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GEF1 is a Ciliary Sec7 GEF of *Tetrahymena thermophila*

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Abstract

Ciliary guanine nucleotide exchange factors (GEFs) potentially activate G proteins in intraflagellar transport (IFT) cargo release. Several classes of GEFs have been localized to cilia or basal bodies and shown to be functionally important in the prevention of ciliopathies, but ciliary Arl-type Sec 7 related GEFs have not been well characterized. Nair et al. (1999) identified a *Paramecium* ciliary Sec7 GEF, PSec7. In *Tetrahymena*, Gef1p (GEF1), tentatively identified by PSec7 antibody, possesses ciliary and nuclear targeting sequences and like PSec7 localizes to cilia and macronuclei. Upregulation of *GEF1* RNA followed deciliation and subsequent ciliary regrowth. Corresponding to similar Psec7 domains, GEF1 domains contain IQ-like motifs and putative PH domains, in addition to GBF/BIG canonical motifs. Genomic analysis identified two additional *Tetrahymena* GBF/BIG Sec7 family GEFs (GEF2, GEF3), which do not possess ciliary targeting sequences. GEF1 and GEF2 were HA modified to determine cellular localization. Cells transformed to produce appropriately truncated GEF1-HA showed localization to somatic and oral cilia, but not to macronuclei. Subtle defects in ciliary stability and function were detected. GEF2-HA localized near basal bodies but not to cilia. These results indicate that GEF1 is the resident *Tetrahymena* ciliary protein orthologous to PSec7.

Keywords

IFT; GEF; cilia; Arl G proteins; PH domains; IQ motifs

Introduction

The Sec7 family of guanine nucleotide exchange factors (GEFs) is responsible for activating small G proteins of the Arf (ADP ribosylation factors) family (reviewed by Casanova, 2007). Arf G proteins and the related Arf-like G proteins (Arls) are predominantly responsible for membrane trafficking and actin cytoskeleton remodeling. Once activated by the appropriate GEF, Arf G proteins carry out their specific functions until deactivated by a GTPase activating protein (GAPs). Five main families of Sec7 GEFs are currently known: Golgi BFA-resistance factor 1/BFA inhibited GEF (GBF/BIG), Arf nucleotide binding site opener (ARNO)/cytohesin, Brefeldin-resistant Arf GEF (BRAG), exchange factor for Arf6 (EFA6) and F-box only protein 8 (FBX8) (Casanova 2007). A sixth family is unique to fungi while a seventh family, TBC-Sec7 (TBS), is probably unique to alveolates (Mouratou et al. 2005).

A Sec7 GEF in *Paramecium* (P_{Sec7}, the designation used throughout this paper) was identified and cloned in a screen for ciliary proteins (Nair et al. 1999). This protein, renamed GGG2, was later described by others (Mouratou et al. 2005). Nair et al. concluded that P_{Sec7} was a ciliary protein; a peptide antibody made against P_{Sec7} confirmed ciliary, basal body and macronuclear localization (Figs 1a,b) through immunofluorescence experiments. P_{Sec7} is characterized by six IQ-like motifs (that bind proteins with EF hand domains, such as calmodulin or centrin), two pleckstrin homology (PH) domains, and a potential protein kinase A phosphorylation site. Further analysis indicates that P_{Sec7} contains a small ciliary localization signal, VxPx, which Deretic et al. (1998) found to be necessary for localization of rhodopsin into the connecting cilium and rod outer segment, as well as a bipartite nuclear localization signal (Dingwall and Laskey 1991).

Since the BRAG family of Sec7 GEFs is characterized by the presence of IQ motifs and PH domains (Casanova 2007), the presence of these motifs in other Sec7 GEFs is not surprising. In addition to IQ and IQ-like motifs, other putative calmodulin (or perhaps centrin) binding motifs have been identified in Sec7 GEFs, using the Calmodulin Target Database predict program (Yap et al. 2000). Table S1 lists IQ and calmodulin binding motifs in Sec7 proteins of *Paramecium* and *Tetrahymena*. PH domains have also been found in other GEFs (Chardin et al. 1996; Chen et al. 1997; Haslam et al. 1993; Hattori et al. 2007; Inaba et al. 2004; Klarlund et al. 1997; Kolanus et al. 1996; Michiels et al. 1997; Nair et al. 1999). While many PH domains bind phosphoinositides, which are resident in cell membranes, only about 10% of those found in the human proteome are phosphoinositide specific (Lemmon 2008; Yu et al. 2004).

Five GBF/BIG and two TBS Sec7 family GEFs in *Paramecium* have been described further (Mouratou et al. 2005). Additional conserved domains present in GBF/BIG family members include: DCB (Dimerization/Cyclophilin Binding region, ~150 aa), HUS (Homology Upstream of Sec7, ~170 aa), HDS1 (Homology Downstream of Sec7, ~130 aa), HDS2 (~160 aa) and HDS3 (~120 aa). These domains can be utilized to identify additional GBF/BIG family members. The DCB domain is possibly involved in dimerization and cyclophilin binding in *Arabidopsis* GNOM (Grebe et al. 2000). While the HDS domains remain poorly characterized, some evidence suggests that they are involved in membrane localization (Chantalat et al. 2003).

In addition to P_{Sec7}, other GEFs of various families have been shown to localize to basal bodies/cilia. These GEFs and/or their associated G proteins are important regulators of protein transport into the cilium (Table S2). Particularly relevant are studies on Arl13b, a mammalian Arf-like G protein that localizes to cilia (Caspary et al. 2007). A mouse mutation, hennin (hnn), a null allele of Arl13b, causes coupled defects in ciliary structure and in Sonic hedgehog (Shh) signaling, which relies on movement of proteins through the cilium. The GEF for this protein is presently unknown. A Ran family GEF, RPGR, localizes to respiratory tract cilia and to the retinal receptor where it is responsible for the regulation of opsin transport (Hong et al. 2003). RPGR knockout mice exhibited cone and rod photoreceptor degeneration. A mutation (631_IVS6+9del) in the human homologue of this gene results in a form of primary ciliary dyskinesia (Moore et al. 2006). The Rab family GEF Rabin8 has been shown to localize to the basal body region in mammalian cells and is likely responsible for the activation of the small G protein Rab8. Activated Rab8 then promotes the docking and fusion of vesicles near the ciliary membrane and allows the movement of Rab8_{GTP} and other associated proteins into the cilium. Disruption of this pathway is probably responsible for the development of Bardet-Biedl syndrome (Nachury et al. 2007).

The PSec7 antibody (PSec7 Ab) recognizes a protein in *Tetrahymena thermophila* cilia. In an effort to identify this unknown protein, we cloned a Sec7 family GEF in *Tetrahymena* (*GEF1*) utilizing degenerate and partial inverse PCR methods. Gef1p (designated GEF1 here) contains a sequence homologous to the peptide sequence used to generate the PSec7 Ab and putative ciliary (Geng et al. 2006) and bipartite nuclear targeting sequences (Dingwall and Laskey 1991) consistent with its immunolocalization by PSec7 Ab. Blast searches against the *Tetrahymena* genome database using the highly conserved Sec7 domain of *Paramecium* Sec7 identified six other potential Sec7 GEF family members (Table S3). Three of these proteins have been identified as Sec7 GEF family members in the GBF/BIG family, while two others appear to belong to the TBS family.

