MULTI-STEP TRAFFICKING PATHWAY FOR REGULATION OF THE PROTEIN COMPOSITION OF THE *CHLAMYDOMONAS* FLAGELLAR MEMBRANE DURING CILIUM-GENERATED SIGNALING

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DEDICATION

To my beloved aunt godmother

"La Tia"

Mercedes Urbaneja, cell biologist (1919 – 2009)

As the person I admire most

She who inspired my insatiable curiosity in biology through her teachings filled with joy and enthusiasm

May your love for biology, your dedication to your loved ones and your infinite generosity remain in our hearts for generations to come

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by

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CARMEN ISABEL HERNANDEZ LARA, B. Sc.

The University of Texas Southwestern Medical Center at Dallas, 2012

WILLIAM J. SNELL, Ph.D.

The primary cilium is a signaling organelle found on most cell types in vertebrates. It is a distinct compartment for membrane and cytoplasmic proteins that mediate multiple sensory processes. Little is known about the cellular and molecular mechanisms that determine regulated ciliary localization of membrane or soluble proteins. Our laboratory uses the biflagellated green alga *Chlamydomonas* as a model system to study flagellar adhesion-induced signaling.

During fertilization, *Chlamydomonas* mating type plus and mating type minus gametes undergo flagellar adhesion through the interaction of flagellar adhesion receptor activities (flagellar agglutinins) encoded by the *SAG1* (*plus* gametes and *SAD1* (*minus* gametes) genes, activating a cAMP-dependent signaling pathway that ultimately leads to cell fusion and zygote formation. Our lab recently developed a *Chlamydomonas* strain expressing a *SAG1-HA* transgene, making it possible to study directly the properties of the SAG1 gene product. We found that SAG1 is cleaved soon after its synthesis to generate a C-terminal 65kDa fragment (SAG1-HA-C65), which we showed is an integral membrane protein. In my studies here, I describe new findings on the properties of SAG1-HA-C65 and its trafficking into and from the flagellar membrane. I found that in resting gametes SAG1-HA-C65 was distributed over the entire surface of the cell body plasma membrane, with little in the flagellar membrane indicating that SAG1-HA-C65 was excluded from the flagellar membrane. After flagellar adhesion-induced gamete activation or after activation of gametes with di-butyryl cAMP, SAG1-HA-C65 rapidly became enriched in the flagellar membrane concomitant with becoming concentrated around the bases of the flagella at the apical end of the cell. Moreover, cytoplasmic microtubules were required for SAG1-HA-C65 apical accumulation and for its significant delivery to the flagella during signaling. Apical concentration and flagellar enrichment of SAG1-HA-C65 occurred in cells conditionally depleted of the anterograde intraflagellar transport

motor protein, kinesin-2. Furthermore, studies on the fate of SAG1-HA-C65 showed that it was shed into the medium in a detergent soluble membrane fraction during flagellar adhesion. This work provides new insights into membrane protein trafficking that regulates the protein composition of the ciliary/flagellar membrane.

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December, 2012

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LIST OF FIGURES

LIST OF ABBREVIATIONS

cAMP: Cyclic adenosine monophosphate
db-cAMP: Di-butyryl cyclic adenosine monophosphate
DIC: Differential interference contrast microscopy
HA: Hemagglutinin
Hh: Hedgehog
IB: Immuno blot
IF: immunofluorescence
IFT: Intraflagellar transport
kDa: Kilo Dalton
PKD: Polycystic kidney disease
PKG: GMP-dependent protein kinase
Ptch1: Patched 1
PTK: Protein tyrosine kinase
RPM: Revolutions per minute
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Smo: Smoothened
wt: Wild type
BBS: Bardet-Biedl Syndrome
NPHP: Nephronophthisis
JBTS: Joubert
MKS: Meckel-Gruber
CH: cycloheximide

CHAPTER ONE

GENERAL INTRODUCTION AND BACKGROUND

GENERAL INTRODUCTION

Once long ignored because of its presumed vestigial function, the primary cilium has recently become recognized as a key organelle in development and homeostasis. This elongated cellular projection is composed of a microtubule-based axoneme enveloped by a specialized plasma membrane, the ciliary membrane. The organelle is assembled by intraflagellar transport (IFT), a two-way system that carries ciliary proteins to the tip and back to the base.

The unique compartment created by the primary cilium mediates many cellular and signaling events. For example, vision in the vertebrate eye occurs when rhodopsin, a membrane protein in the primary cilium, receives photons, triggering a downstream signaling pathway that ultimately integrates in the brain. Defects in primary cilia have been linked to many diseases - - now termed ciliopathies. For example, defective renal cilia cause polycystic kidney disease [1]. And, when the primary cilium is absent during embryonic development, the Hedgehog pathway is non-functional, leading to developmental abnormalities, such as polydactyly and holoprosencephaly [2] . Recent studies have shown that

the primary cilium plays a key regulatory role in orchestrating the dynamic ciliary localization of the receptors and activators of the Hedgehog pathway [3].

The cellular and molecular mechanisms that determine ciliary membrane protein composition are not well understood. To learn more about these important mechanisms, biochemical analysis of the organelle must be integrated with cell biological and genetic approaches. The biflagellate unicellular green alga *Chlamydomonas* is well suited for such studies, in large part because of the ease of carrying out biochemical studies of isolated flagella from this genetically tractable organism [4] [5] [6]. Using *Chlamydomonas* as a model system, our laboratory studies the regulation of the protein composition of the flagellum during cilium-induced signaling.

In this dissertation, I describe my studies on the regulation of cell body-to-flagellar trafficking of a *Chlamydomonas* flagellar membrane protein during fertilization. I have determined that SAG1-HA-C65 in resting gametes is evenly distributed on the surface of the plasma membrane of the cell body and excluded from the flagella; that during signaling the protein undergoes a rapid, microtubule-dependent re-distribution to the apical end of the cell and it enriches in the flagellar membrane; and that SAG1-HA-C65 trafficking is one way, and after enrichment into the flagella it is shed into the medium.

BACKGROUND

Cilia and Flagella

Cilia and flagella function as organelles of motility as well as cellular antennae orchestrating essential cellular events. For example, left-right asymmetry in vertebrates is determined through the combined action of motile cilia in the embryonic node that generate flow of extraembryonic fluid that contains molecules that are sensed by non-motile cilia in the node [7]. And, when the primary cilia of human kidney cells are dysfunctional or absent, the renal cells undergo increased cell growth causing polycystic kidney disease [8] [1]. Also, the primary cilium receives and processes signals responsible for several human senses, including vision and smell. (The terms cilia and flagella refer to the same organelle, and will be used interchangeably.)

Assembly and Structure of the cilium/flagellum

The cilium is formed from the mother centriole in the centrosome, the primary microtubule-organizing center of the cell. A cilium begins to form in the cytoplasm when the distal end of the mother centriole (basal body) attaches by its distal appendages to a membrane vesicle- the ciliary vesicle [9] [10] [11]. The

basal body is composed of nine outer triplet microtubules. Each triplet contains a complete microtubule (the A microtubule) and 2 partial microtubules (the B and C microtubules) that share a common wall with the A microtubule. The doublet microtubules of the axoneme nucleate from the A and B tubules of the triplets of the basal body. This region of transition from 3 to 2 microtubules is known as the transition zone [12] [13]. As the axoneme assembles, the distal appendages of the mother centriole are thought to become transitional fibers that associate with the ciliary membrane forming a deep curvature that will eventually form the ciliary pocket [14], reviewed in [15]. In addition, the ciliary membrane continues expanding by the fusion of secondary vesicles to the ciliary vesicle. This double membrane is composed of an inner and outer membrane: the ciliary membrane and the ciliary sheath. As the axoneme continues to assemble, the ciliary sheath reaches the plasma membrane and fuses with it, exposing the primary cilium to the environment as the cilium becomes fixed in the cell membrane.

Intraflagellar Transport

Cilia and flagella are assembled, maintained, and disassembled by a process termed intraflagellar transport (IFT) [16] [17] [18] [19]. IFT is a bi-directional transport system that carries cargo from the ciliary base to the tip (anterograde) and back to the base (retrograde). Microtubule molecular motors and their associated IFT particles transport cargo in the space between the outer

doublet microtubules and the flagellar membrane [16]. The IFT particles, originally discovered in *Chlamydomonas*, are composed of two protein complexes: IFT-A defined by 6 protein subunits and IFT-B by 13 protein subunits. Kinesin-2, the anterograde microtubule motor is thought to associate primarily with IFT-B particles to deliver protein cargo - - components of the axoneme - - to the flagellar tip. The retrograde motor, cytoplasmic dynein 1b/2 is thought to associate primarily with IFT-A particles to transport protein cargo back to the ciliary base [18] [20] [21] [22]. Proteins responsible for anterograde transport are essential for ciliogenesis. On the other hand, proteins involved in the retrograde system appear to be dispensable for ciliary formation. However, mutations in this later set of proteins result in a short, bulky primary cilium [2] [23] [24] [25] [26].

Structure of the cilium/flagellum

The core building blocks of the axoneme, microtubules, are elongated cylindrical structures composed of 13 protofilaments. Each protofilament is made up of α-tubulin and β-tubulin heterodimers. The peri-centriolar material associated with the sides and bottom of the basal body nucleates the formation of singlet cytoplasmic microtubules that extend into the cytoplasm.

As the most proximal region of the axoneme, structures associated with the transition zone define the distinct compartment of the primary cilium. Within

the transition zone is a distinctive structure, first visualized in freeze-fracture electron microscopy, called the ciliary necklace where transmission electron microscopy shows Y-shaped connectors linking the outer doublets to the ciliary membrane [27]. Many ciliary proteins required for ciliary signaling and ciliogenesis localize in the transition zone [28] [29] [30].

Because of the cylindrical organization of the nine outer doublet microtubules plus a central pair of microtubules, this arrangement is often referred to as the "9 + 2" structure. In addition, the outer doublet microtubules are associated with nexin links, radial spokes, outer and inner dynein arms that together with the central pair render motility to the flagellum. The axoneme of non-motile cilia is made up of only nine outer microtubules without the central microtubule doublet - - "9 + 0".

Ciliopathies

Due to its recent link to human disease, the primary cilium has become much more recognized as an important signaling organelle in the field of cell biology. Seminal studies done in *Chlamydomonas* identified key features about cilium generated signaling and ciliary structure and assembly. The connection between primary cilia and disease was made when murine mutants in IFT proteins or ciliary structural proteins, which lacked or had defective primary cilia,

developed phenotypes related to many human diseases [2] [1]. These include polycystic kidney disease (PKD), Bardet-Biedl (BBS), Nephronophthisis (NPHP), Joubert (JBTS), and Meckel-Gruber (MKS) syndromes and are associated with retinal degeneration, renal cysts, polydactyly, mental retardation and obesity. Many ciliopathy proteins have been identified to localize and signal at the transition zone of the primary cilia [31] [32] [33] [28] [29].

In renal cells, the primary cilium functions as a mechanosensory organelle by sensing the fluid flow in the kidney tubules. The fluid flow bends the primary cilium causing an intracellular calcium increase. Polycystins (PC) 1 and 2 are membrane proteins embedded in the ciliary membrane that mediate the calcium flux [31]. PKD1 and PKD2 genes encode PC1 and 2 respectively. Polycystic Kidney Disease is caused by mutations in these genes. Mutant PC1 and 2 fail to sense the calcium change causing increased cell proliferation and renal cysts formation [34].

Bardet-Biedl Syndrome (BBS) is a rare genetic disorder associated with many clinical phenotypes. These include obesity, retinitis pigmentosa, renal problems, mental retardation and learning disabilities. Defects in a group of at least 12 BBS genes are known to underlie this syndrome. Strong evidence indicates that ciliary defects cause the clinical phenotypes found in BBS. All the BBS proteins localize at the primary cilium and are implicated in ciliary formation and proper localization of other ciliary proteins such as somatostatin

receptor type 3 and melanin-concentrating hormone receptor 1 [35] [36] [37] [38] [39].

Rhodopsin- example a membrane protein that traffics through the cilium

As previously noted, the primary cilium plays a key role in receiving environmental signals and translating them into sensation in vertebrates. For example, specialized light sensing primary cilia (the rod outer segments) on photoreceptor cells in the vertebrate eye are essential for vision. The membrane protein rhodopsin is a G protein-coupled receptor found in these modified cilia that can be activated by light. Rhodopsin absorbs photons triggering a signaling transduction cascade that ultimately translates into a neurotransmitter signaling pathway that integrates the message into the brain's visual cortex [40] [41] [42]. An important feature of the visual system is that rhodopsin and its associated membranes are shed daily and undergo phagocytosis by the retinal pigment epithelial cells [43]. The daily replenishment of rhodopsin occurs by rhodopsin transport carriers that mobilize from the cell body to the ciliary base and fuse at the ciliary pocket to deliver new rhodopsin proteins to the primary cilia.

Hedgehog pathway - - example of regulated protein trafficking at the cilium

The molecular mechanisms that establish the regulated, dynamic trafficking of ciliary proteins are not well understood. The Hedgehog signaling pathway is a good example of the importance of studying the primary cilium. Studied so much for its role in animal development and for its recent link with cancer, the Hedgehog signaling pathway is coordinated at the primary cilium. Genetic studies in *Drosophila* established that Patched (Ptch), a multi-pass transmembrane protein, was the receptor of the Hedgehog ligand (Hh) and the repressor of the pathway that inhibited Smoothened (Smo), the 7 transmembrane protein activator of the pathway. In addition, the Gli transcription factors activate downstream cell proliferation and cell survival genes as well as upregulate Ptch transcription as part of the negative feedback loop of the pathway.

The cellular mechanism of the Hedgehog Pathway

The Hedgehog signaling pathway is mainly active during vertebrate development. The Hedgehog proteins determine growth pattern and tissue differentiation. For example, they act during embryonic development in the formation of the neural tube and the central nervous system. In humans, mutations in the Hh pathway result in developmental abnormalities such as polydactyly or holoprosencephaly. Recently studies have shown in many types of cancer the upregulation of the activators (Smo) as well as non-functional mutations in the repressors of the pathway (Ptch). Basal cell carcinoma is a type of cancer involving mutations in Hedgehog signaling proteins.

The initial connection between the Hedgehog pathway and the primary cilium was made when IFT mutant mice, which lacked cilia, were also unresponsive to the Hedgehog pathway and developed Hh mutant associated phenotypes [2]. The current model in the field is that in the absence of Hh ligand, Smo is constantly trafficking through the primary cilium and ciliary Ptch prevents its accumulation. Upon stimulation, Ptch binds Hh and moves out of the cilium. In the absence of Ptch, Smo becomes trapped in the cilium to activate the Gli transcription factors. These become enriched at the ciliary tip and translocate into the nucleus activating a series of genes involved in cell survival and cell cycle transition [44] [45] [3] [46] (Figure 1.1)

Many questions still remain about the Hedgehog pathway. For example, the molecular function of Ptch and how it represses Smo are still unknown. Recent studies addressed the mechanism of enrichment of Smo in the primary cilium upon activation of the pathway. These groups arrived at different conclusions. Milenkovic *et* al. showed that Smo translocated laterally from the plasma into the ciliary membrane when cells were stimulated with the Hh ligand [47]. On other hand, Wang et al showed that ciliary Smo came from an intracellular pool [48].

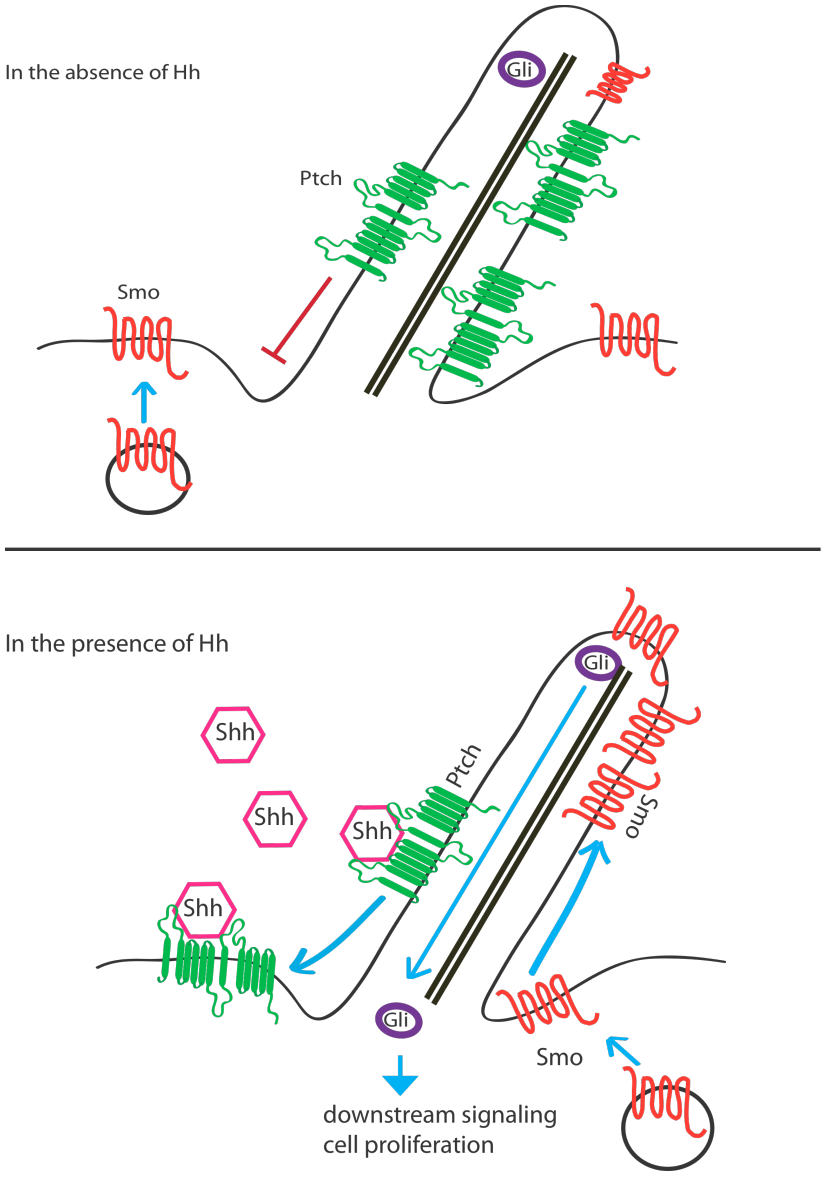
Figure 1.1

Diagram of the Hedgehog Pathway

In the absence of Hh ligand, Ptch and Gli transcription factors are found in the primary cilium, Smo is in the cell body and gene transcription is off. In the presence of Hh ligand, Ptch binds Hh and moves out of the cilium, Smo moves into the cilium and Gli transcription factors localize to the nucleus to turn gene transcription on.

