Introduction

Polycystin-2, also known as TRPP2, is a member of the transient receptor potential (TRP) family of channels that are present in organisms from yeast (Palmer et al., 2005) to humans (Mochizuki et al., 1996; Barr and Sternberg, 1999; Gao et al., 2003; Watnick et al., 2003; Sun et al., 2004). Characteristic of TPR channels, polycystin-2 contains six transmembrane domains and functions as a Ca\(^{2+}\)-permeable, nonselective cation channel (Mochizuki et al., 1996; Hanaoka et al., 2000; Luo et al., 2003; Tsiokas et al., 2007). This channel is found predominantly in the ER, but also in the basolateral plasma membrane, mitotic spindles (Torres and Harris, 2006), and, notably, primary cilia (Pazour et al., 2002; Yoder et al., 2002). Polycystin-2 is of particular interest because mutations affecting it or polycystin-1, with which it interacts, are the primary cause of autosomal dominant polycystic kidney disease (PKD), a genetic disorder characterized by the formation of cysts, particularly in the ducts of the kidney and liver (Gabow, 1993; Torres and Harris, 2006).

Polycystin-2 located on primary cilia in the kidney is a pivotal factor in the etiology of PKD. The first clue linking PKD and cilia came from the characterization of intraflagellar transport (IFT) subunits in Chlamydomonas reinhardtii (Cole et al., 1998). IFT is a transport mechanism responsible for the assembly and maintenance of eukaryotic flagella (Kozminski et al., 1993; Rosenbaum and Witman, 2002; Scholey, 2003). The IFT machinery consists of the anterograde motor kinesin-2, the retrograde motor dynein 1b (dynein 2 in mammals), and IFT particles comprised of several proteins. These IFT components are conserved in organisms with cilia including humans, mice, zebrafish, Drosophila melanogaster, and Caenorhabditis elegans (Cole, 2003; Avidor-Reiss et al., 2004; Li et al., 2004). The link between PKD and cilia emerged when the mouse homologue of one IFT subunit, IFT88, was found to be encoded by Tg737 (Pazour et al., 2000), a gene previously shown to cause kidney defects very similar to those seen in humans with autosomal recessive PKD (Moyer et al., 1994). The tie between PKD and cilia became clearer as homologues of polycystin-1 and -2 were found on the cilia of sensory neurons of C. elegans (Barr and Sternberg, 1999) and the primary cilia of mouse kidney (Pazour et al., 2002; Yoder et al., 2002). These and subsequent studies have shown that almost all the proteins associated with the PKD phenotype (polycystin-1, polycystin-2, fibrocystin, nephrocystins, inversin, cystin, IFT88/polaris/Tg737, and Bardet-Biedl syndrome proteins) are present on cilia (Davenport and Yoder, 2005).

Understanding the importance of cilia in maintaining homeostasis of the kidney has stimulated the study of the sensory flagellar membrane. When IFT was blocked, fluorescence recovery after photobleaching of flagellar CrPKD2-GFP was attenuated and CrPKD2 accumulated in the flagella. Flagellar CrPKD2 increased fourfold during gametogenesis, and several CrPKD2 RNA interference strains showed defects in flagella-dependent mating. These results suggest that the CrPKD2 cation channel is involved in coupling flagellar adhesion at the beginning of mating to the increase in flagellar calcium required for subsequent steps in mating.

To analyze the function of ciliary polycystic kidney disease 2 (PKD2) and its relationship to intraflagellar transport (IFT), we cloned the gene encoding Chlamydomonas reinhardtii PKD2 (CrPKD2), a protein with the characteristics of PKD2 family members. Three forms of this protein (210, 120, and 90 kD) were detected in whole cells; the two smaller forms are cleavage products of the 210-kD protein and were the predominant forms in flagella. In cells expressing CrPKD2–GFP, about 10% of flagellar CrPKD2–GFP was observed moving in the flagellar membrane. When IFT was blocked, fluorescence recovery after photobleaching of flagellar CrPKD2–GFP was attenuated and CrPKD2 accumulated in the flagella. Flagellar CrPKD2 increased fourfold during gametogenesis, and several CrPKD2 RNA interference strains showed defects in flagella-dependent mating. These results suggest that the CrPKD2 cation channel is involved in coupling flagellar adhesion at the beginning of mating to the increase in flagellar calcium required for subsequent steps in mating.

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Abbreviations used in this paper: CrPKD2, Chlamydomonas reinhardtii PKD2; DIC, differential interference contrast; FMG, flagellar membrane glycoprotein; IFT, intraflagellar transport; mt, mating type; NaPPi, sodium pyrophosphate; PKD, polycystic kidney disease; TRP, transient receptor potential; UTR, untranslated region.

The online version of this paper contains supplemental material.
function and signaling pathways of kidney cilia. These studies support a model in which polycystin-1 and -2 comprise a mechanosensory complex that translates deflection of the cilium into signals associated with the control of the cell cycle (Chauvet et al., 2004; Low et al., 2006). How the signals generated by ciliary PKD2 are transferred to the cell body before entering flagella and is enriched in the flagellar membrane. Most of the C. reinhardtii PKD2 (CrPKD2) remains static in the flagella, but a fraction (<10%) moves at a rate similar to IFT. RNAi knockdown of CrPKD2 results in a decrease in the efficiency of mating, a process initiated by the contact of flagella of gametes of opposite mating types (mts), which activates a calcium signaling pathway required for the fusion of the gametes. These observations provide new insights into the function and dynamics of PKD2 in cilia.

Results

PKD2 homologue in C. reinhardtii

A predicted protein in the C. reinhardtii genome sequence database (C_590099 in assembly two) showed significant similarity to human polycystin-2 (e−6 × 10−11), and 10 peptide fragments of this protein were found in the flagellar proteome (Pazour et al., 2005). The available EST clones corresponding to this protein (Asamizu et al., 1999) were sequenced, and one, AV595567, encoded most of the protein as well as the 3′ untranslated region (UTR), including a polyadenylation signal (TGTA) typical for a C. reinhardtii gene. No EST clone encoded the putative transcriptional start site of this gene, so several sets of primers were designed based on genomic sequence to be used for RT-PCR. According to the sequence of these PCR products, the cDNA begins 410 bp upstream from the transcriptional site predicted by the C_590099 gene model (the cDNA sequence is available from GenBank/EMBL/DDJB under accession no. EF446617). The CrPKD2 gene (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200704069/DC1) encodes a protein of 1,626 aa (Fig. S1 B) with a molecular mass of 181 kD and a theoretical isoelectric point of 4.86.

