THE PROTEIN COMPOSITION OF THE *CHLAMYDOMONAS* FLAGELLAR MEMBRANE IS DYNAMICALLY REGULATED BY CILIUM-GENERATED SIGNALING DURING FERTILIZATION

APPROVED BY SUPERVISORY COMMITTEE

William J. Snell, Ph.D.

Dean Smith, M.D., Ph.D.

Frederick Grinnell, Ph.D.

Lawrence Lum, Ph.D.

DEDICATION

To my parents and sisters,

Yves Belzile
Solange Bérubé
Amélie Belzile
Justine Belzile

May we continue to enjoy all our wonderful,
growing family for many years to come

To Siriya,

My love

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by

OLIVIER BELZILE, B. Sc., M. Sc.

DISSERTATION

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OLIVIER BELZILE, B. Sc., M. Sc.

The University of Texas Southwestern Medical Center at Dallas, 2012

WILLIAM J. SNELL, Ph.D.

The cellular and molecular mechanisms specifying the membrane protein composition of cilia and flagella at steady state and during cilium-generated signaling are poorly understood. Our laboratory uses the biflagellated green alga *Chlamydomonas* as a model system to study regulated movement of the flagellar adhesion receptor SAG1 from the cell body to the flagella. Interactions between the *plus* flagellar receptor (agglutinin) SAG1 and its cognate

receptor SAD1 on flagella of *minus* gametes induces flagellar adhesion and activation of a cAMP-dependent signaling pathway ultimately leading to cell-cell fusion. Although previous work from our laboratory and others suggested that pathway activation triggers mobilization of a pool of SAG1 from the cell body to the flagella, those studies depended on indirect adhesion bioassays detecting the activity of *SAG1*, not the protein. Here, I report use of new tools to study directly the regulation of SAG1 localization. I show that the *SAG1* gene bearing a C-terminal HA tag rescues flagellar adhesion and cell fusion in the flagellar adhesion mutant, *sag1-5*. Immunofluorescence studies of resting SAG1-HA/*sag1-5* mt+ *plus* gametes show that the protein is present mostly on cell bodies. Biochemical studies show that only gametes express SAG1-HA. Detection of precursor forms indicates SAG1 undergoes cleavage soon after its synthesis to yield an HA-tagged 65 kDa, C-terminal portion (SAG1-HA-C65), and that SAG1-HA-C65 is on the cell surface. Consistent with the predicted 3 transmembrane domains at the C-terminus, release of SAG1-HA-C65 in a soluble form requires detergent and is not achieved upon mechanical disruption, high salt, or high pH treatments. Cell fractionation demonstrates that in resting gametes the majority of SAG1-HA-C65 is present on cell bodies and only a small amount is on flagella. Within minutes after signaling is triggered by flagellar adhesion, however, SAG1-HA-C65 is mobilized from the cell body to the flagella and the organelles become highly enriched in the protein, where it forms large detergent-resistant complexes.

I show that *Chlamydomonas* can regulate the amount of SAG1 in the flagella through cilium-generated signaling, and thus provides the first system for studying regulation of the membrane protein composition of the cilium/flagellum in a biochemically tractable system.

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May 2012

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Belzile, O., Gulemetova, R., and Kinkead, R. (2007) Effects of medullary Raphe stimulation on fictive lung ventilation during development in *Rana catesbeiana*. *J Exp Biol*. **210**: 2046-56.

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LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

bp:	Base pair(s)
cAMP:	Cyclic adenosine monophosphate
db-cAMP:	Di-butyryl cyclic adenosine monophosphate
DIC:	Differential interference contrast microscopy
EDTA:	Ethylenediaminetetraacetic acid
EM:	Electron microscopy
g:	Earth's gravitational acceleration
GMP:	Guanosine monophosphate
HA:	Hemagglutinin
Hh:	Hedgehog
IB:	Immuno blot
IFT:	Intraflagellar transport
kDa:	Kilo Dalton
L:D:	Light : Dark
PCR:	Polymerase chain reaction
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
SuFu:	Suppressor of Fused
wt:	Wild type

Chapter 1

GENERAL INTRODUCTION AND BACKGROUND

GENERAL INTRODUCTION

Biology students learn about the critical role of flagella in animal development early in their education. Indeed it has been long known that human development begins when a flagellum-propelled sperm fuses with the egg to form a zygote. More recent work, however, has demonstrated that almost all animal cells have a single, primary cilium and that this organelle is a key player in multiple stages of animal development (Goetz and Anderson, 2010). For example, primary cilia are essential for patterning of embryonic organs such as the limbs and the central nervous system, they establish left-right asymmetry of the body, and they are essential for normal kidney integrity (Basu and Brueckner, 2008; Bisgrove and Yost, 2006; Huangfu et al., 2003; Yoder, 2007). Ciliary defects have been shown recently to underlie a variety of seemingly disparate developmental diseases, which now are grouped together under the new term, ciliopathies (Badano et al., 2006). Primary, non-motile, single cilia are significantly implicated in several signal transduction pathways. These surprising discoveries have sparked an intense, multidisciplinary interest in cilia and flagella focused on understanding the features of cilia and flagella that have led to their evolutionary exploitation as signaling organelles.

A salient feature of this slender organelle is that it forms a cell compartment that extends into the extracellular milieu and is spatially isolated from the main body of the cell. The ability to regulate the membrane composition of cilia must contribute to their being an ideal site to control signaling cascades (Rohatgi and Snell, 2010). Several signaling molecules and membrane receptors are constitutively present in cilia and flagella (Pazour and Bloodgood, 2008). More interestingly with regard to dynamic signaling, some signaling membrane proteins shuttle in and out of the organelle depending on the signaling state, and ciliary localization of these proteins appears to be highly regulated (Rohatgi et al., 2007). One of these proteins is Smoothened, a critical component of the Sonic Hedgehog (Hh) signaling pathway in mammals. According to immunofluorescence imaging, Smoothened is restricted from accumulation in cilia when the pathway is off, but becomes enriched there once the pathway is activated by binding of Hedgehog ligand to its receptor (Corbit et al., 2005). The receptor itself, Patched, a 12 transmembrane domain protein, does the opposite: it normally localizes at the cilium and moves out upon ligand binding (Rohatgi et al., 2007). The mechanisms by which these membrane proteins translocate in and out of the cilium are not understood. The mammalian system offers challenges for studying regulation of ciliary membrane protein composition due to the difficulty of isolating primary cilia. Thus, only imaging methods are available for quantifying ciliary membrane protein amounts compared to the amounts on the

plasma membrane of the cell body. To learn more about the mechanism allowing regulated changes in ciliary membrane composition, it is important to be able to isolate the organelle to allow biochemical characterization.

Studies on unicellular organisms whose cilia flagellar are much more amenable to biochemical characterization have revealed that the use of cilia to sense and respond to environmental cues is an ancient invention. *Paramecium* cilia respond to touch and potentially toxic materials in their environment (Eckert, 1972). Even though it is unicellular for most of its life, the green alga *Chlamydomonas* absolutely depends on a cell-cell interaction for a critical step in its life cycle: fertilization. This adhesion event is mediated by the flagella of *Chlamydomonas*, which bear the cell adhesion molecules and organize the signaling pathway that is essential for fertilization (Adair et al., 1982; Klis et al., 1985; Mesland et al., 1980; Pan and Snell, 2002; Snell, 1976). A major advantage for using bi-flagellated green alga *Chlamydomonas* to study ciliary biology is that is it particularly well suited for biochemical studies because flagella and cell bodies can be isolated easily. Just as in the Hh pathway, a key feature of ciliary signaling in *Chlamydomonas* is the ability to regulate the properties of a membrane proteins central to the pathway. These membrane proteins in C*hlamydomonas* are the flagellar adhesion molecules (agglutinins) (Ferris et al., 2005; Snell, 1976). These adhesion molecules act as mutual receptor-ligands to

initiate the first contact between the flagella of gametes of opposite mating type, thereby triggering a complex signaling transduction cascade leading to gamete activation and ultimately cell-cell fusion to form a zygote. Previous work using bioassays to detect agglutinin activity has shown that a large portion of total cellular agglutinin activity is present in an inactive form on the plasma membrane of the cell body. During flagellar adhesion-induced signaling, the activity of the agglutinin on the flagella increases several-fold (Goodenough, 1989).

In this dissertation, I describe my studies on regulation of the membrane protein composition of *Chlamydomonas* flagella, focusing on a polypeptide encoded by the *plus* agglutinin gene, *SAG1*. I propose a model for SAG1 in which the protein is cleaved into fragments soon after synthesis, one large fragment lacking transmembrane domains and a C-terminal integral membrane fragment. I describe rescue of flagellar adhesion in a *SAG1* mutant with an epitope-tagged form of the *SAG1* gene, and characterize SAG1 properties in resting gametes both biochemically and by immunofluorescence. I then show that during fertilization, SAG1-HA-C65 is rapidly enriched on flagella where it forms large detergent-resistant complexes that may contribute to its flagellar accumulation.

BACKGROUND

In this section I will review evidence that several signaling membrane proteins localize to primary cilia, some constitutively, others in a regulated fashion, then I will give an overview of the *Chlamydomonas* life cycle, its flagella and sexual signaling, and finally I will review early work on the agglutinins (or agglutinin activity) and highlight the main current questions about these molecules.

Signaling membrane proteins are present in cilia/flagella of other organisms

The relation between signal transduction and the elongated microtubule-based organelle has been known for a long time in *Chlamydomonas*. However, over the last decade or so the vertebrate cilium, especially the non-motile version, the primary cilium, became notorious for being specialized signaling structures. The primary cilium, long regarded as useless vestigial structure, is a single paralyzed cilium present on most cell types of the mammalian body. They differ from motile cilia by lacking a central pair of microtubules and dynein arms (components required for cilia beating) (Satir and

Sleigh, 1990). Not only many signaling molecules and non-membrane associated proteins are found in primary cilia, for example the gli transcription factors and SuFu of the hedgehog pathway (Haycraft et al., 2005), but also there have been several recent reports of signaling membrane proteins constitutively present in ciliary membranes (see chapter two). Rosenbaum and Witman proposed several years ago that membrane proteins are transported directly from the Golgi (via vesicles) to the base of the cilium during ciliary assembly where they are integrated into the membrane of the elongating organelle (Rosenbaum and Witman, 2002).

Membrane signaling protein trafficking in and out of vertebrate cilia, the Hedgehog system as an example

For my project, the vertebrate signaling proteins that are the most interesting are the membrane proteins that are translocating to the cilium or out of the cilium, because earlier evidence indicated that SAG1 undergoes similar translocation events in *Chlamydomonas*. Perhaps the most studied proteins corresponding to these descriptions are two upstream players of the sonic hedgehog signaling pathway: Patched and Smoothened. In this critical signal transduction pathway, essential for normal development but also involved in cancers when misregulated, the primary cilium appears to be essential for normal

functioning of the pathway (Huangfu et al., 2003). When the signaling is off (when the ligand is not present), the 12 transmembrane domain receptor Patched is greatly enriched in primary cilia while the 7 transmembrane domain downstream protein Smoothened is undetectable in the organelle (Rohatgi et al., 2007). Once the sonic hedgehog soluble ligand is secreted from neighboring cells and diffuses toward hedgehog responding cells, it binds to Patched, resulting in Patched leaving the primary cilium and Smoothened invading the ciliary membrane (Rohatgi et al., 2007). This ciliary Smoothened enrichment promotes the accumulation of Gli2 and Gli3 at the cilium and results in shifting the ratio of Gli proteins from mostly repressor forms to activator forms (reviewed in (Drummond, 2012)).