Abbreviations:
Shh: Hh ligand
Ptch: Patched
Smo: Smoothened
Gli: Gli transcription factors

Figure 1.1


In the absence of Hh
Gli
Ptch
Smo
In the presence of Hh
Shh
Shh
Shh
Shh
Ptch
Smo
Gli
Gli
Smo
downstream signaling
cell proliferation

Protein Composition of the Ciliary Membrane

The molecular mechanisms that determine the protein composition of the ciliary membrane, and therefore the sensory properties of the organelle, are not well understood. Membrane proteins may localize in the flagellar membrane in a constitutive or dynamic manner. Several receptors localize constitutively in the ciliary membrane. The platelet-derived growth factor α α appears in the primary cilium when fibroblasts undergo growth arrest and assemble the organelle [49]. Polycystin-1 and -2 are constitutively in the ciliary membrane, sensing fluid flow and mediating mechanotransduction in kidney cells [32] [33] [31]. Examples of other proteins that localize constitutively in the ciliary membrane include fibrocystin, serotonin receptor 6, notch receptor, somatostatin receptor type 3, dopamine receptor 1, melanin-concentrating hormone receptor 1, and the angiopoietin receptor tyrosine kinases Tie-1 and Tie-2 [50] [51] [52] [53] [54] [55] [56] [57].

The presence in the cilium of a small number of other ciliary membrane proteins is dynamic, and is regulated by signals that originate in the organelle itself. In the Hedgehog pathway, the Hh effector protein Smo is de-enriched from the primary cilium in the absence of the ligand (Hh). Upon binding of Hh to its ciliary receptor Ptch, the ciliary amounts of Smo increase dramatically correlated

with a de-enrichment of Ptch in the primary cilium. Another example of ciliary dynamic localization of receptors is that of the dopamine receptor 1. This protein traffics in and out of the cilium upon external cues of the environment [55].

Proposed Models for regulating the protein composition of the ciliary membrane; Flagellar membrane protein composition in the absence of a signal

The primary cilium has a defined protein composition during steady state. A possibility that explains the constitutive localization of ciliary proteins is that when the primary cilium is assembly, a defined set of proteins is delivered to constitute the protein composition of the flagellar membrane. Another possible explanation is that these membrane proteins are constantly trafficking through the ciliary membrane.

Targeted delivery of freely mobile membrane proteins with a barrier: One model to explain the presence of constitutively localized ciliary membrane proteins would be that a barrier of some sort exists in the membrane near the base of the cilium. For proteins that are enriched in the ciliary membrane, golgi-derived membrane vesicles containing newly synthesized membrane proteins would undergo targeted delivery to the base of the cilium distal to the barrier. In the same way, proteins to be excluded from the ciliary membrane would be in vesicles targeted proximal to the barrier. The BBSome, a coat complex composed

of seven BBS proteins, could be involved in the targeted delivery of membrane proteins for ciliary enrichment. This complex functions at the ciliary membrane and is linked to Rab8, a small GTPase involved in targeted delivery of post-golgi vesicles [35]. A physical gate composed of septins at the base of the cilium could be preventing freely mobile ciliary membrane proteins from diffusing back into the plasma membrane. Septins are GTP-binding proteins that polymerize into filaments and are considered components of the cytoskeleton. A septin ring is found at the base of the cilium and forms a diffusion barrier that restricts protein movement between the plasma and ciliary membrane. This septin ring is required for ciliogenesis and it maintains ciliary protein distribution. For example, ciliary Smo accumulation was reduced in the absence of the septin ring [58]. The physical gate could also be composed of proteins found in the transition zone that constitute the structure of Y-shaped connectors, such as CEP290 and the NPHP1-4-8 complex (Nphp1, Nphp2 and Nphp8) [30] [29]. *Chlamydomonas* CEP290 mutant has defective Y-shaped connectors and possesses abnormal amounts of flagellar membrane protein polycystin-2 [30]. RNAi of Nphp1, Nphp2 and Nphp8 cause defective apical junctions in spheroid growth, a system used to study cystic kidney diseases [29].

Compartment-specific immobilization and no barrier. An alternative, formally possible model for enriching or excluding proteins from the ciliary membrane

would be to posit that no barrier exists at the base of the cilium. Rather, initially freely mobile membrane proteins are delivered to the plasma membrane and then freely exchange with the ciliary membrane. Enrichment could occur in the plasma membrane or the ciliary membrane if a membrane protein was immobilized by interaction with proteins or lipids specific to either membrane compartment. For example, peripheral membrane proteins associated with cytoskeletal elements could bind to integral membrane proteins to immobilize them. This mechanism, possibly mediated by Ptch, may be keeping Smo freely mobile in the plasma and ciliary membranes in the absence of the Hh ligand. Upon Hh ligand biding to Ptch, Smo may become immobilized in the primary cilium in the absence of Ptch.

Flagellar membrane protein composition in the presence of a signal

Models for signaling-controlled specification of the protein composition of existing cilia emerge from the models above. One model from the Hh pathway is related to the immobilization model. According to this idea, Smo constantly trafficks through the primary cilium in the absence of Hh ligand. Binding of Hh to Ptch, however, leads to changes in the ciliary membrane environment that lead to accumulation of Smo there [46].

In other models that more directly incorporate a structural barrier, a signal could cause modification of the barrier that would allow freely mobile proteins to move between the two membrane compartments. Such a model is supported by

the evidence that signaling in the Hh pathway triggers lateral movement of pre-existing Smo from the plasma membrane to the ciliary membrane. Or, as has been proposed in other models for Smo, the barrier might remain intact, and signaling would trigger targeted delivery of Smo-containing endosome-derived membrane vesicles to the base of the cilium, distal to the barrier. In a more complex barrier/transport model, signaling could modify the barrier and at the same time trigger a mechanism for actively moving proteins from the plasma membrane into the ciliary membrane. For example, Smo may be using this delivery mechanism upon Hh binding to Ptch [48].

The role of IFT in transporting membrane proteins into the cilium

The role of IFT in the determination of membrane protein composition of the ciliary membrane is not well understood. Some studies show that membrane proteins, like Smo and *Chlamydomonas* PKD2, accumulate in the cilia of certain IFT mutants [59] [46]. A recent study identified IFT25 to be essential for proper Hedgehog signaling. IFT25 mutant mice possess normal primary cilia but have defective Hedgehog ciliary component distribution and show Hedgehog mutant associated phenotypes [60]. IFT particles have also been implicated in cellular activities within the cytoplasm. IFT20, part of the IFT-B complex, was implicated in protein trafficking from the Golgi to the basal body [61]. In addition, IFT20 was shown to be essential for the proper transport of rhodopsin out of the Golgi

[62]. Perhaps, IFT may contribute to the protein composition of the ciliary membrane by regulating the proteins that are delivered to the ciliary base. In spite of these findings relating IFT and ciliary membrane proteins, definitive experiments are lacking that address whether IFT is required for the movement of membrane proteins from the plasma membrane to the ciliary membrane.

The role of cytoplasmic microtubules in the delivery of ciliary proteins

It also could be that cytoplasmic microtubules are involved in the regulation of ciliary membrane protein composition. The singlet microtubules of the cytoplasm have been implicated in the trafficking of soluble proteins to the ciliary base. In *Xenopus laevis*, microtubules and the dynein-dynactin complex are essential in the delivery of centriolar components to the centrioles [63] [64] [65] [66] [67]. Kim et al. found that cytoplasmic microtubules are required for the Gli2 movement into the primary cilia [46]. In addition to delivery of soluble proteins, perhaps, cytoplasmic microtubules are involved in the targeted delivery of select membrane proteins from the plasma membrane to ciliary membrane upon a signal.

Advantages and disadvantages of the vertebrate system

Some aspects of ciliary assembly and its structural components have been discovered. On the other hand, little is known about how select membrane proteins traffic in and out of the cilium. To study the molecular mechanisms occurring in the mammalian primary cilium, biochemical methods are essential. The vertebrate system offers immunofluorescence techniques and genetic approaches but biochemical analysis is limited because a primary cilia isolation technique has yet to be developed. Therefore, it is challenging to uncover the cellular mechanisms that occur in such a small proportion of the cell without biochemical methods. To learn more about the important signaling mechanisms occurring in the primary cilium biochemical approaches need to be integrated with immunofluorescence microscopy and molecular biology.

Chlamydomonas system

The eukaryote unicellular green alga *Chlamydomonas reinhardtii*, is amenable to several techniques and approaches including molecular biology, genetics and biochemistry and is an ideal model system to study the biology of cilia and flagella. *Chlamydomonas* possesses two flagella used for motility and signaling during fertilization for cell-cell interaction. Biochemical methods are well established in this model organism and their flagella can be easily isolated.

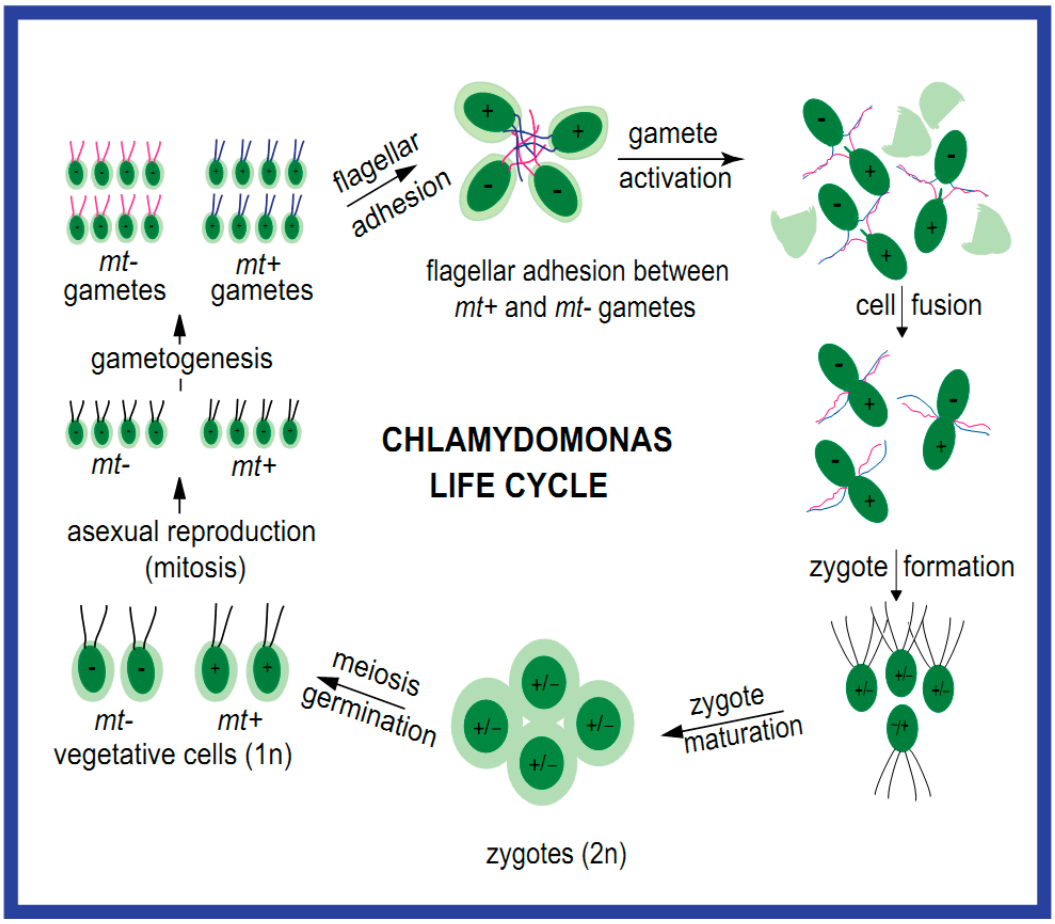
Using *Chlamydomonas* as a model system, our laboratory studies the regulation of the protein composition of the flagella during fertilization. When nutrients are available, mating type minus (mt-) and mating type plus (mt +) cells grow vegetatively. In the absence of resources, vegetative cells undergo gametogenesis [68]. During fertilization, gametes of opposite mating type adhere to each other through their flagella, resulting in gamete fusion to form a zygote. During zygote formation, a thick wall develops to protect the newly formed zygote from harsh conditions. The main purpose of the gametes is to fuse and form a zygote in order to survive [69] (Figure 1.2).

Figure 1.2

Diagram of the *Chlamydomonas* life cycle

Vegetative cells undergo asexual reproduction. They undergo gametogenesis upon nitrogen deprivation. Gametes of the opposite mating type adhere through their flagella and undergo gamete activation (cell wall loss, activation of the mating structure, flagellar tip activation). This cell-cell interaction leads to cell fusion and zygote formation.
(Image courtesy of William J. Snell)

Figure 1.2


CHLAMYDOMONAS
LIFE CYCLE
mt-
gametes
mt+
gametes
flagellar
adhesion
flagellar adhesion between
mt+ and mt- gametes
gamete
activation
cell fusion
zygote formation
zygote
maturation
zygotes (2n)
meiosis
germination
mt-
mt+
vegetative cells (1n)
gametogenesis
mt-
mt+
asexual reproduction
(mitosis)

Interactions between the mt+ flagellar adhesion receptor (agglutinin) encoded by the *SAG1* gene and its equivalent receptor encoded by the *SAD1* gene on the flagella of mt- gametes bring about flagellar adhesion and trigger the activation of an IFT-dependent flagellar signaling cascade that results in cell fusion and zygote formation [70] [71]. Agglutinins interact and activate a protein kinase pathway that results in an increase of cAMP, gamete activation and replenishment of agglutinin activity in the flagella [71] [72] [73] [74] (Figure 1.3).

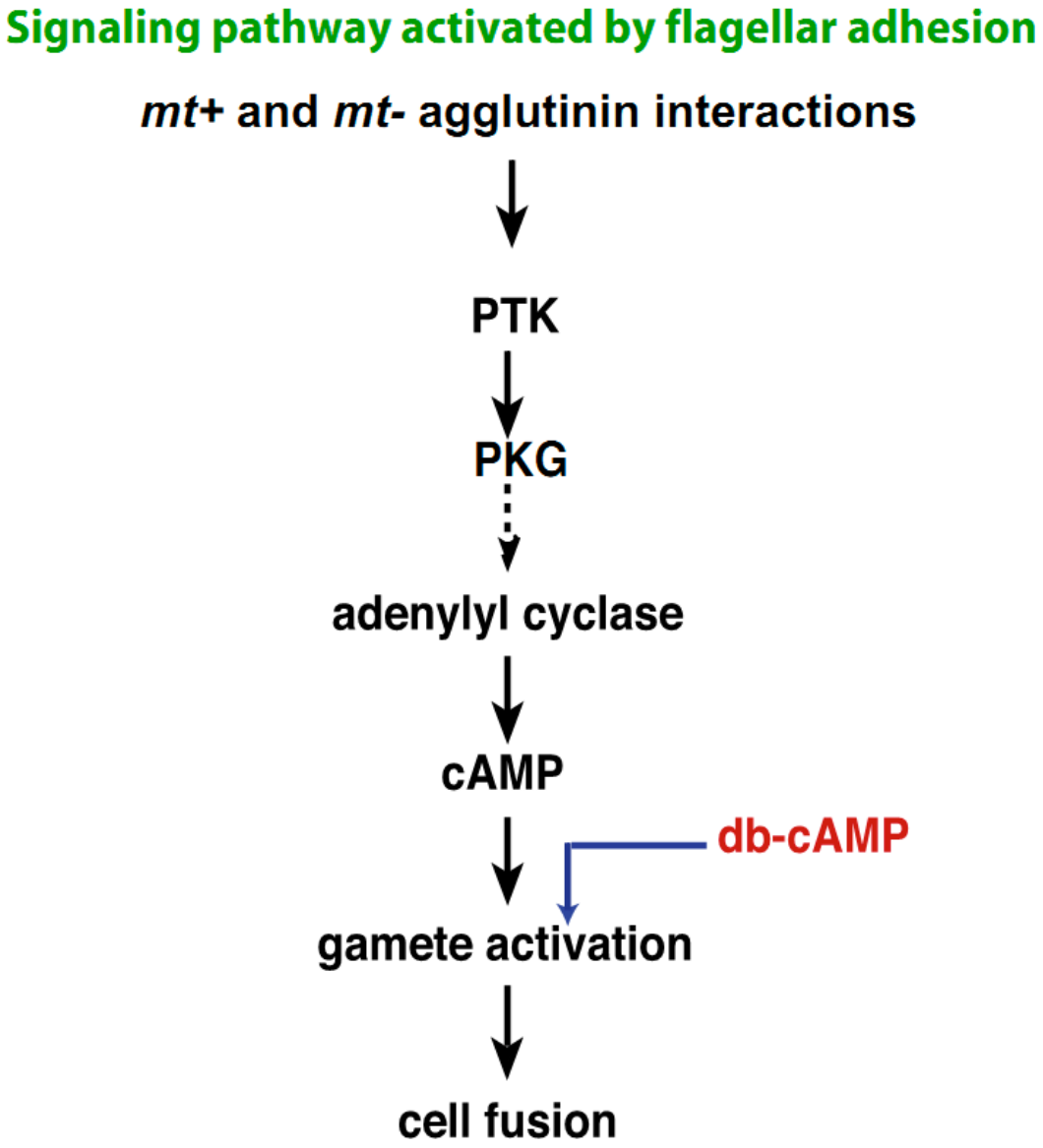
Figure 1.3

Signaling cascade triggered in the adhering flagella of *plus* and *minus* *Chlamydomonas* gametes

Plus and *minus* flagella of gametes of opposite mating type interact and generate a signaling pathway in the flagella activating kinases and resulting in a increase of cyclic AMP levels.

Abbreviations:
PTK: Protein tyrosine kinase
PKG: cyclic GMP dependent protein kinase
cAMP: cyclic AMP
db-cAMP: di-buryryl cyclic AMP

Figure 1.3


Signaling pathway activated by flagellar adhesion
mt+ and *mt*- agglutinin interactions
PTK
PKG
adenylyl cyclase
cAMP
db-cAMP
gamete activation
cell fusion

SAG1- the flagellar adhesion molecule

In 1983, Adair *et* al. used biochemical methods combined with bioassays to characterize a polypeptide with flagellar adhesion activity that was termed the *plus* agglutinin [75]. The adhesion-active polypeptide was reported to be a high molecular weight, water-soluble peripheral membrane glycoprotein [76] [74]. In studies to characterize the SAG1 flagellar adhesion activity, our laboratory used biochemical methods and bioassays to study flagellar adhesion. We also found that a polypeptide with flagellar adhesion activity could be purified in an aqueous soluble form from cell lysates. Using bioassays (rather than direct probes, which were lacking) for the activity, we determined that the majority of the agglutinin activity was associated with the cell body and little was present in the flagella [74]. We reported that agglutinin activity was present on the external surface of the plasma membrane of the cell body and that the cell body activity in resting gametes did not exchange with the flagellar activity. The simplest interpretation of these results was that an uncharacterized barrier blocked movement of the agglutinin activity from the cell body to the flagella. Furthermore, addition of di-butyryl cAMP resulted in the movement of agglutinin activity from the cell body to the flagella [74]. These findings were interpreted to indicate the existence of a mechanism that regulated the protein composition of the flagellar membrane during signaling.

