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Proteomic analysis of the cilia membrane of *Paramecium tetraurelia*

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ABSTRACT

Channels, pumps, receptors, cyclases and other membrane proteins modulate the motility and sensory function of cilia, but these proteins are generally under-represented in proteomic analyses of cilia. Studies of these ciliary membrane proteins would benefit from a protocol to greatly enrich for integral and lipidated membrane proteins. We used LC–MS/MS to compare the proteomes of unfractionated cilia (C), the ciliary membrane (CM) and the ciliary membrane in the detergent phase (DP) of Triton X-114 phase separation. 55% of the proteins in DP were membrane proteins (i.e. predicted transmembrane or membrane-associated through lipid modifications) and 31% were transmembrane. This is to be compared to 23% membrane proteins with 9% transmembrane in CM and 9% membrane proteins with 3% transmembrane in C. 78% of the transmembrane proteins in the DP were found uniquely in DP, and not in C or CM. There were ion channels, cyclases, plasma membrane pumps, Ca^{2+} dependent protein kinases, and Rab GTPases involved in the signal transduction in DP that were not identified in the other C and CM preparations. Of 267 proteins unique to the DP, 147 were novel, i.e. not found in other proteomic and genomic studies of cilia.

1. Introduction

Cilia and flagella are found on a wide range of cells and tissues from unicellular to multicellular organisms. When they are motile, they play a variety of roles in cell or fluid movement, but regardless of motility, they play sensory roles in chemosensing, mechanosensing, cell growth, and development [1–3]. Just a few examples of sensory cilia include olfactory cilia whose signal transduction components including receptors, cyclic nucleotide gated channels and adenylyl cyclase III are almost exclusively expressed in the ciliary compartment [4]; primary cilia of kidney epithelial cells whose transient receptor potential type P (TRPP) channels polycystin 1 and 2 play critical roles in sensing of fluid flow [5]; and the motile cilia of human airway epithelia whose expression of bitter taste receptors allows them to monitor the external environment [6].

The development of a procedure for enrichment of ciliary integral membrane and membrane-associated proteins would facilitate studies of signaling proteins of cilia, especially the ciliary channels and receptors of *Paramecium* that control ciliary beat and swimming behavior and allow the cells to react to their environment [7–12]. These components can be characterized by electrophysiology [10] and genomic analysis [13,14], but the gene products have proven to be elusive and a challenge for protein isolation and identification including by mass spectrometry. Mass spectrometry of membrane proteins

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in general has proven to be more difficult than for soluble proteins because of the lower abundance of the membrane proteins, their insolubility and other physical chemical properties [15]. For example, the photoreceptor sensory cilium has signal transduction components as shown in the recent proteome analysis that identified rhodopsin, transducin, cGMP-specific phosphodiesterase, and ion channels involved in phototransduction signaling [16,17]. However, among the proteins identified only 10% were transmembrane. In the olfactory sensory cilium proteome, many components of the signal transduction were identified by mass spectrometry (G-protein $G_{olf,}$ adenylyl cyclase, and the cyclic nucleotide gated channel), but the less abundant membrane GPCR olfactory receptors were not [4,18]. In the cilia or flagella proteomes of *Chlamydomonas*, *Tetrahymena*, *Paramecium*, *Trypanosoma*, and human motile and primary cilia, axonemal and other components predominate over ciliary membrane proteins [19–24].

To facilitate studies of membrane proteins of the cilia, we sought methods to separate these proteins from the cytoskeletal components and from the glycosylphosphatidyl inositol (GPI)-anchored proteins including the surface antigen that are the majority of ciliary membrane proteins in *Paramecium* [25–27]. Specifically, the surface antigens represent about two-thirds of ciliary membrane proteins by mass [28]. Here we show our comparison of the proteins identified by liquid chromatography-tandem mass spectrometry (LC–MS/MS) from isolated but unfractionated cilia (C), the ciliary membrane fraction (CM), and the Triton X-114 detergent phase of the ciliary membrane fraction (DP). The results show that in the Triton X-114 DP the number of proteins that were predicted to be membrane proteins (integral, GPI-anchored, N-myristoylated, or prenylated) was greatly increased, as compared to those in the CM and C. Importantly, many of the transmembrane proteins including ion channels that were not found in C or CM preparations could be found in the DP and many of these were novel, i.e. not found in other proteomic or genomic studies of cilia. Western blots (Supplementary Fig. 1) confirmed the presence of some of these novel proteins from the cilia DP, such as the voltage gated calcium channel (VGCC1c), XNTA protein (probable Mg^{2+} channel-like exchanger [29]), and a small conductance calcium activated K^+ channel from DP. We also confirmed that a protein from the DP that was previously found but not further studied as an important ciliary component, Rab GTPase (Rab_A10), is present in cilia.

2. Materials and methods

2.1. Cells and culturing

Paramecium tetraurelia strain 51s (sensitive to killer) was cultured in the wheat grass infusion medium inoculated with *Aerobactor aerogenes* at 22 °C [30,31].

2.2. Preparation of cilia and ciliary membrane

The experimental procedure is shown in Fig. 1. The cilia (C) and ciliary membrane (CM) fraction of the cilia were prepared by the protocols described by Adoutte et al. [32].