A comparison of the predicted gene product with a variety of TRP channels shows that it clusters with the TRPP subfamily (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200704069/DC1) and exhibits the signatures typical of PKD2: it has six transmembrane domains that show homology to ion transport proteins, a coiled-coil domain at the C terminus, and an EF hand domain (Fig. 1 A). The six residues in the CrPKD2 sequence predicted to coordinate Ca2+ binding in the EF hand (positions 1, 3, 5, 7, 9, and 12) are allowable (Fig. 1 B): X (D), Y (DNS), Z (DENSTG), −Y (GP), −X (DENQSTAGC), and −Z (DE) (CrPKD2 residues are underlined; Prosite documentation). Moreover, the C terminus of CrPKD2 contains two acidic clusters; one has the casein kinase II phosphorylation motif (EGDDKDD), where S is the phosphorylation site predicted by NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/), and the other one does not (DEDEDDD; Figs. 1 A and S1). Acidic clusters bind phosphofurin acidic cluster sorting proteins, which are involved in routing PKD2 between the ER, Golgi apparatus, and plasma membrane in mammalian cells (Kottgen et al., 2005). Because acidic clusters are characteristic of mammalian PKD2, but not PKD2-like proteins and other PKD-related proteins were identified by comparative genomic and flagellar proteomic analyses (Li et al., 2004; Pazour et al., 2005). In this paper, we cloned a C. reinhardtii gene encoding a member of the PKD2 family. The protein is cleaved in the cell body before entering flagella and is enriched in the flagellar membrane. Most of the C. reinhardtii PKD2 (CrPKD2) remains static in the flagella, but a fraction (<10%) moves at a rate similar to IFT. RNAi knockdown of CrPKD2 results in a decrease in the efficiency of mating, a process initiated by the contact of flagella of gametes of opposite mating types (mts), which activates a calcium signaling pathway required for the fusion of the gametes. These observations provide new insights into the function and dynamics of PKD2 in cilia.

Figure 1. CrPKD2 is a member of the TRPP2 family. [A] Secondary structure of CrPKD2. CrPKD2 contains a coiled-coil domain at the N and C termini, an EF hand domain, six transmembrane domains (numbers denote amino acid positions), and acidic amino acid cluster domains. The pore region (P) is shown, between aa 1,342–3,377, by comparison to human and D. melanogaster PKD2 (Tsiekos et al., 1999; Vengerlitz et al., 2004). Not drawn to scale. [B] The EF hand domain of CrPKD2 contains the six conserved residues (coordination vertices) involved in calcium binding (positions 1, 3, 5, 7, 9, and 12).
(Kottna et al., 2005). CrPKD2 may be an orthologue of PKD2; however, this trait alone cannot unambiguously distinguish CrPKD2 from other PKD2 family members (i.e., PKD2L1 and PKD2L2). In addition, the casein kinase II phosphorylation site that controls targeting and function of PKD2 in the sensory cilia of C. elegans (Hu et al., 2006) is also present in CrPKD2.

Unlike other PKD2s, however, CrPKD2 is predicted to have a coiled-coil domain at its N terminus. Another distinguishing characteristic of CrPKD2 is that its first extracellular loop is atypically long (1,085 aa). Whereas two subfamilies of TRP channels, TRPP and TRPML, have an extracellular loop between the first two transmembrane domains, this loop is typically only ~200 aa residues long (Montell, 2005).

**CrPKD2 is cleaved**

Two polyclonal antibodies were generated against CrPKD2. One antibody (L1-PKD2) was directed against the first predicted loop (aa 358–1,019) expressed in *Escherichia coli* (Fig. 2 A) and the other (C-PKD2) was generated against a C-terminal peptide (aa 1,444–1,462). Immunoblots of whole cells probed with the L1-PKD2 revealed three proteins of 210, 120, and 90 kD (Fig. 2 B, lane 1). Only the two smaller fragments (120 and 90 kD) were present in flagella (Fig. 2 B, lane 2). The smaller forms of CrPKD2 do not appear to arise from alternate mRNA splicing because only one 6-kbp transcript, corresponding to the 210-kD form, hybridizes to CrPKD2 probes (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200704069/DC1). When immunoblots were probed with C-PKD2, two forms, 210 and 90 kD, were detected in whole cells (Fig. 2 B, lane 3) and only the 90-kD form was present in flagella (Fig. 2 B, lane 4). C-PKD2 did not recognize the 120-kD protein, suggesting that this species lacks the C terminus. The simplest explanation of the multiple forms of CrPKD2 is that posttranslational cleavage of 210-kD full-length CrPKD2 produces a 120-kD N-terminal fragment and a 90-kD C-terminal fragment. The epitope recognized by L1-PKD2 spans the cleavage site, so this antibody recognizes both fragments, whereas the C-PKD2 epitope is unique to the C-terminal fragment (Fig. 2 A). To test this explanation, an N-terminal peptide of the first loop (aa 358–583) was used to affinity purify an antibody from the L1-PKD2 antiserum. This antibody (N-PKD2) was predicted to only react with the 210- and 120-kD forms of CrPKD2. As expected, only these two bands were seen in immunoblots of whole cells and only the 120-kD band appeared in flagella (Fig. 2 B, lanes 5 and 6).

The identities of the 210- and 90-kD bands were further corroborated by tagging the C terminus of CrPKD2 with GFP and expressing the fusion protein in *C. reinhardtii* along with endogenous CrPKD2. Probing immunoblots of cells expressing CrPKD2-GFP with an antibody against GFP revealed two bands of 240 and 120 kD (Fig. 2 C, lane 1). These represent the 210-kD full-length CrPKD2 plus the 30-kD GFP tag and the 90-kD C-terminal fragment plus the 30-kD GFP tag. Only the 120-kD band was present in flagella (Fig. 2 C, lane 2). The identity of the GFP-tagged bands in whole cells was confirmed with the C-PKD2 antibody, which detected both GFP-tagged bands along with the two endogenous untagged bands (Fig. 2 C, lane 3).