Mechanisms for targeting and delivering ciliary membrane proteins to the cilium: 3 models

Milenkovic *et al.* worked to establish by what route Smoothened is delivered to the plasma membrane (Milenkovic et al., 2009). The delivery model of Rosenbaum and Witman mentioned above addressed newly synthesized membrane proteins during flagella assembly (Rosenbaum and Witman, 2002). Regarding existing membrane protein localized to the plasma membrane on the cell body (such as Smoothened), one model partially based on the Rosenbaum and

Witman model proposed that the proteins are first delivered to the plasma membrane where they stay until an event triggers them to be endocytosed into vesicles that would be targeted to the base of the cilium to be integrated into the ciliary membrane like the newly synthesized proteins during cilia assembly (Milenkovic et al., 2009). An alternative model is that the existing protein would diffuse laterally from the plasma membrane to the cilia membrane by getting past a functional barrier (Hunnicutt et al., 1990). Using imaging and pulse-chase experiments, it has been demonstrated that this second route was the one used by smoothened (Milenkovic et al., 2009). This discovery highlights the fact that different ciliary membrane proteins use different routes to reach their destination. There is however some controversy in the field. Wang *et al.* propose a different model in which the majority of Smoothened that localize at the cilium originates from an intracellular source, much like the Rosenbaum and Witman model, except that in that case, the cilium is already assembled (Wang et al., 2009).

Role of intraflagellar transport in movement of membrane proteins

Intraflagellar transport (IFT) is a special kind of motility, unrelated of flagellar beating, that goes on bi-directionally between the outer microtubule doublets of the axoneme and the flagellar membrane (Kozminski et al., 1993). IFT is microtubule-based motility driven by molecular motors kinesin II

(anterograde IFT) and cytoplasmic dynein 1b (retrograde IFT) (Kozminski et al., 1995; Pazour et al., 1998; Signor et al., 1999). These motors carry IFT particles composed of at least 16 different proteins rich in protein-protein interaction motifs and forming large complexes (A and B) (Cole, 2003). These IFT particle proteins bind cargo and are required for ciliary/flagellar assembly at the tip of the organelles (Cole, 2003; Cole et al., 1998; Qin et al., 2004). Assembly of primary cilia by IFT has been reviewed recently by Ishikawa and Marshall (Ishikawa and Marshall, 2011). IFT is not only required for flagellar assembly but it also participates directly in signaling events in flagella (Wang et al., 2006). Furthermore, some evidence suggests that IFT is required for movement of some membrane proteins along cilia (Qin et al., 2005; Zhao and Malicki, 2011). Limitations of mammalian cells in ciliary research make it difficult to determine whether Smoothened or Patched require IFT for their movement in and out of primary cilia. On the other hand, *Chlamydomonas* seems like a promising system to study the possible requirement of IFT for transport of membrane signaling proteins because it is possible to isolate their flagella for biochemical analysis and because a conditional IFT mutant is available (Pan and Snell, 2003; Wang et al., 2006).

Overview of the *Chlamydomonas* life cycle

Recognition and adhesion of two opposite sex gametes is a phenomenon shared from unicellular green algae to humans. *Chlamydomonas reinhardtii* is a bi-flagellated green alga found mostly in freshwaters and soil. When conditions are favorable, Chlamydomonas lives under a "vegetative" state and undergo mitosis to reproduce. The cells are surrounded by an extracellular matrix called a cell wall, however the cell wall does not cover the flagella. In *Chlamydomonas*, *plus* and *minus* gametes are produced under unfavorable conditions (e.g. lack of a source of nitrogen) (Sager and Granick, 1954). The opposite mating type gametes recognize and adhere to each other by their flagella, leading to a cell-cell fusion event to produce a zygote surrounded by a thick cell wall and resistant to extreme temperatures and drying up of a pond (Harris, 1988).

The flagella of *Chlamydomonas* : more than just a swimming device

Chlamydomonas cells possess two flagella of equal length (around 12 μm) originating from basal bodies located at the apical end of the cell. Flagella have a central pair of microtubules and dynein arms between the 9 outer doublets and the central pair. With no surprise, with such an axonemal organization, these flagella are highly motile (Witman et al., 1978). And attesting to the usefulness of *Chlamydomonas* as a model organism, much of what we know about the motile functions of eukaryotic cilia and flagella comes from coupled ultrastructural and

biochemical studies of wild type and paralyzed flagellar mutants of *Chlamydomonas* (Bui et al., 2008, 2009; Mitchell, 1994; Mitchell and Nakatsugawa, 2004; Ostrowski et al., 2011; Patel-King and King, 2009; Rupp et al., 1996; Takazaki et al., 2010; White et al., 2005).

These organelles also have another critical function: they are directly responsible for cell-cell recognition and adhesion during mating of two opposite mating type gametes. The signaling cascade that leads to zygote formation starts and takes place in the flagella. Flagella are assembled by a special type of microtubule-based motility, intraflagellar transport (IFT) (Kozminski et al., 1993). The content of the flagella differs from the rest of the cell and comparative genomics have identified a basal body and flagellar proteome (Dutcher, 1995; Li et al., 2004). Mechanisms that are not well understood allow certain molecules to access the flagellar matrix or membrane but block other components from entering these same flagellar compartments (Craige et al., 2010; Hunnicutt et al., 1990).

Figure 1

Overview of the *Chlamydomonas* life cycle.

Upon nitrogen starvation, vegetative cells undergo gametogenesis. Gametes of the opposite mating type adhere by their flagella, flagella-generated signaling occurs, and gametes become activated (cell wall loss, increase in flagellar agglutinin activity, activation of mating structures), and eventually cell fusion to form a zygote.
(Image courtesy of William J. Snell)

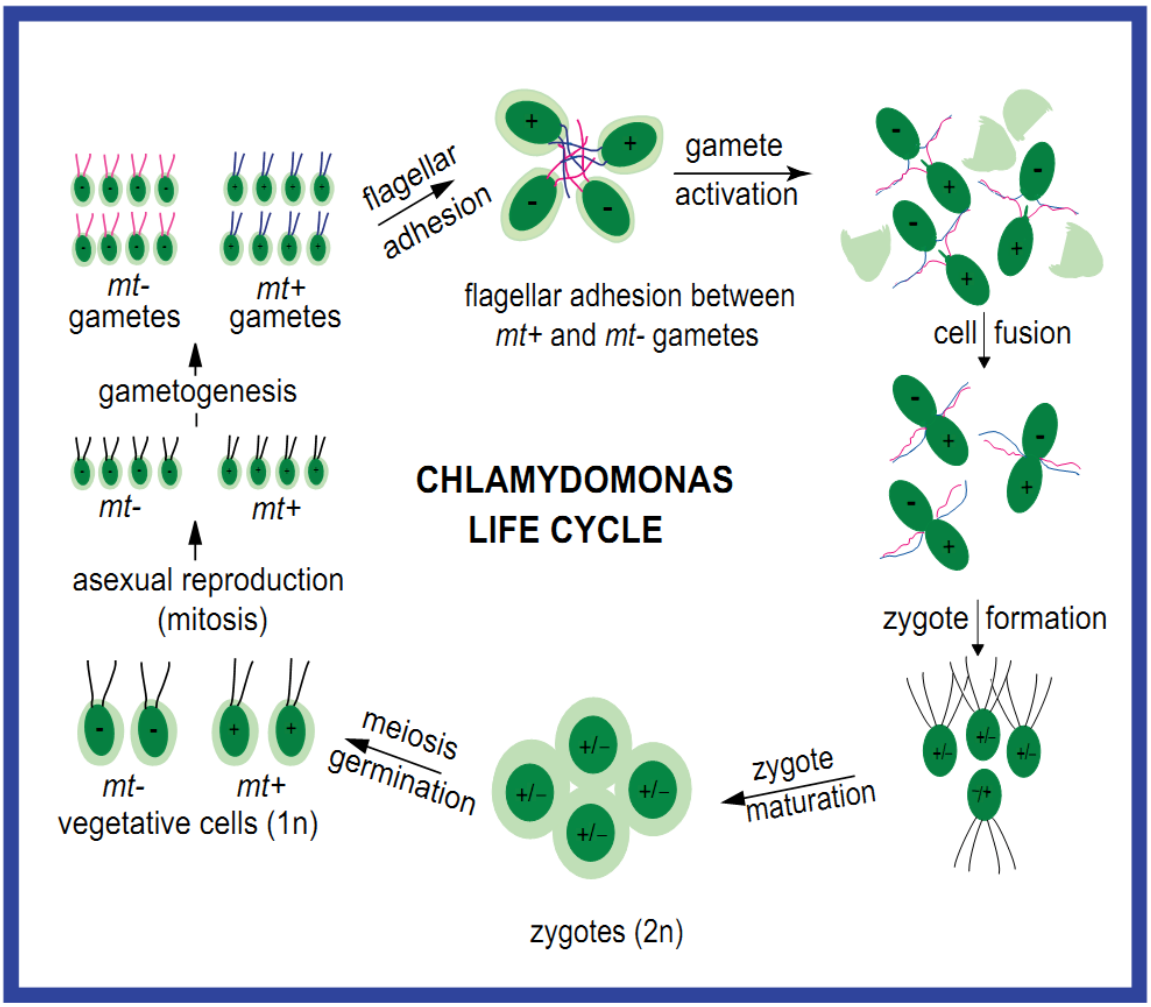

CHLAMYDOMONAS
LIFE CYCLE
mt-
gametes
mt+
gametes
flagellar
adhesion
flagellar adhesion between
mt+ and mt- gametes
gamete
activation
cell fusion
zygote formation
zygote
maturation
meiosis
germination
mt-
mt+
vegetative cells (1n)
zygotes (2n)
gametogenesis
asexual reproduction
(mitosis)