2.3. Treatment of ciliary membrane with Triton X-114

The ciliary membrane proteins were solubilized with 1 ml of 1% Triton X-114 in Tris buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5) or membrane buffer (10 mM Tris buffer, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA) at 4 °C for 2 h. The sample was centrifuged at 14,000 ×*g* for 10 min at 4 °C. The pellet was suspended in membrane buffer. The supernatant was loaded over 100 μl of 6% sucrose in TBS and incubated at 37 °C for 4 min. The sample was then centrifuged

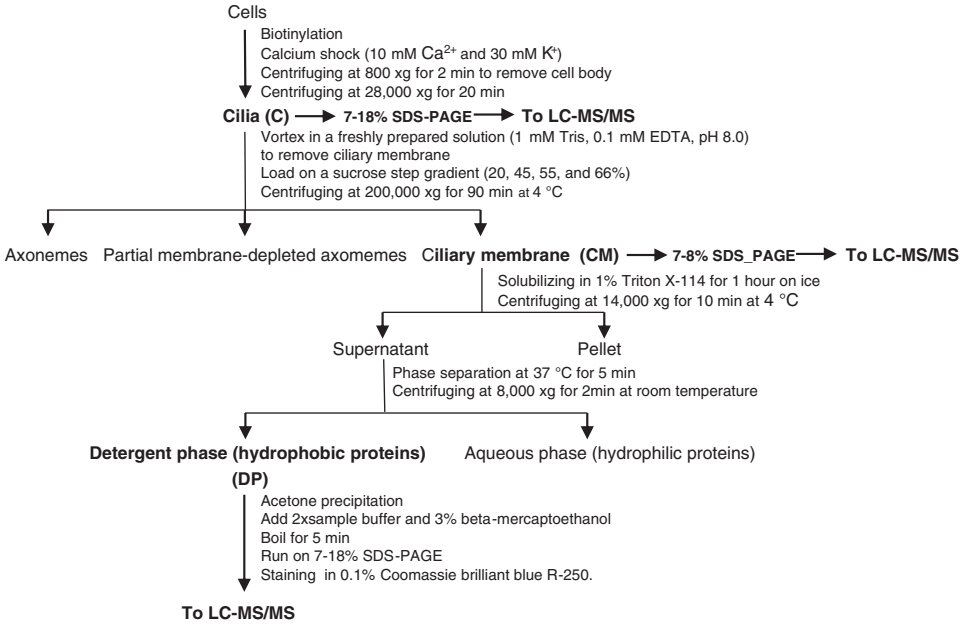

Fig. 1 – Experimental protocol.

at 8,000 ×*g* for 2 min at room temperature. The sample from the aqueous phase (top layer) was collected and 1 ml of TBS was added to the detergent phase (bottom layer) for a second round of phase separation. The detergent phase was then collected and the proteins were precipitated in cold acetone at 4 °C for 30 min, centrifuged at 14,000 ×*g* for 15 min at 4 °C, and washed in cold acetone three times. After being air dried, the proteins were dissolved in 80 μl of membrane buffer.

2.4. Biotinylation of ciliary surface proteins

We used biotinylation of intact cells to confirm that the ciliary surface proteins were harvested in our CM preparation and not left behind with the axoneme. One milliliter of packed cells was suspended in 5 ml of a phosphate buffer (1 mM Na_2HPO_4, 1 mM NaH_2PO_4, 2 mM KCl, 0.01 mM EDTA, 50 μg $CaCl_2$, pH 7.8). Six milligrams of EZ-link Sulfo-NHS-Biotin (Pierce) was added to the cell suspension, and incubated for 30 min at room temperature. As a control, the same treatment without biotin was carried out. After washing the cells three times in Dryl's solution (1 mM Na_2HPO_4, 1 mM NaH_2PO_4, 2 mM trisodium citrate, 1.5 mM $CaCl_2$), we followed procedures for isolating cilia and ciliary membrane. The proteins collected from the three bands in the sucrose step gradient (CM, partially demembranated axonemes and axonemes [32]) were separated on 7–18% gradient SDS–PAGE and analyzed by Western blotting. The biotinylated proteins were detected by ImmunoPureR Streptavidin, alkaline phosphatase conjugated (1:10,000 dilution, Thermo Scientific). GPI-anchored surface antigens were detected with antisera against surface antigens (1:20,000 dilution, gifts from Dr. Jim Forney). Tubulin was detected with an anti-α tubulin monoclonal antibody (1:10,000 dilution, Sigma) on the Western blots.

2.5. SDS–PAGE and LC–MS/MS

The protein concentrations were determined using the Pierce protein assay. The proteins obtained from the C (200 μg), CM (660 μg), and the DP (200 μg) in Triton X-114 phase separation were mixed with SDS sample buffer with a final concentration of 3% β-mercaptoethanol (BME) and then boiled for 5 min. The proteins were separated on 7–18% gradient SDS–PAGE and stained with 0.1% Coomassie brilliant blue R-250, 30% methanol, and 5% acetic acid. The gel lanes with proteins from the C, CM, and the DP of the Triton X-114 phase separation were cut into 23, 23, and 37 regions respectively so as to minimize the number of dominant proteins per region. The number of segments was larger for the gels of DP proteins because of the more numerous bands compared to CM or C. Each region of the gel was diced and subjected to in-gel digestion with trypsin and the resulting peptides were analyzed by LC–MS/MS in an LTQ-XL linear ion trap mass spectrometer as described below. Briefly, the gel pieces were sliced into 1 mm cubes, washed with 1 ml HPLC-grade water and incubated in 1 ml destain solution (50 mM ammonium bicarbonate and 50% acetonitrile (MeCN)) for 30 min at 37 °C. After destaining again, the gel pieces were dehydrated in 200 μl of 100% MeCN for 10 min. The gel pieces were further dried in a speed vacuum for 15 min. Proteins were digested overnight in the gel pieces using sequencing grade modified trypsin (Promega, Madison, WI) at a concentration of 6 ng/μl in 50 mM ammonium bicarbonate at 37 °C after an initial re-swelling of dried gel pieces on ice for 30 min at 12.5 ng/μl trypsin. The digests were centrifuged for 5 min at 13,000 ×*g* and the supernatant was transferred to a 0.6 ml tube. Next, the gel pieces were incubated in 200 μl of extraction buffer (50% MeCN, 2.5% formic acid (FA)) and spun again at 13,000 ×*g* for 15 min. The peptides extracted in the supernatant from each gel slice were pooled and dried in a speed vacuum. The peptides were resuspended in 2.5% FA and 2.5% MeCN and loaded using a Micro AS autosampler onto a microcapillary column of 100 μm inner diameter packed with 12 cm of reverse-phase magic C18 packing material (5 μm, 200 Å; Michrom Bioresources, Inc., Auburn, CA). After a 14.5 min isocratic loading in 2.5% MeCN, 0.15% FA (Solvent A) peptides were eluted using a 5–35% gradient of Solvent B (99% MeCN, 0.15% FA) over 30 min (flow rate averaged 400 nl/min provided across a flow splitter) and electrosprayed (1.8 kV) into a LTQ-XL linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) operating in the positive ion mode. The precursor scan was followed by ten collision-induced dissociation (CID) tandem mass spectra for the top 10 most abundant ions. Instrument control was managed using Xcalibur software, Version 1.4 SR1 (Thermo Electron) which generated RAW files which were, prior to searching, converted to mzXML formats using ReAdW (Thermo Electron). Charge state rejection was enabled for singly-charged precursors. Dynamic exclusion of precursors chosen for MS/MS was enabled with a repeat count of three, duration of 180 s, and an exclusion *m*/*z* width of +/−1.5. The AGC setting was 30,000 and precursors were scanned with one microscan and a 10 ms maximum injection time. MS/MS scans had 100 ms maximum injection time, normalized collision energy of 35, activation Q of 0.25, activation time of 30 s and an isolation *m*/*z* width of +/−1. Peptides from each gel segment were subjected to LC–MS/MS in duplicate for the cilia or triplicate for the other preparations.