These data fit the model that the 210-kD initial CrPKD2 product is

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**Figure 2. Flagellar CrPKD2 is cleaved.** (A) Diagram of the CrPKD2 protein showing the regions used to generate the Loop 1 and N- and C-terminal antibodies. The arrow marks the approximate location of the CrPKD2 cleavage site that generates fragments of 120 and 90 kD. (B) Immunoblots from whole cells (Cell) and flagella (F) of wild-type cells were probed with antibodies against Loop 1 or the N or C termini of CrPKD2. The Loop 1 antibody reacts with three bands (210, 120, and 90 kD; lane 1) in whole cells, but only the smaller two bands are present in flagella (lane 2). The C-terminal antibody does not react with the 120-kD N-terminal fragment (lanes 3 and 4), demonstrating that this fragment does not include the C terminus of the protein. The N-terminal antibody reacts with two bands (210 and 120 kD) in whole cells and the 120-kD form in the flagella (lanes 3 and 6). Thus, full-length CrPKD2 is cleaved into two fragments (120 and 90 kD) that are present in flagella. (C) Immunoblot of transformant expressing CrPKD2-GFP. GFP-tagging results in two bands that react with the GFP antibody (lane 1): a 240-kD band (the 210-kD full-length PKD2 plus 30 kD GFP) and a 120-kD band (the 90-kD C-terminal fragment plus 30 kD GFP). Only the smaller band is present in flagella (lane 2). Both bands also react with the C-PKD2 antibody (lane 3), as do the untagged CrPKD2 bands also present in these cells. (D) Immunoblots of transformant expressing CrPKD2-GFP probed with the anti–C-PKD2 antibody. The flagellar form of CrPKD2 is present in the cell bodies, indicating that cleavage occurs in the cell body. The same blot was probed with α-tubulin as a loading control.
cleaved near the C end of the first extracellular loop (aa 583–1,019) to produce a 120-kD N-terminal fragment and a 90-kD C-terminal fragment. A more detailed examination of immunoblots of the CrPKD2–GFP cells is presented in Fig. S4 (available at http://www.jcb.org/cgi/content/full/jcb.200704069/DC1). Further evidence that the 210-, 120-, and 90-kD bands are all derived from CrPKD2 came from analysis of CrPKD2 RNAi knock-down strains, in which the levels of all three bands were reduced (see Function of CrPKD2 in the mating of C. reinhardtii).

To determine whether the cleavage occurs in the cell body or flagella, we isolated extracts from bld1 cells, which have only short flagellar stubs because of a mutation in the gene encoding IFT52, a component of IFT complex B (Brazelton et al., 2001; Deane et al., 2001), and bld2 cells, which form basal bodies consisting of microtubule singlets instead of triplets because of a deficiency in α-tubulin (Dutcher et al., 2002). These aberrant basal bodies do not attach to the cell surface, form transitional fibers, nor nucleate flagellar microtubules (Dutcher et al., 2002). We also isolated extracts from wild-type cell bodies, i.e., cell bodies from which the flagella had been detached. Both full-length and cleaved CrPKD2 were present in bld1, bld2, and wild-type cell bodies (Fig. 2 D), indicating that CrPKD2 can be cleaved in the cell body before entering the flagella.

CrPKD2 is located in the flagellar membrane

When C. reinhardtii cells were stained with the N-PKD2 or C-PKD2 antibody, CrPKD2 was detected both in the cell body and flagella of vegetative cells and gametes (Fig. 3 A). No difference was detected in the distribution of CrPKD2 with the two antibodies. Immunoblot analysis of the flagella of cells undergoing gametogenesis showed that a fourfold increase in the amount of CrPKD2 gradually occurred during gametic differentiation induced when cells are starved for nitrogen (Fig. 3 B).

In the flagellar proteomic analysis, most of the CrPKD2 peptides were obtained from the axonemal fraction rather than the membrane and matrix fraction (Pazour et al., 2005). This was an unexpected result for a transmembrane protein, which raised the possibility that CrPKD2 might be a membrane protein tethered to the axonemal microtubules. To investigate this further, we developed methods for the isolation and purification of flagellar membranes. Membranes were released from isolated flagella with 0.1% NP-40 (4 °C for 30 min), and the axonemes were removed by centrifugation. The resultant membrane and matrix fraction contained membrane vesicles in addition to soluble flagellar proteins. Membranes either were sedimented directly from the membrane and matrix fraction to recover all the membranes present or further purified by density gradient centrifugation using Optiprep to obtain a band of uniform density (see Materials and methods). EM (Fig. 4 A) and SDS-PAGE analysis (Fig. 4 B) of the resultant membrane fractions showed they contained little axonemal contamination and were enriched in the major flagellar membrane glycoprotein (FMG) 1 (Bloodgood et al., 1986). CrPKD2 was concentrated in the flagellar membrane fraction (Fig. 4 B), confirming that it is a membrane protein. Interestingly, IFT proteins also were found in these membrane fractions.

Because some CrPKD2 remained on the axonemes after detergent extraction, we used more stringent conditions in an attempt to solubilize more CrPKD2. Freezing flagella in liquid nitrogen and thawing releases the matrix and a small amount of membrane, but much of the membrane remains in the axonemal pellet (not depicted). Such axonemes were extracted twice with 0.1 or 1% NP-40 at room temperature for 30 min, and after low speed centrifugation, the soluble fraction contained the membrane and the pellet contained the axonemes. No vesicles or other membrane remained associated with the axonemes after extraction in 1% NP-40 (Fig. 4 D), and all the membrane...
glycoprotein FMG-1 was released (Fig. 4 C); however, 20–25% of the CrPKD2 still remained in the axonemal fraction. Thus, these two membrane proteins show different patterns of solubilization in the flagella: FMG-1 is completely released by nonionic detergent, whereas flagellar CrPKD2 is present in two pools, one that is easily released by nonionic detergent and one that is not.

**Movement of flagellar CrPKD2**

To examine the movement of CrPKD2 in the flagellum, we made a construct designed to express CrPKD2 fused to GFP via a flexible linker of 10 aa. After transformation of cells with the construct, the fluorescence of PKD–GFP was detected in flagella. Like the immunofluorescence localization of CrPKD2 (Fig. 3 A), flagellar CrPKD2–GFP was concentrated in puncta (Fig. 5 A), which may represent clustered channels. When visualized by time-lapse imaging, <10% of the PKD–GFP particles moved continuously to the tip of the flagella at a velocity of ~1.6 μm/s, even though most of the puncta did not move (Fig. 5 and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200704069/DC1). Movement of CrPKD2–GFP toward the base was not detected, perhaps because the faint fluorescence of CrPKD2–GFP was bleached as the protein moved to the tip and became too faint to see as it returned to the cell body.
To assay the movement of CrPKD2–GFP in the absence of IFT, we generated a strain that expressed CrPKD2–GFP in a fla10 background. Because fla10 is a temperature-sensitive mutant of the IFT anterograde motor, we could monitor CrPKD2–GFP movement in the presence and absence of IFT at permissive and restrictive temperatures, respectively. 50 videos were generated of fla10 flagella at permissive and nonpermissive temperatures, and kymographs of these were analyzed for movement. In 10 sequences taken at permissive temperature, movement in the presence and absence of IFT at permissive and restrictive temperatures, respectively. 50 videos were generated for the movement of CrPKD2–GFP in flagella, but because some recovery of fluorescence occurred in the absence of IFT, other mechanisms may be involved.