These interpretations were based on assays for agglutinin activity and were inconclusive about whether the cAMP-induced restoration of flagellar agglutinin activity resulted from re-activation *in situ* of agglutinin activity on the flagella, or from the movement of the protein from the cell body to the flagella. Thus, a direct biochemical tool for immunoblotting and immunofluorecence is required to further characterize SAG1 protein properties.

In 2005, the agglutinin genes were cloned - - *SAG1*- the *plus* agglutinin and *SAD1*- the *minus* agglutinin [77]. At the time, no transmembrane domains were reported to be present in the proteins encoded by the *SAG1* and *SAD1* genes. This was consistent with the biochemical studies that showed that the polypeptides with flagellar adhesion activities were membrane associated, were aqueous-soluble and could be removed from the membrane without use of detergents [76] [74].

To summarize

The distinctive sub-cellular compartment of the primary cilium accommodates signaling receptors essential for cell-cell interactions and cellular homeostasis. The signaling function of the primary cilium is determined by its dynamic membrane protein constitution. Key molecular and cellular mechanisms about the regulation of ciliary membrane protein composition remain to be

understood. As described below, my studies on the fate of the membrane protein SAG1-HA-C65 during its dynamic trafficking in the cell body and flagella provide new insights into a cellular and molecular mechanism that regulates the protein composition of the flagellar membrane.

CHAPTER TWO

MULTI-STEP TRAFFICKING PATHWAY FOR REGULATION OF THE PROTEIN COMPOSITION OF THE *CHLAMYDOMONAS* FLAGELLAR MEMBRANE DURING CILIUM-GENERATED SIGNALING

INTRODUCTION

The primary cilium is a non-motile elongated organelle found in most cell types in vertebrates and functions as a signaling organelle coordinating the cell with its environment. The cilium comprises a distinct compartment containing membrane and cytoplasmic proteins that mediate multiple sensory processes, including phototransduction, odorant sensing, and several key developmental pathways. Defects in primary cilia have been linked to many diseases, now called the ciliopathies, demonstrating the key role of the primary cilium in humans [1] [2]. The use of cilia and flagella as signaling organelles is an ancient invention, and unicellular organisms such as the green alga *Chlamydomonas* use them not only for motility, but also for cell-cell adhesion and intercellular signaling during fertilization (as reviewed in [78]).

Little is known about the cellular and molecular mechanisms that determine either constitutive or regulated ciliary localization of membrane proteins. Previous studies in our laboratory showed that a peripheral membrane

polypeptide with flagellar adhesion activity encoded by the *SAG1* gene was present on both the cell body and the flagellar membrane of *plus* gametes, and that cells possessed a mechanism for preventing free protein exchange between the two compartments. In the absence of signaling, the agglutinin activity was present on the cell body and very little in the flagella. Upon signaling, the agglutinin activity moved from the cell body to the flagella [74]. In addition, previous studies in our laboratory demonstrated that the agglutinin activity moved into the flagella independently of IFT [79]. Although the results were intriguing, the studies suffered from the absence of probes that could directly detect the polypeptide and instead relied on indirect bioassays that detected the flagellar adhesion activity. Thus, changes in the activity of the protein rather than the amount of the protein per se could have influenced the interpretation of the results. Furthermore, because the polypeptide with adhesion activity is a peripheral membrane protein, the movement from the cell body to the flagella could have been due to its release from the cell body membrane and binding to the flagellar membrane. A direct biochemical probe was necessary to proceed in our studies about the biochemical properties and cellular behavior of the SAG1 gene product.

Very recently our laboratory has re-focused on the properties of the SAG1 gene product. Using more recent protein structure prediction analysis methods, we discovered that the C-terminal half of the protein predicted to be encoded by the

SAG1 gene likely has multiple transmembrane domains. Furthermore, the N-terminal half of the protein alone could account for the biochemical and structural properties of the *plus* agglutinin polypeptide that had been studied by the Goodenough laboratory and ours.

To learn more about the SAG1 gene product, our laboratory generated a C-terminal HA-tagged *SAG1* transgene and used it to rescue flagellar adhesion in a *SAG1* adhesion-defective mutant, thereby allowing us to detect SAG1 directly by use of anti-HA antibodies. Consistent with our new observation of the presence of several transmembrane domains in the SAG1 gene product, we discovered that the 2005 model [77] for the SAG1 protein was incorrect. Based on biochemical analysis, we showed that the SAG1 protein undergoes cleavage soon after its synthesis, to generate an HA-tagged, 65 kDa, C-terminal portion (SAG1-HA-C65). Presumably, the N-terminal half of the protein that lacks transmembrane domains and is predicted to form a large, rod shaped molecule represents the agglutinin activity that was previously characterized. The studies reported here focus on the HA-tagged C-terminal 65 kD polypeptide predicted to contain 3 transmembrane domains. In addition, we show that SAG1-HA-C65 has the biochemical properties of an integral membrane protein and that the protein is on the external face of the plasma membrane (Figure 2.1). Thus, we have established a system with a direct biochemical probe to study a bona fide

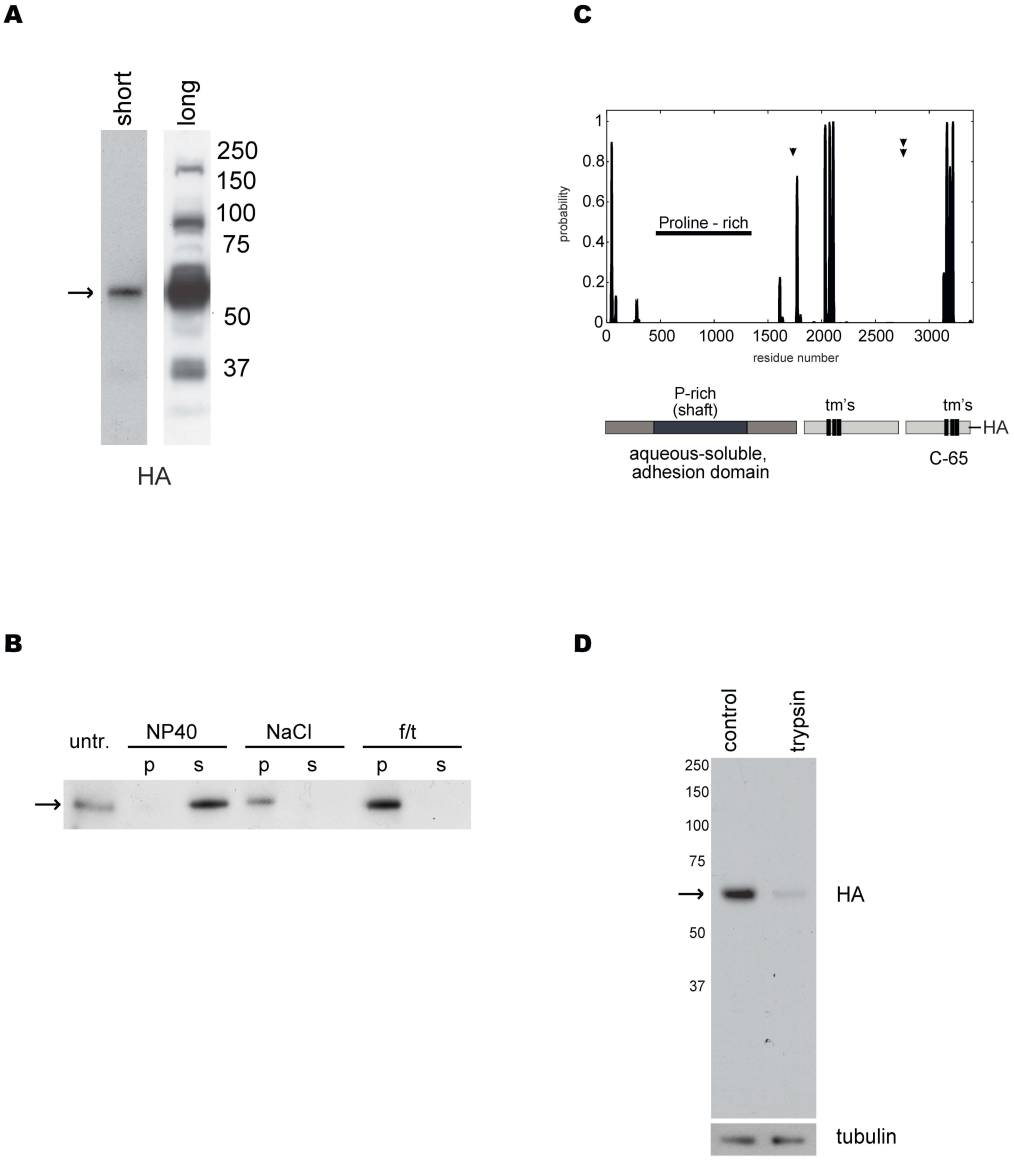
transmembrane polypeptide derived from *SAG1* that can be used to learn more about the regulated movement of an integral membrane protein into the cilium.

Figure 2.1

SAG1-HA-C65 is a 65 kDa C-terminal fragment that behaves as an integral membrane protein found in the cell surface

A) SAG1-HA-C65 is a 65 kDa C-terminal fragment**.** Immunobloting with anti-HA antibody combined with use of short and long exposures showing the 65 kDa band as the major species but also higher molecular bands can be detected in cell bodies of SAG1-HA gametes upon activation with db-cAMP. B) SAG1-HA-C65 has biochemical properties of an integral membrane protein. Cell body samples from resting *SAG1-HA* gametes were treated with 1% NP40 for 30 minutes on ice, 500 mM NaCl for 30 minutes on ice, or 4 cycles of rapid freeze-thaw mechanical disruption. Untr: untreated. f/t: freeze-thaw. C) B: Graphical illustration of transmembrane domains in SAG1 predicted by the TMHMM. Arrowheads indicate cleavage sites that possibly generate an N-terminal soluble fragment and a C-terminal fragment with 3 transmembrane domains (top panel); diagram of 3 predicted fragments when SAG1 gene product undergoes cleavage events (bottom panel). D) SAG1-HA-C65 is found at the cell surface. Immunobloting of control *SAG1-HA* gametes and trypsin treated gametes. Gametes were incubated for 5 min with 0.01% trypsin. Tubulin was used as a loading control.

Figure 2.1


A
short
long
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150
100
75
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37
HA
C
probability
Proline - rich
residue number
P-rich
(shaft)
tm's
tm's
HA
aqueous-soluble,
adhesion domain
C-65
B
untr.
NP40
NaCl
f/t
p
s
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s
p
s
D
control
trypsin
250
150
100
75
50
37
HA
tubulin

Here, I report new findings about the properties of SAG1-HA-C65 in resting and adhering gametes. First, in resting gametes SAG1-HA-C65 is excluded from the flagellar membrane in the absence of cilium-generated signaling. Second, upon signaling, SAG1-HA-C65 rapidly re-distributes to the peri-flagellar region of the plasma membrane in a cytoplasmic microtubule-dependent cellular process. Third, upon signaling, SAG1-HA-C65 is enriched in the flagellar membrane. Fourth, SAG1-HA-C65 is shed from the flagella into the medium during flagellar adhesion. To summarize, the cell possesses multiple mechanisms to regulate its flagellar protein composition: by excluding the membrane protein from the flagellar membrane; by enriching the membrane protein in the flagellar membrane; by concentrating it at the flagellar base facilitating its entry into the flagellar membrane; and by releasing the membrane protein from the flagellar membrane into the medium.

MATERIALS AND METHODS

Cell strains, cell media and reagents

Chlamydomonas reinhardtii strains *hap2* mutant (mt-; Liu [80]*et al*. 2008), 21gr (mt+) (CC-1690), *SAG1-HA* (mt+); *SAG1-HA/fla10* (mt+) were used in these experiments. Cells were plated and maintained on TAP medium agar plates at 12°C for long-term storage. Cells were inoculated in Minimal medium liquid (250 ml) culture at 23°C in a 12:12 h light:dark cycle. Minimal medium (M media) is the Medium I described by Sager and Granick (Sager [68]and Granick, 1954). All chemicals and reagents used are reagent grade from major providers unless noted differently.

Gametogenesis

To trigger gametogenesis, vegetatively growing cells in liquid culture were centrifuged in the early afternoon and resuspended in nitrogen-depleted medium (M-N medium), and aerated by bubbling under continuous illumination for 18-22 hours. M-N medium is the same as M medium minus the ammonium nitrate, the nitrogen source.

Cell Body and Flagella Isolation

Cell bodies and flagella were isolated as previously described (Wang [71]et

al., 2006; Witman [4]et al., 1972). Cells were harvested by low speed centrifugation and resuspended at a concentration of $1x10^8$ cells/ml) into a ice-cold solution of 20 mM Tris containing 5% sucrose (pH 7.2) in a beaker that contained a magnetic stirrer. While the cells were being stirred, the pH was lowered from 7.2 to 4.5 by adding drops of 0.5 M acetic acid. Once the pH was stable at 4.5, flagellar detachment was confirmed by phase contrast microscopy. Immediately, the pH was brought back to 7.2 by adding 0.5 M potassium hydroxide. After deflagellation, samples (30ml) were distributed into 50ml polycarbonate conical tubes (Nalgene) and placed on ice to underlay the samples with ice cold 20 mM Tris with 25% sucrose (pH 7.2). Cell bodies were obtained from the pellet after centrifugation at 3000 rpm for 9 minutes (rotor number, H6000A, Sorvall RC-30 (DuPont Instruments). The flagella were obtained from the supernatant carefully not to disrupt the cell body-containing pellet. This flagella-containing supernatant was under layered one more time with ice cold 20 mM Tris with 25% sucrose (pH 7.2) to remove any trace of cell bodies and centrifuged again. The flagella-containing supernatant was transferred to 50 ml round-bottomed polycarbonate tubes and centrifuged at 12,000 rpm for 20 minutes (SA-600 rotor; Sorvall). The flagella-containing pellet was resuspended in 10 mM Tris containing EDTA-free protease inhibitor from Roche (Catalog number 04693159001) to 1-2 mg/ml. Cell bodies were resuspended in 10 mM Tris containing EDTA-free protease inhibitor from Roche (Catalog number

04693159001) to 5-6 mg/ml. Both sets of samples were stored at -80 °C.

Immunobloting

Protein concentrations were measured with the Bio-Rad Protein Assay (Catalog number 500-0006) as described by the manufacturer. SDS-PAGE and immunoblotting were performed as previously described (Wang [71]et al., 2006). Samples were mixed with 1x SDS sample buffer (from a 4X stock solution: 62.5mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.1M DTT final concentration) boiled for 5 min, placed on ice for 1 minute, centrifuged for 2 minutes at 14,000 rpm (tabletop centrifuge 5417R Eppendorf), and proteins were separated by SDS-PAGE using commercially available 4-20% polyacrylamide gels following the manufacturer's protocol (GenScript USA). Proteins were transferred to a PVDF membrane at 100 V for 90 minutes. The membrane was blocked in a 30 min incubation with TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween 20; pH 7.6) containing 5% dry milk. After blocking, the membrane was incubated overnight under constant slow agitation at 4°C with a 1/10,000 dilution of Rat anti HA antibody (Roche catalog number: 11 867 423 001) in 3% dry milk in TBST. The membrane was then washed 3 times with TBST (7 minutes each wash), and incubated with an anti rat HRP-conjugated secondary antibody (Calbiochem # 401416; dilution 1/5000) at room temperature for 30 minutes. The rest of the procedure was performed as described previously (Misamore [81]et al., 2003).

The membranes were then washed 3 times for 7 minutes with TBST, incubated with enhanced chemiluminescence immunoblotting reagents (Thermo Scientific #34095) for 1 minute as described by the manufacturer, exposed to autoradiography films (GeneMate #F-9029 – 8X10), and the films were developed in an automatic film processor.

Immunofluorescence

The immunofluorescence experiments were performed in 8 well immunofluorescence slides that were coated with 60µl (in each well) of Poly-L-Lysine solution (Sigma-Aldrich Catalog number P8920) for 20 minutes. The solution was aspirated with an air vacuum device. The slides were dried under a fume hood for 3 minutes, washed with water and dried under the hood until use. Cells were counted and resuspended to a concentration of $5x10^7$ cells/ml - $1x10^8$ cells/ml. Live cell samples (60µl) of cells were placed in wells for 5 minutes. Cells were fixed vertically in ice-cold 100% methanol for 10-20 minutes at -20°C. Slides were dried for 2 minutes and then immersed in phosphate buffer solution (PBS) (20mM phosphate; 150mM NaCl; pH 7.4) for 10 minutes. Slides were dried very well and blocked for 30 minutes with 60 µl of Blocking Serum I (BS I) which contains 1% cold water fish gelatin (Sigma-Aldrich Catalog number G7765), 5 % BSA (Sigma-Aldrich Catalog number A7906) in PBS. BS1 was aspirated and slides were blocked again for 30 minutes with Blocking Serum 2

(BS 2) which is 90% BS I + 10% goat serum. BS2 was aspirated and slides were incubated with primary antibodies (1:100) in a solution of PBS that contained 20% of BS2 for 2 hours at 37°C in a humid chamber. Primaries antibodies include Rat anti-HA antibody (Roche catalog number: 11 867 423 001), Mouse anti-alpha tubulin (Sigma-Aldrich catalog number T9026), and Mouse anti-acetylated tubulin (Sigma-Aldrich catalog number T6793). Primary antibodies were aspirated and slides were washed 3 times with 20% BS2 in PBS solution for 5 minutes each wash. Slides were then incubated with secondary antibodies (1:400) for 2 hours at 37°C in a humid chamber. Secondary antibodies include Alexa Fluor 488 goat anti-rat IgG (H+L) (Invitrogen catalog number A11006) and Alexa Fluor 488 goat anti mouse IgG (H+L) conjugate (Molecular Probes catalog number A11001). Secondary antibodies were aspirated and slides were washed 3 times with 20% BS2 in PBS solution for 5 minutes each wash. Slides were washed once with PBS for 5 minutes and mounted with several drops of Fluoromount-G (SouthernBiotech catalog number 0100-01) to cover the entire slide. A cover slip was applied to each slide, sealed with nail polish, and dried for at least 10-15 minutes in darkness before being examined under the microscope. The microscopes used include a digital light microscope (Carl Zeiss Axioplan 2E, motorized focus drive with Hamamatsu monochrome digital camera) and a confocal microscope (Leica TCS SP5). OpenLab software was used to capture images. ImageJ software was used to analyze and process images.

Generating *SAG1-HA/fla10* mutant cell line

SAG1-HA (mt+) and *fla10* (mt-) gametes were pre-incubated with 15mM db-cAMP and 15uM papaverine for activation [73]. Activated *SAG1-HA* and *fla10* gametes were mixed together for 2 hour. Cell fusion was confirmed under the microscope by the presence of quadriflagellated zygotes. Mixed cells were plated in nitrogen-depleted medium agar plates and incubated under constant light overnight. The following day, plates were wrapped in aluminum foil to be kept in the dark at 4 degrees for a week. After a week, a sterilized razor blade was used to gently scrape the plated cells and the plate was exposed to chloroform for 45 minutes. A piece of agar that contained zygotes was cut and transferred to a new M medium plate by carefully rubbing the piece of agar on the surface of the new plate. The M medium plate with the transferred zygotes was placed under constant light for zygote germination until colonies could be detected with the naked eye. Several colonies were transferred to 100 µl of M medium overnight. The following day, a small volume of cell culture (10-50 µl) was plated in M medium plate and placed under constant light. Single colonies were picked into 24 well plates and grown until the concentration was $1x10^5$ cell/ml. Cells were placed at 33 degrees for 2 hours to screen for *fla10* phenotype by identifying those that had shortened or lost their flagella. Cells with *fla10* phenotype were screened for mating type and SAG1-HA expression by inducing gametogenesis and analyzing through immunoblotting using GSP1 and HA antibodies [82]. *SAG1-*

HA positive cells were confirmed to be *fla10* by the shortening or loss of the their flagella at 33 degrees.