2.6. Protein identification and bioinformatic analysis

The data were searched simultaneously against the *P. tetraurelia* forward (target) and reverse (decoy) proteome databases (http://aiaia.cgm.cnrs-gif.fr/download/fasta/ Ptetraurelia_peptide_v1.fasta) using SEQUEST (Thermo Electron, Version 27, Revision 12) with a precursor tolerance of 2 Da and a fragment ion tolerance of 0.5 Da. In our experience the majority of cysteine residues following reducing conditions and SDS–PAGE are found identified with an acrylamide adduction. For increased throughput and simplicity we therefore conducted searches with a static increase in 71.0 Da for acrylamide adduction. Differential modification of 16.0 Da on methionine residues was permitted. The search results were filtered using a dCn score of 0.2 and Xcorr values of 1.5, 2.0, and 2.5 for singly, doubly and triply charged ions respectively. Proteins on these filtered lists that had two or more peptides were retained.

In *P. tetraurelia*, many genes were retained after whole-genome duplications [33], and in many cases two or more proteins (homologs or paralogs) show high amino acid identity. Thus, when a peptide uniquely matched to one protein, its ParameciumDB ID name is given as the reference. When a peptide matched to two or more proteins belonging to a single protein family, as was true in many cases, only one ID is given

as a representative of the family. However, the number of protein entries having an identical peptide match is listed for each peptide in the column "redu" for redundancy (Supplementary Table 1). We used the ParameciumDB [13,14] and NCBI BLAST for the annotation. When the proper name was not found, it is classified as a hypothetical protein. The transmembrane proteins, number of transmembrane domains, and localization were determined by using ParameciumDB, TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), and PSORT (http://psort.ims.u-tokyo.ac.jp/), respectively. The GPI-anchored proteins were determined using big-PI Predictor (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) and GPI-SOM (http://gpi.unibe.ch/). Whether the protein is posttranslationally modified by the myristoylation at its N-terminus or prenylated at the C-terminus for associating with the membrane was examined by using NMT (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm) and PrePS (http://mendel.imp.ac.at/sat/PrePS/index.html), respectively. The palmitoylation site was predicted by CSS-Palm 2.0 [34].

2.7. Construction of the plasmid for expressing the tagged-proteins

We expressed epitope-tagged proteins to demonstrate that some proteins obtained from the MS analysis are localized in the cilia. The open reading frames of a voltage gated calcium channel (VGCC1c, GSPATG00017333001), XNTA (probable Mg^{2+}- specific channel-like exchanger [29], PTETG43000 06001), a small conductance calcium activated potassium channel (GSPATG00031195001), and a small GTPase Rab_A10 (GSPATG00031195001) were amplified from the genomic macronuclear DNA by the primers listed in Supplementary Table 2. The DNA sequences amplified by PCR were inserted to pPXV [35] constructed for 3xFLAG sequence at the N or C terminus of the expressed protein, or for 3xMyc at the C terminus.

2.8. Microinjection

Linearized plasmid was prepared following the protocol of Haynes et al. [35] for a concentration of 5 μg/μl. Five to ten picoliter were injected into macronucleus at three to five fissions after autogamy. Clonal cell lines were kept in 10 ml bacterized culture medium at 15 °C. The cells from an injected cell line were cultured at 22 °C for immunoprecipitation and Western blot analysis.

2.9. Immunoprecipitation

The cilia from cells expressing FLAG or the FLAG-tagged proteins were isolated as above, solubilized with 1% Triton X-100 or 1% Triton X-114 in the membrane buffer at 4 °C for 1 h, and centrifuged at 14,000 ×*g* at 4 °C for 10 min. The supernatant was then clarified with 20 μl protein A beads (Amersham Pharmacia/GE healthCare) that had been washed in 1% Triton in membrane buffer with 0.1% (w/v) BSA. Beads were then incubated with the supernatant at 4 °C for 60 min before removing them by centrifugation. The clarified supernatant was then incubated with 20 μl of anti-FLAG M2 agarose beads (Sigma) that had been previously washed in the membrane buffer with 1% Triton X-100 or X-114 containing 0.1% BSA at 4 °C for 1 h. Next, the anti-FLAG beads were washed in the membrane buffer with 1% Triton and 0.1% BSA three times and three additional times in membrane buffer without Triton and BSA. The beads were suspended in 50 μl of 2 × SDS buffer with 3% BME and boiled for 10 min. After centrifuging, the supernatant was loaded onto 7% (or 5)–18% gradient gels for SDS–PAGE.