In order to demonstrate that the resident *Tetrahymena* ciliary PSec 7-like GEF was GEF1, we devised a truncated *GEF1-HA* construct that would place a hemagglutinin (HA) sequence at the end of a truncated GEF1 protein. The truncated GEF1 protein, comprised of the first 498 amino acids of GEF1 followed by the HA sequence, would contain the putative N-terminal ciliary targeting sequences, but not the Sec7 and nuclear targeting sequences or C-terminal amino acids that might be involved in ciliary targeting (Deretic et al. 1998; Sloboda and Rosenbaum 2007). The construct was used to transform *Tetrahymena* by biolistic transformation with appropriate drug selection procedures, so that cells which overproduced the truncated protein were selected. We anticipated that this construct might act as a dominant/negative knockdown of the wildtype gene, allowing us to observe any aberrant phenotypes in ciliary behavior as a result of *GEF1* knockdown. Also, provided that the ciliary localization signals in the truncated protein acted as previously predicted and were sufficient for localization, we hypothesized that ciliary localization of the truncated GEF1 protein (corresponding to the ciliary localization of the PSec7 Ab) should be maintained. Moreover, because the bipartite nuclear targeting sequences have been ablated in the truncated protein, the macronuclear localization observed with the PSec7 Ab should be abolished. Additionally, a full length *GEF2-HA* construct was produced using identical protocols, which would have Sec7 GEF activity but not the relevant localization signals (i.e. would show no ciliary localization). Here we show that these expectations are largely correct, and discuss implications for ciliary targeting and GEF function.

Results

Immunolocalization

The Ab generated against the *Paramecium* GEF PSec7 exhibited ciliary, basal body and macronuclear localization both in *Paramecium* (Figs 1a,b) and in *Tetrahymena* (Fig. 2a–c). Ciliary localization was often punctate along the length of the organelle (Figs 2c), suggesting transport by IFT complexes. Immunoelectron microscopy studies in *Tetrahymena* confirmed the localization (Figs 3a–f). Localization was seen adjacent to and along ciliary basal bodies and along the ciliary axoneme in both the oral and somatic ciliature (Fig. 3a–d). In addition, immunogold localization was often observed clustered around the base of the cilium. Localization was not apparent at the parasomal sac (Fig. 3c). In the macronucleus, immunogold localized primarily to regions corresponding to heterochromatin (Fig. 3e–f). No localization was present in the absence of primary Ab. These results suggest that in both ciliates the PSec7 Ab is recognizing a molecule or molecules with ciliary and macronuclear targeting signals.

In *Tetrahymena*, approximately 2–3% of cells in light microscopy exhibited a distinctive macronuclear localization pattern where label was localized to one portion of the macronucleus as a ring, cap or filament (Figs 2d–g). Cells observed with this macronuclear localization pattern were in the early stages of cell division. This pattern has not yet been observed in TEM due to the relatively few cells examined with this technique.

Identification and Domain Analysis of *Tetrahymena* GEFs

Degenerate PCR and partial inverse PCR techniques were used to sequence DNA corresponding to the *Tetrahymena* GEF protein, most probably recognized by the PSec7 Ab. The complete genomic sequence of *GEF1* is 8619 bp and contains 16 introns (Fig. S1). The translated amino acid sequence, GEF1 is 2053 amino acids in length with a calculated molecular mass of 238.4 kDa (Fig. S2). A sequence (ILLSCHFNLEQERNNS) homologous to the sequence used to produce the PSec7 Ab (33% identical, 53% homologous) is present.

Blast searches against the *Tetrahymena* genome database (Stover et al. 2006) then revealed that seven proteins from at least three Sec7 GEF related subfamilies exist in *Tetrahymena thermophila* (Table S3). The DNASTAR program MegAlign was used to align the seven Sec7-related *Tetrahymena* proteins with known GBF/BIG1 family members from *Paramecium tetraurelia*, *Homo sapiens*, *Anopheles gambiae* and *Drosophila melanogaster*. Aside from GEF1, two other proteins (designated GEF2 and GEF3) had high homology to PSec7. GEF2 showed a higher amino acid sequence identity to PSec7 than GEF1 in Blast searches.

The *Tetrahymena* EST library (amoebidia.bcm.umontreal.ca/pepdb/searches/welcome.php) was searched to determine whether the genes for the proteins were expressed. Three of the seven proteins produced significant hits: GEF1 - 459×10^{-129} , 411×10^{-115} , 203×10^{-52} ; GEF2 - 96×10^{-20} ; THERM_01082910 - 306×10^{-83} . A search of the *Paramecium* EST library (Arnaiz et al. 2007) for PSec7 yielded one exact sequence match over a 170 amino acid stretch, corresponding to the C-terminal end of PSec7. A search for the remaining four GBF/BIG family GEFs yielded only one significant hit for GGG1 (192×10^{-48}).

Based on sequence homology (Fig. S3), GEF1 homologs are phylogenetically widely distributed. Conserved domains common to proteins in this family are schematized in Figure S4. GEF1, GEF2 and GEF3 all possess DCB, HUS box and HDS domains. IQ domains and several other putative calmodulin binding motifs are present in all the proteins (Table S1). PSec7 contains two PH domains that roughly correspond to HDS domains. Domain analysis suggests that two PH domains are present in GEF1 that correspond to the location of the HDS2 and HDS3 domains (Fig. S2). GEF3 has a single PH domain toward the C-terminus of the protein; GEF2 has no PH domains.

Ciliary targeting sequences were detected in GEF1 but not GEF2 or GEF3. While PSec7 has a single potential ciliary targeting sequence, GEF1 has two RVxP ciliary targeting sequences in the N-terminal region (Fig. S2). These targeting motifs are consistent with the ciliary localization of PSec7 and GEF1 as identified by PSec7 Ab.

Both PSec7 and GEF1 possess bipartite nuclear targeting sequences (Dingwall and Laskey 1991) toward the C-terminus ends of the proteins, consistent with the immunofluorescence and immunoelectron microscopy results in Figures 1 and 2. GEF2 and GEF3 both have shorter nuclear targeting sequences at their N-terminal ends. Toward their N-terminal ends, PSec7 and GEF1 both contain PxxP motifs that bind SH3 domains of signaling and cytoskeletal proteins (Dalgarno et al. 1997; Kay et al. 2000; Sudol 1998). Neither GEF2 nor GEF3 possess PxxP motifs. On the basis of domain analysis and predicted targeting sequences, we conclude that GEF1 is the probable *Tetrahymena* orthologue of PSec7.