Further evidence for a role of IFT in the movement of CrPKD2 was obtained from biochemical analysis of the flagella of fla10 cells after incubation at 32°C for 1 or 1.5 h in 10 mM Hepes, pH 7.2. At these times, IFT proteins were dramatically decreased (Fig. 7 A), no IFT particles could be observed moving by differential interference contrast (DIC) microscopy (Kozmiński et al., 1995), and the flagella had shortened to about half length (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200704069/DC1). Under these conditions, the concentration of flagellar CrPKD2 increased relative to total flagellar protein (Fig. 7 A). To determine if this increase was caused by the absence of IFT or simply flagellar shortening, the amount of CrPKD2 was examined in flagella induced to shorten by the addition of sodium pyrophosphate (NaPPi; Lefebvre et al., 1978), which causes the flagella to shorten as IFT continues unabated (Dentler, 2005; Pan and Snell, 2005). In this case, the concentration of flagellar CrPKD2 did not change (Fig. 7 B) as the flagella shortened. Thus, flagellar shortening does not per se affect the concentration of flagellar CrPKD2 when IFT is functioning properly; however, when IFT is defective, the concentration of CrPKD2 in the flagella increases, perhaps because it cannot be removed from the flagella in the absence of IFT.

**Function of CrPKD2 in the mating of C. reinhardtii**

The observation that CrPKD2 increases in the flagella of gametes (Fig. 3 B) suggests that CrPKD2 may have some function in the calcium-dependent steps of mating. The sexual cycle of *C. reinhardtii* begins with gametogenesis (Fig. 8 A). Vegetative (haploid) cells differentiate into gametes when they are starved for nitrogen. When gametes of m+ and – strains are mixed, the flagella of gametes of opposite mts adhere to each other, mediated by mt-specific agglutinins on the flagellar membrane (Bergman et al., 1975). Flagellar adhesion is followed by a cascade of signaling events that result in the shedding of the cell wall, formation of mating structures on mt+ and – gametes, and ultimately cell fusion (Fig. 8 A; Pasquale and Goodenough, 1987; Quarmbry, 1994; Pan and Snell, 2000; Wang et al., 2006). The early stages of this cascade are blocked by drugs that interfere with Ca2+ signaling (Snell et al., 1982; Bloodgood and Levin, 1983; Goodenough et al., 1993), suggesting that an influx of Ca2+ is required as an early step in mating. This influx is...
followed by the phosphorylation and activation of a cyclic GMP-dependent protein kinase (CrPKG), which in turn increases the concentration of flagellar cAMP by up-regulating an adenylate cyclase in the flagellar membrane (Fig. 8 B; Pasquale and Goodenough, 1987; Wang and Snell, 2003; Wang et al., 2006). Ultimately, the cAMP concentration in the cell body rises and the mt+ and − gametes elaborate mating structures that touch to initiate cell fusion.

We took advantage of RNAi to knockdown the endogenous CrPKD2 and examine the function of CrPKD2 in mating. The RNAi construct consisted of genomic CrPKD2 DNA including its native promoter with the corresponding cDNA in reverse orientation (Fig. 9 A). This strategy has been shown to efficiently knockdown endogenous proteins in *C. reinhardtii* (Fuhrmann et al., 2001; Huang and Beck, 2003). Transformants were obtained in which CrPKD2 was decreased to 10% of wild-type

**Figure 6.** CrPKD2–GFP FRAP is reduced in the flagella of fla10 cells at restrictive temperature. (A) An ~2-μm segment of a fla10 flagellum containing CrPKD2–GFP is shown (box in DIC panel) before bleaching, after bleaching, and after 2 min of recovery. The rest of the flagellum is not visible because it is not illuminated. (B) The fluorescence in the bleached area increased in the flagellum of fla10 cells at non-restrictive temperature after 1 and 2 min after photobleaching. In fla10 cells at restrictive temperature, recovery only reached 9.4 versus 19.3% at permissive temperature. (C) In control (pf18) cells at 22 and 32°C, fluorescence recovery is similar to that of fla10 cells at room temperature. Bar graphs represent mean ± SEM (statistics determined by t test).

**Figure 7.** Flagellar CrPKD2 increases when IFT is blocked. (A) Wild-type and fla10 cells were shifted to 32°C for 1 or 1.5 h, and immunoblots of isolated flagella were probed with antibodies as listed. Note the rise in CrPKD2 when IFT proteins disappear in flagella from fla10 cells. (B) Cells were treated with 20 mM NaPPi to induce flagellar resorption, and flagellar proteins were probed on immunoblots as in A. CrPKD2 did not increase in these flagella during resorption under this condition.

**Figure 8.** (A) A scheme of the mating process in *C. reinhardtii*. (B) An electron micrograph of a wild-type flagellum containing the CUB-PKD GFP fusion protein is shown. Bar, 1 μm.

**Figure 9.** RNAi knockdown of CrPKD2. (A) A scheme of the RNAi strategy used to knockdown endogenous CrPKD2. The RNAi construct consisted of genomic DNA including its native promoter and corresponding cDNA in reverse orientation. Transformants were obtained in which CrPKD2 was decreased to 10% of wild-type.

**Figure 10.** Fluorescence recovery measurements of CrPKD2–GFP FRAP in fla10 cells. (A) A scheme of the RNAi strategy used to knockdown endogenous CrPKD2. The RNAi construct consisted of genomic DNA including its native promoter and corresponding cDNA in reverse orientation. Transformants were obtained in which CrPKD2 was decreased to 10% of wild-type.

**Figure 11.** Immunoblots of isolated flagella from wild-type and fla10 cells. Note the rise in CrPKD2 when IFT proteins disappear in flagella from fla10 cells.
levels (Fig. 9 B). Mating efficiency was measured as the percentage of gametes fused to form quadriflagellates after mixing mt+ gametes of wild-type or CrPKD2-depleted strains with mt− wild-type gametes (see Materials and methods). Compared with wild-type gametes, the RNAi cells showed up to a 75% reduction in mating efficiency, and the severity of the defect correlated with the extent of CrPKD2 depletion (Fig. 9, B and C).