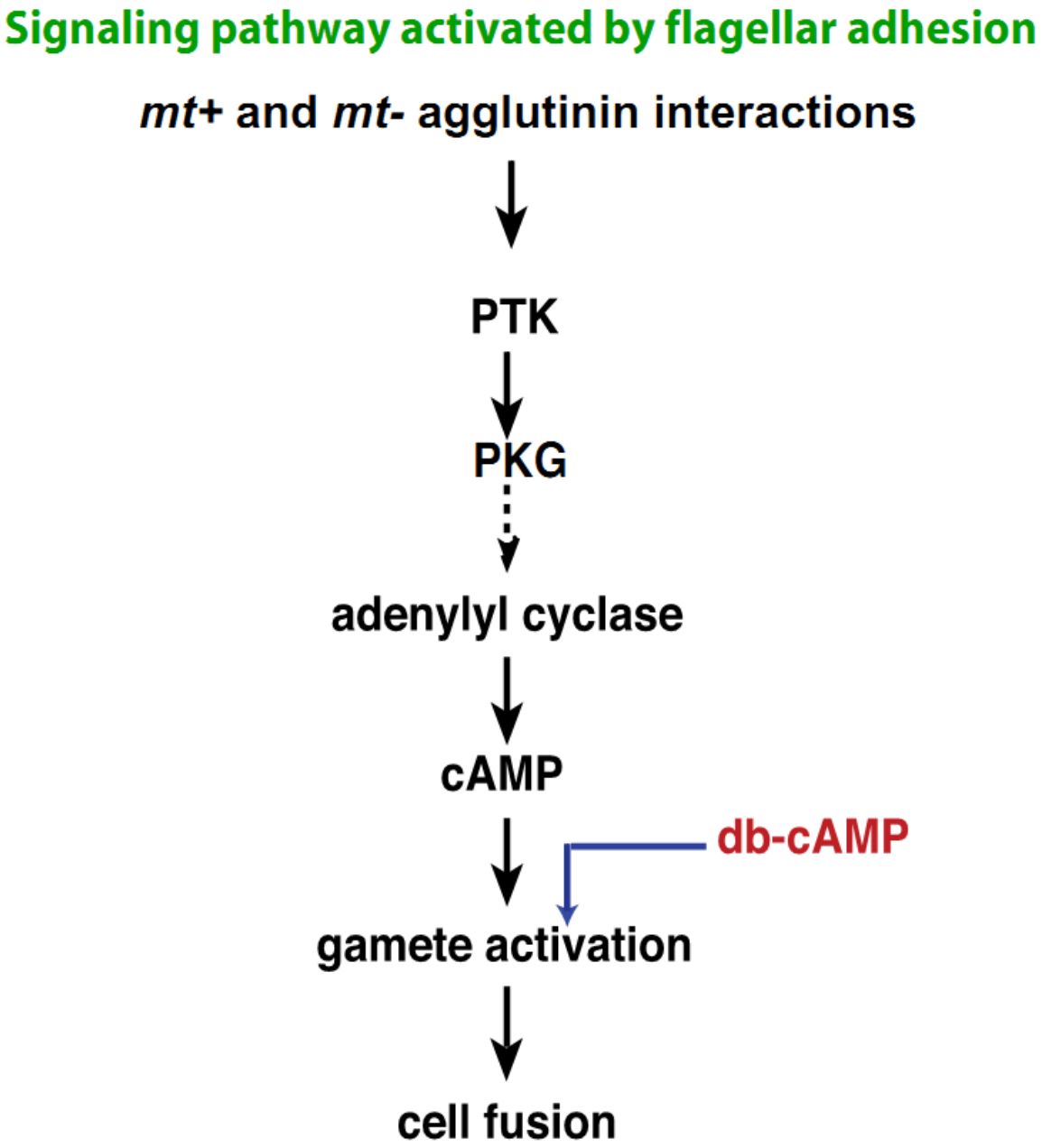
Figure 2

Signaling pathway activated by flagellar adhesion between the flagella of *plus* and *minus Chlamydomonas* gametes.

Initial interactions between *plus* and *minus* flagella of competent gametes triggers a complex signaling cascade within flagella involving kinases and an important elevation in cyclic AMP concentration.

Abbreviations:

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


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

Flagellum-generated signaling cascade during fertilization

Adhesion between flagellar agglutinins is the first step of a complex signaling cascade occurring within the flagella and activating protein kinases, an adenylyl cyclase, and the intraflagellar transport machinery. The interactions between *plus* and *minus* agglutinins can be considered a ligand-receptor interaction. The first biochemically detectable step triggered by this interaction is the activation of a protein tyrosine kinase (Wang and Snell, 2003) that phosphorylates a cGMP-dependent protein kinase (PKG) (Wang et al., 2006). Following the phosphorylation of this PKG, an adenylyl cyclase is activated (Zhang and Snell, 1994), leading to an increase of cyclic AMP (Pasquale and Goodenough, 1987) which leads to several responses including appearance of more agglutinin activity on the flagella, loss of the cell wall, and activation of mating structures (reviewed in (Wilson, 2008)). These responses constitute gamete activation and are required for cell fusion. The molecular events that couple agglutinin interactions to the downstream events of the signaling cascade are not clear, therefore understanding the properties of these molecules is critical for understanding transmittal of the signal from outside to inside the flagella. The rationale for this dissertation is that this flagellum-based signaling pathway in *Chlamydomonas* provides a powerful system for dissecting the unique properties

of cilia and flagella that favored their extensive use as signaling organelles or cellular antennae.

The agglutinins are responsible for gamete recognition and adhesion

Identification of the molecules responsible for gamete recognition in *Chlamydomonas* was a long process. In 1954, Forster and Weise discovered that medium from gametes of one mating type contained a factor that would cause gametes of the opposite mating type to adhere to each other (a phenomenon termed isoagglutination) (Forster and Wiese, 1954). It was later shown that flagellar vesicles in the medium were responsible for the isoagglutination (reviewed in (Hunnicutt, 1989)). When the media from vegetative and gametic cell cultures were compared by SDS PAGE and coomassie blue staining, a so-called "U" band of around 70 kDa was shown to be present only in the sample from the *plus* gametes (Snell, 1976). The identity of this "U" was unknown and experiments to study it were not pursued.

Significant progress was achieved in 1982 when Adair *et al.* discovered that the agglutinin activity in detergent extracts of isolated flagella could be detected by use of a novel *in vitro* bioassay: the dried spot assay. The extracts, which lost their flagellar adhesion activity during the detergent extraction

procedure (they were unable to cause isoagglutination of gametes of the opposite mating type nor they were able to block adhesion of gametes of the opposite mating type), regained flagellar adhesion activity if the extract was allowed to dry on a glass slide (Adair et al., 1982). Presumably, the cryptic adhesion activity of the agglutinin was exposed upon the denaturation that occurred upon binding to the glass substratum (Adair et al., 1982; Hunnicutt et al., 1990). This ability to support binding of cells of the opposite mating type by their flagella was defined as agglutinin activity, and served as a bioassay to measure the agglutinin activity of fractions of isolated flagellar samples. Polypeptides with bioassayable 'agglutinin activity' were then purified and studied by several labs. Although early work used detergent to extract the agglutinin activity, it has been shown that the polypeptide carrying this activity could be solubilized by other detergent-free methods (EDTA, sonication, passage through a French press) (Goodenough et al., 1985) (Hunnicutt et al., 1990), indicating that the adhesion-active polypeptide lacked transmembrane domains.

By use of the dried spot assay (to follow what was then thought to be the full length agglutinin protein), several groups identified a heavily glycosylated polypeptide whose migration in SDS-PAGE gels was very low, yielded an apparent molecular mass of greater than 500 kDa (Adair et al., 1983; Hunnicutt et al., 1990). Adair *et al.* performed a de-glycosylation by anhydrous hydrogen

fluoride and concluded that the core polypeptide had a size of 480 kDa; although the carbohydrate stripping might not have been fully achieved. Interestingly, once the carbohydrates were lost; the polypeptides lost their agglutinin activity, suggesting an essential role of glycosylation for the agglutinin activity.

In 1985, these polypeptides carrying the agglutinin activity for the *plus* and *minus* gametes were visualized by electron microscopy and were described as long cane-shaped molecules (Goodenough et al., 1985). Diagrams of these molecules can be found in figure 3. Interestingly, in this same study, the authors discovered similar but shorter versions of these fibrous molecules and (although they did not favor the idea) they raised the possibility that those shorter molecules might be fragments of the long cane-shaped molecules possessing the agglutinin activity. (Goodenough et al., 1985). These shorter molecules did not possess agglutinin activity.

Evidence that the *plus* agglutinin interacted with the *minus* agglutinin came from studies in which monoclonal antibodies were raised against the semi purified agglutinins (Homan et al., 1988; Snell et al., 1986). These antibodies were able to block the adhesiveness of gametes, isolated flagella and extracts of these flagella (Snell et al., 1986). Blocking either one of the agglutinins by antibodies blocked gamete adhesion, leading to the most parsimonious

interpretation that the agglutinins must bind to each other. Other antibodies were able to elicit some gamete activation reactions (Homan et al., 1988). Also, Goodenough *et al.* (Goodenough et al., 1978) used antiflagellar antibodies to get the *SAG1* and *SAD1* mutants to become activated and fuse, thereby allowing them to establish that several of the mutants strains had mutations in the same gene (allelic). They specifically showed that most mutations affecting the *plus* agglutination also map to the *SAG1* locus (Goodenough et al., 1978).

The majority of agglutinin activity in resting gametes is on the cell body and inactive *in situ*

Previous work in our lab and others using the dried spot bioassay developed by Adair et al. (Adair et al., 1982) aimed to characterize *plus* agglutinin activity. For example, it has been reported that only around 10% of the total *plus* agglutinin was present on flagella of resting *plus* gametes while 90% of it was present on the cell body but as an inactive form (Hunnicutt et al., 1990).

Work in our lab demonstrated that the flagellar plus agglutinin activity could be removed from flagella using a monoclonal antibody and that in these circumstances the cell body agglutinin as detected by the dried spot assay was unchanged from control cells. Because the cell body agglutinin activity was on

the cell surface (i. e., sensitive to trypsin treatment of live gametes), these results suggested that a functional barrier prevented movement from the cell body to the flagella. Moreover, addition of db-cAMP led to restoration of the agglutinin activity on the flagella and reduction of agglutinin activity on the cell bodies (Hunnicutt et al., 1990). The favored interpretation of those results was that the cell body agglutinin protein moved to the flagella. On the other hand, these experiments provided only indirect evidence about the behavior of the protein, and it was possible that the db-cAMP treatment altered the activity of the proteins without changing their location. Thus, a full understanding of the signaling regulated location of the *plus* agglutinin requires development of methods to detect protein directly, as opposed to following its activity (Chapter 2).

Since removal of cell wall is a consequence of gamete activation with dibutyryl cAMP, Hunnicutt *et al.* did a critical control in which they had cells in parallel treated with autolysin (a cell wall removing protease) instead of dibutyryl cAMP, and they did not agglutinate (Hunnicutt et al., 1990). This confirmed that the cell body agglutinins were inactive *in situ*. Also using autolysin, the authors showed that the cell wall was not the barrier preventing movement from cell bodies to flagella.

The dried spot bioassay used in most experiments described above is not a very precise method and its effectiveness is limited. It remains to be seen if these results can be confirmed using more quantitative biochemical methods. In addition, no attempt to visualize the protein responsible for the *plus* agglutinin activity by immunofluorescence has been achieved in *Chlamydomonas reinhardtii* and such studies should give more precise information about the exact localization of the polypeptide causing the agglutinin activity than what can be interpreted by old bioassays.

In adhering/activated gametic cells, adhesiveness of flagella is enhanced

In cell bodies, quantification of agglutinin activity and use of radiolabeling methods showed an adhesion-triggered decrease in agglutinin activity and in the amount of a high molecular weight polypeptide proposed to be the adhesion-active agglutinin. These results were consistent with the idea of the adhesion triggered recruitment of agglutinin from the cell body to the flagella (with the cell body population later being replenished by protein synthesis) (Hunnicutt et al., 1990). Consistent with this hypothesis, Goodenough (Goodenough, 1989) showed that cyclic AMP stimulation of *plus* gametes resulted in enhanced adhesiveness in flagella. But these results did not rule out other hypotheses, for example, that adhesion/cyclic AMP stimulation could

somehow enhance the adhesiveness of existing flagellar agglutinins without requiring an influx of additional molecules from the cell body.

