Sequencing Project. Further studies of a third overlapping cosmid and analysis by PCR determined that these cosmids lay immediately end to end (Fig. 1a) and contained a single region of homology with polycystin-1 extending over 6 kb. The contiguous nature of the homology between genomic DNA and PKD1 protein sequence initially suggested a pseudo-gene; however, further analysis indicated that this was a functional locus, *PKDREJ*. The evidence for functionality was, firstly, that an open reading frame (ORF) of 6759 bp was maintained in genomic DNA and that the 5′ end of the ORF lay at a GC rich, CpG island (Fig. 1a). Secondly, Southern analysis of genomic DNA showed that the sequence was single copy (data not shown), and northern blotting revealed expression; however, unlike the ADPKD genes which are ubiquitously expressed in human organs (17,19), the *PKDREJ* mRNA was entirely restricted to testis (Fig. 1b). The observed size of the transcript, ~8 kb was consistent with the detected ORF plus 5′ and 3′ UTRs.

To understand better the significance of this unusual locus a P1 clone containing the mouse orthologue was identified and the gene containing region subcloned and sequenced. This revealed a similar structure to that found in man with a CpG island and ORF in genomic DNA of 6378 bp (Fig. 1a), transcribing a testis expressed mRNA.

Cloning human *PKDREJ*

To confirm the apparent intronless nature of *PKDREJ* a cDNA contig encompassing the human ORF and 3′ UTR was constructed by screening a testis cDNA library and RT–PCR from testis RNA (Fig. 1a). A cDNA contig of 7686 bp was assembled including a 3′UTR of 900 bp terminating with the atypical polyadenylation signal ATTAAA and a poly(A) tail. The first in-frame Met codon in the ORF is proposed as the start codon, because of its correspondence to the Kozak consensus and conservation between the predicted human and murine proteins. The majority of cDNAs confirmed the intronless nature of the gene. However, two possible alternatively spliced clones, alt g2 and alt g3, were characterized (Fig. 1a) that contain in-frame deletions of 768 and 273 nt respectively, each flanked by consensus splice sites. To determine if these clones represented the usual form of the transcript 'intronic' probes were designed and hybridized to a northern blot. In both cases these probes detected the ~8 kb transcript (data not shown) confirming that these regions were usually present in the *PKDREJ* mRNA, although they may represent a minor alternatively spliced product (Fig. 1b).

To analyse when the murine ortholog *Pkdrej* was expressed, mouse testis samples from newborn to adult stages were analysed by northern blotting (Fig. 1c). This experiment showed that expression began at ~2 weeks and continued into adult life, mirroring the production of mature spermatozoa (20).

The PKDREJ protein

The predicted human PKDREJ protein has 2253 amino acids and a calculated unglycosylated molecular mass of 255 kDa; the corresponding mouse protein is 2126 amino acids and 241 kDa. The human and murine proteins show 64% identity and 78% similarity. The PKDREJ proteins have predicted cleavable signal peptides and a region of sequence homology of ~1000 amino acids with polycystin-1 and the suREJ protein, corresponding to the previously defined REJ module (Fig. 2) (14,15). The remaining C-terminal region of the protein (apart from the predicted C-terminal tail) shows contiguous homology to polycystin-1 with the carboxy region also similar to polycystin-2 (Fig. 2). Analysis of hydrophobicity through this region indicated a similar integral membrane structure to polycystin-1 with 11 transmembrane regions proposed; the last six of which are also homologous to those found in polycystin-2 (Figs 2 and 3). The suREJ has just a single transmembrane domain, corresponding to the first found in PKDREJ and polycystin-1. No clear homology has been found with the N-terminal 170 amino acids of human PKDREJ between the signal peptide and REJ module and this region differs between humans and mouse with the murine protein 113 amino acids shorter. The proposed structures of PKDREJ, suREJ and the polycystin-1 protein are summarized in Figure 4.