In addition to GEF1, GEF2 and GEF3, two *Tetrahymena* proteins, referred to here as TST1 (*Tetrahymena* Sec7 and TBC) and TST2, belong to the group of Sec7 related TBS (TBC-Sec7) proteins, first described in *Paramecium*, that seems to be unique to alveolates (Mouratou et al. 2005). Proteins in this family possess a TBC (Tre/Bub2/Cdc16) domain following the Sec7 domain. The TBC domain is predicted to harbor a GAP (GTPase

activating protein) specific toward small G proteins of the Rab family (Rak et al. 2000). A protein with both Arf G protein activation and Rab G protein inactivation properties has also been described (ArfGEF STY1 in yeast (Jones et al. 1999)). The remaining two Sec7-containing GEFs uncovered in the Blast search belong to an unidentified family, possibly related to the cytohesins.

Upregulation of *GEF1* upon deciliation

Experiments using RT-PCR with *GEF1* specific primers were performed on cells undergoing ciliogenesis during recovery from dibucaine-induced deciliation. The results were compared to upregulation of *PSec7* and *HSP10* RNA. The latter, a heat shock protein RNA, is not upregulated at deciliation. Like other RNAs of ciliome proteins including *PSec7*, *GEF1* is upregulated (Fig. 4a), increasing over 4 fold from 30–120 minutes of ciliary regrowth. β -tubulin specific primers were used as a control for RNA upregulation (Fig. 4b). As expected, β -tubulin was found to be upregulated, as was P13 Kinase. Another protein, *Tetrahymena thermophila* protein tyrosine kinase 1 (TtPTK1), a putative insulin-like receptor that localizes to cilia (Christensen et al. 2003), was not found to be upregulated during ciliogenesis (Fig. 4b). Heat shock and gene specific primers against a heat shock protein (HSP) were used as a control for general stress-inducing gene expression. HSP was not upregulated during ciliogenesis but was upregulated following a heat shock (Fig. 4c). Both tubulin and P13 kinase were not upregulated following heat shock (Guerra 2007).

Localization of truncated *GEF1*-HA

We used a gene replacement strategy (Gaertig and Kapler 2000) to produce and localize a truncated *GEF1* modified with an HA sequence. A hemagglutinin (*HA*) sequence (TATCCTTATGATGTTCTGATTATGCT) was placed at the C-terminal end of a target gene sequence, which was then integrated into the wild type target gene locus in the macronucleus via biolistic transformation. Paramomycin resistance was used to select for transformants in which the modified protein was expressed.

First, a truncated *GEF1*-HA was constructed and used for transformation. The resultant gene product (calculated Mr 58.62 kDa), the first 498 amino acids of *GEF1* followed by the HA sequence (Fig. S2, dotted box), would contain the DCB domain, ciliary targeting sequences and HA sequence. It would lack most of the HUS domain and the macronuclear localization sequence, Sec7, HDS and PH domains, including the sequence recognized by *PSec7* Ab.

Eight days following shooting of the truncated *GEF1*-HA construct into *Tetrahymena* SB255, clones resistant to paramomycin (100 μ g/ml) were evident. The concentration of paramomycin was gradually increased to and maintained at 400 μ g/ml - which selects for cells with continued replacement of wt *GEF1* genes in the macronucleus. Because there are ca. 45 copies of each macronuclear gene, replacement was rarely complete.

To verify integration of the construct into the correct gene locus, we isolated genomic DNA from the drug resistant clones and performed PCR using an anti-sense primer specific for the *HA* sequence and a sense primer specific to *GEF1* sequence 100 bps 5' of the sequence used for targeting the locus. A PCR product of the correct size was produced, subcloned using a Topo TA Cloning kit (Invitrogen, Carlsbad, CA) and sequenced to confirm that the anticipated nucleotide sequence was present. Immunoblot experiments with an HA Ab were also performed to verify the presence of the HA modified protein in the transformed cells. A single HA positive protein of approximately the expected size was detected. In untransformed cells, no proteins were recognized by the HA Ab.

IF of cells transformed with the truncated *GEF1*-HA construct demonstrated ciliary localization of the HA modified protein with a lack of macronuclear localization (Figs 2h–

o). There was also faint staining of the longitudinal microtubule (MT) bands and basal bodies in the cell cortex. Somatic cilia labeled along their length, but they were difficult to observe because they often were shed from the cells during preparation for immunofluorescence, although oral cilia were maintained. In the absence of primary HA and tubulin Abs, no localization was seen (Figs 2x–z). Labeling of transformed cells with PSec7 Ab (Figs 2p–w) showed similar oral and somatic ciliary and basal body localization (comparable to Figure 2a–c) as well as macronuclear localization, presumably from residual endogenous GEF1 (Fig. 2t) where both full length and truncated GEF1 are present in the cells. In these cells, PSec7 Ab did not localize to the longitudinal MT bands that were recognized by the HA Ab. Since the HA modified protein localizes to cilia but not macronuclei, we conclude that the putative ciliary localization signal containing RVxP is sufficient for ciliary localization, and that GEF1 is a ciliary GEF. We also suggest that the bipartite nuclear localization signal of PSec7 and presumably GEF1 is probably responsible for macronuclear localization.

The shedding of somatic ciliature from the truncated *GEF1-HA* cells was confirmed using scanning electron microscopy (Fig. 5a–f). Cells were prepared for SEM using the protocol used for IF preparation with the exception of two 0.1 M sodium phosphate buffer washes at the end of the protocol. Both SB255 cells (Figs 5a–b) and *GEF2-HA* (Figs 5c–d) cells were compared to truncated *GEF1-HA* cells (Figs 5e–f). Truncated *GEF1-HA* cells were generally similar in size to SB255 and *GEF2-HA* cells but many were quite bald, although oral cilia were present. Thirteen cells from each of the three cell types were chosen at random and the visible somatic cilia were counted (Fig. 6). Truncated *GEF1-HA* cells had an average of 75 somatic cilia per cell compared to 255 cilia for SB255 cells and 314 cilia for *GEF2-HA* cells.

Localization of GEF2-HA

Because of the high homology with PSec7 seen in Blast searches (Table S3), we considered the possibility that the PSec7 Ab recognized GEF2. To eliminate this possibility, we constructed *GEF2-HA* to determine the protein's localization in *Tetrahymena*. Transformation of SB255 cells with a *GEF2-HA* construct was performed in the same manner as with the truncated *GEF1-HA* construct. Resistant clones were isolated 9 days after biolistic transformation and maintained at 400 µg/ml paramomycin. PCR analysis of *GEF2-HA* confirmed that the HA construct was integrated into the correct gene locus and a HA modified protein within the expected size range was produced, although a faint ca 97 kDa band, presumably a breakdown product, was also present.