Cell wall loss assay

In order to measure cell wall loss, a small amount of cells (20 - 100µl; $1x10^8$ cells/ml were incubated with 1ml of cell wall loss buffer (.075% of Triton X-100, 5mM EDTA, pH 8. Samples were vortexed for 15 seconds and centrifuged at 6000 rpm for 30 seconds (MiniSpin plus centrifuge, Eppendorf). Cells with walls would be sedimented under these conditions, yielding a green pellet, whereas cells that had lost their walls would be disrupted by the detergent, yielding a green, chlorophyll-containing supernatant and a small white pellet [83].

Conditioned medium treatment (Lysin)

Conditioned medium containing lysin, a metalloproteinase released in an active form from cells during gamete activation, was obtained as previously described in [83]. Briefly, $1x10^8$ cell/ml of 21gr (mt+) and $1x10^8$ cell/ml of fusion mutant *hap2* were mixed for 30 minutes with aerated under constant light to induce gamete activation, lysin release, and cell wall loss. Cell wall loss was confirmed using the wall loss assay. Cells were centrifuged at 3000 rpm for 5 minutes to obtain the supernatant (adhering medium). This supernatant was ultra centrifuged at 200,000g (rotor 70ti) for 30 minutes at 4 °C. Cell samples were

incubated with the resulting supernatant for 20 minutes to induce cell wall loss, which was confirmed with the cell wall loss assay.

Recovery of SAG1-HA-C65 from the medium

Shed SAG1-HA-C65 was harvested from the medium of gametes of cells that were resting, mixed with *hap2* mutant gametes, mixed with *hap2* mutant gametes in 10 μg/ml cycloheximide final concentration, incubated with adhering medium, or activated with di-butyryl cAMP. To obtain shed SAG1-HA-C65 from cell culture medium, cells were removed from the medium by centrifugation twice at 3,000 rpm for 5 minutes in a table top centrifuge (AccuSpin 3R) at 4°C. This supernatant was then centrifuged at 11,500 rpm (rotor SA-600) for 30 minutes at 4°C in round-bottomed 50 ml polycarbonate centrifuge tubes. This low speed supernatant was then centrifuged at 133,000 g (49,600 rpm/ rotor TLA 100.3) for 1 hour at 4°C to sediment particulate material, which was resuspended in 100-200 μl of 10mM Tris pH 7.6 and stored at -80 °C.

Detergent fractionation of shed SAG1-HA-C65

The high-speed pellet of material shed from adhering gametes was thawed and an equal volume of 2X HEMDEK (20mM Hepes, 50mM KCl, 10mM MgCl2, 1mM EDTA, 2mM DTT) or 2X HEMDEK 4% Triton X-100 was added. Samples were incubated in ice for 20 minutes and centrifuged for 30 minutes at 150,000 g

(58,700 rpm; rotor TLA 120.1). Supernatants and pellets were analyzed with immunoblotting.

Gamete activation

Flagellar adhesion-induced activation: Gametes were also activated by mixing equal numbers of *plus* and *minus* cells in M-N media. Cells were bubbled under constant light. Flagellar adhesion was confirmed under the microscope.

db-cAMP-induced activation: To induce gamete activation with db-cAMP, gametes were incubated in a solution containing 15mM di-butyryl cyclic AMP and 0.15mM papaverine final concentration in M-N media. Cells were bubbled under constant light.

RESULTS

SAG1-HA-C65 is evenly distributed in the cell body plasma membrane and little is present on the flagellar membrane of resting gametes.

To determine the distribution of SAG1-HA-C65 in resting gametes, I used an anti-HA antibody and indirect immunofluoresence (IF) with *sag1-5* gametes expressing the *SAG1-HA* transgene. As shown in Figure 2.2A, whereas the control (*hap2*) gametes showed only low, background levels of staining, the gametes expressing the transgene were strongly stained with the antibody. The cell body plasma membrane was most strongly stained, with only low levels of signal evident on flagella (Figure 2.2B). The distribution in the plasma membrane had a characteristic ring staining. This specific sub-cellular localization in the cell body plasma membrane suggested that the protein was membrane associated, consistent with the biochemical results shown above that document SAG1-HA-C65 as an integral membrane protein on the cell surface. The low level of the polypeptide in the flagellar membrane compared to the plasma membrane of the cell body indicated that the protein was excluded from the flagella. This observation was confirmed with biochemical methods. As shown in Figure 2.2C and subsequent figures, the amount of SAG1-HA-C65 in flagella of resting cells was very low and sometimes undetected under our standard immunoblotting conditions, compared to the amount of SAG1-HA-C65 in the cell body. Thus, SAG1-HA-

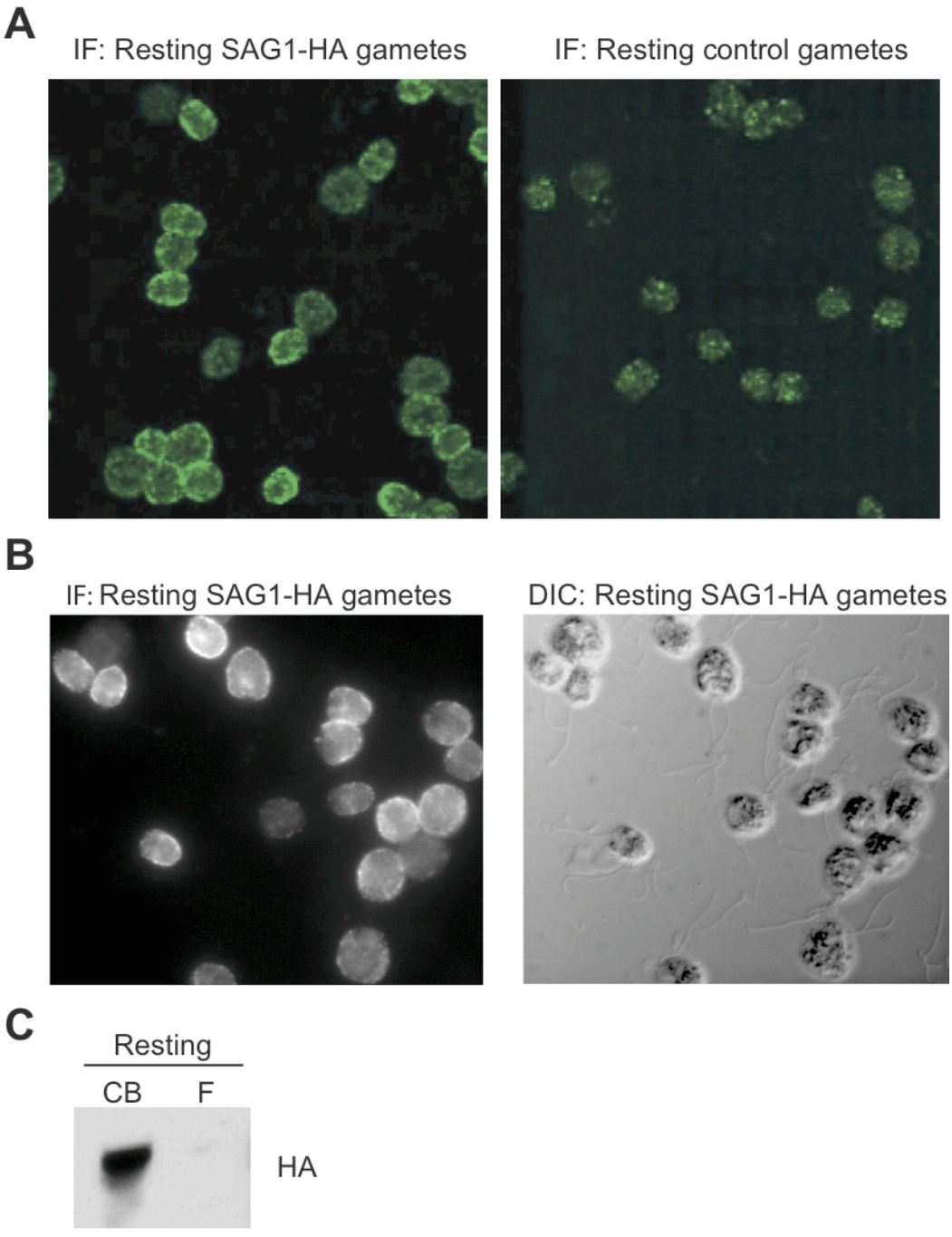
C65 was de-enriched in the flagellar membrane compared to the plasma membrane of the cell body, indicating the existence of a cellular mechanism for excluding the protein from flagella. In addition, SAG1-HA-C65 seemed to be distributed in puncta or clusters along the plasma membrane.

Figure 2.2

SAG1-HA-C65 is evenly distributed in the cell body plasma membrane and little is present on the flagellar membrane of resting gametes.

A) SAG1-HA-C65 is present in the cell body plasma membrane. Confocal images of IF resting *SAG1-HA* gametes staining mostly localized in the cell body (left panel). Absence of HA staining in *hap2* mutant gametes (right panel). Single stack image. B) IF images of resting *SAG1-HA* gametes anti-HA immunofluorescence (left panel). DIC images of the same cells (right panel) C) Immunoblotting with anti-HA antibody of resting *SAG1-HA* gametic cell bodies and flagella showing de-enrichment of SAG1-HA-C65 in the flagella compared to cell bodies. 2 μgs of protein were loaded per lane.

Figure 2.2


A
IF: Resting SAG1-HA gametes
IF: Resting control gametes
B
IF: Resting SAG1-HA gametes
DIC: Resting SAG1-HA gametes
C
Resting
CB
F
HA

SAG1-HA-C65 is enriched rapidly in the flagella during cilium-generated signaling.

To determine whether signaling modified the sub-cellular localization of SAG1-HA-C65, *SAG1-HA* gametes were treated with db-cAMP for 30 minutes to trigger gamete activation. As illustrated in Figure 2.3A, immunofluorescence results showed that the flagella of gametes activated by incubation in db-cAMP had become much more enriched in SAG1-HA-C65. The HA signal was distributed along the flagella and sometimes appeared to be enriched at the flagellar tips.

In collaborative studies with Dr. Olivier Belzile in our laboratory, we also took advantage of the ease of using biochemical approaches with *Chlamydomonas* to obtain a more quantitative view of SAG1-HA-C65 distribution before and after signaling. *SAG1-HA* gametes can be mixed with fusion-defective *hap2* minus gametes for flagellar adhesion-induced signaling. Gametes of the *hap2* mutant (a *minus* strain) undergo normal flagellar adhesion and cilium-generated signaling, but fail to fuse to form zygotes [80]. Therefore mixing *SAG1-HA* gametes with *hap2* gametes allows us to study flagellar adhesion induced-signaling without the interference of cell fusion. *SAG1-HA* and *hap2* gametes were mixed for 10 minutes followed by cell fractionation and immunoblotting. As expected from the IF results and illustrated in Figure 2.3B, we observed small amounts SAG1-HA-

C65 in the flagella of resting gametes. Upon flagellar adhesion, the amount of SAG1-HA-C65 in the flagella increased dramatically and rapidly.

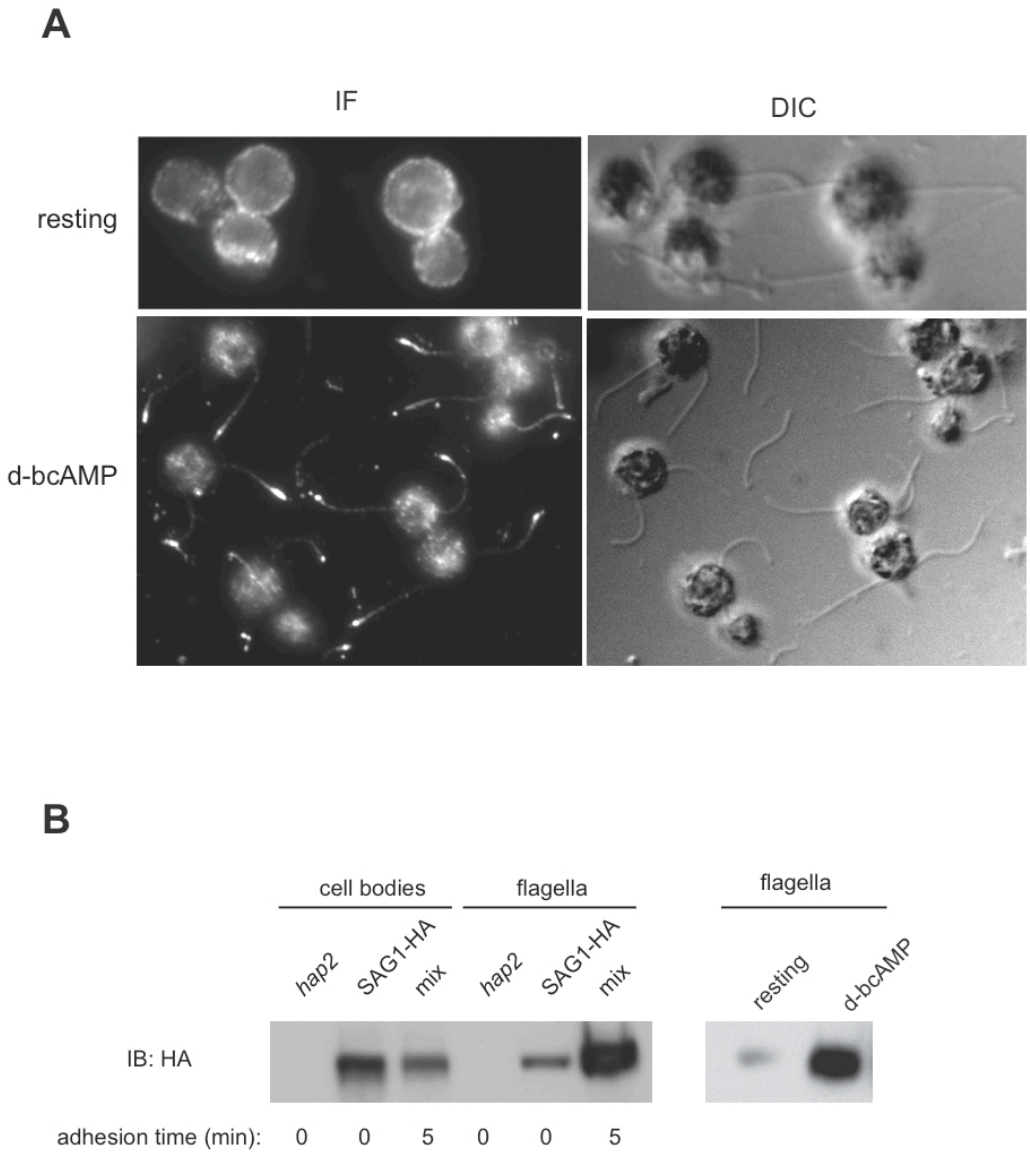
In addition, *SAG1-HA* gametes were incubated with db-cAMP for 5 minutes to induce signaling followed by cell fractionation and immunoblotting. Gamete activation was sufficient for SAG1-HA-C65 translocation into the flagella (Figure 2.3B). Immunofluorescence and biochemical methods both confirmed that SAG1-HA-C65 became enriched in the flagella during cilium-generated signaling. This time scale of signaling-induced enrichment of SAG1-HA-C65 in the flagellar membrane was similar (albeit somewhat shorter) to that observed previously in IF experiments on Hh-triggered Smo enrichment in the primary cilium. Rohatgi *et* al. detected Smo accumulation using immunofluorescence within 1 hour after addition of the Hh ligand [3].

Figure 2.3

SAG1-HA-C65 is enriched rapidly in the flagella during cilium-generated signaling.

A) SAG1-HA-C65 localizes to flagella upon signaling. Immunofluorescence images of resting and db-cAMP treated gametes showing enrichment of SAG1-HA-C65 in the flagella (left panel). DIC images of the same cells (right panel). Gametes were treated for 30 minutes with db-cAMP. B) Immunoblot showing *SAG1-HA* mixing with *hap2* gametes results in SAG1-HA-C65 enrichment in the flagella. Gametes were mixed for 10 minutes. 9μgs of protein were loaded per lane (left panel). Immunoblot of flagellar samples of *SAG1-HA* gametes treated with db-cAMP for 5 minutes showing that signaling is sufficient for flagellar SAG1-HA-C65 enrichment. Equal cell equivalents of flagella were loaded.

Figure 2.3


A
IF
DIC
resting
d-bcAMP
B
cell bodies
flagella
flagella
hap2
SAG1-HA
mix
hap2
SAG1-HA
mix
resting
d-bcAMP
IB: HA
adhesion time (min):
0
0
5
0
0
5

Testing for a possible role of cytoplasmic microtubules in the distribution of SAG1-HA-C65 in resting and activated gametes.