DISCUSSION

From screening genomic sequence we have identified a gene predicted to encode a protein, PKDREJ, which has significant sequence similarity to polycystin molecules and the suREJ protein. Although the sequence identity with each protein is low (21–25%)—and barely detectable at the nucleic acid level—evidence of homology over a large region (over ~2000 amino acids with polycystin-1) and similar hydropathy profiles indicates that these are related proteins with related functions. The structure of PKDREJ, intermediate in size and complexity between polycystin-1 and -2 (Figs 2–4), initially suggested that PKDREJ may be associated with polycystic kidney disease. This possibility was excluded, however, by analysis of its expression pattern. The ADPKD proteins are widely expressed (17,19), consistent with the systemic nature of the disease (21), but *PKDREJ* is only expressed in testis. The pattern of expression, temporally and spatially coincident with sperm maturation, and the sequence similarity to the suREJ protein suggests that PKDREJ may be a human homologue of the sea urchin protein.

Characterization of the human and murine *PKDREJ* genes has revealed an unusual intronless structure; this is one of the largest intronless human coding regions described to date. Interestingly, there appears to be an enrichment of intronless genes expressed only in testis (22). Some of these are retrosposons of X encoded

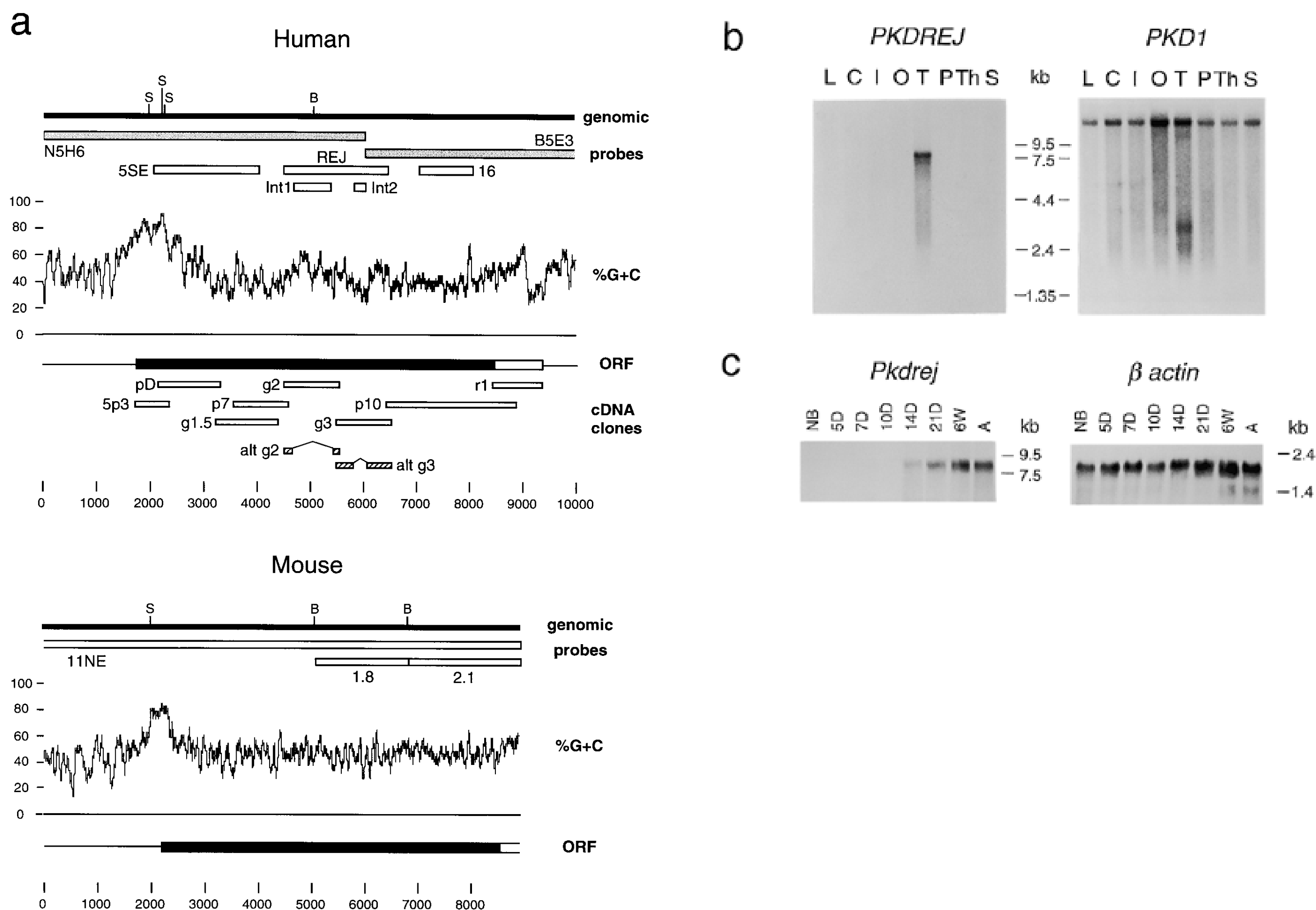

Figure 1. Structure and expression of *PKDREJ*. (**a**) Map of the genomic *PKDREJ* locus in human (top) and mouse (bottom) showing restriction sites for *Bam*HI (B) and *Sac*II (S). Genomic probes are indicated as: cosmid (shaded) or plasmid (open boxes) including the 'intronic' probes (Int) to test alternative splicing; GC content illustrating CpG islands at the 5′ ends of the genes; the open reading frames (ORFs; solid boxes) in genomic DNA and 3′UTRs (contiguous unshaded regions); cDNA clones (open boxes) including alternatively spliced clones (hatched). (**b**) Northern blot (2 µg poly(A)$^+$ RNA per lane) hybridized with the *PKDREJ* probe, REJ, and control *PKD1* probe (3A3) against adult human tissues: peripheral blood leukocytes (L); colon (C); small intestine (I); ovary (O); testis (T); prostate (P); thymus (Th) and spleen (S). *PKDREJ* was found to be expressed as a ~8 kb mRNA only in testis. Negative results were also obtained for *PKDREJ* in adult heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, adrenal, thyroid and stomach, and fetal brain, lung, liver and kidney (data not shown). A weak ~7.5 kb band seen with *PKDREJ* in testis was not seen with the Int1 probe suggesting that this may be a rare alternatively spliced product. The ~14 kb *PKD1* transcript is seen in all tissues. (**c**) 20 µg total mouse testis RNA from newborn (NB), 5 (5D),7 (7D), 10 (10D), 14 (14D) and 21 day (21D), 6 weeks (6W) and adult hybridized with the mouse *Pkdrej* probe, 2.1 (left) and rehybridized with β actin control (right).

genes, inserted into an autosome apparently to overcome the problem of expression in post meiotic spermatoids (22–24). Others however, like *PKDREJ*, show no evidence of retro-transposition and it is unclear whether these simplified gene structures may be favoured in the specialized transcriptional and post-transcriptional processing found during spermiogenesis (25).

Although the cloning and expression studies indicate that this is generally an intronless gene, two examples of possible alternatively spliced forms of *PKDREJ* were characterized. In both cases the alternative forms have in-frame deletions, so may generate protein products. However, they would encode proteins missing important regions; an area of the REJ module and the first transmembrane domain (changing the topology of the protein) for the alt g2 product and transmembrane regions 2 and 3 for the alt g3 transcript. Further work with PKDREJ antibodies will be required to see if these products are generated within the testis.

The role of the suREJ protein in fertilization has been shown both by its ability to bind egg jelly (13) and induction of the acrosome reaction by a suREJ mAb (14). A related role for PKDREJ in mammals is suggested from its testis specific expression; however, there are some differences in the structure of PKDREJ and suREJ (Fig. 4). PKDREJ does not contain the lectins found in suREJ, which probably interact with carbohydrates in the egg glycoprotein coat (14), so it is not clear whether PKDREJ could interact directly with the ZP. The N-terminal region of human PKDREJ does not appear to encode any particular motif and the difference between these areas in the human and murine proteins is interesting. It is possible that this area may play a role in species specific recognition of the egg. Further analysis of this area in other mammalian species would be informative.