Immunofluorescence of *GEF2-HA* cells (Figs 7a–d) revealed punctate labeling, possibly of parasomal sacs adjacent to the longitudinal MT bands, absent in controls which showed only minimal non-specific cell labeling below the oral apparatus (Figs 7q–x). Although most of the somatic ciliature of the *GEF1-HA* transformed cells was consistently lost, consistent with the SEM results, *GEF2-HA* transformed cells retained their somatic ciliature in these IF preparations (Fig. 7b). Cilia were not labeled by HA Ab (Fig. 7d, h). Labeling of *GEF2-HA* transformed cells with PSec7 Ab showed oral and somatic ciliary and basal body localization (Fig. 7i–l, p) as well as macronuclear localization (Fig. 7m–o), which was comparable to Figure 2a–c and p–w. PSec7 Ab localization and the *GEF2-HA* localization were not overlapping, indicating that the PSec7 Ab was not recognizing GEF2.

Transformed Cell Biology

Cells transformed with the *GEF1-HA* construct observed microscopically after washing in inorganic media had no obvious morphological abnormalities and swam normally. Dividing cells were seen in the population and cilia were of normal length and number. Therefore, the

production of truncated GEF1 did not act as a lethal dominant negative. Cells transformed with the *GEF2-HA* construct also swam normally and had pear-shaped morphology. However, following a 24 hour starvation period in inorganic medium, transformed cells appeared slightly larger than SB255 cells and seemed to swim more slowly.

These observations suggested that transformed cells might behave differently from the non-transformed parental strain (SB255) if swimming were challenged, for example by subjecting the cells briefly to centrifugal force. Cells with swimming defects would be expected to pellet more easily at lower speeds than the parental strain (Fig. 8). SB255 experiments, used as controls for swimming competency, showed that the percentage of surface swimming cells (SSCs) seen at 300 g gradually decreased with increasing force until all cells were pelleted above 850 g. *GEF2-HA* transformants were utilized as controls for truncated *GEF1-HA* transformation and paramomycin exposure. Both the truncated *GEF1-HA* and *GEF2-HA* transformants pelleted significantly faster than untransformed SB255 cells at all speeds below 850 g, so that only a few SSCs were seen. T-test analysis comparing the percent SSC after pelleting of *GEF2-HA* cells vs truncated *GEF1-HA* cells were statistically significant between 450 and 750 g ($p < 0.05$), where truncated *GEF1-HA* cells pelleted more completely than *GEF2-HA* cells. These observations suggest that a subtle impairment of ciliary assembly or function is present in the truncated *GEF1-HA* cells.

Discussion

The conserved domain architecture within the GBF/BIG Arf GEF family facilitated discovery of three members of this family present in *Tetrahymena thermophila*, designated GEF1, GEF2 and GEF3. GEF1 and GEF2 are significantly expressed in the cells. Two other GEFs discovered in the *Tetrahymena* genome database, TST1 and TST2, belong to the TBS family. A focus of this study was to determine which of the three GBF/BIG family GEFs present in *Tetrahymena* is the orthologue of PSec7, the ciliary GEF discovered in *Paramecium*. A peptide antibody made against PSec7 was used to verify that PSec7 localizes to both the cilia and macronucleus in *Paramecium*. The Ab was subsequently used for IF and immunoEM experiments in *Tetrahymena* and was also found to localize to the cilia and macronucleus. Although all three *Tetrahymena* GBF/BIG family GEFs share a 53% homology to the PSec7 Ab recognition site, only GEF1 contains ciliary targeting sequences and bipartite macronuclear targeting sequences comparable to those of PSec7.

Furthermore, sequence analysis reveals similarities between PSec7 motifs and corresponding motifs in GEF1 not present in the other GEFs. For example, only GEF1 has two PH domains in approximately the same positions as the PH domains found in PSec7. GEF3 has a single PH domain toward the C-terminus of the protein while no PH domains were found in GEF2. In addition, both PSec7 and GEF1 contain PxxP motifs toward the N-terminal ends of the proteins, whereas GEF2 and GEF3 do not. Finally, both PSec7 and GEF1 have putative IQ motifs, both full length and truncated, present in comparable positions. The presence of IQ motifs suggests a role for calmodulin or centrin binding in the activation of these proteins, including GEF1 activation within the cilium. Both calmodulin and centrin have been shown to be expressed in *Tetrahymena* and localized to cilia (Guerra et al. 2003; Jamieson et al. 1979; Stemm-Wolf et al. 2005). Some researchers have suggested that complete IQ motifs might not require Ca^{2+} to bind to calmodulin or centrin, whereas truncated IQ motifs would be Ca^{2+} dependent (Houdusse and Cohen 1995; Munshi et al. 1996).

GEF2 also has two putative leucine zipper motifs present at the C-terminal end of the protein, unlike PSec7 or any other *Tetrahymena* GBF/BIG family GEFs. Although GEF2 showed a higher amino acid sequence identity to PSec7 than GEF1 in Blast searches, GEF2

does not localize to cilia, as shown by IF results. Therefore GEF2 cannot be the protein localized by PSec7 Ab and indeed HA vs PSec7 Ab localization in *GEF2-HA* transformed cells gives quite different results. In contrast, HA vs PSec7Ab localization in cells transformed with a truncated *GEF1-HA* gives the expected ciliary and macronuclear localizations. This domain and targeting sequence analysis together with the IF and immuno EM results support the conclusion that *GEF1* is the *Tetrahymena* orthologue of PSec7. Further, like other proteins of the ciliome and like *PSec7* (Nair et al. 1999), *GEF1* was upregulated over four fold upon deciliation and subsequent regrowth of cilia. To confirm this conclusion definitively, it would be useful to co-localize the GEF1 and HA Abs along the same cilium; we hope to do this in future work.

PSec7 Ab also localized to rows of puncta just below the longitudinal MT bands. The puncta are in the position of basal bodies and the adjacent parasomal sacs (coated pits). Immunoelectron microscopy revealed that the puncta definitively corresponded to basal bodies. Puncta seen with GEF2-HA Ab were in a slightly different and more irregular pattern. Given that most GBF/BIG GEFs are involved in the regulation of vesicular transport, the most likely site for GEF2 localization is the parasomal sacs, known to be involved in endocytosis (Nilsson and van Deurs 1983).

GEF1 and PSec7 exhibit both ciliary and macronuclear localization. Several GEFs or small G proteins that operate with GEFs have been localized to cilia, while others localize to nuclei. Localization in both organelles simultaneously has not previously been demonstrated for the same GEF, but is highly probable on the basis of our results.