Current model of the agglutinins' structure: strengths and weaknesses

Genes encoding the agglutinins were cloned in 2005 by Ferris *et al.* to identify the *SAG1* locus (Ferris et al., 2005). The gene product of *SAG1* and *SAD1* are predicted to be large (3409 amino acids for SAG1, 3889 amino acids for SAD1) proteins with several putative glycosylation sites. Both proteins contain a region rich in hydroxyprolines (Ferris et al., 2005).

The overall domain architecture of the SAG1 and SAD1 gene products is similar (figure 3). Both have a region of around 900 amino acids very rich in proline residues (mostly hydroxylated) near the N-terminus, both have presumed globular domains before and after the proline-rich region, and both are predicted to possess 6 transmembrane domains in their C-terminal halves. Neither gene product is similar to genes in other species.

Based on their electron microscopic analysis of the purified flagellar polypeptides that possessed flagellar adhesion activity as detected with the dried spot assay Goodenough's group concluded that the *plus* and the *minus* agglutinins had a

similar overall morphology, consisting of a central shaft region, a hook region and a globular head region (Ferris et al., 2005) (Goodenough et al., 1985).

In their 2005 paper, they integrated the ultrastructural information with the predicted sequence of the proteins encoded by SAG1 and SAD1 to propose a model of the structures of the two. In their model, the 900 amino acid proline rich region corresponded to the shaft region seen in their EM images (Goodenough et al., 1985), and they proposed that the hooks corresponded to the N-terminus of the proteins, and that the heads corresponded to the C-terminal half of the gene product. Ferris et al. (Goodenough et al., 1985) mention that no predicted transmembrane domains were present in the shaft region, but they did not comment about the presence of such domains in other regions of the full-length protein predicted to be encoded by the SAG1 gene. Furthermore, because the polypeptides with agglutinin activity were soluble without detergents (Hunnicutt et al., 1990), Ferris and Goodenough proposed that the SAG1 and SAD1 gene products must be associated with the membrane through interactions with unidentified integral membrane proteins.

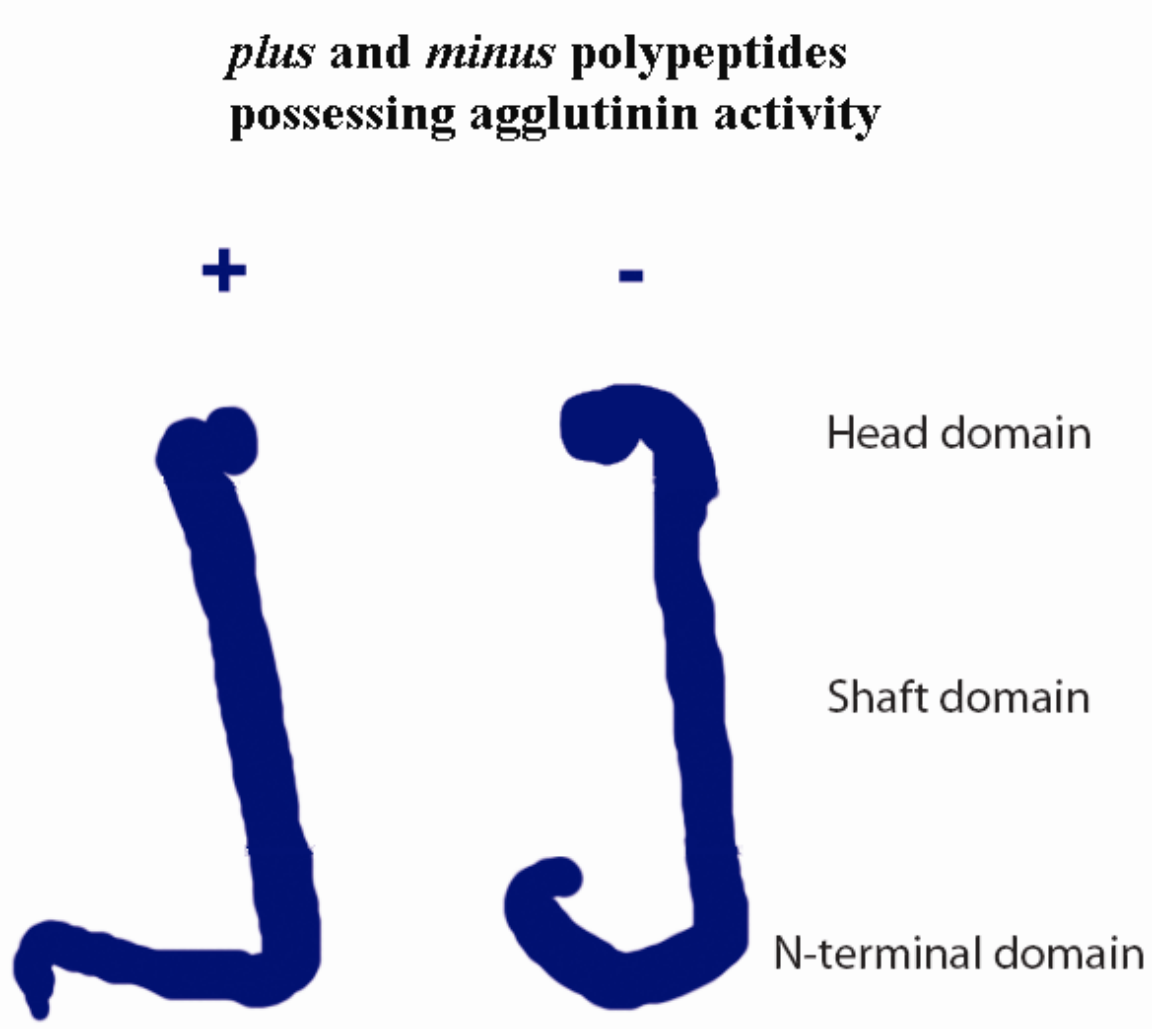
While intriguing, several features of the model are not supported by current evidence. First, the heads seen in the EM images do not appear to be significantly larger than the hook regions, even though the head is predicted to

contain approximately 4 times as many amino acids as the hook, and are supposed to account for more than half of the total amino acids in the agglutinins. Weakening the model even more, however, is my new observation that several transmembrane domain prediction algorithms strongly predict at least 6 transmembrane domains in both agglutinins. As I will outline below, my discovery of the predicted presence of transmembrane domains in the protein makes it likely that the domain of the SAG1 gene product that possesses the flagellar adhesion activity is a large N-terminal fragment, which lacks predicted transmembrane domains.

Figure 3

Domain architecture of the *plus* and *minus* agglutinins.

Diagram of agglutinins depicting the similar domain architecture of the *plus* and *minus* agglutinins (adapted from Ferris *et al.* 2005 (Ferris et al., 2005)). The diagram shows the 3 major domains of the agglutinins: head, shaft, and N-terminal hook.


plus and *minus* polypeptides
possessing agglutinin activity
+
-
Head domain
Shaft domain
N-terminal domain

Amino acid sequence of SAG1:

MSRATHSSSSACPAGRLRLSRARMVRASVARNRPLQTGSLAWLVLLTFSLAWVTIPVSDALQFPV
NVSVTSSAATDAPSAIAYIGALLDVLWNVPWQPNCINDTRRSYDAPWPSRCALPAVQYGYYDETY
DATVYSGNSLRPFYSTCRYPASSDPYAFLEPYSMPWVDDFGIYQPVLDGFDMWWGFTVNTVDSNL
KWIDPAWTAQGAWLGGQDFRDAVWNFGTHYCSWPFVECSSCENYDIADPYDPDKIANGIVPAVIT
ALDFRNASLYIYYLDFGMFPAGSLDNVWYLNLAYNFIGGPLPANLPTLLPSLQHLALDHCRATPD
VRGTASLQYGWQQYPSTGQPYEYCSDGDVTGSDGTEYVISGMIPDEWGDAVAAAASDSSLPWANL
RTVRLSNQALYGPIPEGLRSASSSISSWRLQGNTELCGPLPEFAAPINSLLYLGTKLGTHTWVWH
DPADHTQGGECLAPPPPPSPPSPRPPRPPPLPPSPPPPLLPPSPPVPPPSPPSPPSPPPSPPEPP
SPPPLPPSPPSPTPVARCIQVGGICDSPSPMPPSPRPPQPPSPPPPPPPRPPPRAPRPSPPFHPPS
PDSPPASSVPPSPEPPSPKPPSPAPPSPAPPSPPPPSPAPPSPAPPSPAPPSPQPPSPVPPQPPS
PVPPSPKPPSPAPPSPVPPSPAPPSPAPPSPAPPNPAPPSPAPPLPPSPEPPSPAPPSPEPPSPA
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PPSPAPPSPSPPAPPSPEPPSPAPLLPPSPDPPSPAPPSPMPPPLPTSPPSPEPVPPTPPPSPPA
PPSPAPPSPQPLEPPSPEPPSPAPPSPAPPSPAPPSPEPPSPEPPSPEPPSPAPPSPAPPSPAPP
SPAPPSPAPPSPAPPSPQPPSPAPPSPEPPSPAPPSPAPPSPAPPSPAPPSPAPPSPAPPSPEPP
SPAPPQPPSPVPPSPAPPSPTPPAPAPAAALPPLPPSPAPPLPVPPASPAPSPSPLRPPQPQTPA
MPPPSPAPPSPAPPSPAPPGVPPPPPTPTPPLAPLPPDCTLLAQAALLSIPDAANSVFVVSAGLP
TSVAPSTPPELLASFCTVCSCQLTATAISLVGGSSRGNNTNGSSSGGNYYSNGGDAAIQRIPAGN
STDRGGSSSGSGGSSSWGPGTETAAEWAVDAVQDGTYQLQLSIGGVTYTRTVVVDRTPPSVSGNV
TLSANRIKQEPSAVGEASLNALGSKQAMLLTISFSEPVPAFDPAASLIVTGALVAEWVAAADKMT
FYVLAMTLPAELVATAAGSSSSSGTSRSGNGNGTAAAAAAAAPPPPAGTTGRRRALQQQAAAPPP
PASGSSSSLSGAATANQQQRHVHFLLPATAYADAARNPGRNDLSLSVELTDNAVASPAVGEALAT
TARVTAATYPAVAATTTLVAAASSSFAQAIRAKGSLLQGSYHIQMLTMSLYLASRGVGREYGEYA
VEFKYAVLGVKGNLGPAEAAMPTNEKEVTAAEQARQVGGDLWPIGNDLLGGSSNTTASGSSSGSS
SSSNSPPRRPPPPPAAGSTGLLFSNADASPPPLAVATPALPAPLPSTIAAAATAAPPRLPSPPPP
AVGSSTGVLPRRHLMQQMLQPPAAAVAAPPPPPASSSALVLQPSPPPPPPPSQLLIQQASATYVS
DMQDLLYTLVVAAMLITAVAAGRLIAAVLYRLLVSPEPHPFLAFPRLETTIAGLILVALTFYSCM
ALGGPAADWHGSRTAAYCVLTIAVVPYAAFLWWLALARAWMVPQFTLVEPMTTSSYASPRPSAFE
RADRTSPRPSTVSIATAADIGGTATGGGGGAASGACADVGAMAAPATVAAGADVPSEDDRYARGP
HWKQFDGVLPPTVASGVGGSGGGVVPLPPLVALPGSGRHLPLPPLLPAAGAATAAGGGAATAPGS
PRGAAAGEDTHQFGPHWRKFSVPDDGAATAGVGVDAAAGGAGGGGGRGSRGGSTDGVRFANGTRT
PSDDGAKGRSSSHGGGNGGNAMSSGAASFGASAGGGGAAAASRLGSRSGSPGDGDYNAMSSGAAM
RGGGAGASRFGPAGSRAGSPPEGAGGYAGGANAMFAGTAGRAGAISPAHDGSSRPGSGSNEGGSR
QMSYGSNNAMTAGAVGVGGGVRRPAGSLRNASDGDAPPPLPAPGTVPLPFANLLTSGADVRSRRA
LNAEAVAAAAAGGGGGGGTSRSGSGNSNGSRAIGLASVPGTADRQSLNVLPEEAAAVASQMPGFQ
KEQLVASGGSGGGSVAGSVVGGSVGGASRRVLAASASRPGSLSGLAGGGIAAAGPVNGGAVRPRV
GGMSLGGGGAADVNDAPGSPRQPRPRVGSTPTNVPAAVAASAGAPRPPRITEVDEMGIAVVSAAE
VAATAAASSSPPSPPLSPPMAGKRGFFLPTPPPASLQAAAAAATAAANASAAAAERQAAGTVGMA
PLPSTASARAAAGAGGGASLQRSRTSLAPAASSEPVYGDGLGGAAGMLARRARVRRMVTLGDAAS
PGGDGSTAAAAAADRRGLRFGSVPVATPSADAAAAKPPQKQARSNGWLNNDAGGSAAAGPVAEAT