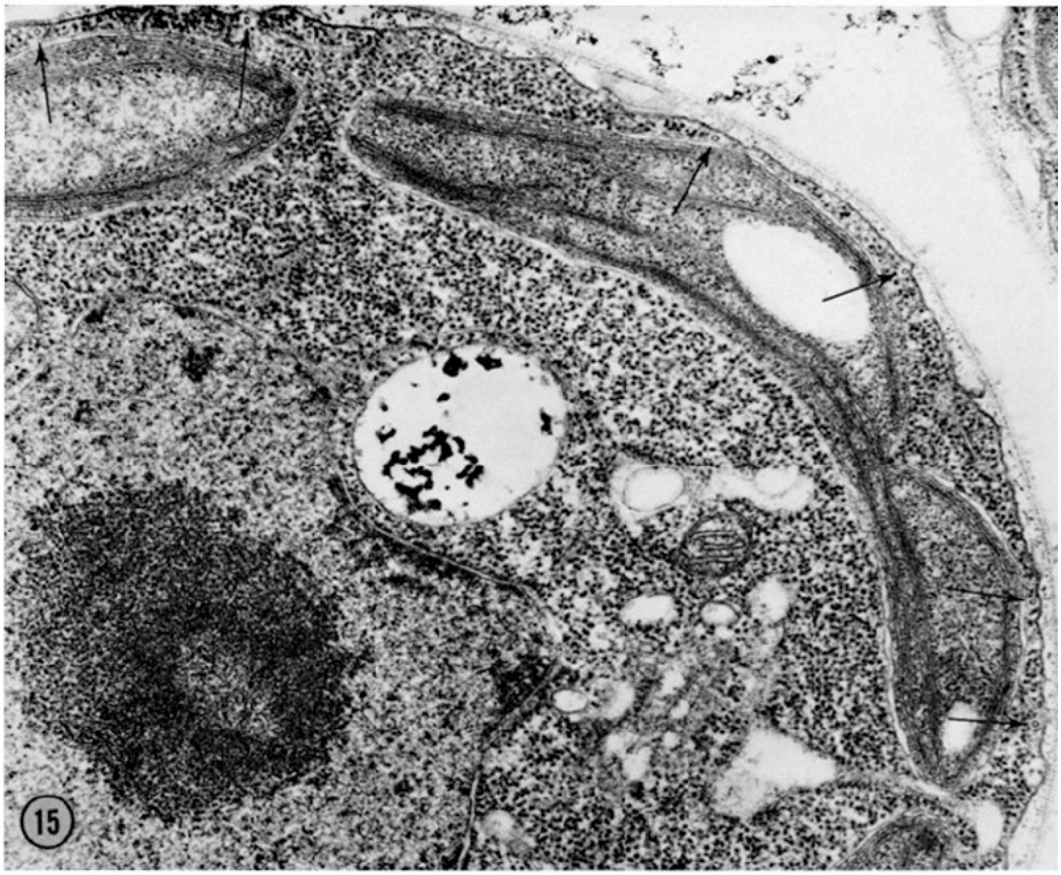
The striking rapidity of the signaling-induced enrichment of SAG1-HA-C65 in flagella raised the possibility that enrichment might be somehow facilitated by processes and structures at the cell body. One possible system that could participate in enrichment is the organized array of 15-20 microtubules that originates in the pericentriolar/peri-basal body region and extends to the basal end of the cell. As is the case for cytoplasmic microtubules that originate from the pericentriolar material in most other cells, it is likely that the microtubules in the array all have equivalent polarity, presumably with their + ends directed towards the basal ends of the cell. Consistent with this interpretation, when the microtubules are disrupted by microtubule destabilizing agents, they shorten from their distal ends, and re-grow from the same end when the agents are removed [84]. Importantly, Ringo's classic transmission electron microscopic analysis showed that each of these singlet microtubules is intimately associated with the plasma membrane (Figure 2.4; taken from Figure 15 of [85]). In addition, *Chlamydomonas* possesses two sets of microtubule rootlets that also project from the peri-basal body region and extend a short distance along the plasma membrane [85].

Figure 2.4

Cytoplasmic microtubules are found underneath the plasma membrane.

Electron microscope image of a transverse section of *Chlamydomonas* cell. Arrows point out six cytoplasmic microtubules and show that they are right underneath the plasma membrane. Image was obtained from [85].

Figure 2.4



(obtained from Figure 15 of [85])

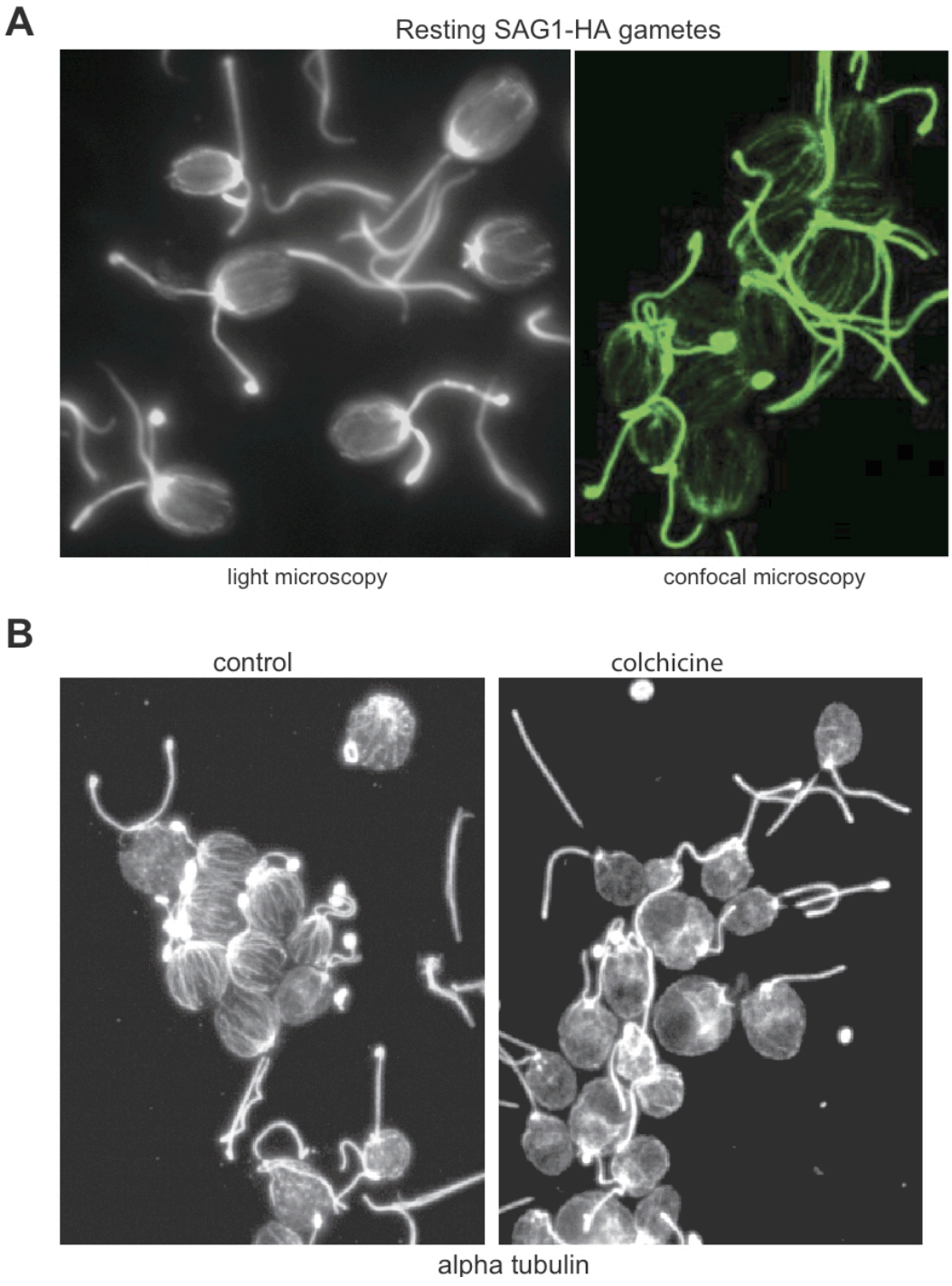
To determine whether cytoplasmic microtubules participated in signaling-induced enrichment of SAG1-HA-C65 in the flagella, I tested whether disrupting them would interfere with enrichment. IF with an anti-tubulin antibody demonstrated the submembranous array in *Chlamydomonas* gametes (Figure 2.5A). This unique subcellular localization of the cytoplasmic microtubules was similar in resting and db-cAMP activated gametes, showing that the microtubules remained intact during activation. Furthermore, incubation of cells in colchicine, demonstrated that indeed the cytoplasmic microtubules were depleted from the majority of the cells (Figure 2.5B).

Figure 2.5

***Chlamydomonas* has a colchicine-sensitive submembranous array of cytoplasmic microtubules**

A) *Chlamydomonas* has a submembranous array of cytoplasmic microtubules. IF images using alpha tubulin antibody. Resting *SAG1-HA* gametes show a submembranous array of cytoplasmic microtubules (left panel). Confocal images of the same samples (right panel). Single image. B) Colchicine disrupts cytoplasmic microtubules. 2D projection of Z-stacks of confocal images of IF against alpha tubulin antibody. Control and colchicine treated *SAG1-HA* gametes showing cytoplasmic microtubule depletion upon colchicine treatment. Cells were incubated with the microtubule inhibitor colchicine (Sigma catalog number C9754) for 100 minutes at a final concentration of 2 mg/ml.

Figure 2.5


A
Resting SAG1-HA gametes
light microscopy
confocal microscopy
B
control
colchicine
alpha tubulin

To examine whether the cytoplasmic microtubules participated in enrichment of SAG1-HA-C65 in flagella, I pre-incubated *SAG1-HA* gametes with colchicine followed by incubating gametes in db-AMP in the continued presence of colchicine. IF examination of the resting control and microtubule-depleted gametes showed a similar sub-cellular localization of SAG1-HA-C65, with strong staining in the cell body and little in the flagella (Figure 2.6A). This suggested that it is unlikely that cytoplasmic microtubules participate in excluding SAG1-HA-C65 from the flagellar membrane. On the other hand, immunofluorescence of activated gametes treated with colchicine led to a substantial reduction in their flagellar staining compared to the activated control gametes (Figure 2.6A).

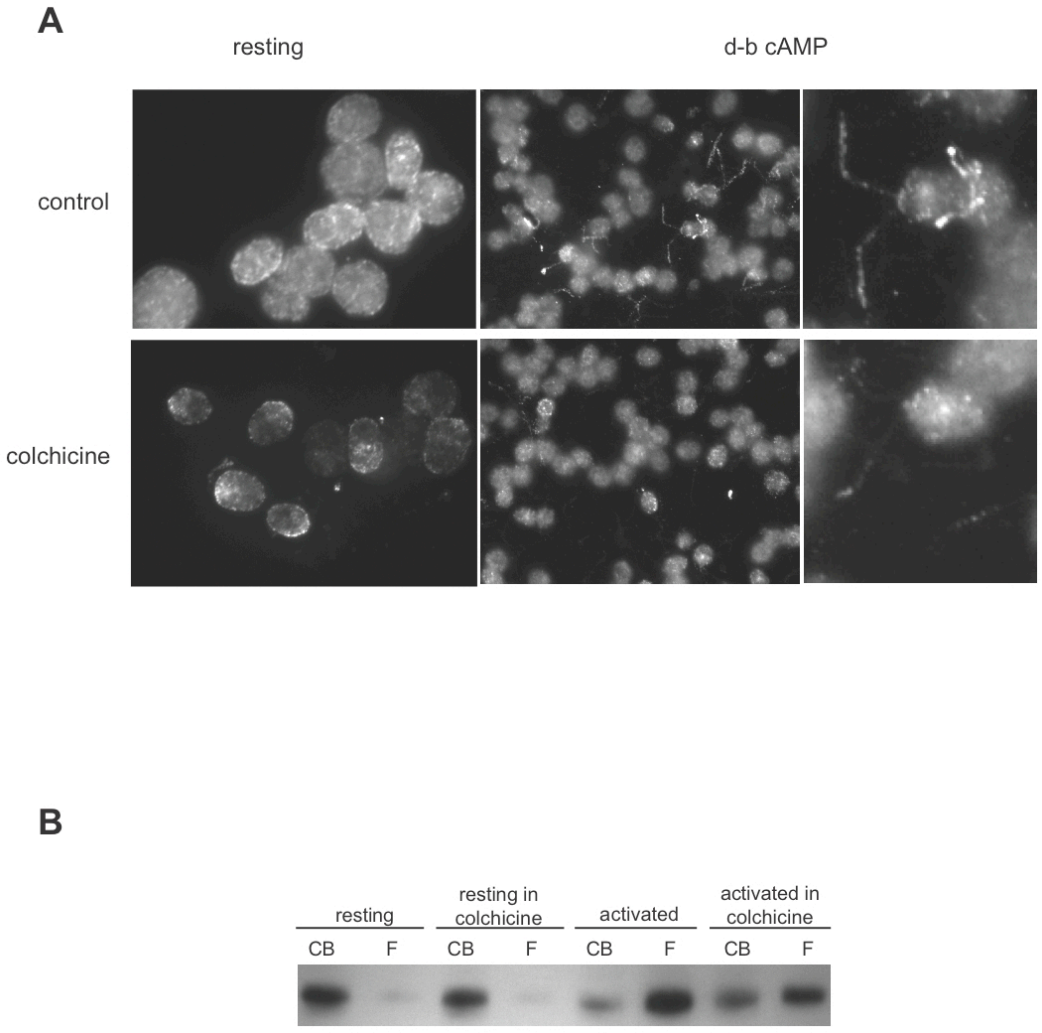
Similar results were obtained with cell fractionation. Cell bodies and flagella isolated from resting or activated with db-cAMP in the presence or absence of colchicine were analyzed by immunobloting. Figure 2.6B shows that the rapid, db-cAMP induced increase in flagellar SAG1-HA-C65 was reduced in cells depleted of cytoplasmic microtubules. In addition, SAG1-HA-C65 did not increase in the flagella when resting gametes were treated with colchicine. Both immunofluorescence and biochemical methods confirmed that the resting SAG1-HA-C65 distribution in the cell body was not dependent on cytoplasmic microtubules. These experiments indicated that, although SAG1-HA-C65 enrichment in flagella was not entirely dependent on cytoplasmic microtubules, the cytoskeletal elements facilitated SAG1-HA-C65 enrichment in flagella.

Figure 2.6

Cytoplasmic microtubules participate in SAG1-HA-C65 flagellar enrichment

A) IF images of control and colchicine treated *SAG1-HA* gametes showing exclusion of SAG1-HA-C65 from the flagella (left panel). Activated gametes treated with db-cAMP and colchicine showed decreased flagellar enrichment of SAG1-HA-C65 compared to activated control cells (center panel). Higher magnification view of region of the images in the central panel (right panel). B) *SAG1-HA* gametes were activated with db-cAMP in the presence or absence of colchicine. Cell bodies and flagella were isolated and analyzed with immunoblotting and showed a decrease of flagellar enrichment of SAG1-HA-C65 in activated gametes treated with db-cAMP and colchicine compared to activated control cells. Cells were activated with db-cAMP for 10 minutes. 2 μgs of protein were loaded per well.

Figure 2.6


A
resting
d-b cAMP
control
colchicine
B
resting
resting in colchicine
activated
activated in colchicine
CB
F
CB
F
CB
F
CB
F

During flagellar adhesion-induced signaling, SAG1-HA-C65 rapidly becomes concentrated at the flagellar base concomitant with SAG1-HA-C65 enrichment in the flagellar membrane.

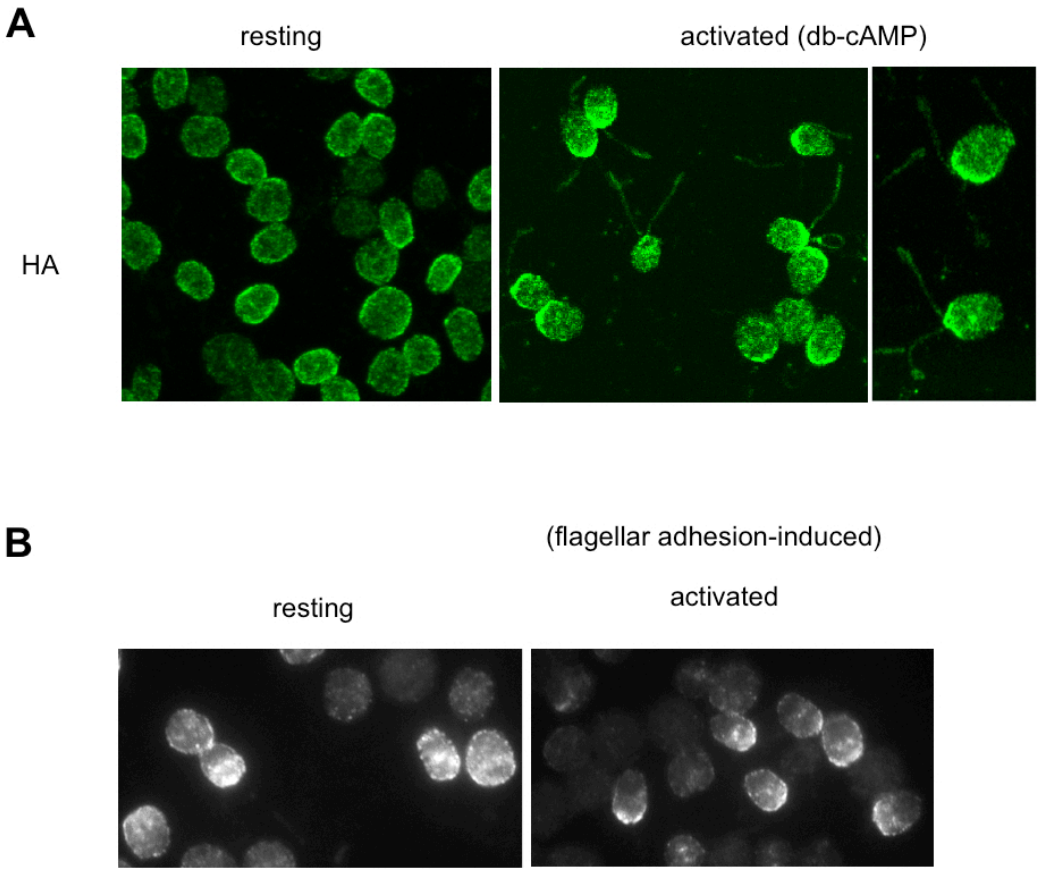
I looked more closely at cells during signaling to learn more about the molecular mechanism of flagellar enrichment of SAG1-HA-C65. *SAG1-HA* gametes were mixed with *hap2* gametes for 20 minutes and analyzed by IF. I noticed that in many cells, not only was SAG1-HA-C65 enriched in the flagella, but it was also concentrated in the peri-flagellar region of the plasma membrane (Figure 2.7B). I found even more striking results in *SAG1-HA* gametes activated with db-cAMP. In cells incubated for 5 minutes with db-cAMP, SAG1-HA-C65 was a almost completely from the basal 2/3 of the cell and was highly enriched in the apical plasma membrane around the bases of the flagella. In addition to an obvious redistribution of the protein in the cell body, flagellar staining was also very prominent (Figure 2.7A). This apical accumulation was observed in almost every single cell. These experiments indicated that very early during gamete activation, SAG1-HA-C65 underwent a rapid reorganization from its even distribution in the plasma membrane to strong enrichment in the periflagellar plasma membrane concomitant with its enrichment in the flagellar membrane.

Figure 2.7

SAG1-HA-C65 rapidly becomes concentrated at the flagellar base concomitant with SAG1-HA-C65 enrichment in the flagellar membrane upon cilium-generated signaling.

A) Confocal images of IF of resting and db-cAMP treated *SAG1-HA* gametes showing SAG1-HA-C65 redistribution to the apical end upon activation. Cells were treated with db-cAMP for 10 minutes. Higher magnification view of region of the images in the central panel (right panel). Single image. B) IF images of resting and mixed *SAG1-HA* gametes showing apical accumulation of SAG1-HA-C65 upon flagellar adhesion. *SAG1-HA* and *hap2* mutant gametes were mixed for 20 minutes.