2.10. Western blot analysis

The proteins that were separated on gels were transferred to BioTrace™ nitrocellulose blotting membrane (PALL Life Sciences). Anti-FLAG or -Myc polyclonal antibody (Sigma and GenScript, respectively) was used in dilution of 1:2500 for detection; 200 μg of a peptide with the FLAG or Myc epitope (GST-CBD-3xFLAG or GST-CBD-3xMYC) was used to pre-absorb the anti-FLAG or anti-Myc antibodies as a control. (CBD is the calmodulin binding domain from plasma membrane calcium pump (PMCA2) in *Paramecium*.) Blotted membranes that were to be developed with alkaline phosphatase were incubated in blocking buffer with 5% non-fat milk, 3% normal goat serum (Vector) and 2% Teleost fish gelatin (Sigma) in TBST buffer (16 mM Tris HCl, 4 mM Tris, 137 mM NaCl, and 0.1 % Tween 20, pH 7.5) for 1 h. Alternatively blots to be developed with Enhanced ChemiLuminescence (ECL, Perkin Elmer) were blocked with 5% non-fat milk in TBST. The blotted membranes were incubated in the respective blocking buffer with antibody or the pre-absorbed antibody overnight at 4 °C. Alkaline phosphatase conjugated goat anti-rabbit antibody or horseradish peroxidase linked goat anti-rabbit antibody were used to visualize the primary antibodies on the blots following the protocol of Moss Inc. for alkaline phosphatase, or Perkin Elmer for ECL.

The SDS–PAGE gels of supernatant or lysate before treatment with antibody-conjugated beads were also blotted, blocked, developed as above with anti-tubulin monoclonal antibody and alkaline phosphatase (Santa Cruz Biotechnology).

3. Results

3.1. Purification of the ciliary membrane proteins

Using the experimental procedure shown in Fig. 1, we isolated cilia (C) and further purified the ciliary membrane (CM). We examined whether the CM preparation effectively captured the membrane proteins and did not leave them behind with the axoneme. We biotinylated intact cells to label those membrane proteins that were exposed to the outside of the cell and compared labeled proteins in the CM, partially demembranated axonemes (PDA) and axonemes (AX) that were collected from three interfaces of the same sucrose step gradient [32] (Fig. 1). We used alkaline-phosphatase conjugated strepavidin (Fig. 2A) and anti-surface antigen antisera (Fig. 2B) to identify the surface proteins and the GPI-anchored proteins that are a subset of surface exposed proteins. Lane CM in Fig. 2A and B shows many surface proteins, while lanes PDA and AX in Fig. 2A and B show few to none. Blots in Fig. 2A and B show that the ciliary membrane efficiently stripped off the axoneme were enriched in the CM fraction.

The blot Fig. 2C shows the α-tubulin in each preparation, and provides evidence that although there are few to no biotinylated proteins in the partially demembranated and pure axonemes,

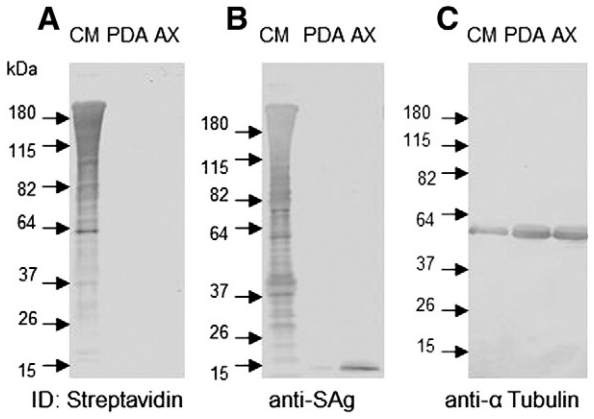

Fig. 2 – Biotinylated ciliary membrane proteins. Whole cells were biotinylated and three cilia fractions, ciliary membrane (CM), partially membrane-depleted axonemes (PDA), and axonemes (AX) were prepared. Proteins were detected on the Western blots by alkaline-phosphatase-conjugated streptavidin (A), anti-surface antigen antibodies (B), and anti-α tubulin antibody (C). The biotinylated surface proteins and the surface antigens were detected on lane CM in A and B, but are not detected in the other two fractions. Arrows show locations of molecular mass markers.

protein was loaded in each lane. It is not uncommon to find α-tubulin in membrane preparations (lane CM in Fig. 2C) possibly through a link from the outer doublet microtubules to a membrane-associated dynein-like ATPase [36,37].

3.2. *Partition of the ciliary membrane proteins into the detergent phase in Triton X-114 phase separation*

While there are not many different GPI-anchored proteins in the *Paramecium* ciliary membranes, including the large surface antigens, they are numerous and make up the majority of ciliary membrane proteins by mass [25]. The GPI-anchored proteins are cleaved from the lipid moiety inserted into the membrane by an endogenous lipase during the Triton X-114 treatment. As a result, the proteins, which lack the lipid moiety (hydrophobic domain), partition into the aqueous phase with Triton X-114 phase separation [26]. Our goal was to enrich peptides from integral membrane proteins and increase their relative abundance compared to the peptides from GPI-anchored proteins in the preparation. By removing many of the GPI-anchored proteins into the AP, we made it easier to find peptides prepared for mass spectrometry from the less abundant proteins.

We examined the distribution of proteins by Western blot analysis of proteins from the detergent insoluble pellet (P), the aqueous phase (AP) and detergent phase (DP) of Triton X-114 phase separation of the CM (Fig. 3). As expected, the GPI-anchored proteins were enriched in the AP compared to DP and P as shown on blots with antibodies against surface antigens [26] (Fig. 3A). In contrast, when we probed for a specific integral membrane protein, plasma membrane calcium ATPase 2 (PMCA2), we found that it was much more enriched in the DP compared to the AP and that there was no evidence of it in the P (Fig. 3B). Thus, the Triton X-114 DP seemed to provide an approach to enrich for integral ciliary membrane proteins for use in proteomic analysis.