Inefficient mating could be caused by a failure of cells to form gametes when starved for nitrogen. To determine whether CrPKD2-depleted cells expressed agglutinins on their flagellar surface during gametogenesis, aggregation of mt+ and − gametes was observed. RNAi knockdown gametes appeared to adhere normally to wild-type gametes of the complementary mt, demonstrating that there was no obvious defect in gametogenesis and that the flagellar agglutinins were expressed normally on the flagella of the CrPKD2-depleted gametes. For a more complete assessment of the ability of the CrPKD2-depleted gametes to mate, mating was done in the presence of cAMP. The addition of cAMP to the mating reaction bypasses the initial stages of the signaling pathway where a calcium influx is required (Fig. 8 B; Goodenough, 1989). The mating efficiency was restored in CrPKD2-depleted gametes by the addition of 15 mM dibutyryl-cAMP and 0.15 mM papaverine, indicating that these gametes were competent to mate. To investigate the effect of CrPKD2 depletion on signaling events upstream of the increase in cAMP, the phosphorylation state of a CrPKG, a protein known to be phosphorylated after flagellar adhesion in wild-type cells (Wang and Snell, 2003; Wang et al., 2006), was assayed. Phosphorylation of CrPKG was reduced in matings of the RNAi46 strain to the wild type (Fig. 9 D), suggesting that CrPKD2 functions in mating between flagellar adhesion and CrPKG phosphorylation. The residual phosphorylation of CrPKG in these matings was caused by its phosphorylation in the wild-type gametes used to mate with the CrPKD2-depleted gametes.

Discussion

In this study, the C. reinhardtii homologue of a PKD2 family member was cloned. CrPKD2 was found to have the hallmarks of vertebrate polycystin-2, a cation channel located on primary cilia (Pazour et al., 2002; Yoder et al., 2002). We have exploited various advantages of this organism to investigate the flagellar form of CrPKD2 and examine its signaling function. This work shows that CrPKD2 is cleaved in the cell body, that the cleavage products can be found in isolated, purified flagellar membranes, that some of the CrPKD2 is immobilized, possibly by linkage to the flagellar axoneme, and that a small portion of the total CrPKD2 in the flagella moves in an IFT-dependent manner. By use of RNAi CrPKD2 knockdowns, we have also shown that the CrPKD2 channel plays a role in the initial steps of the well-characterized signaling cascade that occurs in the flagella during mating.
Flagellar CrPKD2 is cleaved

Analysis of the *C. reinhardtii* flagellar proteome indicated that a PKD2 family homologue was present in *C. reinhardtii* flagella (Pazour et al., 2005). Cloning and sequencing this gene indicated that this protein has the hallmarks of PKD2, including the six transmembrane domains showing homology to cation channels. Immunoblots of purified flagella confirmed that the protein is present in flagella. Interestingly, the full-length (210-kD) form of CrPKD2 was not present in the flagella; rather, two smaller forms (120 and 90 kD) were present. This is the first study showing cleavage of a PKD2 family member. These fragments can be generated in the cell body by cleavage of full-length CrPKD2. Although it seems likely that the CrPKD2 fragments move into the flagella after cleavage, we cannot rule out the possibility that full-length CrPKD2 moves into the flagella and is rapidly cleaved.

Like CrPKD2, other ciliary membrane proteins involved in PKD are known to undergo cleavage. For example, the long extracellular N terminus of PKD1 is cut near the first transmembrane domain and reattached to the C-terminal fragment (Qian et al., 2002). Likewise, fibrocystin undergoes several proteolytic cleavages, resulting in an extracellular fragment that is shed from the primary cilium (Kaimori et al., 2007). Proteolysis is also involved in the maturation of channels such as the heterotrimeric epithelial Na\(^+\) channel, in which the cleavage of the extracellular loops occurs at two sites within the \(\alpha\) subunit and at a single site within the \(\gamma\) subunit (Carattino et al., 2006). The inositol 1,4,5-trisphosphate receptor is cleaved by caspase 3, removing a regulatory element from the channel, and thereby allowing a rapid increase in cytosolic Ca\(^{2+}\) during apoptosis (Assefa et al., 2004; Nakayama et al., 2004). One member of the TRP family of channels belonging to the TRPML class is also known to undergo cleavage (Kiselyov et al., 2005; Miedel et al., 2006). This class, like CrPKD2, is characterized by a large extracellular loop, and in both cases proteolysis occurs in this loop. Thus, cleavage is emerging as a mechanism for regulation of channel activity and may be a general feature of TRP channels with large extracellular loops, including members of the TRPP2 family.

To further study flagellar CrPKD2, we optimized methods for isolating flagellar membranes using nonionic detergent, either with or without freeze–thaw treatment, to remove the membranes from isolated flagella followed by purification of the released membranes on Optiprep density gradients. The resultant membranes had little axonemal contamination as determined by thin section EM and immunoblots of the membrane fractions with axonemal antibodies and contained most of the flagellar CrPKD2. The remainder of the CrPKD2 appeared to be bound to the axoneme, and the two CrPKD2 fragments partitioned together in both the membrane and axonemal fractions. Cofractionation leaves open the possibility that the two fragments interact with each other, as is the case with TRPML-1 (Kiselyov et al., 2005), but such an interaction has not yet been demonstrated. Although we do not know the significance of the apparent attachment of CrPKD2 to the axoneme, it may be instrumental in CrPKD2 function. Interactions of kinesin-2 and tubulin with PKD2 have been shown to affect PKD2 activity in vitro (Wu et al., 2006; Montalbetti et al., 2007).

Figure 9. CrPKD2 is involved in mating.

To further study flagellar CrPKD2, we optimized methods for isolating flagellar membranes using nonionic detergent, either with or without freeze–thaw treatment, to remove the membranes from isolated flagella followed by purification of the released membranes on Optiprep density gradients. The resultant membranes had little axonemal contamination as determined by thin section EM and immunoblots of the membrane fractions with axonemal antibodies and contained most of the flagellar CrPKD2. The remainder of the CrPKD2 appeared to be bound to the axoneme, and the two CrPKD2 fragments partitioned together in both the membrane and axonemal fractions. Cofractionation leaves open the possibility that the two fragments interact with each other, as is the case with TRPML-1 (Kiselyov et al., 2005), but such an interaction has not yet been demonstrated. Although we do not know the significance of the apparent attachment of CrPKD2 to the axoneme, it may be instrumental in CrPKD2 function. Interactions of kinesin-2 and tubulin with PKD2 have been shown to affect PKD2 activity in vitro (Wu et al., 2006; Montalbetti et al., 2007).
PKD2 out. Indeed, KLP-6 is required to move PKD2 into cilia. The accumulation of PKD2 in the cilia of all three organisms would implicate calcium as a key player in the initial stages of this process.