Figure 2.7


A
resting
activated (db-cAMP)
HA
B
(flagellar adhesion-induced)
resting
activated

Cytoplasmic microtubules are essential for SAG1-HA-C65 apical localization

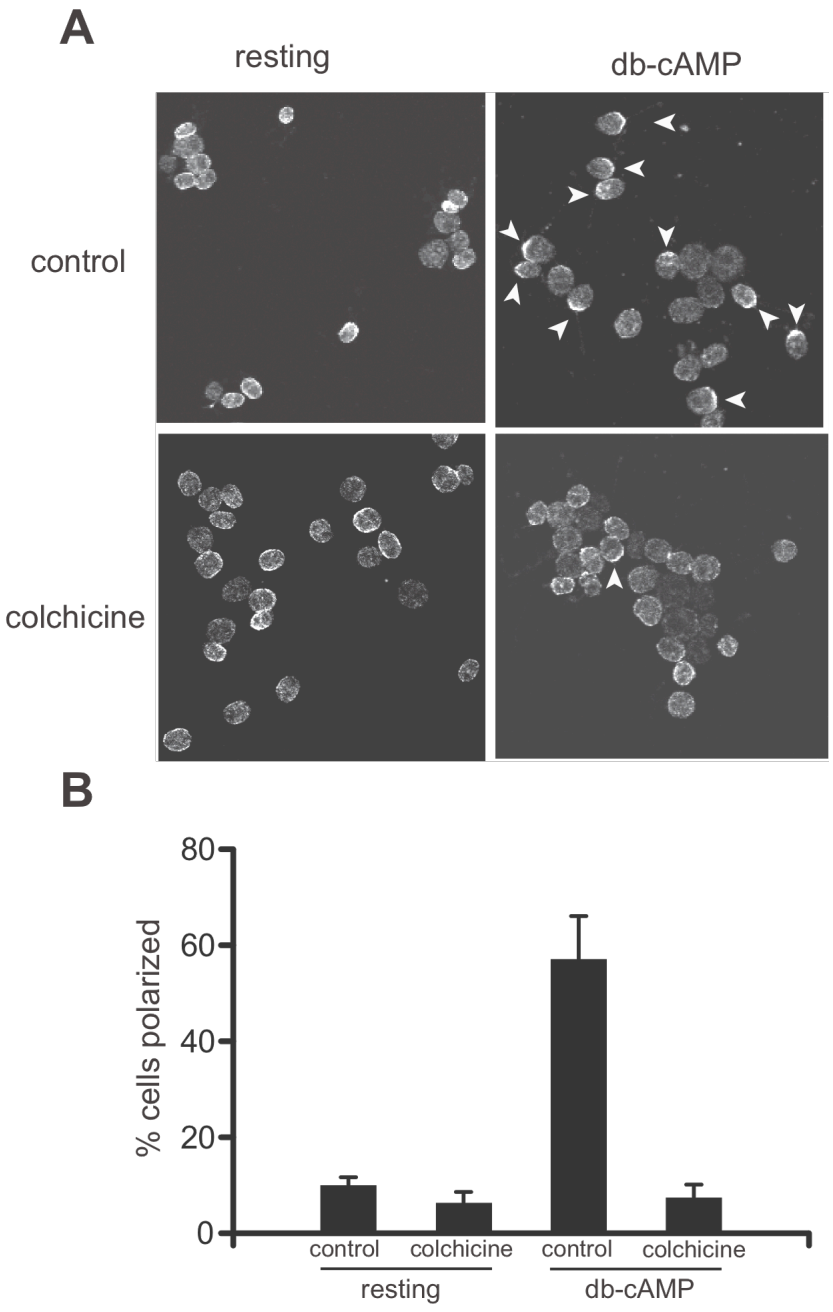
Having established a system to disrupt cytoplasmic microtubules *in vivo* and knowing that cytoplasmic microtubules participate in the delivery of SAG1-HA-C65 to the flagella, I tested their role in the apical accumulation of SAG1-HA-C65 during activation. *SAG1-HA* gametes were treated with db-cAMP in the presence or absence of colchicine. I found with IF that the apical redistribution of SAG1-HA-C65 protein failed to occur in the db-cAMP activated gametes whose cytoplasmic microtubules had been depleted by incubation in colchicine (Figure 2.8A). The proportion of cells with apical accumulation was decreased dramatically compared to the control, activated sample (Figure 2.8B). These experiments indicated that cytoplasmic microtubules were essential for the rapid, regulated enrichment of SAG1-HA-C65 in the peri-flagellar membrane.

Figure 2.8

Cytoplasmic microtubules are essential for SAG1-HA-C65 apical localization

A) *SAG1-HA* gametes were activated with db-cAMP in the presence or absence of colchicine and confocal IF images show the inhibition of SAG1-HA-C65 apical accumulation in the absence of cytoplasmic microtubules. Arrows indicate apical localization of SAG1-HA-C65. B) Graph showing quantification of the number of cells that exhibited apical enrichment of SAG1-HA-C65. A cell was scored as having apical accumulation of SAG1-HA-C65, when SAG1-HA-C65 had been depleted from the ~ lower 2/3 of the cell body plasma membrane and was concentrated at the apical end of the cell. Data were obtained from 3 independent experiments and 100 cells were scored for each condition. Error bars indicate standard error of the mean.

Figure 2.8


A
resting
db-cAMP
control
colchicine
B
% cells polarized
80
60
40
20
0
control
colchicine
control
colchicine
resting
db-cAMP

Apical accumulation of SAG1-HA-C65 occurs independently of kinesin-2.

The result that cytoplasmic microtubules were essential for the apical accumulation of SAG1-HA-C65, raised the possibility that kinesin-2 might participate as part of a transport cycle with a retrograde motor in the signaling-induced peri-flagellar enrichment of SAG1-HA-C65. To determine the role of kinesin-2 in the apical accumulation of SAG1-HA-C65 during signaling, in collaborative studies with Dr. Qian Wang in our laboratory, we generated a *SAG1-HA/ fla10* mutant cell line in a *sag1-5* background by crossing *SAG1-HA* and *fla10* gametes. The *fla10* strain has a temperature sensitive mutation in kinesin-2 [20] [86]. At the permissive temperature (22 degrees), *fla10* can undergo normal flagellar adhesion, cilium-induced signaling, and cell fusion. When transferred to the non-permissive temperature (32 degrees) for 45 minutes, however, kinesin-2 becomes non-functional, as evidenced by its failure to transport IFT particles into flagella [18] [79] [19]. Although the *SAG1-HA*/*fla10* cells exhibited slightly lower levels of SAG1-HA-C65 staining than the *SAG1-HA* parental cells, overall the distribution was similar, and the *SAG1-HA*/*fla10* cells showed similar patterns to parental cells of apical enrichment in activated gamete (Figure 2.9A). Importantly, when *SAG1-HA*/*fla10* cells that had been incubated at the non-permissive temperatures for 45 minutes to inactivate kinesin2 were subsequently incubated with db-cAMP at the non-permissive temperature, they retained the ability to undergo apical localization of SAG1-HA-C65 (Figure 2.9A

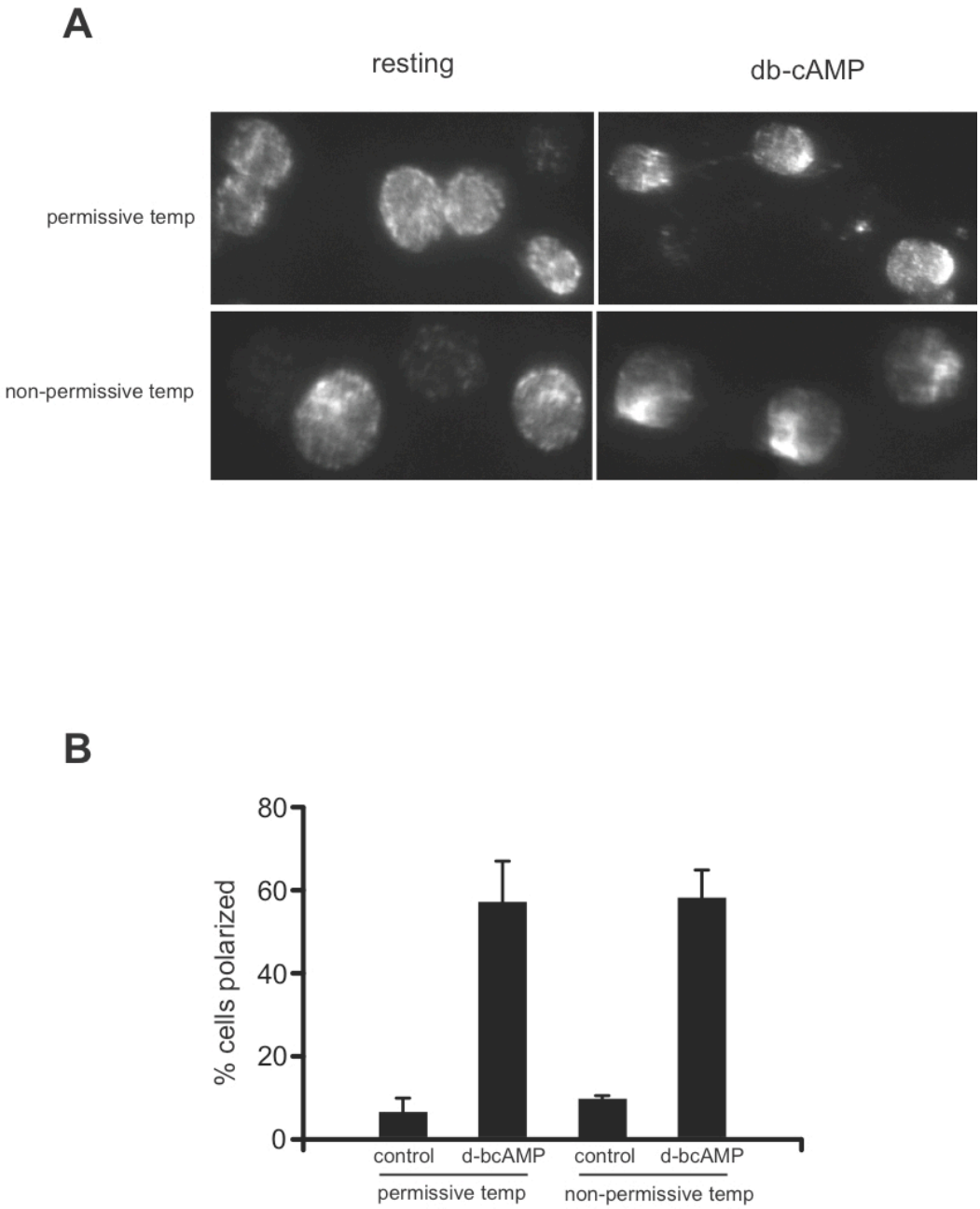
and 2.9B). Therefore, this microtubule-dependent cellular process of membrane protein delivery to the flagellar base occurs independently of the anterograde motor kinesin-2.

Figure 2.9

Apical accumulation of SAG1-HA-C65 occurs independently of kinesin-2.

A) IF images of resting or activated *SAG1-HA/fla10* gametes at the permissive and non-permissive temperature. Apical accumulation of SAG1-HA-C65 was observed at the non-permissive temperature. Cells were incubated at their respective temperatures for 45 minutes. Activated cells were treated with db-cAMP for 10 minutes. B) Graph quantifying the numbers of cells exhibiting apical accumulation in each condition. Apical accumulation was scored when 2/3 of SAG1-HA-C65 had been depleted from the ~ lower 2/3 of the cell body plasma membrane and was concentrated at the apical end of the cell. Data obtained from 2 independent experiments. 100 cells were counted in duplicate samples for each condition. Error bars indicate standard error of the mean.

Figure 2.9


A
resting
db-cAMP
permissive temp
non-permissive temp
B
% cells polarized
80
60
40
20
0
control
d-bcAMP
control
d-bcAMP
permissive temp
non-permissive temp

The anterograde IFT system is not required for enrichment of SAG1-HA-C65 in the flagellar membrane

Several workers showed previously that when *fla10* mutants gametes are shifted to the non-permissive temperature for 45 min, the flagella of *fla10* mutants become depleted of kinesin-2 and IFT particles- the anterograde system [18] [79] [19]. After ~1.5 hours at the non-permissive temperature the *fla10* mutant flagella begin to shorten, but a window of ~45-60 minutes exists in which the flagella are of full length, but they lack the IFT machinery.

To test for a possible role of IFT and kinesin-2 in the regulated movement of SAG1-HA-C65 into the flagella, *SAG1-HA/fla10* cells were assayed for SAG1-HA-C65 enrichment in the flagella in the presence or absence of the anterograde IFT system. *SAG1-HA/fla10* cells were incubated at the permissive and non-permissive temperatures for 45 minutes, treated briefly (10 minutes) with db-cAMP and assayed for flagellar SAG1-HA-C65 staining with IF. Figure 2.10A shows that resting SAG1-HA-C65 distribution was similar at the permissive and non-permissive temperatures. Upon db-cAMP addition, SAG1-HA-C65, despite the absence of IFT particles and kinesin-2, became enriched in the flagella.

Cell fractionation and immunoblotting confirmed the immunofluorescence observations. *SAG1-HA/fla10* gametes incubated at the permissive and non-permissive temperatures for 45 minutes were treated for 5 minutes with db-cAMP and subjected to cell fractionation and immunoblotting. As shown in Figure

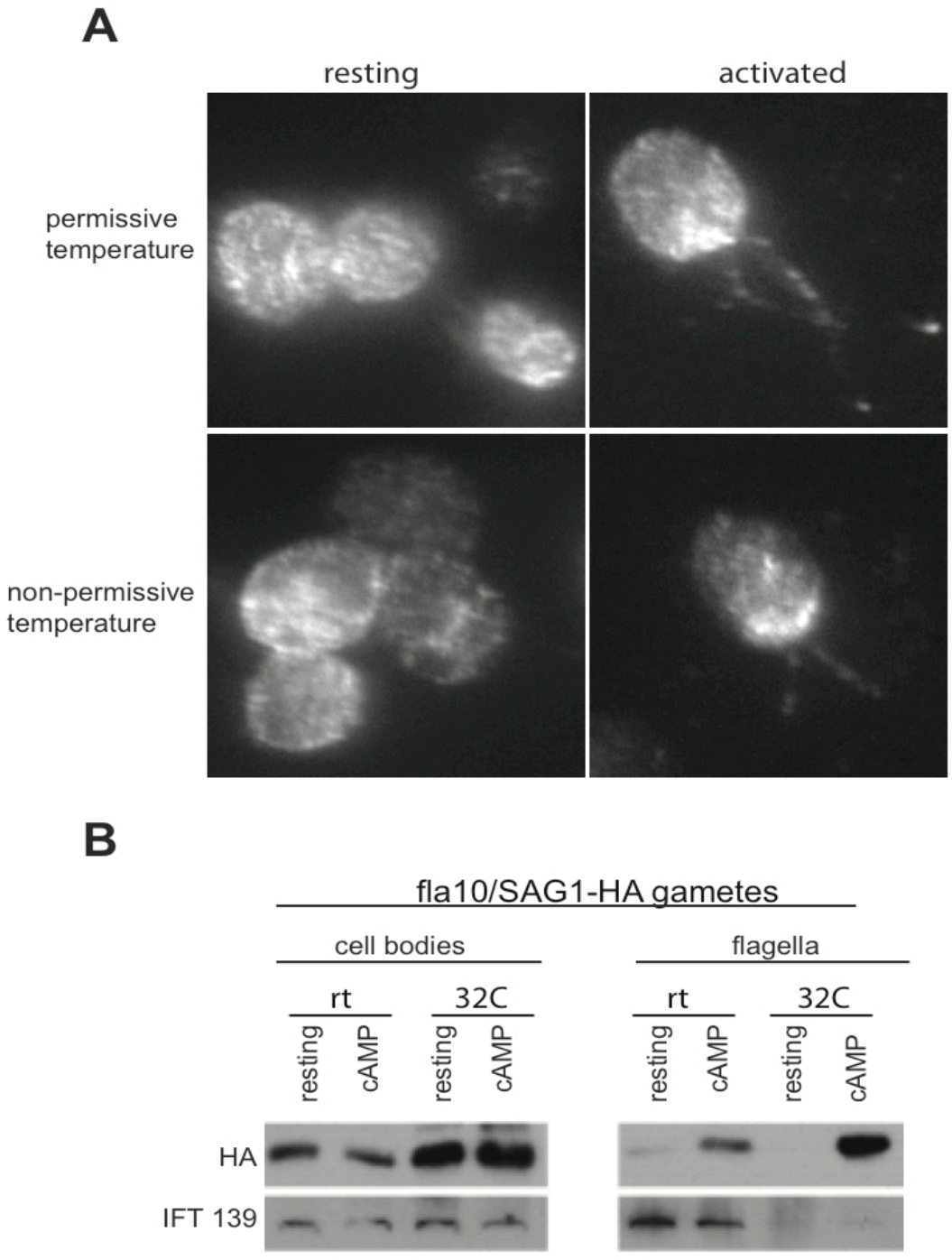
2.10B, SAG1-HA-C65 underwent the typical, dramatic increased in flagella at both the permissive and non-permissive temperatures, even though the IFT machinery was depleted from the flagella at the non-permissive temperature. Thus, both immunofluorescence and biochemical methods indicated enrichment of SAG1-HA-C65 in flagella does not depend on IFT.

Figure 2.10

The anterograde IFT system is not required in the enrichment of SAG1-HA-C65 in the flagellar membrane

A) IF images of resting and activated *SAG1-HA/ fla10* gametes at the permissive or non-permissive temperature show SAG1-HA-C65 flagellar enrichment at the non-permissive temperature upon activation. Cells were incubated at the indicated temperatures for 45 minutes. Cells were treated with db-cAMP for 10 minutes for gamete activation. B) *SAG1-HA/ fla10* resting or activated cells were incubated at the permissive or non-permissive temperature for 45 minutes in a concentration of $9.3x10^7$ cells/ml. Cells were activated for 5 minutes with db-cAMP. Cell bodies and flagella were isolated and assayed by immunoblotting. Anti-HA and IFT139 antibodies were used. 5 μgs of protein were loaded per lane.

Figure 2.10


A
resting
activated
permissive temperature
non-permissive temperature
B
fla10/SAG1-HA gametes
cell bodies
flagella
rt
32C
rt
32C
resting
cAMP
resting
cAMP
resting
cAMP
resting
cAMP
HA
IFT 139

SAG1-HA-C65 is lost from the cell body and the flagella during flagellar adhesion-induced signaling.

The rapid enrichment of SAG1-HA-C65 in the flagella during signaling suggested that it originated from a pre-synthesized pool of SAG1-HA-C65 in the cell body. Previous studies in our laboratory using bioassays to detect the agglutinin activity (which we now believe represented the N-terminal half of the protein encoded by *SAG1*) had suggested that cells possess pre-synthesized agglutinin activity that could move to the flagella. To test this idea using our direct probe, *SAG1-HA* gametes were mixed with *hap2* in the presence and absence of the protein synthesis inhibitor cycloheximide (CH). Cell bodies and flagella were isolated at the indicated times and analyzed with immunoblotting. The SAG1-HA-C65 in control samples adhering in the absence of CH underwent a modest decrease in the cell bodies and the typical increase in amounts in flagella during adhesion (Figure 2.11A) at the 30 minutes time point. I obtained similar results at the 30 minutes time point in the presence of CH.