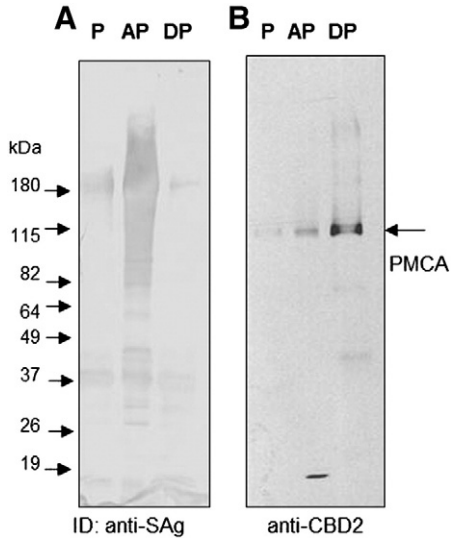

Fig. 3 – Ciliary membrane proteins partitioned into aqueous and detergent phase in Triton X-114 phase separation. Total proteins from each fraction were loaded on the gels. A. GPI-anchored proteins were detected on the Western blots by anti-surface antigen antisera (anti-SAg). B. After stripping the blot A, the plasma membrane calcium ATPases (PMCA, arrow) were detected using ECL and antibodies (anti-CBD2) against the calmodulin binding domain of PMCA2. P, proteins in the detergent insoluble pellet. AP, proteins partitioned into the aqueous phase. DP, proteins partitioned into detergent phase.

3.3. *Proteomic analysis of detergent phase from Triton X-114 phase separation*

The ciliary proteins from the C, CM, and DP obtained by phase separation of Triton X-114 were separated by SDS polyacrylamide electrophoresis, and sections of the Coomassie brilliant

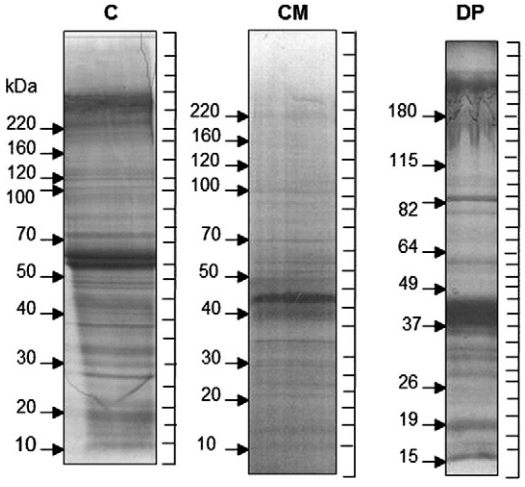

Fig. 4 – Samples from cilia (C), ciliary membrane (CM), and ciliary proteins partitioned into Triton X-114 detergent phase (DP) were run on 7–18% SDS–PAGE gels. The gels were stained in 0.1% Coomassie brilliant blue R-250. The lines show where each gel was cut.

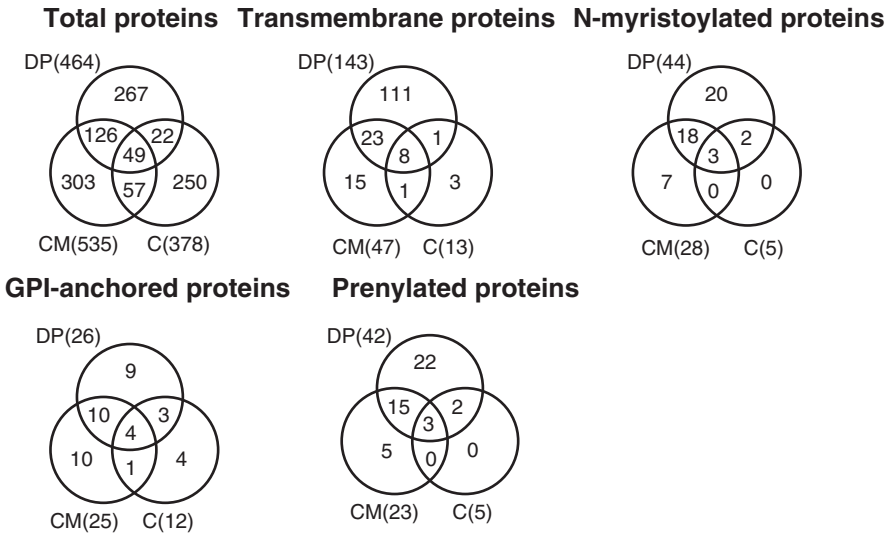

Fig. 5 – Venn diagrams showing the proteins (total proteins, transmembrane proteins, N-myristoylated proteins, GPI-anchored proteins, and prenylated proteins) identified from three preparations, cilia (C), ciliary membrane (CM), and the detergent phase of Triton X-114 phase separation (DP). The parentheses show the total number of proteins detected from each sample.

blue stained gels were analyzed by LC–MS/MS. Fig. 4 shows the gel lanes for C, CM, and DP and where each gel was cut. Following the protocol in Materials and methods, we found 378 proteins in C, 535 proteins in CM and 464 proteins in DP with two or more different peptides. Reverse sequences of *Paramecium* genome were used to identify false peptides. The calculated false-positive ratio was 0.06% in C, 0.32% in CM, and 1.35% in DP.

Fig. 5 shows the relative distributions of the proteins identified in each preparation C, CM and DP. Note that all classes of predicted membrane proteins (transmembrane, N-myristoylated, prenylated) except GPI-anchored are more highly represented in the DP compared to C and CM. In the Triton X-114 DP, 55% of the 464 proteins detected by MS were predicted membrane proteins. This is compared to only 23% of the 535 proteins identified from the CM and 9% of the 378 total proteins from C. 31% of the proteins in the DP were predicted to be transmembrane, compared to 9% in CM and 3% in C. Also of importance, 78% of the transmembrane proteins in the DP (111 of 143) were found uniquely in DP, and not in C or CM (Supplementary Table 3 shows unique proteins in DP).