CrPKD2 is involved in the mating signal pathway
PKD2 plays a role in the mating of several organisms as well as C. reinhardtii: the acrosomal reaction triggered when sea urchin sperm bind egg jelly requires PKD1 and 2 (Neill et al., 2004); the proper entrance of sperm into the sperm storage organ of female D. melanogaster is dependent on PKD2 (Gao et al., 2003; Watnick et al., 2003); and LOV1, an OSM-3 homologue that is also found in the C. reinhardtii genome, transports cyclic nucleotide-gated channels to the cilia in murine cells (Jenkins et al., 2006). Support for a mechanism other than IFT would accumulate there in the absence of removal by retrograde IFT, which is also defective in fla10 at restrictive temperature (presumably because the retrograde motor is no longer being carried to the distal tip of the flagellum; Cole et al., 1998; Iomini et al., 2001). Although no retrograde movement of CrPKD2–GFP was detected, such movement may occur beneath our level of detection.

One of the early stages of the signaling pathway, phosphorylation of CrPKG, does not occur after inactivation of IFT (Wang and Snell, 2003; Wang et al., 2006) or depletion of CrPKD2 (this paper). When gametes of the temperature-sensitive kinesin-2 mutant fla10 are incubated at 32°C before mixing with gametes of the opposite mt, flagellar adhesion occurs, but the activation of CrPKG is abolished (Pan and Snell, 2002; Wang and Snell, 2003; Wang et al., 2006) and the cells are unable to fuse. Likewise, in this study, CrPKG was not activated in CrPKD2-depleted gametes even though their flagella adhered to those of gametes of the opposite mt. Given that PKD2 can act as a mechanosensitive cation channel, it is reasonable to speculate that agglutinin binding and flagellar adhesion serve as the stimuli that cause CrPKD2 to admit a Ca\(^{2+}\) influx into the flagellar compartment and initiate the signaling pathway. Because there is no clear homologue of PKD1 in the C. reinhardtii genome, it may be that agglutinins assume the role of regulating the channel activity of CrPKD2 during the mating of C. reinhardtii.

Several issues about ciliary signaling are highlighted by this work on flagellar PKD2. First, is cleavage of ciliary PKD2 a widespread phenomenon, and what is its role in the function of the protein? Second, how is the signal that is initiated by increased Ca\(^{2+}\) in the cell transmitted to the cell body? It is doubtful that this occurs by calcium diffusion because free calcium ions are generally unable to diffuse more than 0.5 μm in the cell given the typical concentration of calcium-binding proteins (Chlamham, 1995). Propagation of the signal to the cell body may require secondary messengers, such as cAMP, which is known to become elevated in the flagella of C. reinhardtii during the mating reaction. Third, what are the roles of IFT in signal transduction pathways in cilia? IFT is known to be essential for the formation of this sensory organelle and also appears to regulate the concentration of sensory molecules, like PKD2, in cilia (Follit et al., 2006). In addition, IFT may be directly involved in the completion of signaling pathways by transporting the signal itself, possibly in the form of a posttranslationally modified protein (Chauvet et al., 2004; Low et al., 2006). Thus, it will be of great interest to elucidate further details of the ciliary PKD2 signaling pathway and its relationship to other PKD-related proteins and to develop C. reinhardtii as an in vivo assay for screening compounds for therapeutic activity in treating PKD.

The first clues that cilia were involved in the pathogenesis of PKD came from the characterization of the IFT-dependent flagellar assembly process in C. reinhardtii (Pazour et al., 2000). The presence of CrPKD2 along with homologues of many PKD-related proteins in the flagella of C. reinhardtii demonstrates that this organism will continue to act as an attractive model for the study of the cilium as a sensory antenna and of its involvement in a wide spectrum of cilia-related diseases (Pan et al., 2005; Pazour et al., 2005).

Materials and methods

Cell and culture media
The C. reinhardtii wildtype strains CC-125 (mt+), and CC-124 (mt−), para-lyzed flagella strain pFL18 (CC-1297, mt−), temperature-sensitive flagellar assembly mutant fla10 (fla10−1) allele, CC-1919, mt−) and arg− strain CC3681 (arg−, mt−) were obtained from the Chlamydomonas Genetics Center.
Flagella were isolated from a C. reinhardtii wild-type strain. The C. reinhardtii genome sequence database (http://www.jgi-psf.org/chre2/chre2.home.html) was used to design primers. The mRNA was isolated and reverse transcribed as described previously (Huang et al., 2002). The RT-PCR products were cloned into PCRIITOPO (Invitrogen), generating plasmids pHK4 and pHK5, respectively. The inbred by genomic DNA was not present in the PCR products.

**Antibody production**

Loop1 CrPKD2 antibodies were generated against a His-tagged fragment of CrPKD2 (aa 358–1,019) expressed in E. coli and purified by affinity chromatography using nickel–nitrilotriacetic acid agarose according to the manufacturer’s instructions (QIAGEN). The purified protein was used for antibody production in rabbits at Pocono Rabbit Farm and Laboratory Inc. A peptide (AGEGDKDDKSPVEERKK, corresponding to aa 1,444–1,462 of CrPKD2) was synthesized and used to produce a second antibody by the same company. The N-PKD2 antibody was affinity purified from the Loop1 antisera using a peptide containing aa 358–583 of CrPKD2, which was expressed in E. coli and purified using nickel–nitrilotriacetic acid agarose.