EEDPAAAGGGAAAGAAGAWRTRYASQLAAAGGAATDTTDSDGEGTQPWNRDSFRRRDRDAAAAAA
ARRRGKAPLPPGADPHQFSGGAAIISTAAGEDLFVLPDADADAAARRKFSSHEDIVLQTDGGGGA
AAAGGGGRRSVALVTGYRYSRGYGRDGGGGCSRLLLPSPRLMAMFEFLFEDMLGSEPEQQLTRAQ
RPARLVATALNFTHKAGCAAALGYWGLREQSWAQLGTLLGLQVVMLVYLLAVWPYAEWQLAAMEV
VCHAAELGIFVAATAVANRFVHSSAATFAMIALFFLTLAAVLLYEVRRLYLLLKEVWAVLRTKIA
SCRGSGAGSGGGGDGGGGCFGRFGGRGGGAAAAQEQGGKNAFADVHPPAAILSAPQPVLLPQLQA
AATAAGGDAAAAVASGPMGGMEAAAAAAAAQTRHPSVGNATTGAPASATAAPAASATAAPAASAT
SATAAAVAATATHAQAATVGGRASTSNV

Amino acid sequence of SAD1:

MMLRKSTGKAGRHDLRRVAMALAFATLFFLPDLASTQTTYGPWNATEDLNEQHKGLLAFILSGDT
SFWSRPEVATRLGFGTAPWRCISNCQTQIFTSDQQCAPDCESRTYCEPGGAALGSENTTCCALSL
LDQTYASAQPPSSTQPAWCSTYPGWGARPRPSVCDFNVFAARGTTPPAGADDPDLAVSCSSGTVP
TYSISGTRYRQNDTVYRIMHNGAVTNPANVTRNQVKSIKLRHSAMWHHPSVNITSPPEFSLVSEL
ACLPLEEIFLEDVALRADYSVLMAQPNTTLDNGKVFNLYDPSTYPFNVTGLYNNVFSMLIEPSFM
LHSSVSVKASSFNPGLALRNAALRTCNFAPFTELHAMRWTGQAQLDWSAEYAQALSYVRDGSQLR
PRGWLPPVIEPVSARLLLPDTLRRLTIRRTRDEQHGAVQWTSRPLITGPLPGEWALLRNLEYLDL
SDEMETGAIVGPIPSTWLMMSHLRVINMTGHHNFCRDWHKIISWQIRMYYRAATHEPNLNVPHYY
GPWGGNNGNMTRYNISVYDLSGHGWQWYDEVTTEAGFVEVIAPHGQCCWDKWSQTIKDNNYEILY
PDGSRFGNNVVQDEIYGGFYQDEEWCEPTSPPQPPPPPAPPSPPSPPTTPDVPPMPPSSPPAPVM
PPAPPPQPPIPPASPLTPAAPPRPPLPPTWPGKWEGAWPFRPPIPPRPPRPPPPPLPPSPPLPPV
TPSPPPRPPPPKSPPPPKPSPPPRPSPPPRPPPRPLPPSPPPPPPLPPNPPSPAPPSPPPPPSPS
PPPSPGPPSPEPPSPAPPSAAPPSPMPPSPAPPSPDPPSPKPPSPVPPSPLPPSPEPPSPVPPSP
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EPPSPAPPSPPPPSPEPPSPAPSPPPPSPEPPSPAPPSPPPPSPEPPSPAPPLPPPPSPEPPSPA
PPSPPPPSPEPPSPAPLPPPPSPEPPSPAPPSPPPPSPEPPSPAPPSPPPPSPEPPSPAPPSPPP
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SGGLDAAWRLTPGATGDYLLRLKVADQEWWRWVSVDIDPPRAAGQLLLARRTGGSSSSSNSTSGS
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RQPSTPPPAPPPMPALSLSGDRDVLVSWLQQVGAIGSGSNSSGGGSATSDSASVGAYSLGGAAAS
PRGDVVVLPDGQLGLLGPGGSAPPPMQPGSGSGGQQSSGASVATDTTIHTNVQDLLYTLAIAALL
MVALVAAHLLVIGLWRLAVMYDVCGAAESGVEGLHPVLRFPRAEMVLGGLLLVALTFYSALTLSG
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GGAGAGGDGGGHGASALPAVAGFGGTGGGGGVAFAACQAEPGSGDGGSGGGEDGYGLGPHWALAP

AGAKTLDYESIASPAAGAGSAAGATRTDAAGSTVGGAQSGKGRPRTLRSTDVTAGGAAAGTAAAA
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APATAARKMAGSDDGAAGERRRANPHARKGSADGADGGAGDPQAAGRPLLRRGVSYGDNLLTAGA
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RGAAMGSNAYTAGASRFGGPRTRADSPPRAASPPPEPASPLSGDAGAISEVGFNAMVSGGSRPPR
SDSPTMEDEAAAIRIQQDAADPAVEANRSRRKQAGSVEEQQVAINPMAVPRASVLERPDVAASLA
AAVAASGAPPQGTVPLPPITEATGPNGLGSSPRDGRVMTTASVSHGRDTSRLLHPMARPRSLGAS
LVPASATTNGGSGNSSGNGRESGSGSTSGGAPAAMPRPRVGGAPSNASAAAAAAAAAAAAQPAAH
QDLFGELDTTVLTSKMTADAGAASAPPNLPSPMMRLGSRLGLVRGGSNSGAAQVAPASPLPAKAP
SPPAAAARPRPPGAVTTNAAVPMPPPQKVFRSPSEPANARAQGAAPPAAAAVANGGGRELKQQPA
VQAVSSTTSSIRSNSSGSGNPLARALRYMSVPKPGAQPSTASNAAPAAAEAEPAAPPSRHTAWGG
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PVWPARRRNNRAAIMDEDDLVAEGGGAAGPVAEGAEAPDSAGHYFGGWEDEDPRLLLREAPRSPV
MPPPPPLRAGGAARGSSTGGMSAAAVSVHSYSPSSSARGGGSTAAGHYMTYNPLSLPSAVSAAAE
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AMLVYLAAVRPFREALLQAVELTCHALEAGLFACALALVNAQPNNGAATTYVMVACFFGVALLVI
VYELRRLALLVMALWRMWRERGTQARRASHAIIDEMEGGGVSAAAAAAAAASRTPSALAPPVSIT
ARRSSALKEGAVRTGVASSLSDGGGSTPTSPLGAQPQPSAAAPTRRSRRSSAAGGSSSSDGGTAG
RQAAAAIAELAQGQDGPGGDAVGEAATAQPETSTAAGEIAMRGATATGAGAAR

Flagellum-generated signaling molecules change their properties during gamete adhesion

Proteins participating in sexual signaling in *Chlamydomonas* can undergo significant biochemical changes during signal transduction events. A good example of this is was demonstrated in our lab a few years ago when it was shown that not only intraflagellar transport was required for the signal transduction (Pan and Snell, 2002), but also that a cyclic GMP dependent protein kinase (PKG) was involved in the signaling cascade and at least two IFT particle proteins (IFT 81 and IFT 139) changed their properties and ran differently during

differential centrifugation experiments (Wang et al., 2006). Wang *et al.* also showed that flagellar PKG was being phosphorylated during adhesion-induced signaling. Shortly after these discoveries, the group of Joel Rosenbaum made interesting discoveries about another signaling protein localizing to cilia and involved with a serious kidney disease when mutated: PKD2. They identified a *Chlamydomonas* homolog and showed that this membrane protein undergoes cleavage events and that apparently only some cleaved forms are able to localize at the flagellum (Huang et al., 2007). It is not known if the SAG1 gene product undergoes cleavage events or changes its properties during signaling as assessed by differential centrifugations.

To summarize

Some big unanswered questions remain about ciliary biology, among them, how is the composition of the ciliary membrane regulated during signaling? How do ciliary membrane proteins alter their properties and activity during signaling? What is their role in the signaling cascade? My work on *Chlamydomonas* and future work inspired by my results will certainly help answer some of those questions in both green algae and animals.

Chapter 2

THE PROTEIN COMPOSITION OF THE *CHLAMYDOMONAS* FLAGELLAR MEMBRANE IS DYNAMICALLY REGULATED BY CILIUM-GENERATED SIGNALING DURING FERTILIZATION.

INTRODUCTION

Key signaling pathways in eukaryotes depend on the cellular compartment created by the primary cilium. The relatively small size of the this organelle belies its importance, and in multicellular organisms, the primary cilium is a sensory and signaling center essential for establishment of left-right asymmetry, kidney development, Notch-regulated morphogenesis of the epidermis, and Sonic Hedgehog patterning of embryonic organs and tissues. Attesting to the ancient function of the cilium as a sensory organelle, its cognate organelle, the flagellum, is essential for gamete recognition and signaling during fertilization in the biflagellated green alga *Chlamydomonas*. A major goal of current work on the biology of cilia and flagella is to discern the unique properties of the organelles that have led to their widespread exploitation as signaling nodes.

We are just beginning to learn about the cellular and molecular mechanisms that regulate the localization of membrane proteins in this slender 3 – 6 um long structure whose membrane likely represents less than 0.5% of total cellular plasma membrane. In vertebrate cells, immunolocalization studies have shown that several membrane proteins are constitutively localized in the ciliary membrane, including polycystins 1 and 2 (Pazour et al., 2002; Yoder et al., 2002),

adenylyl cyclase type 3 (Wong et al., 2000), the Notch receptor (Ezratty et al., 2011), platelet-derived growth factor receptor αα (Schneider et al., 2005); the angiopoietin receptor tyrosine kinases Tie-1 and Tie-2 (Teilmann and Christensen, 2005); dopamine receptor 1 (Domire et al., 2011; Domire and Mykytyn, 2009), melanin-concentrating hormone receptor 1 (Berbari et al., 2008b); serotonin receptor 6 (Berbari et al., 2008a), somatostatin receptor type 3 (Berbari et al., 2008a), and fibrocystin (Wang et al., 2004). Several studies have identified ciliary localization motifs in ciliary membrane proteins (Berbari et al., 2008a; Corbit et al., 2005; Geng et al., 2006). And, recent studies have shown that septin is essential to establish a diffusion barrier that both retains proteins within the ciliary membrane and prevents entry of proteins into the organelle from the adjacent apical plasma membrane of the cell (Hu et al., 2010).