Also shown in Fig. 5 are the proteins predicted to be GPI-anchored, prenylated, or N-myristoylated. As for transmembrane proteins, the DP contained more lipid modified membrane proteins (24% of 464) than found in CM and C (14% of 535 and 6% of 378 respectively). About half of these lipid modified proteins (46% of 112) in DP were not found in C or CM.

One of our goals was to reduce the contribution by mass of GPI-anchored proteins and enrich for transmembrane and other lipidated membrane proteins. Fig. 5 and Supplementary Table 4A show that the identified GPI-anchored proteins in slightly higher number in the DP preparation (5.6% of total proteins) compared to CM (4.7%) and C (3.2%). However, when the abundance of *peptides* in the preparation is taken into account, the GPI-anchored proteins contribute 5.0% to the total 4739 peptides in the DP, but 17.2% to the 4766 peptides in CM and 14.3% to the 2333 peptides in C. Many of the GPI-anchored proteins are very large (>200 kDa), and contribute many peptides but few proteins to be identified in the preparations. Peptides from the GPI-anchored large (>200 kDa) surface antigens were reduced to 0.9% in DP compared to 15.2% and 13.8% in CM and C. In contrast, the peptide numbers from transmembrane proteins in DP increased to 36.9% of the total, as compared to 11.0% in CM and 5.6% in C (Supplementary Table 4B). The reduction of these very large and abundant GPI-anchored proteins in the preparation, which present about two-thirds of ciliary membrane protein by mass [28], seems to have been useful in the enrichment of other membrane proteins in DP.

For comparisons among preparations, we chose the 300 proteins in each preparation that ranked highest by total peptides identified (spectral counts) and then by average Xcorr value. The peptide false-positive ratios for these the

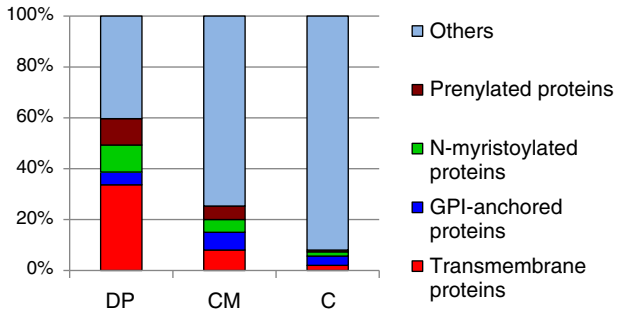

Fig. 6 – Frequency of integral membrane proteins identified from the 300 most abundant proteins from each preparation, cilia (C), ciliary membrane (CM), and the detergent phase of Triton X-114 phase separation (DP). Membrane proteins are the transmembrane or lipidated proteins including the N-myristoylated, prenylated, or GPI-anchored proteins. Others are proteins not included in our definition of membrane proteins.

proteins, 300 per preparation, were 0.00%, 0.18%, and 0.67% for C, CM, and DP, respectively. These selected proteins were compared across fractions as a first approximation comparison of relative abundance. Among the 900 total proteins, we found 728 individual proteins. Among these 728, 225 proteins in C, 175 in CM, and 192 in DP that were unique to those preparations. Only 36 proteins were in common among all three.

Fig. 6 shows the relative distribution of the 300 most abundant membrane protein classes in C, CM, and DP. In DP, 60% of the top 300 identified proteins were membrane proteins (transmembrane, lipidated, GPI-anchored) compared to 25% and 8% for CM and C respectively (Fig. 6). 34% of the most abundant DP proteins detected were transmembrane including PMCAs, adenylyl cyclases, sodium pumps, and ABC transporters (Supplementary Table 5).

Fig. 7 shows that transmembrane proteins from the DP fraction have the highest numbers of transmembrane domains. P-type ATPases with 10 transmembrane domains such as PMCAs and (amino) phospholipid transporters were abundant in DP, with 10 PMCAs alone. The largest number of transmembrane domains in the identified transmembrane proteins was 24 in the voltage gated calcium channel (VGCC). Many of these transmembrane proteins were not found in C or CM (Supplementary Tables 3 and 5).

The annotation of the 300 most abundant ciliary proteins in each preparation by predicted function is shown in Fig. 8 (Supplementary Tables 5–12). In C, ciliary axonemal components such as dynein motors (heavy, intermediate, light chain), microtubules (α-, β-tubulin), and radial spokes made up 20% of the 300 proteins. Forty (13%) proteins were *Paramecium* homologues or orthologues of flagellar associated proteins found from the proteomics of *Chlamydomonas* flagella [19]. Their functions are largely unknown, although some of the flagellar associated proteins are reported to be involved in the ciliogenesis and ciliary function in *C. reinhardtii* [38,39].

Many CM proteins are those involved in the signal transduction and vesicle transport (20% of the 300 proteins are protein kinases, protein phosphatases, small G-proteins), in biogenesis and metabolism (7%), and in the protein synthesis (tRNA synthetases, ribosomes, and elongation factors, 8%) (Fig. 8). Of proteins in signal transduction, 29% were protein kinases, with many EF hand proteins among

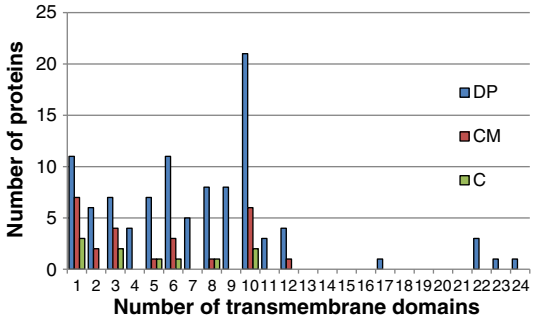

Fig. 7 – Frequency of transmembrane proteins identified from the 300 most abundant proteins from each preparation: cilia (C), ciliary membrane (CM), and the detergent phase of Triton X-114 phase separation (DP).