**Flagellar isolation and flagellar membrane vesicle isolation**

Flagella were isolated from C. reinhardtii by pH shock as described previously (Witman et al., 1972) and modified by Cole et al. (1998). The flagella from 40–48 liters of wild-type cells were resuspended in 6 ml HMDEK buffer [10 mM Hepes, pH 7.2, 5 mM MgSO4, 1 mM DTT, 0.5 mM EDTA, 25 mM KCl, including the protease inhibitors 1.0 mM PMSF, 50 μg/ml soybean trypsin inhibitor, 1 μg/ml of pepstatin A, 2 μg/ml aprotinin, and 1 μg/ml leupeptin] for a protein concentration of ~3–4 μg/μl, and NP-40 (Calbiochem) was added to a final concentration of 0.1%. The mixture was incubated at 4°C for 30 min with shaking. The membrane vesicles were separated from the axonemes by centrifugation at 16,000 g for 10 min at 4°C, the supernatant was centrifuged again to remove any remaining axonemes, and the membranes were harvested by centrifugation at 228,000 g for 30 min (TLA 120.2 rotor, Optima Ultracentrifuge; Beckman Coulter). The pellet was resuspended in 1 ml of 0.1% NP-40 HMDEK buffer and centrifuged again to obtain the final pellet. Alternatively, membranes were further purified by density gradient sedimentation. For this, 1/3 volume of 60% iodixanol (Optiprep density medium; Sigma-Aldrich) was added to the membrane-containing supernatant for a final concentration of 15%, and 0.9 ml of the mixture was added to a 1.5 ml-tube. After layering with 0.3 ml of 30% iodixanol in HMDEK + 0.1% NP-40 buffer, the sample was centrifuged for 1 h and 11 min at 431,000 g. A white band of membrane vesicles in the middle of the tube was collected and diluted 10 times with 0.1% NP-40 HMDEK buffer. The mixture was centrifuged as before, sedimenting the membrane vesicles. One membrane pellet was used for EM and the other was resuspended in 100–200 μl HMDEK buffer with 0.1% NP-40 for immunoblotting.

To remove all the flagellar membrane from the axoneme, the flagella were freeze–thawed twice to release the matrix and a small amount of membrane. After the membrane was removed, the axonemes were treated with 0.1% NP-40 at room temperature for 30 min, and after centrifugation at 16,000 g for 10 min, the soluble fractions contained the membrane and the pelleted contained axonemes. The membranes were sedimented at 280,000 g for 30 min.

**Protein extraction, concentration determination, and immunoblot analysis**

Preparation 3 of the C. reinhardtii whole cell extract, determination of the protein concentration, PAGE, and immunoblotting were performed as described previously (Huang et al., 2002). Immunoblots were scanned and the relative protein concentrations were determined using ImageJ (National Institutes of Health).

**GFP constructs and transformation with CrPKD2-GFP**

To make the CrPKD2–GFP fusion, the C. reinhardtii bacterial artificial chromosome clone 18K16 (https://www.genome.clemson.edu/cgi-bin/orders) was cut with EcoRV and Spel, generating an ~18 kb fragment, which included the entire CrPKD2 gene and its promoter. This fragment was cloned into the EcoRV and Spel sites of pBluescript II KS+ (Stratagene), generating a plasmid named pHK25. This plasmid was cut with HindIII, generating three fragments. One of these fragments, an 8 kb fragment containing the promoter and most of the genomic DNA encoding CrPKD2, was cloned into the HindIII site of the pBluescript KS+, generating pHK28. A second fragment, a 5 kb fragment containing the last two introns, exons, the 3’ UTR of CrPKD2, and the KS+ vector, was ligated to produce pHK26.

To tag the CrPKD2 gene, we cloned the GFP gene into a unique EcoRI site in intron 11, flanked by the first intron of RBCS2 (Goldschmidt-Clermont and Rahire, 1986). For this, the two ends of the intron were subcloned as follows: using the genomic DNA as template, the 3’ end of this intron was amplified with the primers CGGAATTCTAGTGACCGAGCCGCC and GTGAATCTCTCTGCAATAGAAAC. EcoRI and BamHI sites (added sites in bold) were added to the primers and the PCR product was cut with EcoRI and BamHI and the mixture was inserted into the KS+ vector (pHk31). Another set of primers was used to amplify the 5’ end of the first intron of the RBCS2: CAGGATCCCCGAGTTGACCGACCCATCGGAGCAGAAG including a BamHI site and CTCTAGAGAACATCAAGGAGCCCAGG including XbaI and EcoRI sites. The PCR product was cut with BamHI and XbaI and cloned into pHK31 BamHI and XbaI sites, generating pHK32. The BamHI fragment of the pGFP (Entelechon GmbH), which contains the C. reinhardtii codon-adopted GFP ending with a stop codon, was inserted into the BamHI site of pHK32, generating pHK35. The sense orientation of this and following inserts was identified by restriction enzyme digests and confirmed by sequencing. The EcoRI fragment from pHK35 was inserted into the EcoRI site in intron 11 of the CrPKD2 gene of pHK26, generating pHK37. The 8 kb fragment that contains the promoter and 5’ end of CrPKD2 was inserted in the HindIII site of pHK37 in the sense orientation, generating pHK38.

To remove the stop codon at the end of GFP from pHK38, two primers, AGGGTGACTTCAAGATGCCGCTGCCATCCGG, both containing a BamHI site, were used to amplify the GFP fragment. The PCR product, which did not have the GFP stop codon, was cloned into the TOPO-PCR vector (Invitrogen), generating plasmid pHK34. The BamHI fragment of the pHK34 was exchanged with the BamHI fragment of pHK37 (which has a stop codon at the end of the GFP gene), generating plasmid pHK39. The HindIII fragment of pHK28 was inserted into pHK39 in the sense orientation to generate pHK41. Plasmids pHK38 and pHK41 were linearized with SpeI before transformation.

Because we did not observe fluorescence in transformants that harbor pHK38 or 41, these cells were only used for immunoblots, and the overlap PCR method was used to make another construct in which GFP was fused to the end of CrPKD2 through a flexible linker. Using the left (GGAGGAAACCTTTGTTTIGG, HindIII) and right primers (CGGCCGCGAGGC- GCCGTGCGAGGGCGGCGCGCGCGAATCTCATCATCA) and pHK26 as the template to amplify the C terminus of CrPKD2, the PCR product containing the fragment from the HindIII site in the last intron to the stop codon of CrPKD2 was obtained. The nucleotide sequence GGGCCGCTCCGCGAGGCCGGCCTCCGGCGGATGTTTIGG, which corresponds to a flexible protein linker GASGGAGSA, was included in the right primer. Another set of primers (GGGGGTCCTCCCGAGGGCGGAGCCCGCGCGCCGCCGCGACCGAGGGCGGAGCTGIGGACCC, KpnI) was used to amplify GFP and the 3’ UTR of RBCS2 from the plasmid pcGFP. The sequence encoding the protein linker was included in the left primer. Both PCR products were purified using a PCR purification kit (QIAGEN), and equal molar amounts of the products were used as templates to fuse these two PCR products together. For this purpose, primers [GGGAGAAAGCCTTTGGTTTTIGG (HindIII) and CTGGATCCCTCGATTTACG] were designed to amplify the new PCR product and the sequence containing the fragment from the HindIII site into the pBluescript II KS+ vector (HindIII and KpnI sites). The resulting plasmid was named pHK49. The HindIII fragment from pHK28 was inserted into the HindIII site of pHK49 in the sense orientation, generating pHK52. pHK52 was linearized with SpeI before transformation.