Several putative ciliary targeting sequences have been reported recently (Deretic et al. 1998; Ersfeld and Gull 2001; Geng et al. 2006; Godsel and Engman 1999; Nasser and Landfear 2004; Piper et al. 1995; Tull et al. 2004). Geng et al (2006) described the trafficking of polycystin-2 to the cilium in *Pkd1^{-/-}* mice and in MDCK and LLC-PK1 cells, where mutation of a RVxP motif at the N-terminal end of polycystin-2 resulted in accumulation of the protein around the base of the cilium, instead of typical ciliary localization. These results were subsequently verified by for the localization of olfactory CNG channels (Jenkins et al. 2006). Similarly, Deretic et al. (1998) has demonstrated that the C-terminal sequence VxPx of rhodopsin is necessary for localization of rhodopsin into the connecting cilium and rod outer segment (Deretic et al. 1998; Deretic et al. 2004). Our experiments with truncated *GEF1-HA* and *GEF2-HA* constructs have shown that the protein with RVxP motifs localizes to the cilia, while a related protein without these motifs does not, suggesting that the RVxP motif may be necessary for ciliary targeting in this situation but perhaps by itself not sufficient. Due to the small size of the RVxP-type ciliary targeting sequences, it is likely that these four amino acid sequences are also found in proteins that are not associated with cilia. However, we think that additional amino acid residues in close proximity to the conserved VxPx or RVxP sequence are likely required in order to form a functional ciliary targeting motif. These additional residues might be in the form of hydrophobic residues that lack sequence conservation (much like Pleckstrin Homology domains), which makes identification of the complete ciliary targeting motif difficult. Alternatively, Pazour's group suggested that a C-terminal hydrophobic sequence was important for ciliary localization of membrane proteins (Sloboda and Rosenbaum 2007). This sequence is missing from the truncated GEF1 and cannot be responsible for its ciliary targeting.

A search for ciliary targeting sequences in human BIG1, BIG2 and GBF1 proteins revealed that both BIG1 and BIG2 proteins contain four putative ciliary targeting sequences of the VxPx variety while GBF1 contains eight. The localization of these GEFs to cilia has not been reported but might be worth further investigation. For ciliary Arf-like G proteins such

as Arl13b (Casparly et al. 2007) we would predict that there would be a BIG Sec7 GEF orthologous to PSec7 and GEF1.

Cells producing HA modified truncated GEF1 showed subtle physiological effects. The truncated GEF1 protein was predicted to function as a knockdown of wildtype GEF1, leading to a dominant negative phenotype. Instead, the truncated protein did not act as a dominant negative in the presence of endogenous GEF1. Since knockdown was incomplete, the truncated molecule did not replace endogenous GEF1 completely and only a mild swimming defect could be detected. During centrifugation, both living *GEF2-HA* cells and truncated *GEF1-HA* cells pelleted at significantly lower speeds - that is more easily - than wildtype cells, which suggests a correlation between paramomycin exposure and/or HA transformation and swimming competency. However, direct comparison of the transformed cells revealed that *GEF1-HA* cells pelleted more easily than *GEF2-HA* cells, a difference that was statistically significant ($p < 0.05$). Subjected to a force, cells containing the truncated GEF1 swim against the force less successfully than cells transformed to produce GEF2. These experimental results coupled with the observation that fixed truncated *GEF1-HA* cells shed somatic ciliature, unlike the *GEF2-HA* cells, suggest that ciliary function is impaired in the truncated GEF1 transformants, despite the normal appearance and behavior of these cells in culture. In addition, compared to PSec7 Ab, which probably recognizes endogenous GEF1, truncated GEF1-HA exhibited basal body localization as well as mislocalization to the longitudinal MT bands in the transformed cells.

GEF1 coimmunoprecipitates with and is transported by the *Tetrahymena* intraciliary transport homodimeric kinesin motor protein Kin5 (Awan, A., personal communication). Consistent with transport by a homodimeric IFT kinesin, our images show that GEF1 is found all along the cilium and that the truncated GEF1 is transported to the cilium tip. Normally after IFT (probably by Kin5) GEF1, like PSec7, becomes resident along the cilium. We propose that GEF1 and PSec7 function to regulate the transport of proteins in cilia in a similar manner to the resident nuclear GEF RCC1. The transport of nuclear proteins via the small G protein Ran utilizes RCC1 localized to the nucleus and Ran GAP (a GTPase activating protein) in the cytoplasm. Because of this, Ran is in an inactive GDP bound state in the cytoplasm and an active GTP bound state in the nucleus, releasing cargo. This gradient drives nuclear transport (Gorlich and Kutay 1999; Macara 2000; Mattaj and Englmeier 1998; Pemberton et al. 1998). Similarly, the localization of GEF1 to the cilium could activate cargo release from the IFT apparatus.

As an alternative or additional function of GEF1 ciliary localization, a GEF1-centrin complex located in the ciliary necklace region might be responsible for the finding that somatic cilia are easily shed by the truncated GEF1 transformants during fixation. This centrin-containing region above the necklace is activated by Ca^{2+} shock to produce cilia shedding (Sanders and Salisbury 1989). We propose that upon binding of calcium, GEF1-centrin becomes activated. Subsequent activation of the corresponding small G protein (Arl?) results in the uncoupling of proteins destined for the cilium from a G protein complex. This proposed mechanism of calcium regulation of GEF1 is not without precedent. Ras-GRF1 and Ras-GRF2 are bifunctional Ca^{2+} /calmodulin-dependent Ras GEFs that activate both Ras and Rac (Walker et al. 2003). As with GEF1 and PSec7, Ras-GRF1 and Ras-GRF2 possess two PH domains and an IQ motif (Buchsbaum et al. 1996; Fam et al. 1997). However, Someya et al. (2001) utilized a binding assay to illustrate that the presence of calmodulin (with or without Ca^{2+}) did not accelerate the binding of GTP γ S by ARF6 in the presence of an Arf family Sec7 GEF (ARF-GEP(100)).

While these proposed functions of GEF1 are still speculative, the data presented here strongly support the suggestion that GEF1 is a resident ciliary protein that, like other ciliary

GEFs (Table S2), plays a role in the transport of other proteins into the cilium. The corresponding small G protein and the mammalian orthologues of the GEF1/G-protein system remain to be identified. Table S2 is unlikely to be an exhaustive list. Because several classes of GEFs have been localized to cilia or basal bodies and shown to be functionally important in prevention of ciliopathies, it is probable that distinct classes of GEFs have different roles in the transport and localization of proteins, including the different membrane receptors and signaling proteins, which localize to cilia.

Materials and Methods

Cloning of GEF1

Whole genomic *Tetrahymena* DNA was isolated using the protocol of Gaertig et al. (1994a). Standard degenerate PCR protocols were used to generate the initial 50 base pair PCR fragment corresponding to the highly conserved Sec7 domain. The fragment was then subcloned into a pBluescript vector (Stratagene, Cedar Creek, Tx) and subsequently sequenced. Partial inverse PCR (PI-PCR) was used (Pang and Knecht 1997) to determine an additional 1500 base pairs of the *GEF1* gene sequence. The remaining genomic sequence was found using the *Tetrahymena* genome database. RT-PCR was used to determine the location of introns.

Tetrahymena Genome Analysis

Blast searches of the *Tetrahymena* genome (Stover et al. 2006) were performed against the *PSec7*, *Sec7* or *GEF1 Sec7* sequence as determined above. With the results identified (Table S3), both the *Tetrahymena* EST library (amoebidia.bcm.umontreal.ca/pepdb/searches/welcome.php) and the *Paramecium* EST library (Arnaiz et al. 2007) were searched.