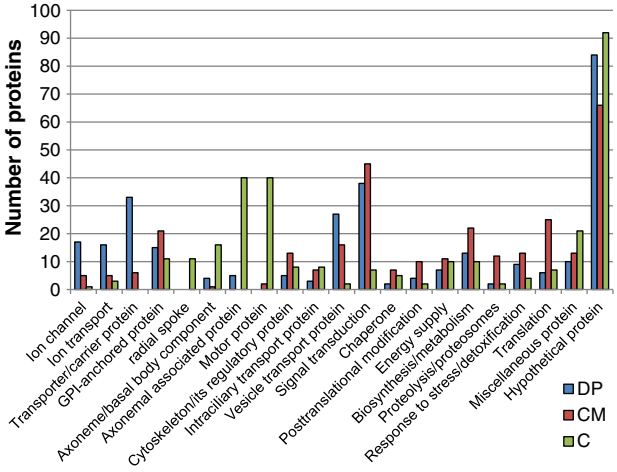

Fig. 8 – Frequency of proteins organized by function from the 300 most abundant proteins from each preparation: cilia (C), ciliary membrane (CM), and the detergent phase of Triton X-114 phase separation (DP).

them (Supplementary Table 8). Axonemal components such as dynein were dramatically decreased in CM, in comparison to cytoskeletal elements such as tubulin (α, β) and actin1 that were enriched in CM compared to DP but not compared to C (Fig. 8).

In DP, ion channels (17), ion transporters (16), and other transporters (33) were more highly represented (Supplementary Table 5). Likewise, proteins involved in the vesicle transport such as small GTPases and signal transduction such as protein kinases and phosphatases numbered 9% and 13% of the 300 DP proteins respectively.

3.4. *Validation of proteins detected from MS analysis*

We examined the 267 proteins that were uniquely found in the Triton X-114 detergent phase and were absent from the other preparations (Fig. 5 and Supplementary Table 3) for whether they were also identified in other analyses. We included studies that used proteomics, comparative genomics, gene expression analysis during re-ciliation, and promoter analysis (x-box) related to primary and motile cilia, and Cildb V2.1 [21] (see Supplementary Table 3). Of 267 proteins, 147 proteins were novel, i.e. not detected in other studies of cilia or ciliary membrane. Of these novel proteins, 51 had orthologs or homologs in other species (Table 1). They included guanylyl cyclases, cation channel family (IP_3 receptors), ion channels, and ABC transporter family members. Among the remaining 120 proteins which were not novel, Arl6 (ptArl_B64), PACRG-like protein, B9 protein (B9D1), and Rabl5 (ptRab_B78) have been described as having important ciliary functions in the other organisms [40–43]. Still other non-novel proteins have not been characterized further for function in cilia, such as Rab GTPase Rab_A10 [44].

To confirm with a method other than mass spectrometry that novel proteins from the DP were in the cilia, we chose Western blots to detect a small conductance Ca^{2+} activated K^+ channel (SK1a channel), a voltage gated calcium channel (VGCC1c), and a probable Mg^{2+} channel-like exchanger (XNTA). We also used Western blots to confirm ciliary location of Rab

Table 1 – Novel proteins unique to the DP preparation, not identified in other proteomics or genomics studies, and with orthologs or homologs in other species.

Reference	Description	Reference	Description
		GSPATP00025553001	Cyclin Y
GSPATP00018908001	Adenylate and Guanylate cyclase catalytic domain containing protein	GSPATP00010358001	Small conductance calcium-activated potassium channel protein 2 isoform a
GSPATP00009387001	Inositol 1,4,5-trisphosphate receptor type 2	GSPATP00008808001	ATP-binding cassette, sub-family C (CFTR/MRP), member 4
GSPATP00002549001	Cation channel family protein	GSPATP00011730001	Small-conductance calcium-activated potassium channel
GSPATP00004213001	Cation channel family protein	GSPATP00013465001	rac_A62
GSPATP00018392001	ATP-binding cassette, sub-family G (WHITE), member 2	GSPATP00013743001	Chromosome 14 open reading frame 135 (Pecanex-like protein)
GSPATP00034265001	Surface antigen 51B	GSPATP00024721001	ADP-ribosylation factor 1
GSPATP00002030001	Hypothetical protein (Predicted permease)	GSPATP00012736001	E3 ubiquitin protein ligase TOM1-like protein
GSPATP00018399001	ATP-binding cassette, sub-family G (WHITE), member 2	GSPATP00031027001	Glycosyl hydrolases family 31 protein
GSPATP00000757001	ATP-binding cassette, sub-family G (WHITE), member 2	GSPATP00022332001	Ubiquitin-activating enzyme e1
GSPATP00014844001	Probable Mg^{2+}-specific channel-like exchanger	GSPATP00016028001	Copine-like protein
GSPATP00024548001	Adenylate and Guanylate cyclase catalytic domain containing protein	GSPATP00025787001	Sodium/hydrogen exchanger family protein
GSPATP00001509001	Cation channel family protein	GSPATP00017042001	Inorganic H+ pyrophosphatase
GSPATP00003683001	ABC transporter, putative	GSPATP00008624001	SNF7 family protein
GSPATP00024438001	Adenylate and Guanylate cyclase catalytic domain containing protein	GSPATP00019709001	Cytidine deaminase
GSPATP00029355001	TROVE domain containing protein	GSPATP00032992001	Mitochondrial carrier; adenine nucleotide translocator
GSPATP00021763001	Protein kinase domain containing protein	GSPATP00008694001	Beta-hexosaminidase
GSPATP00017333001	Calcium channel, voltage-dependent, L type, alpha 1S subunit	GSPATP00023811001	E3 ubiquitin-protein ligase MARCH4 Precursor
GSPATP00010323001	Calcium channel, voltage-dependent, L type, alpha 1S subunit	GSPATP00007277001	Hypothetical protein (Arrestin domain-containing protein 5)
GSPATP00035536001	Silent mating type information regulation 2 (NAD-dependent deacetylase sirtuin-2)	GSPATP00028464001	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN
GSPATP00000989001	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	GSPATP00007930001	Protein kinase domain containing protein
GSPATP00030859001	Vacuolar protein sorting 8 homolog isoform a	GSPATP00021502001	PhoD-like phosphatase
GSPATP00021275001	Mitogen-activated protein kinase 15	GSPATP00016237001	piwi-like 1
GSPATP00001278001	Ammonium transporter	GSPATP00000190001	cAMP-dependent protein kinase, catalytic subunit 3-2
GSPATP00027163001	Guanylyl cyclase	GSPATP00012203001	Small-conductance calcium-activated potassium channel protein
GSPATP00005580001	Solute carrier family 44, member 2	GSPATP00019865001	Cation channel family protein