Not all proteins that function in the cilium are constitutively localized in the organelle. In the well-studied Hedgehog cilium-based signaling pathway in vertebrates, the the Hh ligand regulates the location of two pathway proteins that can function only in the cilium. In the absence of the Hh ligand, the Hh receptor Ptch1 is present in the ciliary membrane, where it suppresses accumulation of the effector membrane protein, Smoothened (Smo). Hh binding to Ptch1 somehow relieves suppression by Ptch1, and Smo accumulates in the organelle followed by pathway activation and depletion of Ptch1 (Rohatgi et al., 2007; Wong and Reiter,

2008). New evidence also suggests that dopamine receptor 1 in neuronal cilia is dynamically regulated (Domire et al., 2011).

Although vertebrate cells are powerful systems for studying ciliary signaling, biochemical analysis of signaling pathways within the organelle is challenging because it is not yet possible to isolate primary cilia. Indeed, the evidence that membrane proteins are localized in the primary cilium comes essentially exclusively from imaging studies. Thus, because of the difficulties inherent in using immunolocalization methods to quantify protein amounts, we have little knowledge of the relative distributions of membrane proteins in the cell body versus the cilium. Furthermore, without being able to isolate the organelles, detecting signaling-induced post-translational modifications or protein-protein interactions that occur only within the context of the membrane of the primary cilium will be challenging if the amount of the protein in the cilium represents only a small portion of total cellular protein.

Cilium-generated signaling in *Chlamydomonas* is highly amenable to biochemical analysis because of the ease of isolating flagella from cells before and during signaling. Furthermore, previous work indicated that flagellar adhesion-induced signaling between gametes of opposite mating type in the sexual phase of the life cycle regulated the activity of a membrane protein and

might regulate the protein composition of the flagellar membrane. During gametogenesis, newly formed *plus* gametes and *minus* gametes (which are cultured separately) acquire the ability to bind to each other by their flagella. When the gametes are mixed together, the ensuing flagellar adhesion engages the intraflagellar transport (IFT) machinery and activates a protein kinase-dependent signaling pathway within the flagella that rapidly leads to 10-20 fold increases in intracellular cAMP levels, thereby triggering gamete activation in preparation for cell-cell fusion (Pasquale and Goodenough, 1987; Pijst et al., 1984; Wang and Snell, 2003). One of the major events that occurs during gamete activation is an increase in the adhesiveness of the flagella.

By use of bioassays that detect flagellar adhesion activity (i. e., cell agglutination) several groups identified polypeptides (agglutinins) from both type of gametes that possessed flagellar adhesion activity. The polypeptides are heavily glycosylated and migrate on SDS-PAGE gels with an apparent molecular mass of >500 kD. Partial deglycosylation yields forms of ~ 480 kDa. Cell biological and biochemical studies carried out several years ago, primarily on the *plus* agglutinin activity, demonstrated that a large pool was associated with the cell body. Because cell bodies never adhere to the flagella or cell bodies of gametes of the opposite mating type (Goodenough, 1991; Hunnicutt et al., 1990; Snell and Moore, 1980; Snell and Roseman, 1979) *in vivo*, the presence of

agglutinin activity in cell bodies was unexpected, and was detected only when the protein fractions were denatured by absorption to a glass surface (Goodenough, 1989; Hunnicutt et al., 1990; Musgrave et al., 1986; Pijst et al., 1983; Saito et al., 1985; Snell and Moore, 1980; Snell and Roseman, 1979). Ultrastructural studies, which showed the purified agglutinin polypeptides to be large fibrous proteins similar to those seen on flagella, failed to detect them on the cell body surface. On the other hand, vectorial labeling methods and protease sensitivity experiments demonstrated that the polypeptide containing the agglutinin activity was surface localized. Another unexpected property of these agglutinin polypeptides, which function *in vivo* on the flagellar membrane, was that they are soluble in aqueous buffers (Adair et al., 1982; Hunnicutt et al., 1990).

More recently, genes that encode the *plus* agglutinin (*SAG1)* and the *minus* agglutinin (*SAD1*) were cloned. Although the predicted mRNA for *Sag1* encodes a protein of 330 kDa and that for *SAD1* encodes a protein of 385 kD (Ferris et al., 2005), the absence of antibodies for immunoblotting or immunolocalization has impaired full characterization of the *SAG1* and *SAD1* gene products. Thus, we do not know whether the agglutinin polypeptides studied to date, which were purified based on their flagellar adhesion activity, represent the entire SAG1 and SAD1 gene products, or if they are cleavage products. Several large ciliary multispan transmembrane proteins, including polycystin 1

(Qian et al., 2002; Woodward et al., 2010) and 2 (Huang et al., 2007) and fibrocystin (Kaimori et al., 2007) are cleaved to yield large N-terminal extracellular fragments and transmembrane domain-containing, C-terminal fragments.

The early studies led to the model that *Chlamydomonas* gametes possess a diffusion barrier functionally similar to the one demonstrated more recently in vertebrate cells that blocks protein exchange between the cell body membrane and the ciliary membrane. We showed that experimental depletion of the *plus* agglutinin activity from flagella of resting gametes using two different methods failed to deplete agglutinin activity from the plasma membrane of the cell body.

In related studies, we found that when *plus* gametes whose flagellar agglutinin activity had been depleted were experimentally induced to undergo gamete activation, the flagella re-acquired agglutinin activity(Hunnicutt et al., 1990; Pan and Snell, 2002). These results were consistent with other studies using other bio-assays for agglutinin activity that showed that the flagellar adhesiveness of gametes increased several-fold during gamete activation (Goodenough, 1989). Thus, based on measurements of agglutinin activity, these earlier studies provided compelling evidence that the cell body and flagellar agglutinins did not exchange

in resting (i. e., non-activated) gametes, and they also showed that agglutinin activity on flagella underwent signaling-induced increases. Although the preferred explanation for these observations was that the cell body polypeptide with agglutinin activity translocated onto the flagella during signalings, an explanation supported by ultrastructural evidence for a modest, signaling-induced increase in flagellar agglutinins (Goodenough, 1989), these *in vitro* bioassays were inconclusive on whether the increase in agglutinin activity was due to an enhancement in the activity of pre-existing polypeptides already on the flagella, or to polypeptides that had translocated from the cell body.

Here, I report rescue of flagellar adhesion in a *SAG1* adhesion-defective mutant using a *SAG1* gene encoding a C-terminal epitope tag. I show that the tag is present on the surface of the plasma membrane of the cell body, with small amounts on flagella. I further report that soon after its synthesis SAG1 is rapidly cleaved to yield a C-terminal 65 kDa fragment with the properties of an integral membrane protein. Finally, for the first time in any system, I use cell fractionation and biochemical methods to demonstrate that a membrane protein indeed undergoes rapid (5 minutes), signaling-induced translocation from the cell body to the flagellar membrane, where it forms large, detergent-resistant complexes.

MATERIALS AND METHODS

Cells, cell culture, and reagents

Chlamydomonas reinhardtii strains *21gr* (mt+; CC-1690), *6145C* (mt-; CC-1961), *sag1-5,* formally known as *imp9* (mt+; CC-1146), and the *hap2* mutant (mt-; Liu *et al.* 2008) were used in this study. Cells were maintained on M medium agar plates at 12°C for long term storage. To perform my experiments, cells were cultured in liquid culture in M media containing flasks at 23°C in a 12:12 h light:dark cycle. To trigger gametogenesis, vegetatively growing cells in liquid culture were centrifuged, resuspended in nitrogen-depleted medium (M-N medium), and aerated by bubbling under continuous illumination for 18-22 hours. M medium is the medium I described by Sager and Granick (Sager and Granick, 1954), M-N medium is the same as M medium minus the ammonium nitrate. Chemicals used are reagent grade from major providers unless otherwise noted.

Generating an HA-tagged version of SAG1

The SAG1 gene was digested out of a Bacterial Artificial Chromosome (BAC 2F3) by using the EcoRI and HindIII restriction sites and then ligated into

pBluescript. This genomic fragment most likely includes the endogenous promoter of SAG1 (nearly 600 base pairs upstream of start codon and downstream of HindIII site). This newly generated plasmid was then digested by BglII and NheI to extract the C-terminal region of SAG1 and clone it into the pGEM T easy vector (Promega). This smaller plasmid allowed us to generate new unique restriction sites and add a 3X HA tag before the stop codon, followed by a terminator (HA tag + terminator from Liu *et al*. 2008). This modified DNA was then re-introduced into pBluescript by ligation to generate the final pBSAG1-HA plasmid used for electroporating into the *sag1-5* flagellar adhesion mutant.

Rescue of *sag1-5* mutant strain

Sag1-5 cells were obtained from the *Chlamydomonas* Center and maintained on M medium agar plates. To prepare for a transformation, a cluster of cells was transferred from a plate into a flask containing 100 mL of sterile TAP medium. This flask was placed under continuous light on a rotating shaker until the culture reached a dark green color (3-4 days). These cells were then transferred into a 2.8 L flask containing 1 L of TAP medium (medium II of Sager and Granick (Sager and Granick, 1954)) and agitated on the rotating shaker in continuous light for 24 hours. Cells ($2 \times 10^{8)}$ in mid-log phase (($\sim 3 \times 10^{6}$/mL) were harvested by centrifugation (5 min at 3000 RPM, in an Accuspin 3R

centrifuge (Fisher Scientific)) in a sterile conical tube, resuspended in 25 mL TAPS medium (TAP medium + 60 mM Sorbitol), washed once in the same medium by centrifugation, and resuspended in 1 mL TAPS. 200 ng of aphVIII plasmid pSI103 (a paromomycin resistance gene (Sizova et al., 2001)) and 1 μg of pBSAG1-HA linearized (by EcoRI) were added to 0.3 mL of the cells in TAPS in 0.4 cm electroporation cuvettes (USA Scientific). The samples were chilled on ice for 5 min and electroporated using a Biorad gene pulser Xcell using the following settings: capacitance, 25 mF; pulse control, ∝; shunt resistor, none; voltage, 2000V/cm (800 V for the 0.4 cm cuvettes); and pulse duration. ~ 7-10 milliseconds. After electroporation, cuvettes were placed on ice for 15 minutes, the samples were transferred to 15 mL conical tubes containing 10 mL of TAPS, and agitated by rocking overnight under low light. The next day, cells were harvested by centrifugation (3000 rpm/ 5 min), re-suspended with the residual supernatant/media by gently tapping, and ~ 300 μL was plated on TAP + paromomycin (7.5μg/mL) agar plates. The plates were kept under low light overnight, and maintained on a L:D cycle until colonies of 1-3 mm in diameter were visible (~10 days).