Sequence analysis

Sequence alignments were done using the DNASTar Lasergene Suite of programs. In addition to *Tetrahymena* genome database (Stover et al. 2006) searches, domain and secondary structure analysis was done through Predict Protein (Rost et al. 2004), WoLF PSORT (Horton et al. 2007), ExPASy Proteomics Server (ca.expasy.org), PROF (www.aber.ac.uk/~phi/www/prof), NNPREPDICTION (alexander.ucsf.edu/~nomi/nnpredict.html), Jpred 3 (Cole et al. 2008), PSORT (psort.nibb.ac.jp) and Prosite (Hulo et al. 2006).

Hemagglutinin (HA) constructs and cell transformation

The truncated GEF1 and full length GEF2 were modified with HA sequence via a gene replacement method. The HA construct consisted of the following DNA fragments: 5' gene specific targeting sequence, HA epitope sequence, β -Tubulin 3' UTR, *Histone 4 promoter*, neocassette, β -Tubulin 3' UTR and 3' gene specific targeting sequence. The β -Tubulin 3' UTR, *Histone 4 promoter*, neocassette and β -Tubulin 3' UTR core fragments were digested out of the pH4T2 vector (Gaertig et al. 1994) and ligated into pBluescript KS (+) vector (Stratagene, Cedar Creek, TX). The 5' and 3' gene specific targets were generated with the appropriate terminal restriction enzyme sites using standard PCR protocols with a Mastercycler Gradient 5331 thermocycler (Eppendorf, Hamburg, Germany). The HA sequence was generated by making two long sense and anti-sense primers with the appropriate restriction enzyme sites and ligated into the HA construct vector. Constructs were subcloned using Top 10 *E. Coli* bacterial cells (Invitrogen, Carlsbad, CA, Carlsbad, CA).

Tetrahymena SB255, a strain that lacks dense core secretory granules (mucocysts) (Orias et al. 1983), was chosen for this experiment. Transformation of SB255 cells with the HA

constructs was performed using a biolistic particle delivery system (Bio-Rad, model PDS-1000/He) following the protocol described by Cassidy-Hanley et al. (1997). Clones resistant to paramomycin appeared within 7–9 days and were subsequently cultured. Paramomycin concentration in the cultures was increased stepwise from 100–400 µg/ml over several days and maintained in 400 µg/ml paramomycin.

PCR analysis of HA transformants

Genomic DNA was isolated from the truncated *GEF1-HA* and full length *GEF2-HA* transformed cells in order to verify integration of the HA sequences into the correct gene loci. An anti-sense primer was made to the HA sequence and gene specific sense primers for *GEF1* and *GEF2* were made to regions just outside of the integration sites. PCR reactions were performed using standard protocols. Amplification products were run out on 1% agarose gels, stained with ethidium bromide and detected with UV. PCR products were then subcloned using a Topo TA cloning kit (Invitrogen, Carlsbad, CA) and sequence analysis was performed to verify the presence of the HA sequence at the correct gene location.

RNA Upregulation Experiments using RT-PCR

Tetrahymena were grown to a density of approximately 1×10^7 cells per ml then placed in starvation media (10 mM Tris HCl, pH 7.4) overnight. Cells were then pelleted and deciliated using the Ficoll method (Calzone and Gorovsky 1982). Cells were passed directly into 50 mls of regeneration media (15 mM Tris-HCl, pH 7.95, 2.0 mM CaCl₂) and allowed to regenerate cilia at 27°C. Approximately 1×10^6 cells were removed every 30 min during ciliogenesis (until 120 min) and snap frozen in liquid nitrogen. cDNA was generated using the Cells-to-cDNA™ II Kit (Ambion, Austin, TX) following the protocol provided. The cDNA was then amplified by standard PCR protocols using *GEF1* gene specific primers. Upregulation was normalized to internal 17S rRNA and standardized against similar experiments performed with primers against *HSP10* a heat shock protein gene that shows no upregulation and compared to upregulation of tubulin cDNA under similar conditions.

Immunoblotting

Samples for immunoblot analysis were prepared by diluting 75 µl of packed cells with 2X sample buffer in 425 µls of ddH₂O and heating to 70°C for 20 min. 20 µls of sample was loaded onto a 4–12% SDS Nupage gel (Invitrogen, Carlsbad, CA) with 7 µl of marker placed in an adjacent lane. Gels were run at 200 V constant for 50 min with Nupage MOPS running buffer (Invitrogen, Carlsbad, CA). Protein was transferred to nitrocellulose and incubated for at least 30 min in blocking buffer (7% non-fat milk in 1X TBST (0.01 M Tris-HCl pH 7.4, 0.15 M NaCl and 0.05% Tween 20)). Blots were then incubated with 1° antibody (1:500 dilution) overnight at 4°C. The membranes were washed 3 times for 5 min each in 1X TBST, then incubated with a 2° antibody coupled to alkaline phosphatase (Sigma) at room temp for 1 hr, again washed 3 times for 5 min each in 1X TBST, then incubated with a BCIP/NBT solution (Kirkegaard and Perry Labs, Gaithersburg, MD) for developing.

Immunofluorescence

Paramecia were harvested, fixed with 2% paraformaldehyde for 30 min at room temperature (RT) and then quenched with 0.1M glycine in PBS for 30 min at RT. The cells were resuspended in PBS and pipetted onto poly-lysine coated coverslips and allowed to settle for ~15 min. The fixed cells were permeabilized using 0.5% TX-100 for 15min and washed with 0.3% Tween-20, 5% BSA in PBS (PBST). The coverslips were incubated with PSec7 antibodies (1:100) overnight at RT in a humid chamber. The coverslips were washed three times with large volumes of PBST and incubated with goat anti-rabbit IgG-Cy3 (1:100) for

one hour at RT. The coverslips were washed as before and mounted in 0.1% N-propyl gallate in 50% glycerol and viewed using the appropriate fluorescence filters in a Biorad confocal microscope.

Tetrahymena were starved overnight in 10 mM Tris-HCl buffer (pH 7.2). Cells were concentrated and then fixed in 2% paraformaldehyde in buffer A (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, 1% Triton X-100, pH 6.9) (Schliwa and van Blerkom 1981) for 30 min. Following three washes in buffer A, cells were washed in buffer B (10mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.01 % Tween-20, 3% bovine serum albumin, 5 mM CaCl₂) prior to incubation in 1° antibody diluted in buffer B (7 hrs, 1:100 dilution). Cells were then washed 3 times in buffer B followed by incubation in 2° antibody (1 hr, 1:500 dilution). Preparations were washed three times in buffer B before a 5 min incubation in DAPI, then mounted in mounting media (1X TBS, 70% glycerol, 2% n-propylgallate). PSec7 Ab was generated in rabbit and affinity purified using the peptide sequence in the first PH domain IQLMGRFDLDEEKDT (aa 854–869) of PSec7. Several commercial HA Abs were tested; only a rabbit peptide Ab to the HA sequence was useful for IF. Both 1° HA Ab and anti-rabbit 2° Ab were supplied by Jackson Labs, ME. Fluorescence imaging was done on an Olympus AX70 microscope.