GTPase Rab_A10. We expressed the FLAG- or MYC-tagged protein in *Paramecium* and showed by immunoprecipitation and/or Western blotting that these specific proteins were present in the cilia. (We had previously shown that the PMCAs were present in the cilia (Fig. 3).) The results are shown in Supplementary Fig. 1.

4. Discussion

To approach the proteome of *Paramecium* cilia with an emphasis on membrane proteins, we have applied our previous experience with Triton X-114 phase separation of cell body and ciliary membranes to segregate transmembrane from axonemal and peripheral proteins [26] including GPI-anchored proteins that are very abundant ciliary membrane proteins in *Paramecium* [25–27]. There are few *Paramecium* proteins that can be classified as GPI-anchored protein, but they make up the largest portion of membrane proteins by mass. For example, while the genome codes for multiple surface GPI-anchored proteins, only one large surface antigen is expressed at a time. This protein makes up the largest portion of the ciliary membrane proteins by mass [25,45]. The contribution from the large surface antigens to the ciliary membrane proteins by mass is estimated to be about two-thirds [28] and other smaller GPI-anchored proteins are about equal to the large surface antigens by mass [25]. These numbers are not completely consistent because methods of analysis differed, but what is clear is that the GPI-anchored proteins, especially the large surface antigen, constitute a large component of the ciliary membrane proteins by mass.

We found as did others [25,26] that Triton X-114 phase separation enriched for *Paramecium* integral membrane proteins. More than twice as many membrane proteins were found among the total DP proteins (55%) compared to CM and C (23% and 9%) and of these more than three times were transmembrane proteins in DP (31%) compared to CM (9%) and C (3%). When one considers the abundance of peptides derived from GPI-anchored proteins rather than the numbers of GPI-anchored proteins identified in each preparation, the DP has far fewer of these peptides than the other preparations (5% in DP, 17% in CM and 14% in C). The peptides from the very large mass GPI-anchored proteins are even more dramatically reduced in DP (less than 1% in DP, 15% in CM and 14% in C, Supplementary Table 4). Therefore, Triton X-114 phase separation appears to be very useful for concentrating the ciliary integral membrane proteins as it has been for microsomal membrane proteins [46].

Among the proteins in the DP, we found many (267) that were not identified in the other preparations (C and CM). Among these, 147 were novel and not found in the 19 genomic and proteomic studies that we examined (see Supplement Table 3). Of the novel proteins, 51 had orthologs or homologs in other species and 96 had no BLAST match. These hypothetical proteins are numerous because of three *Paramecium* whole genome duplications [33] allowing for many genes without orthologs in other species to arise.

Three *Paramecium* whole genome duplications [33] have provided us with many paralogs and ohnologs (genes originating from whole genome duplications) whose functions are surmised from sequence. With this proteomic analysis, we can determine which among many potential gene family products are found in the cilia, within the constraints of the interpretation of negative results when we fail to find a particular gene product. For example, there are 23 genes that code for PMCAs in the *Paramecium* genome [13,14]. We have found 11 of these genes' products in our proteomic analysis of the DP. This is an interesting result in that the other 12 PMCAs might have been missed in our analysis because we found only peptides that they shared with other PMCAs or they function in maintaining Ca^{2+} homeostasis elsewhere in the cell. Previously we had found PMCA 2 and 4 in Western blots of cilia as well as somatic (plasma) membrane (Yano, unpublished data). In our new analysis we found in addition to these PMCAs, PMCA 3, 7, 9, 10, 13, 16, 18, 19, and 21 by unique peptide(s) in the DP.

From past physiological studies, we expect that there will be an α-subunit of a VGCC that is specific to the cilia and is responsible for action potentials [47]. Although there were at least 40 VGCCs coded in the genome [13,14], our analysis of cilia DP peptides identified proteins from a triplet of paralogs that code for the VGCC α- subunits. The remainder of the VGCCs may function in the cell soma where there provide hyperpolarization and depolarization activated Ca^{2+} conductances, but not action potentials [48,49] that are uniquely associated with the voltage gated Ca^{2+} conductances from the cilia.

New knowledge from our proteomic analysis includes identification of a set of calcium dependent K^+ channels that were thought to be in the cilia based on physiological measurements but not identified before. Genomic analysis predicted 17 of these channels [13,14]. There were three in cilia based on our analysis, providing a redundant K^+ channel system to repolarize the cell following an action potential.

5. Conclusion

The use of Triton X-114 phase separation appears to have improved the use of mass spectrometry for identification of the ciliary membrane channels, pumps and other integral membrane proteins that are not as abundant as peripheral proteins and hard to solubilize. In the future other studies may benefit from combining the approach of Triton X-114 phase separation with an aqueous two-phase polymer system or sodium carbonate treatment, which allowed for the recovery of 41–61% membrane proteins in microsomal preparations [50–52].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.09.040.

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