To assay for rescue of flagellar adhesion, colonies were transferred into wells of a 96-well plate (Corning Inc.) containing 100 μL of M-N medium to trigger gametogenesis. A replica of the plate was prepared by transferring 10 μL

from each well into a new 96-well plate containing 100 μL of M media. Following 15-20 hours of shaking under constant light, cells from the wells in the M-N plates were mixed with a similar amount of wild type mt *minus* gametes and examined for flagellar adhesion on an inverted microscope.

Characterization of SAG1-HA/sag1-5 clone

The presence of the pBSAG1-HA plasmid in a SAG1-HA/sag1-5 clone with restored flagellar adhesion was confirmed by PCR of genomic DNA purified by a previously described method (Harris, 1988) using as forward primer : AATTCACTAGTGATTCCGCTACGCTTCTGCGTCTT, which was present in the HA sequence; and as reverse primer: AATTCACTAGTGATTCCGCTACGCTTCTGCGTCTT, which was present in the terminator. I determined by microscopy that the rescued SAG1-HA gametes were able to undergo zygote formation with wild type gametes of the opposite mating type (*6145C* strain). Zygote formation was assessed by visual observation of quadriflagellated cells under the microscope a few minutes after mixing gametes together. The formula to show percentages of zygote formation was: (2 x (number of zygotes)) / (((2 x (number of zygotes)) + number of biflagellaged gametes).

In some experiments, I used *plus* progeny of a backcross between SAG1-HA/*sag1-5* (mt+) and *sag1-5* (mt -). The two generations had identical experimental behavior so there is no indication of distinction between them in this dissertation.

Gametogenesis

Large scale: Cells grown in 6L liquid culture in R medium (a variation of medium I of Sager and Granick (Sager and Granick, 1954) with 3 times the amount of phosphate buffers and supplemented with 3 g/L of sodium acetate trihydrate) were harvested by centrifugation (2000 RPM for 5 minutes in a Sorvall RC-30 centrifuge (DuPont Instruments) in 1 L plastic bottles), resuspended in M-N medium, washed twice in the same medium by centrifugation, and placed in continuous illumination overnight with aeration. Small scale: The procedure was essentially the same except that cells were cultured in M medium in 250 mL flasks and harvested by use of an Accuspin 3R (Fisher Scientific) centrifuge.

Flagella and Cell body isolation

Flagella were isolated as previously described (Wang et al., 2006; Witman et al., 1972) Briefly, cells were harvested by centrifugation and

resuspended at a concentration of~ 2 x 10^8 cells/ mL) into a ice-cold solution of 20 mM HEPES containing 5% sucrose (pH 7.2). All subsequent steps were conducted at 4°C. The cells were transferred into a beaker containing a magnetic stirrer and while being stirred vigorously on a magnetic stirrer, the sample was brought to pH 4.5 by dropwise addition of 0.5 M acetic acid over 45 to 60 seconds, followed by addition of 0.5 M sodium hydroxide to return the pH to the original value of 7.2.

Portions (25- 30 mL) were then transferred into polycarbonate conical tube (Nalgene) and they were underlayed by and an ice-cold solution of 20 mM HEPES containing 25% sucrose (pH 7.2). Cell bodies were sedimented to the bottom of the tube by centrifugation in a swinging bucket rotor at 2000 RPM for 10 minutes (rotor number, H6000A, Sorvall RC-30 (DuPont Instruments).

If necessary to remove remaining cell bodies, the flagella, which were at and above the 5%/25% sucrose interface were transferred to a new tube, underlayed once more with 25% sucrose/ 20 mM HEPES and centrifuged again. Flagella were harvested by centrifugation in round-bottomed polycarbonate tubes (20 minutes, 12,000 RPM, SA-600 rotor (Sorvall). The sedimented flagella (which were white in appearance, thereby attesting to the successful separation from the chlorophyll-fllled cell bodies) were resuspended to 1-4 mg/mL in

HMDEK Buffer (20 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 25 mM KCl; pH 7.2) supplemented with an EDTA-free protease inhibitor cocktail (Roche).

Cell bodies in the 25% sucrose/Hepes fraction were resuspended to a concentration of ~ 6-10 mg/mL in HMDEK buffer supplemented containing the EDTA-free protease inhibitor cocktail. Flagellar and cell body samples were flash frozen and stored in cryotubes in liquid nitrogen.

Immunoblotting

SDS-PAGE and immunoblotting were carried out as previously described (Wang et al., 2006). Briefly, samples were mixed with 4x SDS-Page sample buffer, boiled for 5 min, and proteins were analyzed 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 membranes were blocked by 30 min incubation in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20; pH 7.6) containing 5% dry milk, incubated overnight with agitation at 4°C with a 1/10,000 dilution of Rat anti HA antibody (Roche item number:11 867 423 001) in 3% milk in TBST. After 3 washes with TBST (7 min each), the membranes were incubated for 30 minutes

with rabbit anti rat HRP-conjugated secondary antibody (Calbiochem # 401416; dilution 1/5000) at room temperature. The rest of the procedure was performed as described previously (Misamore et al., 2003). The PVDF membranes were then washed 3 times for 7 min with TBST, incubated in enhanced chemiluminescence immunoblotting reagents (Thermo Scientific #34095) for 1 min as described by the manufacturer, exposed to autoradiography films (GeneMate #F-9029 – 8X10), and the films were developed in an automatic film processor.

Evaluating the solubility of SAG1-HA-C65

Frozen samples were thawed, diluted to ~ 0.6 mg/mL in HMDEK containing one of the following constituents: 1% NP40, 500mM NaCl, or 100 mM Na_2CO_3. Mechanical disruption was achieved by subjecting samples to 4 rounds of rapid freezing in liquid N2 followed by thawing in a room temperature water bath. The samples in 1% NP40 samples were centrifuged at 50,000 RPM in a TLA 100.3 rotor (135300 *g*) and the other samples were subjected to centrifugation at 4°C for 30 minutes at 13,000 RPM. The supernatants and sedimented material were analyzed by SDS-PAGE and immunoblotting.

Trypsin treatment of live gametes

Gametes in M-N medium were incubated in presence of 0,01% trypsin (added from a 5% stock solution also containing 1 mM HCl) for 5-8 min. In control experiments, bovine serum albumin replaced trypsin. Cells were then washed 4 times with 0,05% trypsin inhibitor (type III-O: chicken egg white, SIGMA) in M-N medium.

Gamete activation by di-butyryl cyclic AMP

Di-butyryl cyclic AMP (from a freshly prepared stock in M-N medium) and papaverine (from a 15 mM stock solution in DMSO) were added to gametes to final concentrations of 15 mM and 0.15 mM, respectively and aerated under constant illumination for the times indicated in the figure legends.

Gamete activation by mixing with *hap2* mutant gametes

Equal numbers of freshly washed *plus* and *minus* gametes in M-N were mixed together and aerated under constant illumination for the times indicated in the figure legends. Flagellar adhesion was assessed by phase contrast microscopy.

Evaluation of gamete activation by use of a cell wall loss assay

Cell wall loss was determined essentially as previously described(Snell, 1982). Briefly, 2 x 10^6 cells were added to 1 mL of lysis buffer containing 0,075 % triton X-100 and 5 mM EDTA, pH ,8.0, vortexed briefly, and subjected to centrifugation at 6000 RPM for 30 seconds (MiniSpin plus centrifuge (Eppendorf)). Cells with walls sedimented, yielding a clear supernatant and a green pellet, whereas cells that had lost their walls, were disrupted by the detergent, yielding a green supernatant and a white pellet.

Velocity sedimentation

Flagellar samples (protein concentration: 1-2 mg/mL) were thawed and supplemented with a 10% NP40, HMDEK solution to yield a final detergent concentration of 2%. After 30 min on ice with occasional vortexing, the samples were subjected to centrifugation at 13,000 RPM for 30 minutes at 4 °C. The supernatants containing released SAG1-HA-C65 were overlayed on 5-40% sucrose gradients prepared in 2.2 mL ultracentrifuge tubes (Beckman Coulter # 347357) and containing 0.5% NP40. Ultracentrifugation was carried out at 40,000 RPM (137,000 *g*) for 4 hours at 4°C in a TLS55 swinging bucket rotor (Beckman Coulter). 256 µL fractions were removed from the top and analyzed by SDS-PAGE and immunoblotting.

RESULTS

An epitope-tagged *SAG1* transgene rescues flagellar adhesion in a *SAG1* flagellar adhesion mutant.

I rescued flagellar adhesion in the flagellar adhesion mutant, *sag1-5* (originally generated by UV mutagenesis), by use of a *SAG1* transgene encoding a C-terminal HA tag. PCR analysis confirmed integration of the *SAG1-HA* plasmid into the genome of the rescued clone SAG1HA/*sag1-5* (Fig. 4 A). Flagellar adhesion was restored in the SAG1HA/*sag1-5* gametes, as evidenced by formation of clusters of adhering (agglutinating) cells in mixtures of *minus* gametes with SAG1HA/*sag1-5 plus* gametes (Fig. 4 B, bottom panel). Fig. 4 B, top panel, shows wild type *plus* and *minus* gametes adhering, and the center panel demonstrates the absence of flagellar adhesion in mixtures between wild type *minus* gametes and the parental *sag1-5 plus* gametes. Fig. 4 C documents that the agglutinating cells were adhering to each other by their flagella. In some experiments, the flagellar adhesiveness of the SAG1HA/*sag1-5* gametes was not as intense as wild type gametes, but increased after they were mixed with *minus* gametes, suggesting that adhesion-induced signaling led to an increase in SAG1 activity on the flagella. As expected, the SAG1HA/*sag1-5* gametes, unlike the

parental *sag1-5* gametes were capable of fusing with *minus* gametes to form quadriflagellated zygotes (percent of cells forming zygotes in a typical experiment is indicated next to each panel (Fig. 4B)).

Immunofluorescence shows that HA-tag of SAG1-HA is predominately on the cell body, and during signaling, it moves onto the flagella.

Immunofluorescence with an HA antibody demonstrated that whereas vegetatively growing SAG1HA/*sag1-5* cells lacked anti-HA-staining, the SAG1HA/*sag1-5* gametes stained strongly (Fig 5). Consistent with earlier work showing that most of total cellular agglutinin activity was associated with the cell bodies, HA staining also was most prominent on cell bodies, with small and variable amounts of reactivity on flagella, sometimes barely detectable (Fig 5 B). The signal on the cell bodies was most prominent at the cell periphery, suggesting that it was membrane associated. These results, indicating that at least the C-terminal portion of SAG1-HA was present on the cell bodies, allowed us to test whether the location of the polypeptide was regulated by db-cAMP or flagellar adhesion-induced signaling. As seen in figure 5 C, after 90 minutes of activation with db-cAMP, the flagella were massively stained for HA.