Immunoelectron Microscopy

Tetrahymena were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 hr followed by three 5 min buffer washes. Cells were then dehydrated in an ethanol series, incubated in a 1:1 mixture of ethanol and LR white (London Resin Company) for 2 hr before being embedded in LR white. Sections were cut on a Diatome diamond knife (85 nm thickness) with a Reichert Ultracut E microtome and placed on nickel grids. Grids were incubated for 30 min in TBST (10 mM Tris and 0.1% Tween 20, pH 7.4) and 0.5% bovine serum albumin before incubation in 1° antibody (PSec7 Ab) for 48 hours. Grids were then washed 6 times for 5 min each in TBST before incubation in a gold conjugated 2° antibody (anti-rabbit, 15 nm; Electron Microscopy Sciences, Hatfield, PA). Grids were again washed 6 times for 5 minutes each in TBST before being post fixed in 2% glutaraldehyde in phosphate buffered saline (PBS) for 10 min, followed by three 5 min washes in ddH₂O before post staining in 3% uranyl acetate (dissolved in methanol) for 30 min. Finally, grids were washed in ddH₂O five times for 5 min each and allowed to air dry before viewing in a JEOL 100CXII operated at 80 kv.

Scanning Electron Microscopy (SEM)

The SEM fixation protocol used to view the truncated *GEF1-HA* cells (along with the *GEF2-HA* and SB255 controls) is identical to the protocol used for immunofluorescence with the exception of two 10 minute washes with 0.1 M sodium phosphate buffer (pH 7.2) at the end of the protocol. After a ten minute buffer wash with sodium cacodylate, cells were dehydrated in an increasing ethanol series. Fixed cells were then allowed to settle onto coverslips coated with 0.02 mg/ml DEAE-dextran (Amersham Pharmacia Biotech). The coverslips were then placed in a coverslip holder and dehydrated in an ethanol series before critical point drying in a Tousimis Samdri 790 Critical Point Dryer. Samples were then coated with Gold/Palladium using a Denton Sputter Coater before viewing in a JEOL 6400 Scanning Electron Microscope operated at 10 kv.

Swimming Competency Assay

To assess swimming competency, cells were grown to early stationary phase in 2% proteose peptone medium, then transferred to 10 mM Tris HCl starvation medium overnight. Cell densities were estimated and the cultures diluted to a density of approximately 20,000/ml. One ml of diluted cells from each cell line was aliquoted into 11 1.5 ml Eppendorf tubes. 50

µls of cells were removed from each of the three samples prior to centrifugation in order to calculate the SSC percentage. Each sample was spun for 1 minute at the designated speed and 50 µls of cells was removed from the surface of the sample within 10 seconds of centrifugation. Samples were then fixed 1:1 in Lugol's solution (0.39 M I₂ and 0.6 M KI) before cells were counted with a hemacytometer (Reichert Bright-Line, Rochester, NY). The percent SSC was calculated by dividing the number of cells counted following centrifugation by the number of cells counted prior to centrifugation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1a–b. Immunofluorescence localization of PSec7 Ab in *Paramecium tetraurelia*
a. Localization of PSec7 Ab is evident in the somatic and oral ciliature of *P. tetraurelia* as well as the macronucleus. Bar is equal to 10 μm. b. High magnification of *P. tetraurelia* cell cortex showing punctate staining along the length of the cilia. Bar is equal to 5 μm.

Figure 2a–z. Immunofluorescence of SB255 and truncated GEF1-HA in *Tetrahymena*
a. Double label with tubulin Ab (green) and PSec7 Ab (red). Nuclear staining is evident (arrow). Bar is equal to 2.5 μ m. b–c. Region in box has been magnified (b) and channels offset (c) to illustrate the punctate staining of PSec7 Ab along the cilia. Magnification is identical in both images. Bar is equal to 2.5 μ m. d–g. Nuclear staining in a subpopulation of cells labeled with PSec7 Ab (red) and DAPI (blue). Magnification is identical in all images. Bar is equal to 2.5 μ m. h. Truncated *GEF1-HA* transformant labeled with anti-HA Ab (red). Labeling is evident along the length of the cilia and basal bodies (arrowheads) as well as a faint staining along the longitudinal MT bands (arrows). Bar is equal to 5 μ m. i. Corresponding tubulin (green) labeling exhibiting labeling of cilia, basal bodies

(arrowheads) and longitudinal MT bands (arrows). j. Overlay of h and i. k. Higher magnification of single cilium from boxed region in (j). Anti-HA Ab labeling (red) is evident along the length of the cilium, particularly at the ciliary tip. Bar is equal to 0.25 μm . l. Truncated *GEF1-HA* transformant labeled with anti-HA Ab (red) and DAPI (blue). Labeling is evident along the cilia but not in the nucleus of the cell. m. Corresponding tubulin (green) labeling exhibiting ciliary labeling without nuclear labeling. n. Overlay of l and m. o. Higher magnification of single cilium from boxed region in (n). Anti-HA Ab labeling (red) is evident along the length of the cilium, particularly at the ciliary tip. Bar is equal to 0.25 μm . p. Truncated *GEF1-HA* transformant labeled with anti-PSec7 Ab (red). Labeling is evident along the length of the cilia as well as in distinct puncta in the basal body region. Longitudinal MT bands are not labeled. Bar is equal to 5 μm . q. Corresponding tubulin (green) labeling exhibiting labeling of cilia, basal bodies and longitudinal MT bands. r. Overlay of p and q showing staining along the length of the cilia. Note the punctate PSec7 Ab (red) labeling in the basal body region below the MT bands. s. Higher magnification of single cilium from boxed region in (r). Anti-PSec7 Ab labeling (red) is evident along the length of the cilium. Bar is equal to 0.25 μm . t. Truncated *GEF1-HA* transformant labeled with anti-PSec7 Ab (red) and DAPI (blue). Labeling is evident in both the cilia and the nucleus of the cell. u. Corresponding tubulin (green) labeling exhibiting ciliary labeling without nuclear labeling. v. Overlay of t and u showing labeling of both cilia and the nucleus. w. Higher magnification of cilia from boxed region in (v). Anti-PSec7 Ab labeling (red) is evident along the length of the cilia. Bar is equal to 0.25 μm . x. Truncated *GEF1-HA* transformant minus 1 $^{\circ}$ Ab. y. Truncated *GEF1-HA* transformant minus tubulin 1 $^{\circ}$ Ab. z. Overlay of x and y showing no evidence of background staining from 2 $^{\circ}$ antibodies. Bar is equal to 5 μm .