On the other hand, at the later time points, SAG1-HA-C65 in the cell bodies and in the flagella of the cells in CH decreased dramatically compared to control samples. These results indicated that flagellar adhesion in CH was accompanied by loss of pre-synthesized SAG1-HA-C65 from the cell body and the flagella.

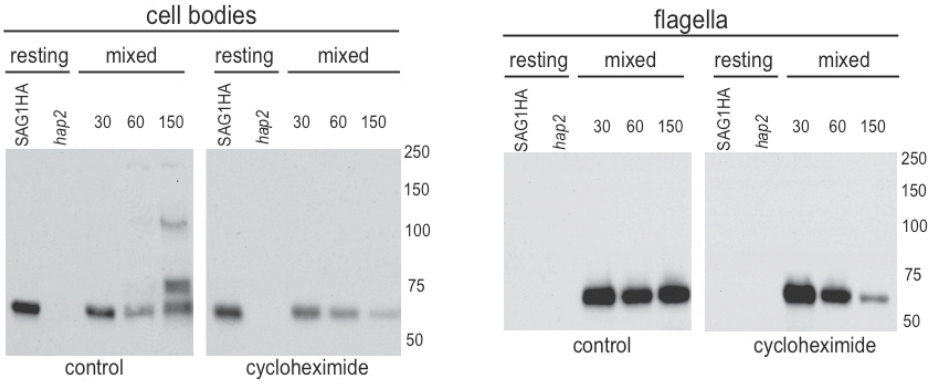
Figure 2.11

SAG1-HA-C65 is lost from the cell body and the flagella during flagellar adhesion-induced signaling.

A) Immunoblot of resting and mixed *SAG1-HA* cell bodies and flagella shows that SAG1-HA-C65 is lost from the flagella during adhesion in the absence of protein synthesis. Equal cell equivalents were loaded: cell bodies: 7.3×10^5, flagella: 2.4×10^7

Figure 2.11

A


cell bodies
flagella
resting
mixed
resting
mixed
SAG1HA
hap2
30
60
150
SAG1HA
hap2
30
60
150
250
150
100
75
50
control
cycloheximide
resting
mixed
resting
mixed
SAG1HA
hap2
30
60
150
SAG1HA
hap2
30
60
150
250
150
100
75
50
control
cycloheximide

Flagellar adhesion but not gamete activation or incubation of cells in conditioned medium causes SAG1-HA-C65 shedding into the medium in a particulate form

The dramatic loss of SAG1-HA-C65 from the cell body and the flagella during adhesion raised the possibility that SAG1-HA-C65 protein was being shed into the medium. Previous work from our lab had indicated that the flagella agglutinin activity was released into the medium [74]. To test for adhesion-induced shedding of SAG1-HA-C65, *SAG1-HA* gametes were mixed with *hap2* for 2 hours with continuous aeration. The medium was cleared of cells by a brief, low speed centrifugation followed by centrifugation at 19,150g, and particulate material was harvested from the supernatant by centrifugation for 1 h at 133,000g. As a control, I fractionated the medium from control, resting *SAG1-HA* gametes not incubated with *hap2* gametes but simply aerated for 2 h. To my surprise, I found that substantial amounts of sedimentable SAG1-HA-C65 were present in the medium of the adhering samples. Little if any was present in the control sample.

During flagellar adhesion-induced gamete activation, cells release several proteins into their medium, including a serine protease, an active cell wall degrading enzyme, along with cell wall components. To test whether factors released into the medium during flagellar adhesion brought about shedding of

SAG1-HA-C65, *SAG1-HA* cells were incubated with conditioned medium obtained from control wild type *plus* and *hap2 minus* gametes undergo flagellar adhesion and gamete activation [83]. As expected, incubation of *SAG1-HA* gametes alone in conditioned medium brought about cell wall loss in the *SAG1-HA* gametes but failed to bring about shedding of SAG1-HA-C65. Finally, to test whether gamete activation alone was sufficient for shedding of SAG1-HA-C65, I subjected the medium from *SAG1-HA* gametes incubated with db-cAMP to the differential centrifugation protocol. As shown in Figure 2.12A, the activated cells failed to shed SAG1-HA-C65. Thus, shedding of SAG1-HA-C65 required flagellar adhesion.

Shed SAG1-HA-C65 is in a membrane compartment.

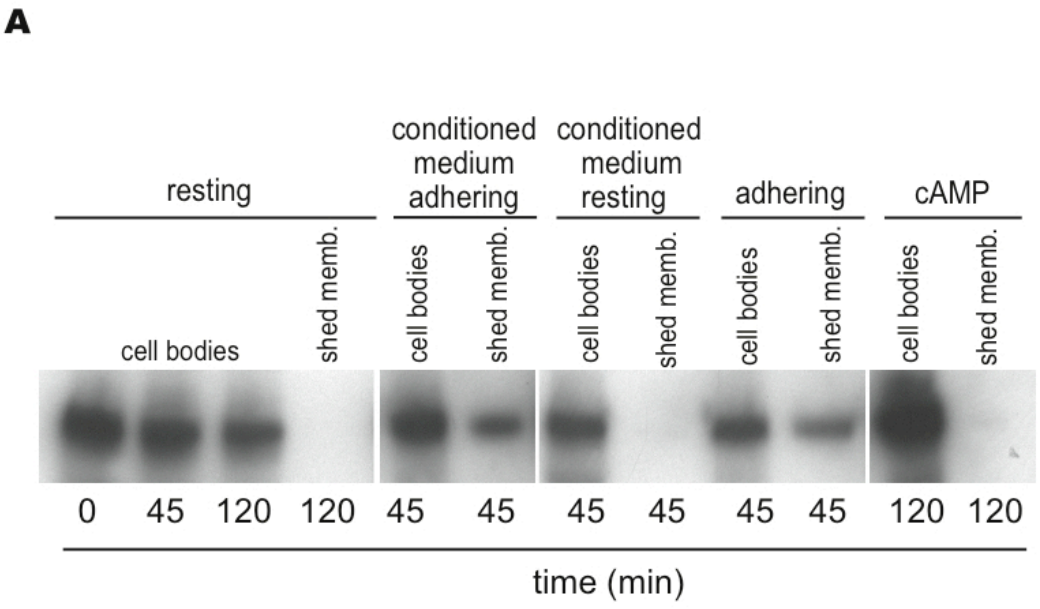
To test if shed SAG1-HA-C65 behaved as an integral membrane protein, I froze and thawed shed samples and incubated it with buffer in the presence or absence of detergent. The samples were subjected to high-speed centrifugation for fractionation and analyzed with immunoblotting. As expected, shed SAG1-HA-C65 remained sedimentable after freeze-thaw, but was rendered soluble upon incubation in detergent (Figure 2.12B). This result suggests that SAG1-HA-C65 was released into the medium in a membrane compartment.

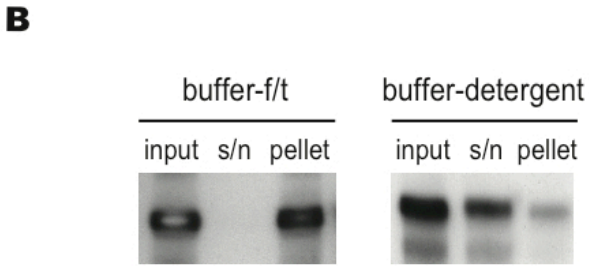
Figure 2.12

SAG1-HA-C65 is shed in a particulate form into the medium during flagellar adhesion in a membrane compartment. Gamete activation or incubation of cells in conditioned medium fail to induce SAG1-HA-C65 shedding.

A) SAG1-HA-C65 is shed in a particulate form into the medium during flagellar adhesion. Gamete activation or incubation of cells in conditioned medium failed to induce SAG1-HA-C65 shedding. Immunoblot of cell bodies and shed membranes extracts obtained from the following types of *SAG1-HA* gamete samples: resting, resting gametes incubated with conditioned medium, resting gametes incubated with conditioned medium followed by mixing with *hap2* gametes to induce activation, gametes activated by flagellar adhesion and db-cAMP treated gametes. Samples were harvested at the indicated times. The conditioned medium was prepared from 21gr(+) wild type *plus* gametes mixed with *hap2* minus gametes for 45 minutes. Cell wall loss was determined with the cell wall loss assay. After the supernatant was pre-cleared, as previously described in [82], resting *SAG1-HA* gametes were resuspended in it and incubated for 20 minutes with aeration. Cell wall loss was determined with the cell wall loss assay. Equal proportions of the samples were subjected to SDS-PAGE and immunoblotting. B) Shed SAG1-HA-C65 is in a membrane compartment. Immunoblot of shed membranes fractionation. Shed membranes were thawed and resuspended in the absence or presence of 2% Triton X-100 in HEMDEK buffer at 4 degrees for 20 minutes. Samples were centrifuged for 30 minutes at 150,000 g (58,700 rpm). Inputs, supernatants and pellets were harvested and equal proportions of the samples were analyzed by immunoblotting.

Figure 2.12


A
resting
conditioned medium adhering
conditioned medium resting
adhering
cAMP
cell bodies
shed memb.
cell bodies
shed memb.
cell bodies
shed memb.
cell bodies
shed memb.
cell bodies
shed memb.
0 45 120 120 45 45 45 45 45 45 120 120
time (min)


B
buffer-f/t
buffer-detergent
input s/n pellet
input s/n pellet

Most of the pre-synthesized SAG1-HA-C65 could be recovered in the medium in a unique membrane compartment.

Because flagellar adhesion was required for shedding of SAG1-HA-C65 membranes, it was possible that the shed membranes would contain flagellar constituents, including tubulin; the major flagellar membrane protein, FMG1; the flagellar membrane-associated cGMP-dependent protein kinase; or IFT particle proteins. To test for other proteins in the shed membrane fraction, I harvested it from adhering medium and performed immunoblotting with several antibodies. Remarkably, the shed membrane fraction was a unique compartment. It was substantially enriched in SAG1-HA-C65, compared to equal amounts of flagellar protein from adhering gametes. On the other hand, tubulin, FMG1, PKG and IFT81 and IFT139 were prominent in flagella, but substantially de-enriched in the shed membrane fraction (Figure 2.13B).

To determine whether SAG1-HA-C65 shedding represented a major event in its trafficking, I followed the fate of pre-existing SAG1-HA-C65 in gametes undergoing prolonged flagellar adhesion in cycloheximide. *SAG1-HA* gametes were mixed with *hap2* in the presence or absence of cycloheximide. After 45 minutes of adhesion, cells were harvested for isolation of cell bodies and flagella, shed membranes were harvested from the medium, and equal cellular proportions were analyzed by SDS-PAGE and immunoblotting. As expected, SAG1-HA-C65 was lost from the cell bodies and from the flagella (Figure 2.13A). And, the

majority of the SAG1-HA-C65 in the starting cells was recovered in the shed membrane fraction.

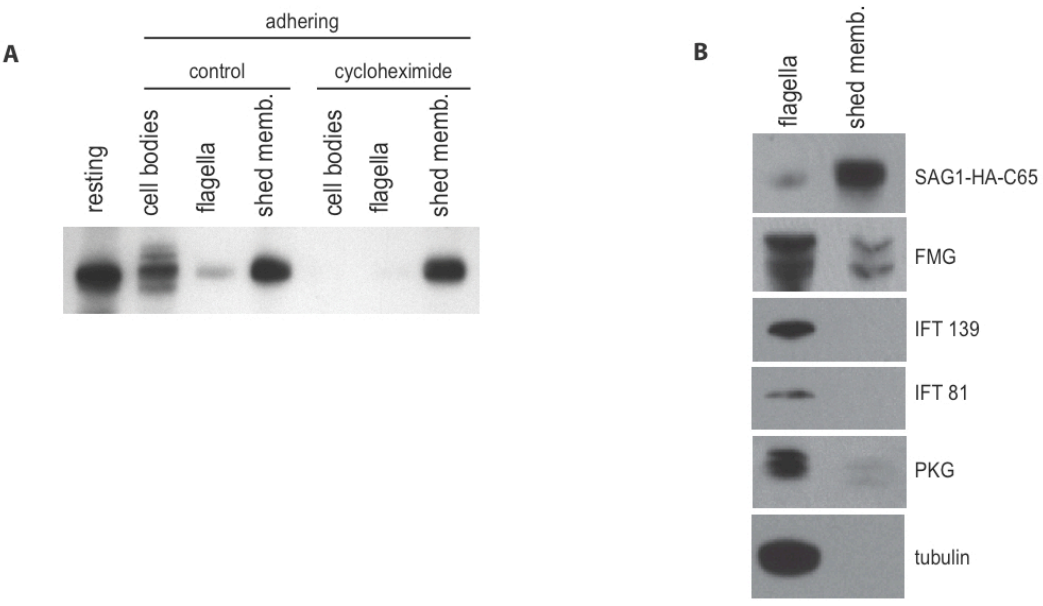
These results indicated that *Chlamydomonas* possesses a mechanism for release of a unique, SAG1-HA-C65-containing membrane compartment. And, they indicated that shedding of the SAG1-HA-C65 membrane fraction was not a minor consequence of flagellar adhesion, but represents the principal fate of this membrane polypeptide during flagellar adhesion.

Figure 2.13

Most of the pre-synthesized SAG1-HA-C65 could be recovered in the medium in a unique membrane compartment

(A) Most of the pre-synthesized SAG1-HA-C65 could be recovered in the medium. Immunoblot of cell bodies, flagella, and shed SAG1-HA-C65-containing membranes from adhering gametes incubated in the absence or presence of cycloheximide. *SAG1-HA plus* gametes and *hap2 minus* gametes were pre-incubated separately for 10 minutes with cycloheximide (final concentration of 10 ug/ml). Control and cycloheximide-treated gametes were mixed for 2 hours and 30 minutes in the continued presence of CH. Cell bodies, flagella and shed membranes were harvested and equal proportions were analyzed by immunoblotting. (B) SAG1-HA-C65 is shed in a unique membrane compartment. Immunoblot of adhering flagella and shed membranes against HA, major flagellar protein (FMG), IFT 139 and IFT 81, PKG and alpha tubulin antibodies. Equal protein was loaded per lane.

Figure 2.13


A
adhering
control
cycloheximide
resting
cell bodies
flagella
shed memb.
cell bodies
flagella
shed memb.
B
flagella
shed memb.
SAG1-HA-C65
FMG
IFT 139
IFT 81
PKG
tubulin

DISCUSSION

My work has examined the cellular fate of the integral membrane polypeptide SAG1-HA-C65. I found that in resting gametes SAG1-HA-C65 was excluded from the flagella. Upon flagellar adhesion-induced signaling, it underwent a dramatic cytoplasmic microtubule dependent redistribution to become concentrated at the apical end of the cell in a peri-flagellar pattern and enriched in the flagellar membrane. SAG1-HA-C65 enrichment in the flagellar membrane occurred independently of IFT. I also found that during flagellar adhesion SAG1-HA-C65 was released from the flagella into the medium in a membrane compartment.

The distribution of SAG1-HA-C65 in resting gametes

Immunofluorescence showed that SAG1-HA-65 was evenly distributed in the cell body plasma membrane and little was present on the flagella of resting gametes. This staining distribution in the plasma membrane was consistent with our recent results that SAG1-HA-C65 is an integral membrane protein exposed on the surface of the plasma membrane. The observation that SAG1-HA-C65 was in the form of clusters as opposed to an even distribution was somewhat surprising, and still unexplained. One possibility to explain this clustered distribution is that

multiple SAG1-HA-C65 molecules aggregate and form clusters in the plasma membrane as part of the ciliary exclusion system active in the absence of flagellar adhesion. The mobility of SAG1-HA-C65 could be tested to determine whether it is found freely mobile or immobile in the plasma membrane in resting gametes.

My results indicated that SAG1-HA-C65 was excluded from the flagellar membrane in resting gametes. Both biochemical and immunofluorescence experiments support this interpretation. One possible explanation for these observations is that SAG1-HA-C65 is excluded from the flagella because a barrier prevents its diffusion into the flagellar membrane. Another possibility is that an array of cytoplasmic microtubules associated with SAG1-HA-C65 in the plasma membrane of the cell body participating in SAG1-HA-C65 exclusion from the flagellar membrane.

Cytoplasmic microtubules do not play a role in SAG1-HA-C65 distribution in resting cells

Figure 2.6A showed that the resting control and microtubule-depleted gametes had similar sub-cellular localization of SAG1-HA-C65, with strong staining in the cell body and little in the flagella. In both conditions, SAG1-HA-C65 remained evenly distributed on the cell membrane in clusters, and continued to be excluded from the flagella. One possibility to explain these results is that cytoplasmic microtubules are not involved in the de-enrichment of SAG1-HA-

C65 from the flagellar membrane. Therefore, they would not be required in the exclusion mechanism responsible of SAG1-HA-C65 de-enrichment from the flagella in the absence of cilium-generated signaling.

One possible model to suggest the molecular mechanism for SAG1-HA-C65 exclusion from the flagella is that in resting gametes, an unidentified protein complex sequesters immobile SAG1-HA-C65 clusters in the plasma membrane thereby preventing its trafficking into the flagella. Another possibility is that SAG1-HA-C65 is freely mobile in the plasma membrane and is restricted from the flagellar membrane by an uncharacterized diffusion barrier resulting in SAG1-HA-C65 exclusive localization in the plasma membrane of the cell body.

Further studies are needed to identify key players of the exclusion mechanism and characterize the components of the diffusion barrier that independently or together may be regulating SAG1-HA-C65 resting distribution in the cell body plasma membrane. Perhaps, in resting gametes, SAG1-HA-C65 associates with a specific protein complex in the plasma membrane to be excluded from the flagellar membrane.

SAG1-HA-C65 apical accumulation during cilium-generated signaling

As shown in Figure 2.7, SAG1-HA-C65 becomes concentrated very quickly at the apical end of the cell during flagellar adhesion-induced and cAMP-induced signaling. To my knowledge, this is a novel cellular mechanism. It is

known that intracellular vesicles can become locally enriched within cells [87], but this does not seem to be the case for membrane proteins found in the plasma membrane.

The role of cytoplasmic microtubules in apical localization of SAG1-HA-C65

Figure 2.8 showed that the rapid apical accumulation of SAG1-HA-C65 on the plasma membrane around the base of the flagella required cytoplasmic microtubules. Since cytoplasmic microtubules are known to move targets within the cell, these data indicate that an active targeted process might be involved during SAG1-HA-C65 enrichment in the flagella. Previous studies have shown that cytoplasmic microtubules mediate the delivery of soluble ciliary proteins [46] [63] [64] [65] [66] [67].

One possibility to explain how SAG1-HA-C65 accumulates at the apical end of the cell is that upon signaling, immobile SAG1-HA-C65 interacts with cytoplasmic microtubules to be delivered toward the ciliary base. Specifically, during signaling the increase of cAMP might activate a PKA that inhibits a putative protein complex that maintains SAG1-HA-C65 immobile in the plasma membrane. Upon release, SAG1-HA-C65 interacts with cytoplasmic dynein, accessory proteins and cytoplasmic microtubules to engage in lateral minus-end directed traffic through the plasma membrane toward the peri-flagellar membrane.