To prepare cells (CC-3681, arg7, and arg2 pf1) for transformation, cell walls were removed with autolysis as described previously (Huang and Beck, 2003). Linearized plasmids pHK38, pHK41, and pHK52, together
with pCB412, which harbors an AGR7 gene as a selectable marker (gift from C.F. Beck, University of Freiburg, Freiburg, Germany), were introduced into the cells using the glass bead method. Transformants were selected on Tri-tris-acetate-phosphate medium plates and tested for GFP by Western blot analysis. GFP antibody was obtained from Roche Applied Science (clones 7.1 and 13.1).

The CA10 (mt+) and pF18 (mt−) strains expressing CrPKD2–GFP, the original CrPKD2–GFP transfomant (mt−) was crossed to CC-125 (mt+) to produce an mt+ strain. This strain was crossed with CA10 and pF18, and the progeny were analyzed to obtain the desired phenotype.

**Nucleic acid manipulations and transformation of CrPKD2**

To make the genomic and cDNA fusion construct for RNAi, primers (GCAGATCTGTCGACTAGTACCGCCCAGTCATCCCGGTTTAAG [corresponding to the cDNA 1,145–1,125]), including a SalI site, were used to amplify the promoter and S′ region of the CrPKD2 gene using genomic DNA as a template. The 2.5-kb cloned into the 3′ SalI site near the EcoRV site of the vector, generating a plasmid pHK3. Another set of primers (AATCAGTCTACATTGTACCGACGCCGACGTAATTGT [corresponding to be 1–25 of the cDNA] and GTCAGACCGCGACGACGTCATCCCGGTTTAAG [corresponding to the cDNA 1,145–1,125]), including a SalI site, were used to amplify the PKD2 cDNA fragment, which corresponds to the genomic fragment in pHK3. The PCR product was cloned into the TOPO TA vector, and a clone with the S′ and near the EcoRV site was identified and named pHK15. The EcoRV and SalI fragment from the plasmid pHK15 was inserted into the same sites in pHK3, creating pHK22. The other two (Ri45 and Ri46) showed a more pronounced phenotype. The PCR product was cloned into the same sites in pHK3, creating pHK22. The other two (Ri45 and Ri46) showed a more substantial deletion of CrPKD2.

**Immunofluorescence light microscopy, live cell imaging, and FRAP**

Wild-type strains were prepared for immunofluorescence light microscopy using methanol fixation, and were stained with primary and Alexa fluor-conjugated secondary antibodies (Invitrogen) as described previously (Pedersen et al., 2003), modified by the addition of 0.05% glutaraldehyde directly to the medium for primary fixation before fixing with methanol at −20°C. Images were recorded with a microscope (Eclipse TE2000; Nikon) equipped with a Plan Apo 100×, 1.4 NA objective lens and a forced-air-cooled camera (Cascade 512B; Photometrics). Photoshop (Adobe) was used to adjust brightness and contrast and crop images. The movement of the GFP-tagged CrPKD2 was recorded as described in the previous paragraph using an argon ion 488 laser controlled by a Mosaic System (Photronics) for illumination and photobleaching experiments. For photobleaching, CA10 cells were immobilized with 0.02-M LiCl (Dentler, 2003) and embedded in 0.75% low-melt agarose. Next, the selected region of the flagellum was bleached for 2 s with laser illumination at 32 mW (Fprebleach). After 1 or 2 min of recovery, the GFP fluorescence of the selected region was recorded for 2 s at low power (Frecovery). Finally, another DIC picture of the flagellum was taken. By comparing the DIC pictures of the same flagellum, videos were selected for further analysis in which the flagellum did not move and the focus did not change. The mean fluorescence of each parameter, Fprebleach, Fbleached, and Frecovery, were used to calculate recovery. % recovery = (Frecovery − Fprebleach)/Fprebleach.

**Measurement of mating efficiency and flagellar protein tyrosine kinase activity**

Gametes were generated by resuspending vegetative cells in MII-N medium at a density of 1–2 × 10⁷ cells/ml. The cells were incubated under continuous light for 16–24 h and gametes (CC-125 and RNAi strains) were adjusted to the same cell density. The gametes to be assayed were mixed with a twofold excess of gametes of the opposite mt (CC-124) to ensure that mating efficiency of the test strains was not limited by depletion of the mating partner. After completion of the mating reaction in 1 h, the cells were fixed by addition of glutaraldehyde, and the number of the quadriflagellate zygotes and biflagellate cells were counted by phase-contrast microscopy (Martin and Goodenough, 1975; Beck and Acker, 1992). The percentage of cells that had mated was then calculated according to the method of Beck and Acker (1992).

To assay flagellar protein tyrosine kinase activity, gametes of CC-125 or Ri46 were mixed with the same number of CC-124 gametes, and flagella were isolated 3 min after mixing. The concentration of the flagellar protein was measured using the Amido black method (Huang et al., 2002), and equivalent amounts of protein were used to assay the protein tyrosine kinase activity by immunoblotting for phosphorylated CrPKG, the substrate of protein tyrosine kinase (Wang and Snell, 2003).

**RNA isolation and Northern blot hybridization**

Gametes were deflagellated using the pH shock method and vigorously aerated under light. Total RNA was isolated from cells before deflagellation and after 10 or 15 min of flagellar regeneration. RNA was blotted and hybridized as described previously (von Gromoff et al., 1989).

**Online supplemental material**

Table S1 lists the lengths of flagella measured from cells induced to resorb their flagella. Fig. S1 shows a diagram of the CrPKD2 gene and the amino acid sequence of the protein. Fig. S2 shows a phylogenetic tree of TRP channels. The Northern blots in Fig. S3 demonstrate that only one 6-kbp transcript is present encoding CrPKD2. Additional immunoblots of the CrPKD2 fragments from cells expressing CrPKD2–GFP are shown in Fig. S4. Video 1 shows the movement of CrPKD2 in the flagella of CA10 gametes at permissive temperature. The online version of this article is available at http://www.jcb.org/cgi/content/full/jcb.200704069/DC1.

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