The overall structure of PKDREJ is more closely related to polycystin-1 than suREJ, because of the inclusion of the multiple

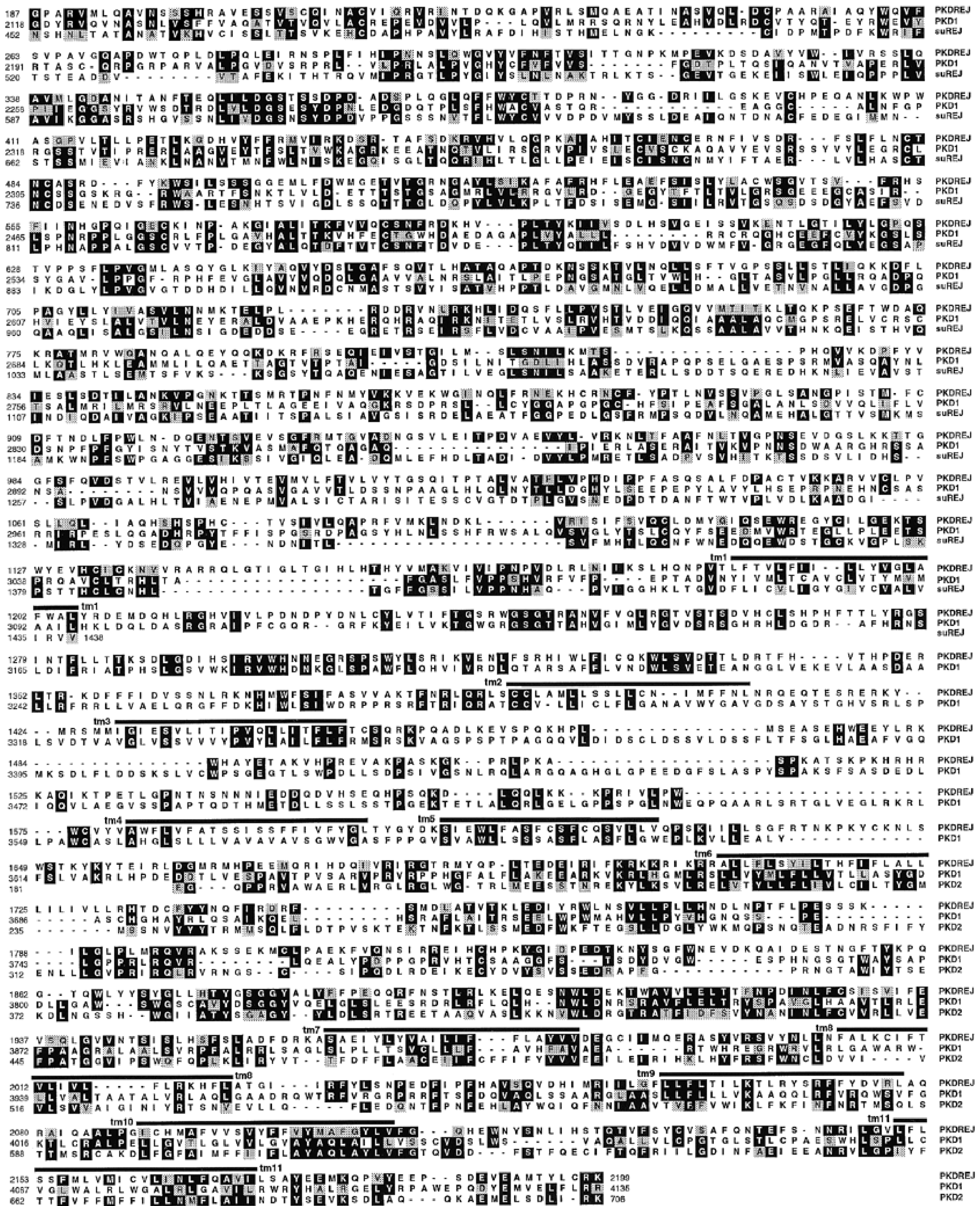

Figure 2. Alignment of human PKDREJ (AF116458) with human polycystin-1 (PKD1; L33243), sea urchin REJ (suREJ; Q26627) and human polycystin-2 (PKD2; U50928) proteins showing identity (solid boxes) and similarity to identity (shaded boxes). The approximate positions of the proposed transmembrane domains (tm) are shown as solid lines above the aligned sequence. PKDREJ and polycystin-1 are homologous over an area of ~2000 amino acids (2012 and 2017 amino acids, respectively) and show identity of 22 and similarity of 44.5%. The N-terminal region includes an area of homology extending over ~1000 amino acids with the suREJ protein (identity of 22.7 and similarity of 46.2% to PKDREJ), corresponding to the previously defined REJ domain (14,15). The C-terminal part of the PKDREJ/polycystin-1 homology is similar to polycystin-2 over a region of 525 amino acids (identity of 23 and similarity of 46.2% to PKDREJ). This region contains the final six proposed transmembrane domains of PKDREJ and polycystin-1 and the corresponding transmembrane domains of polycystin-2.