Another possibility that could explain the molecular mechanism for SAG1-

HA-C65 apical concentration is that freely mobile SAG1-HA-C65 that is excluded from the flagella by a diffusion barrier, engages with cytoplasmic microtubules upon signaling for targeted delivery to the flagellar base. Signaling also causes the breakdown of the barrier that permits freely mobile SAG1-HA-C65 to enrich at the ciliary base.

Even though my results suggest a role for cytoplasmic microtubules in the redistribution process, they do not allow me to distinguish between possible direct or indirect roles. For example, in an indirect role, upon signaling, cytoplasmic microtubules could be delivering a protein complex to the peri-flagellar base that retains free mobile SAG1-HA-C65 there. Further studies will be needed to test possible models and determine whether SAG1-HA-C65 associates with other proteins (retaining/targeted delivery) during its apical re-distribution from the plasma to the peri-flagellar membrane.

The role of cytoplasmic microtubules in facilitating flagellar SAG1-HA-C65 enrichment.

In addition to learning that SAG1-HA-C65 enriched rapidly in the flagella during cilium-generated signaling, I found that cAMP-induced enrichment of flagellar SAG1-HA-C65 levels was reduced in cells depleted of cytoplasmic microtubules compared to control, cAMP-treated gametes. Our system allowed us to confirm this observation with both immunofluorescence and immunoblotting.

Several models can be proposed about the molecular mechanism of SAG1-HA-C65 enrichment in the flagellar membrane. The SAG1-HA-C65 rapid enrichment in the flagella and the fact that cytoplasmic microtubules are involved in the process suggest a regulated and specific active process rather than random diffusion of the protein to the flagellar membrane upon signaling.

One option to explain the role of cytoplasmic microtubules in the flagellar enrichment of SAG1-HA-C65 is that upon signaling, the diffusion barrier collapses and freely mobile SAG1-HA-C65 can flow into the flagellar membrane without cytoplasmic microtubules. Another possibility is that in addition to the collapse of the barrier upon signaling, cytoplasmic microtubules enrich SAG1-HA-C65 at the flagellar base, thereby contributing to its enrichment in the flagella.

Relationship between SAG1 gene products: the N-terminal adhesion domain and the C-terminal polypeptide

Previous studies done in our laboratory showed that in the absence of signaling, the majority of the agglutinin activity was found on the plasma membrane of the cell body. Addition of di-butyryl cAMP resulted in an increase of agglutinin activity in the flagella [74]. My studies focus on the cellular fate of the C-terminal portion and do not address the N-terminal adhesion domain of the SAG1 gene product. A correlation between the increase of agglutinin activity and

the SAG1-HA-C65 enrichment in the flagella upon signaling could be proposed. One possibility is that these two portions of the gene product could be interacting to bring about flagellar adhesion.

IFT independent movement of SAG1-HA-C65 into the flagellar membrane

Biochemical analysis and immunofluorescence showed that IFT system was not required for enrichment of SAG1-HA-C65 in the flagellar membrane (Figure 10). Because the discoveries of constitutive and dynamic ciliary protein localization and the fact that IFT is essential for ciliary assembly, several studies have raised the possibility that membrane proteins associate with IFT particles to move into the flagellar membrane [59] [88].

The role of IFT in determining the ciliary membrane protein composition is not well understood. One study shows flagellar accumulation of the *Chlamydomonas* membrane protein PKD2 in the absence of kinesin-2 and IFT particles with the use of the *fla10* mutant [59]. They concluded that the retrograde system was required for flagellar PKD2 removal, presumably because in the absence of the anterograde system, components of the retrograde system could not be delivered, therefore the retrograde system was non functional as well [59]. Previous studies in our laboratory showed that the agglutinin activity moved into the flagella independently of IFT [79]. Even though this study analyzed the agglutinin activity, one explanation could be that the N-terminal portion of the

SAG1 gene product, associated with the C-terminal polypeptide in the flagella. My studies show that SAG1-HA-C65 enriched in the flagella in the absence of kinesin-2 and IFT particles. This is the first direct biochemical evidence to show that the dynamic enrichment of a membrane protein in the flagellar membrane of an intact flagellum occurs independently of IFT. As previously proposed, an IFT independent mechanism for the flagellar entry of membrane proteins must exist [79] [59]. Perhaps, flagellar adhesion-induced signaling causes the collapse of the diffusion barrier, and the now freely mobile SAG1-HA-C65 can diffuse into the flagellar membrane. Further studies are required to identify whether IFT independent molecular mechanisms exist to enrich membrane proteins in the flagellar membrane.

SAG1-HA-C65 undergoes regulated shedding from the flagella to the medium.

The adhesion-dependent release of pre-existing SAG1-HA-C65 from cells was dramatic and extensive (Figure 11). Moreover, that the particulate shed SAG1-HA-C65 could be rendered soluble by detergent treatment indicated that it was membrane associated (Figure 12 and 13). These results indicated that *Chlamydomonas* possesses a one-way route for membrane protein trafficking in the flagella. Rather than returning to the cell body for degradation or re-use, SAG1HA-C65 was released into the medium. In addition, I discovered that

neither gamete activation nor incubation of cells in conditioned medium induced SAG1-HA-C65 shedding. This evidence indicated that this shedding process requires flagellar adhesion. One possibility to explain shedding is that if in the flagella SAG1 N-terminal adhesion domain is associated with its C-terminus fragment, during flagellar adhesion, SAG1 receptor in the *plus* flagella interacts with the receptor SAD1 in the *minus* flagella triggering the immediate release of the SAG1 complex into the medium including SAG1-HA-C65.

Shedding as a conserved mechanism for regulation of the dynamic protein composition of the flagellar membrane

Biochemical analysis of the shed material showed that SAG1-HA-C65 is shed in a unique membrane compartment. One possibility to explain shedding of SAG1-HA-C65 is that receptor-receptor unique interactions associated with SAG1-HA-C65 mediate its release upon a signal.

Many of the mechanisms that underlie the biology of cilia and flagella are conserved, and many were first found in *Chlamydomonas*. Learning about how *Chlamydomonas* regulates the membrane protein composition of its flagellar membrane can shed some light into the molecular mechanisms utilized by mammalian cells to coordinate the dynamic protein composition of the ciliary membrane during signaling. It is possible that similar mechanisms of ciliary protein accumulation at the flagellar base or shedding through the primary cilium

may be occurring in other organisms. In the presence of a signal the cell can remove membrane proteins that are no longer required in their flagellar membrane by releasing membrane protein containing vesicles into the extracellular environment. Perhaps, Ptch may be getting released into the medium upon binding to Hh ligand. Smo may be shed from the ciliary membrane to regulate its amounts of enrichment in the ciliary membrane depending on the stimulation state of the cell.

Shedding is not specific to *Chlamydomonas*

Several examples of shedding of membrane proteins can be found in cell biology. One example is the daily shedding of the membrane protein rhodopsin from the modified cilia in the photoreceptor cells of the vertebrate eye [43]. Another example is that recent studies have shown that PKD proteins such as PC1, PC2 and fibrocystin are shed in membrane particles in the urine [89]. They also report that these shed urinary vesicles interacted with renal and biliary primary cilia *in vitro* [89]. Another example is that cancer cells release exosomes, small vesicles that contain RNA and proteins, into the environment to inhibit the immune system [90] [91], as reviewed in [92]. Whether shed urinary vesicles or exosomes are released from the primary cilium or the plasma membrane remains to be understood. A possibility is that shedding could be a type of cell-cell communication mechanism. The cell antenna function of the primary cilium could

be a two-way system for sending and receiving signals from the environment. Additional studies are required to characterize the molecular mechanism of the shedding process and test whether it occurs in other types of primary cilium in vertebrates to regulate dynamic ciliary membrane localization.

CHAPTER THREE

GENERAL CONCLUSION, PROPOSED MODELS AND FUTURE DIRECTIONS

Introduction

Many questions still remain to be answered about the molecular mechanisms that regulate the entry and exit of membrane proteins into the primary cilium. My studies presented in the previous chapter have provided new insights into some of these questions. My work has shown that *Chlamydomonas* have cellular mechanisms for regulating the protein composition of the ciliary membrane. In collaborative studies, we found that a membrane protein was excluded from the flagella in the absence of signaling. This membrane protein enriched in the flagellar membrane upon cilium-generated signaling. This enrichment was mediated by its accumulation at the flagellar base. In addition, we found that the membrane protein composition of the flagellar membrane can be regulated by shedding membrane proteins from the flagella into the environment. Even though these mechanisms for regulating the membrane protein composition of the ciliary membrane may be unique to the model organism, this has not been proven to be the case in the past. Basically, most of the biology of cilia and

flagella has been revealed from studies done in *Chlamydomonas*. Biochemical methods combined with molecular biology and cell-imaging techniques make this model system ideal for learning about the biology of the primary cilium.

Below, I will present a proposed model about the regulation of the location and fate of the membrane protein SAG1-HA-C65 and describe futures directions for learning more about the regulation of membrane protein composition of the ciliary membrane.

The proposed model

One possible model about the molecular mechanism of the regulation of membrane protein composition of the flagellar membrane is that in resting gametes, most of the pre-existing integral membrane protein SAG1-HA-C65 is present in clusters in the surface of the cell body plasma membrane. Membrane-associated proteins specific to the plasma membrane (sequesterin protein complex) immobilize SAG1-HA-C65 clusters, resulting in its de-enrichment from the flagellar membrane. Immobilization of SAG1-HA-C65 in the plasma membrane causes the its exclusion from the flagellar membrane. During flagellar adhesion the immobilized SAG1-HA-C65 becomes freely mobile and can associate with the targeted delivery machinery composed of the submembranous cytoplasmic microtubules, cytoplasmic dynein and accessory proteins to engage in lateral minus-end directed traffic through the plasma membrane toward the

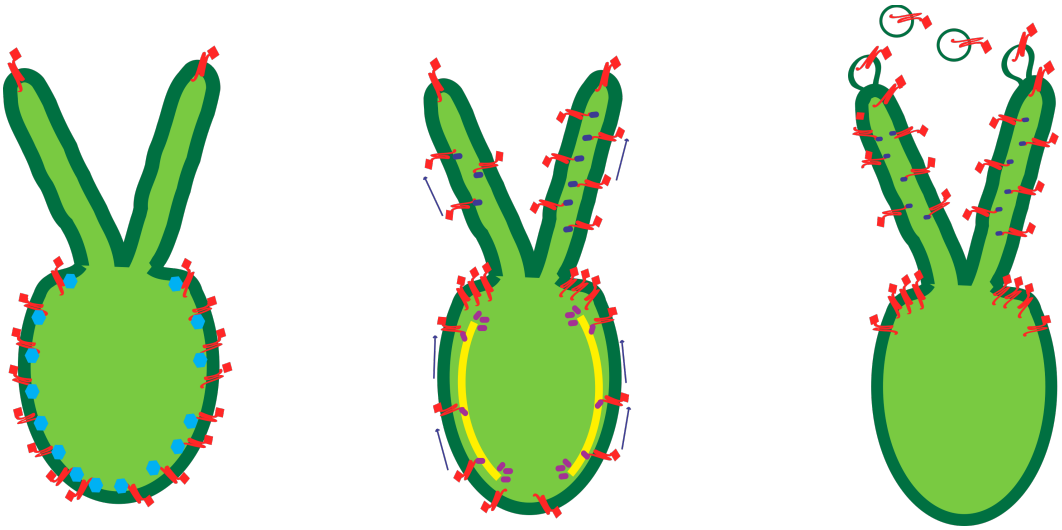
periciliary membrane. Apically accumulated SAG1-HA-C65 can diffuse laterally and efficiently into the flagellar membrane without the IFT machinery. Upon flagellar adhesion, SAG1 (N-terminal adhesion domain associated with C-terminus portion) interacts with SAD1 and triggers their shedding in membrane vesicles into the medium. This process coupled with signaling causes a positive feedback loop to enrich more SAG1 into the flagella for prolonged adhesion (Figure 3.1).

Figure 3.1

Possible model about the molecular mechanism of SAG1-HA-C65 during flagellar adhesion-induced signaling

Resting immobilized SAG1-HA-C65 is present in clusters in the surface of the cell body plasma membrane. SAG1-HA-C65 clusters are sequestered by membrane-associated proteins (sequesterin protein complex) in the plasma membrane, resulting in flagellar SAG1-HA-C65 de-enrichment. During flagellar adhesion the immobilized SAG1-HA-C65 becomes freely mobile and can associate with the targeted delivery machinery composed of the submembranous cytoplasmic micotubules, cytoplasmic dynein and accessory proteins to engage in lateral minus-end directed traffic through the plasma membrane toward the periciliary membrane. SAG1-HA-C65 enriches in the flagellar membrane with an IFT independent transport. Upon flagellar adhesion, SAG1 (N-terminal adhesion domain associated with C-terminus portion) interacts with SAD1 and triggers their shedding in membrane vesicles into the medium. This process coupled with signaling causes a positive feedback loop to enrich more SAG1 into the flagella for prolonged adhesion.

Figure 3.1



Resting Gamete

Early Adhering Gamete

Prolonged Adhering Gamete

SAG1-HA-C65

sequesterin protein complex

targeted delivery machinery

IFT independent flagellar transport

Other possible models

Resting distribution of SAG1-HA-C65

I observed with immunofluorescence that in resting gametes, SAG1-HA-C65 was evenly distributed along the plasma membrane and very little in the flagellar membrane. Cells showed a characteristic ring staining of SAG1-HA-C65 and what appeared to be SAG1-HA-65 clusters. As discussed in chapter 2, one possibility is that SAG1-HA-C65 exclusion from the flagella in resting gametes results from the presence of a barrier at the base that prevents the freely mobile membrane protein from going into the flagellar membrane in the absence of a signal. Another possibility is that a barrier is lacking. According to such a model, SAG1-HA-C65 would not be freely mobile, but would be immobilized in the plasma membrane through an yet uncharacterized mechanism mediated by associated proteins specific in the plasma membrane and absent from the ciliary membrane. Another possibility is the combination of both models where a barrier restricts movement of membrane proteins into the flagellar membrane and SAG1-HA-C65 is immobilized in clusters in the plasma membrane unable to interact with the targeted ciliary delivery machinery in the absence of flagellar adhesion induced signaling.

Cytoplasmic dependent periciliary accumulation and IFT independent enrichment of SAG1-HA-C65 into the flagellar membrane upon adhesion

Several key findings led me to understand a little better the cellular fate of SAG1-HA-C65. First, we learned that the protein was enriched in the flagella upon adhesion. Then, looking more closely at this enrichment event, I observed that SAG1-HA-C65 accumulated rapidly in the periciliary membrane upon flagellar adhesion and, in a more accentuated manner, upon addition of db-cAMP. This distinctive re-distribution was very interesting and led us to discover that cytoplasmic microtubules were essential for this accumulation phenomenon. In addition, with integrated biochemical and *in vivo* approaches offered by our model organism, we were able to discover that SAG1-HA-C65 enriched in the flagellar membrane in an IFT-independent manner.

The cytoplasmic dependent accumulation at the ciliary base and the IFT-indendepent enrichment in the flagella are two important discoveries where several models can be proposed about the molecular mechanism of regulated enrichment of membrane proteins in the flagellar membrane. One possibility is that upon flagellar adhesion SAG1-HA-C65 associates with a targeted delivery machinery composed of cytoplasmic microtubules to reach the ciliary base with a simultaneous collapse of a diffusion barrier. Once accumulated there, SAG1-HA-C65 can diffuse efficiently into the flagellar membrane without the need of IFT. Another possibility is that, without a diffusion barrier to begin with, SAG1-HA-C65 may be found freely mobile in the plasma membrane and then cytoplasmic microtubules mediate the immobilization of SAG1-HA-C65 in the ciliary

membrane upon signaling, resulting in an evident accumulation at the flagellar base. Once there, an IFT independent mechanism could be transporting it into the flagellar membrane. An IFT-independent movement may include SAG1-HA-C65 association with an uncharacterized flagellar microtubule motor and accessory transport proteins. Other options may include all possible combinations of the proposed models above.

Regulated shedding of SAG1-HA-C65 from the flagella to the medium

Another interesting observation is that a membrane protein undergoes regulated flagellar shedding upon a receptor-receptor interaction. Even though this shedding process may be specific to *Chlamydomonas*, recent studies have shown that renal cells shed membrane vesicles that into the urine contain ciliary proteins [89] [93]. Also, recent evidence suggests that cancer cells shed exosomes that, among other molecules, contain membrane proteins to regulate cell-cell interactions (reviewed in [92]). My studies on regulated shedding of SAG1-HA-C65 may play an important potential role in this emerging field of exosomes and cancer signaling. Learning the molecular mechanisms that regulate cancer exosome shedding and signaling and determining its possible link with primary cilia can help not only with cancer diagnosis but also with targeted cancer therapy.

As discussed in chapter 2, a model to explain the shedding process would require that during flagellar adhesion SAG1 N-terminal adhesion domain is associated with its C-terminus portion in the flagella. SAG1 receptor in the *plus* flagella binds to SAD1 receptor in the *minus* flagella. This receptor-receptor binding may function as a receptor-ligand interaction that triggers a conformational change in SAG1 concomitant with the activation of downstream signaling proteins. The conformational change in the receptor generates enough force for the membrane protein to pull away from its tight fit between the axoneme and the ciliary membrane. The pulling force brings about the budding off of SAG1 containing vesicles into the medium. One biological explanation for the shedding process is that gametes have to undergo flagellar adhesion coupled with flagellar de-adhesion [94]. The correlation between loss of agglutinin activity for de-adhesion and the recovery of SAG1-HA-C65 in the medium supports the possibility that SAG1 N-terminal adhesion domain and the C-terminus fragment are associated in the flagella. Thus, the shedding process is a possible molecular mechanism for de-adhesion during flagellar adhesion.

Future Directions

Many questions still remain unanswered about the how the protein composition of the ciliary membrane is determined. Specifically key aspects about the cellular behavior of SAG1-HA-C65 are not well understood. To learn

more about this membrane protein and its regulated enrichment in the flagella, further studies are required. These include the analysis of SAG1-HA-C65 mobility to determine whether the membrane protein is immobile or mobile in the plasma or ciliary membrane before and after signaling. Furthermore, studies are needed to identify the protein composition of the targeted delivery machinery and their association with SAG1-HA-C65 in resting and activated gametes. Also, SAG1-HA-C65 association with specific protein complexes and cytoskeleton components in resting and activated gametes could be tested. Studies to dissect the components of the diffusion barrier and how signaling modifies its blocking function would be interesting. Other required studies include the identification of potential IFT independent mechanisms of flagellar membrane protein transport. In addition, important would be to characterize the molecular mechanism of the shedding process by determining if flagellar motility contributes to the pulling force for membrane vesicles budding into the medium. *Chlamydomonas* motility mutants are available to test this idea. Also, it would be interesting to determine whether this membrane protein shedding process occurs in other types of primary cilium in vertebrates to regulate the protein composition of their ciliary membrane.

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