Figure 3a–d. Immunoelectron microscopy of Psec7 Ab localization in *Tetrahymena* cilia and macronuclei

a–b. Immunogold labeling of the oral ciliature. Bars are equal to 1 μm (a) and 0.5 μm (b). c–d. Immunogold labeling of the somatic ciliature. Note labeling at the basal body of the cilium (arrow) and lack of label in the parasomal sac (asterisk) in (c). Bars are equal to 0.25 μm in (c) and (d). e–f. Immunogold labeling can be seen throughout the macronucleus, particularly in the heterochromatin and in nucleoli. Dotted line demarcates macronuclear border. Bars are equal to 0.5 μm in (e) and (f).

Figure 4a–c. RT-PCR Upregulation of GEF1, Tubulin, TtPTK1, P13 Kinase and Heat Shock Protein (HSP)

a. RT-PCR demonstrating upregulation of GEF1 upon deciliation of *Tetrahymena* cells. Messenger RNA was isolated from *Tetrahymena* cells in 30 minute increments following deciliation. cDNA was generated from the samples in order to perform RT-PCR. Gene specific primers against GEF1 were used to probe the samples. A 360 bp fragment, corresponding to the expected size, was shown to be upregulated during the two hour interval. Deciliation and regrowth of cilia on *Tetrahymena* cells during the two hour period is represented diagrammatically below corresponding lanes. b. RT-PCR controls for upregulation experiment. Both Tubulin and P13 kinase exhibit upregulation during ciliogenesis whereas TtPTK1 (*Tetrahymena thermophila* protein tyrosine kinase1) does not exhibit upregulation. c. HSP (Heat Shock Protein) does not exhibit upregulation following deciliation. However, when cells are subjected to a heat shock, HSP protein is upregulated.

Figure 5. Scanning Electron Microscopy of SB255, *GEF2-HA* and truncated *GEF1-HA* *Tetrahymena* cells

a. SEM of *Tetrahymena* SB255 strain. Bar is equal to 25 μm. b. Higher magnification of SB255 cell. Bar is equal to 5 μm. c. SEM of *GEF2-HA* expressing cells. Bar is equal to 25 μm. d. Higher magnification of *GEF2-HA* expressing cell. Bar is equal to 5 μm. e. SEM of truncated *GEF1-HA* expressing cells exhibiting a significant loss of somatic ciliature. Bar is equal to 25 μm. f. Higher magnification of truncated *GEF1-HA* expressing cell. Bar is equal to 5 μm.

Figure 6. Average Numbers of Cilia on SB255, *GEF2-HA* and Truncated *GEF1-HA* (TGEF1) *Tetrahymena* cells

Thirteen cells from each of the three cell types were randomly selected and the cilia counted. The average number of cilia for each group was calculated. The standard error of the mean for each group was calculated and used to generate error bars. T-test analysis comparing the number of cilia between SB255/TGEF1 and GEF2/TGEF1 were statistically significant (p value $<0.005E-08$ and $<0.005E-05$ respectively) whereas comparison of cilia between SB255/GEF2 was not significant (p value >0.005).

Figure 7a–x. Immunofluorescence of GEF2-HA in *Tetrahymena*

a. *GEF2-HA* transformant labeled with anti-HA Ab (red). Labeling is evident near the basal body regions of the cell cortex. Bar is equal to 5 μ m. b. Corresponding tubulin (green) labeling exhibiting labeling of cilia, longitudinal MT bands and basal bodies. c. Overlay of a and b. *GEF2-HA* label (red) is not evident in the cilia. Many basal bodies (green) also show no *GEF2-HA* label. d. Higher magnification of single cilium from boxed region in (c). Anti-HA Ab labeling (red) is evident near the base of the cilium. Bar is equal to 0.25 μ m. e. *GEF2-HA* transformant labeled with anti-HA Ab (red) and DAPI (blue). Nuclear labeling is not evident. Bar is equal to 5 μ m. f. Corresponding tubulin (green) labeling exhibiting ciliary but not nuclear labeling. g. Overlay of e and f. h. Higher magnification of single cilium from boxed region in (g). Anti-HA Ab labeling (red) is not evident in the cilium. Bar is equal to 0.25 μ m. i. *GEF2-HA* transformant labeled with anti-PSec7 Ab (red). Labeling is evident along the length of the cilia as well as distinct puncta in the basal body region. Bar is equal to 5 μ m. j. Corresponding tubulin (green) labeling exhibiting labeling of cilia, basal bodies and MT bands. k. Overlay of i and j. Basal bodies below the MT bands show prominent PSec7 Ab (red) labeling. l. Higher magnification of single cilium from boxed region in (k). Anti-PSec7 Ab labeling (red) is evident along the length of the cilium. Bar is equal to 0.25

μm. m. *GEF2-HA* transformant labeled with anti-PSec7 Ab (red) and DAPI (blue). Labeling is evident in both the cilia and the nucleus of the cell. Bar is equal to 5 μm. n. Corresponding tubulin (green) labeling exhibiting ciliary labeling without macronuclear labeling. o. Overlay of m and n showing labeling of both cilia and the macronucleus. p. Higher magnification of cilium from boxed region in (o). Anti-PSec7 Ab labeling (red) is evident along the length of the cilium. Bar is equal to 0.25 μm. q. SB255 cells labeled with anti-HA 1° Ab. Staining along the basal body regions of the somatic ciliature is not evident. Bar is equal to 5 μm. r. SB255 cells labeled with anti-tubulin 1° Ab. s. Overlay of Figs q and r. Bar is equal to 5 μm. t. Higher magnification of cilium from boxed region in (t). Staining at the base of the cilium is not evident. Bar is equal to 0.25 μm. u. SB255 labeled with anti-HA Ab (red) and DAPI (blue). Labeling is not evident in the macronucleus of the cell. Bar is equal to 5 μm. v. Corresponding tubulin (green) labeling exhibiting ciliary labeling without macronuclear labeling. w. Overlay of u and v showing lack of macronuclear labeling. x. Higher magnification of single cilium from boxed region in (w). Anti-HA Ab labeling (red) is not evident in the cilium. Bar is equal to 0.25 μm.

Figure 8. Swimming Competency Assay

The number of surface swimming cells was determined at 0 g for SB255 (gray bars), *GEF-2 HA* (GEF2; white bars) and truncated *GEF1-HA* (TGEF1; black bars) cells. Increasing centrifugal force results in a gradual decrease in the percentage of surface swimming cells (SSCs) in SB255. Both *GEF2-HA* and truncated *GEF1-HA* transformants have significantly fewer SSCs relative to SB255. T-test analysis comparing the percentage of SSCs of *GEF2-HA* and truncated *GEF1-HA* transformants were statistically significant ($p < 0.05$) between 450 and 750 g (marked with asterisks), indicating that truncated *GEF1-HA* transformants pelleted at lower centrifugal force than *GEF2-HA* transformants. Each bar for SB255 and *GEF2-HA* represents an average of three experiments whereas each bar for truncated *GEF1-HA* represents the average of four experiments.