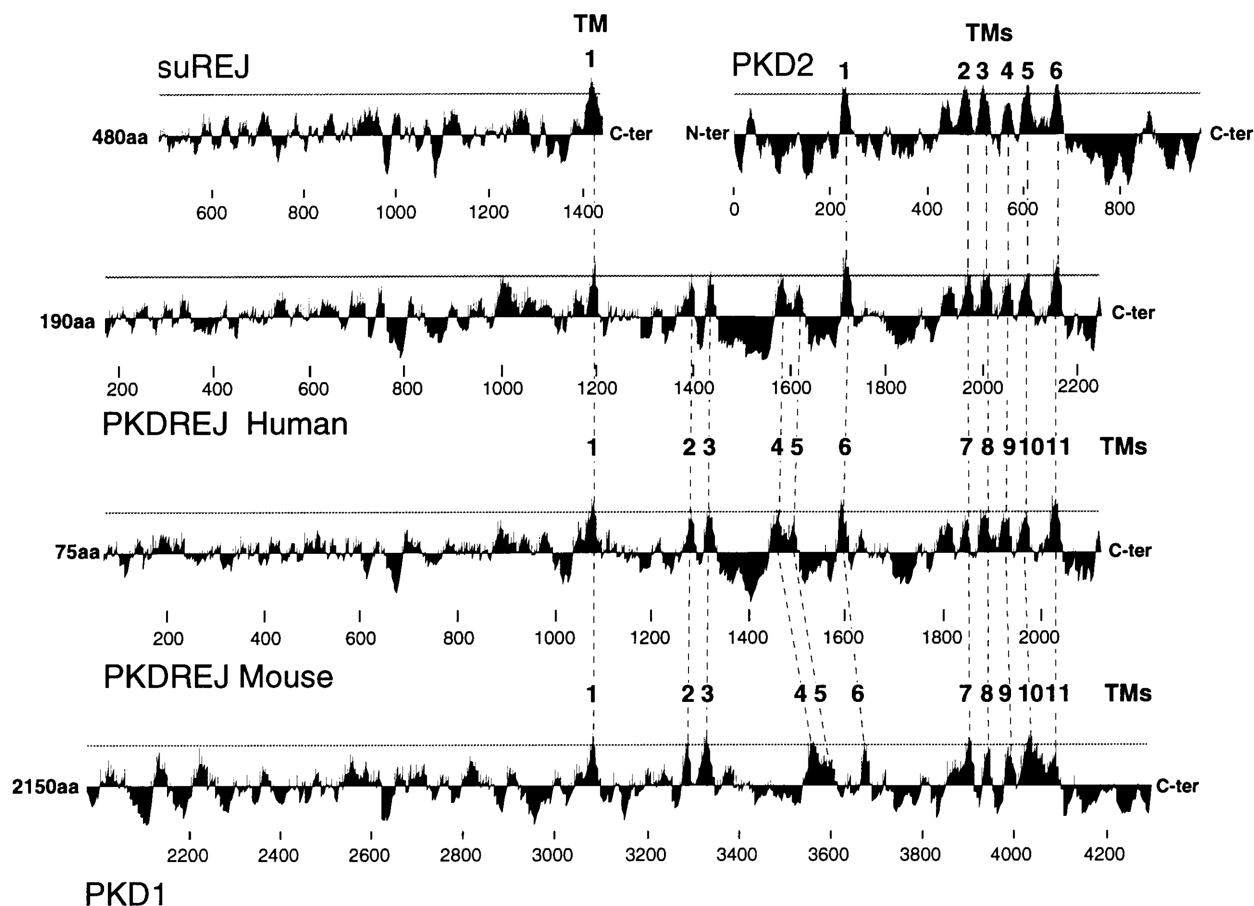

Figure 3. Hydropathy plots of the homologous areas of the suREJ (sea urchin), PKDREJ (human and mouse) and PKD1 and 2 (human) proteins using the Kyte–Doolittle method with a 20 amino acid window. A line shows the +2 point on the hydrophobicity scale and predicted transmembrane domains (TM) are numbered and aligned.

transmembrane domains (Fig. 4). The suREJ is thought to trigger the acrosome reaction by regulation of an ion channel (26) (a potential role of the conserved REJ module) and it is possible that the mammalian PKDREJ protein incorporates a subunit of such a channel in its structure. Previous analysis has shown similarity between the six transmembrane domains of polycystin-2, polycystin-L and the corresponding region of polycystin-1 with voltage gated Ca^{2+} or Na^{+} channel subunits (15,17,18). Although these channels consist of four subunits encoded by the same gene, in other cases, such as the voltage gated K^{+} channels (27) and trp Ca^{2+} channels (28), the subunits are encoded by separate genes and assemble as homo- or hetero-multimers to generate a variety of functional channels (29). It is possible that PKDREJ proteins may come together to generate a Ca^{2+} transporting channel or form part of a heteromultimer in combination with as yet unidentified subunits. Such a complex may be directly involved in initiating the acrosome reaction by transporting Ca^{2+} when triggered by ZP (30). In addition to suggesting a role for PKDREJ in ion transport, by inference, these sequence similarities also further support a role for the polycystin molecules in ion transport or ion regulation.

Further study of PKDREJ is now required, especially immunohistological analysis to determine its precise localization in the testis. Evidence of PKDREJ on mature sperm would support the proposed role as a suREJ homologue and fuller characterization would aid our understanding of human fertilization.

MATERIALS AND METHODS

RNA procedures

RNA was isolated from snap frozen human or mouse testis tissue using the SV Total RNA Isolation System (Promega, Madison, WI). RT–PCR was performed as previously described (31), or modified in GC rich areas by using the RT enzyme Superscript II (Gibco BRL, Paisley, UK) at 50°C in the presence of 10% DMSO with gene specific primers. The combined RT DNA polymerase, RetroAmp (Epicentre Technologies, Madison, WI), was used to amplify the 5′ clone, 5p3. To avoid confusion from genomic contamination of RNA, all samples were DNased prior to RT, and an RT negative control was included during each amplification. Mouse northern blots were prepared as previously described (31) and Clontech (Palo Alto, CA) human filters were employed.

Genomic and cDNA clones

The cosmids cN5H6 (Z93024), cB5E3 (Z79998) and cN98G1 (a bridging clone) were identified, characterized and sequenced by the Human Chromosome 22 Sequencing Group at the Sanger

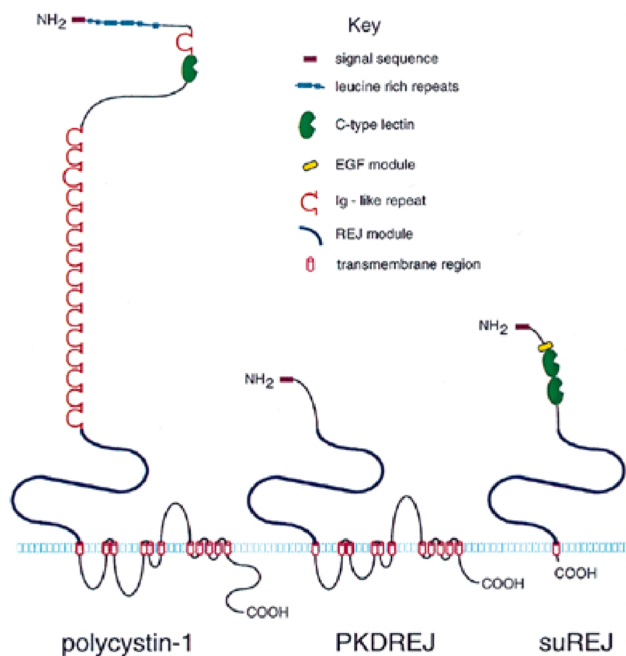

Figure 4. Diagrams of the comparative predicted structures of polycystin-1 (human), PKDREJ (human) and suREJ (sea urchin), see key for details.

Centre and can be obtained at www.sanger.ac.uk/HGP/Chr22/ . The human genomic clones REJ, 16, Int1 and Int2 were amplified from cosmid DNA and contain the following areas of the *PKDREJ* transcript: REJ (1972 bp) 2772–4743 nt; 16 (1012 bp) 5332–6343 nt; Int1 (707 bp) 2963–3669 nt and Int2 (216 bp) 4106–4321 nt. 5SE is a *Sac*I–*Eco*RI fragment (1965 bp) 338–2302 nt. The cDNAs p10 (2469 bp: 4711–7179 nt), p7 (1041 bp: 1837–2877 nt) and pD (1172 bp: 410–1581 nt) were obtained by screening 1.5×10^6 p.f.u. of a human adult testis cDNA library (5′ STRETCH PLUS cDNA library in λgt10; Clontech) by standard hybridization procedures with the genomic probes 16, REJ and 5SE, respectively. The clones g1.5 (1184 bp: 1496–2679 nt), g2 (1052 bp: 2786–3837 nt), g3 (1052 bp: 3768–4819 nt), 5p3 (636 bp: –27–611 nt), alt g2 (285 bp: 2786–2947 and 3716–3877 nt) and alt g3 (779 bp: 3768–4083 and 4357–4819 nt) were generated by RT–PCR. The 3′ cDNA r1 (937 bp: 6723–7659 nt) was isolated by 3′RACE using the primer 5′-CAGAGAAGAACAGCCACCGTTATC-3′ (6683–6706 nt) and testis cDNA (Marathon-Ready; Clontech) with the Expand Long Template PCR System (Boehringer Mannheim, Mannheim, Germany). The *PKD1* probe, 3A3, has been described previously (32).

The mouse P1 (ICRFP70302313Q5) was isolated by hybridizing the mouse P1 library #703 (33) with the human clone 16 at reduced stringency. An 11 kb *Not*I/*Eco*RI (11NE) fragment containing *Pkdrej* was subcloned and *Bam*HI fragments of ~2.1 and ~1.8 kb were subcloned as hybridization probes. The P1 ends within the 3′UTR of *Pkdrej*.

Sequence analysis

Human cDNAs and, where necessary, genomic clones were sequenced from plasmids using DyeDeoxy Terminator Cycle Sequencing (TaqFS or BigDye; ABI) and ABI 373A or 377 Sequencers. The mouse genomic clone 11NE was sonicated and subcloned as ~2 kb fragments into M13 and sequenced as above. Contigs were assembled with the programme Sequencher. Sequence was analysed with MacVector and BESTFIT and homologies sought in the GenEMBL and TREMBL databases using BLAST and FASTA. Alignment of the REJ modules and transmembrane regions was prepared with MegAlign (DNA-STAR) using the Clustal Method. SIGCLEAVE was used to predict signal peptides and cleavage sites. Potential transmembrane regions and protein topology were predicted with alignment with existing proteins (15,17) and using the Tmap programme.

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