SAG1 is expressed exclusively in gametes and is cleaved into smaller fragments soon after its synthesis, generating a 65 kDa C-terminal, membrane associated fragment

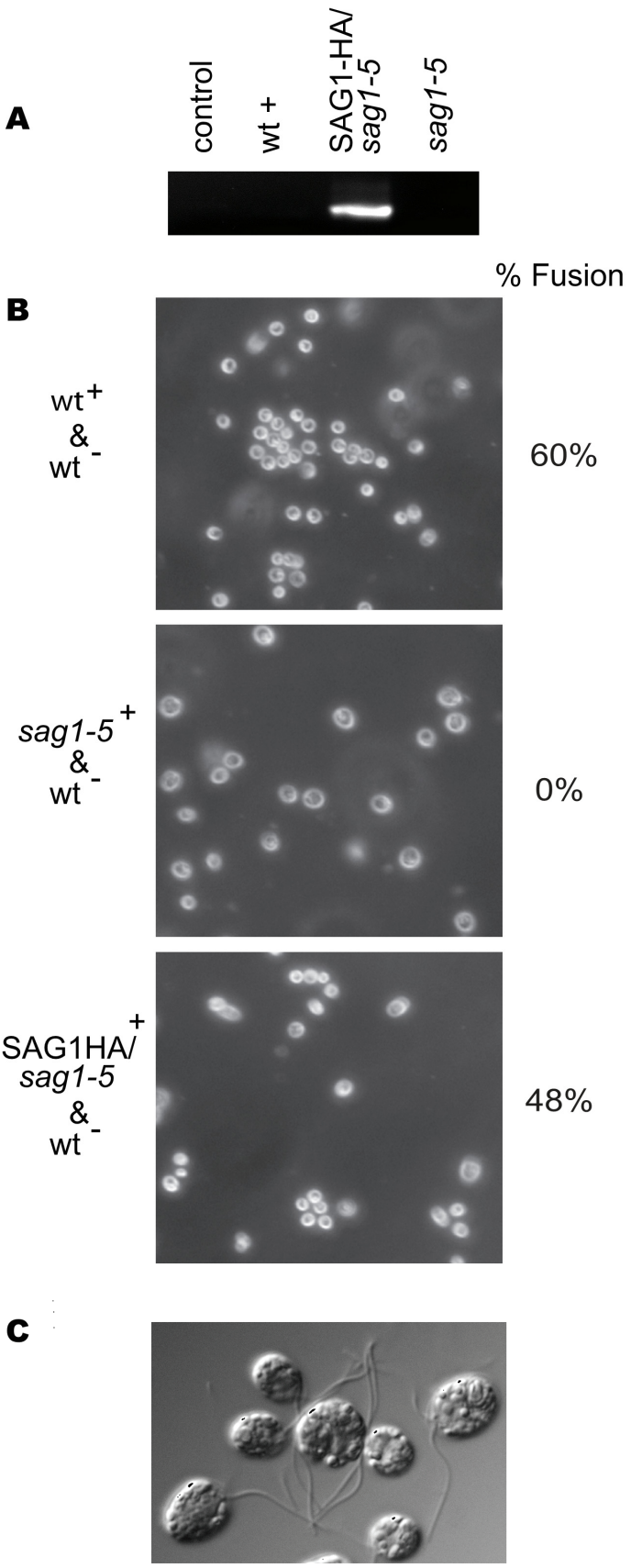
Because the agglutinin activity encoded by the SAG1 gene is associated with the flagellar membrane *in vivo*, I used web-based transmembrane domain prediction programs (TMHMM and PHOBIUS) to search for possible transmembrane domains in SAG1. I found that the full length SAG1 protein is predicted to contain 6-7 transmembrane domains in its C-terminal half (Fig 6 B). This likely presence of transmembrane domains in the predicted SAG1 sequence is at odds with earlier work that showed that the flagellar agglutinin activity was soluble in aqueous buffers (no detergent) and with models of SAG1 protein structure based on ultrastructural analysis (Ferris et al., 2005). One explanation for these apparently conflicting observations is that SAG1 is cleaved soon after its synthesis to generate an N-terminal, aqueous-soluble fragment that contains the flagellar adhesion/agglutinin domain. Based on this new view, I anticipated that biochemical analysis of cells expressing C-terminally tagged SAG1 would yield a C-terminal domain with the properties of a transmembrane protein. Confirming this prediction, HA immunoblotting analysis showed that although higher molecular mass forms of SAG1-HA were detected in immunoblots of SAG1-HA/*sag1-5* gametes, the predominate form was a ~65 kDa polypeptide that I

named SAG1-HA-C65 (Fig 6 A). The higher molecular mass, HA-tagged forms of the protein were detected in overexposed immunoblots, or in gametes actively synthesizing SAG1 (Fig 6 A). These higher molecular mass forms were observed only in cell bodies and were absent from flagellar samples.

Figure 4

Plasmid pBSAG1-HA encoding HA-tagged SAG1 gene rescued flagellar adhesion in a *sag1-5* adhesion-defective mutant.

A: pBSAG1-HA was integrated into the SAG1-HA/*sag1-5* cells. Diagnostic PCR demonstrated that the plasmid was present in the rescued clone but not in the parental mutant (*sag1-5*). B: pBSAG1-HA rescued adhesion in the *sag1-5* mutant. Photos comparing wild type adhesion (top), lack of adhesion of the *sag1-5* gametes (middle), and rescued adhesion in SAG1-HA/*sag1-5* gametes (bottom). % zygote formation are shown on the right side of each photo. C: Flagellar adhesion is rescued by pBSAG1-HA. Micrograph depicting flagellar adhesion between SAG1-HA/*sag1-5* gametes and wild type *minus* gametes.


A
control
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sag1-5+
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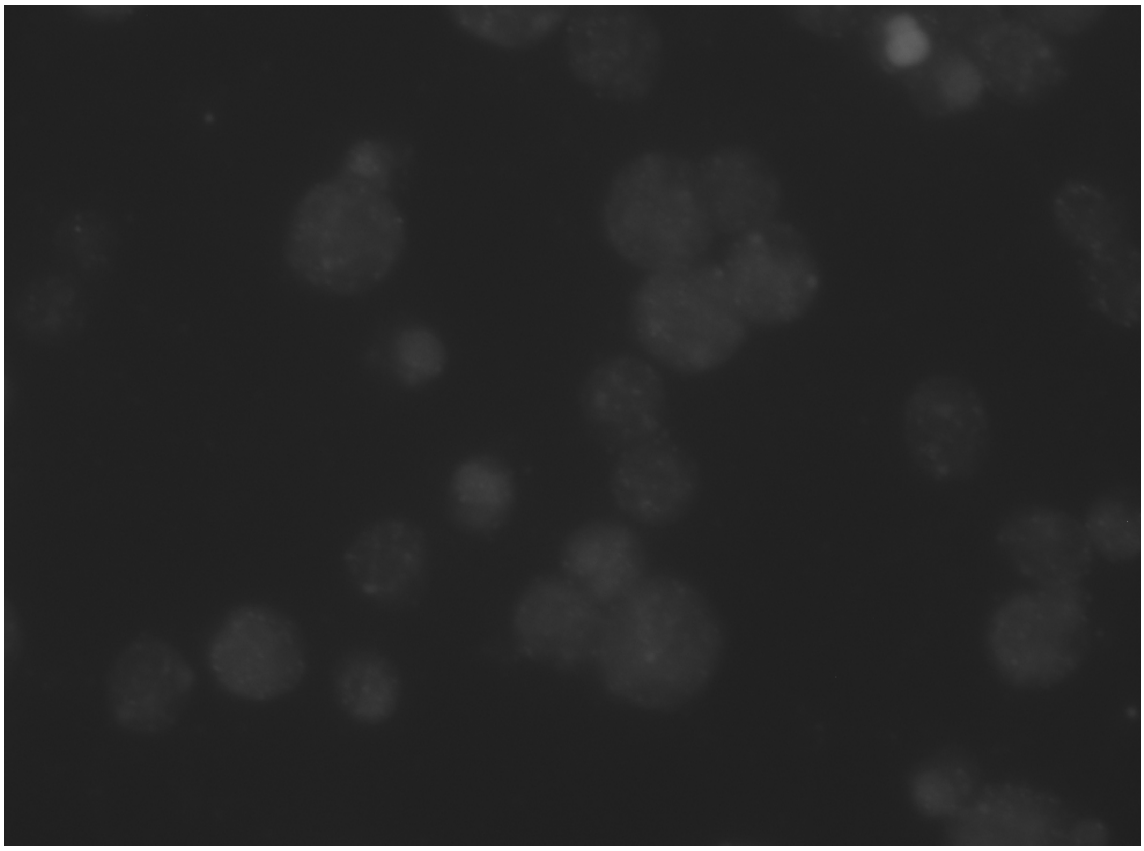
wt-
48%
C

Figure 5

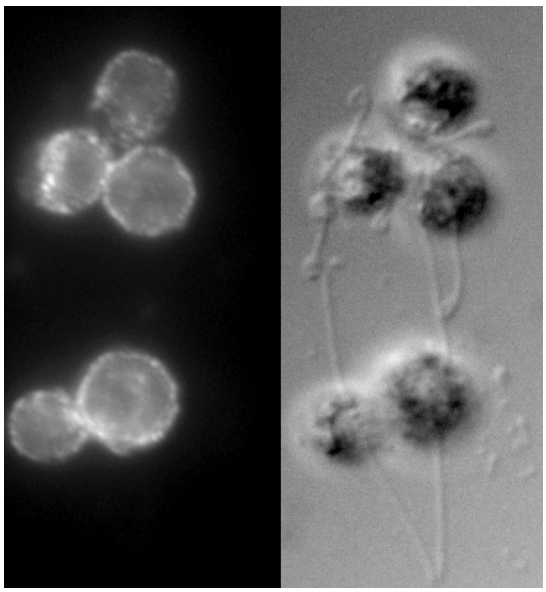
Immunofluorescence images depicting localization of resting and activated SAG1-HA in SAG1-HA/*sag1-5* gametes and absence from *sag1-5* gametic cells.

A: Absence of HA staining in *sag1-5* mutant gametic cells. HA staining in *sag1-5* gametes. B: Images show that resting SAG1-HA/*sag1-5* gametes express the HA-tagged construct mostly in the cell body (Left panel: anti-HA immunofluorescence; right panel: DIC images of the same cells). C: Images show that SAG1-HA/*sag1-5* gametes activated by db-cAMP for 90 min show strong HA staining in their flagella (Left panel: anti-HA immunofluorescence; right panel: DIC images of the same cells). (Images provided by Carmen I. Hernandez-Lara).

A



B



C

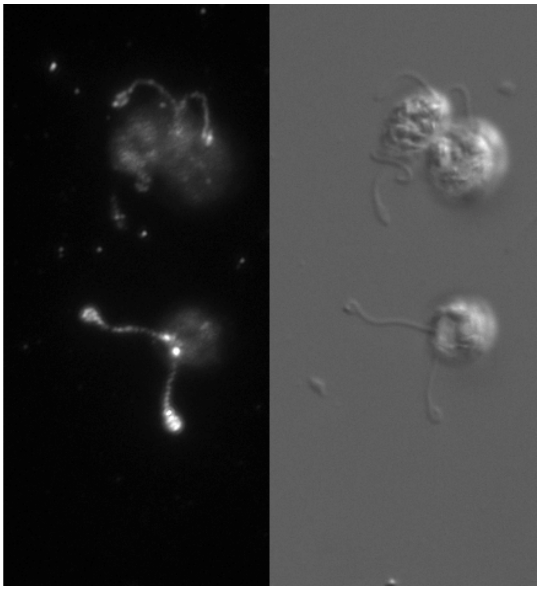
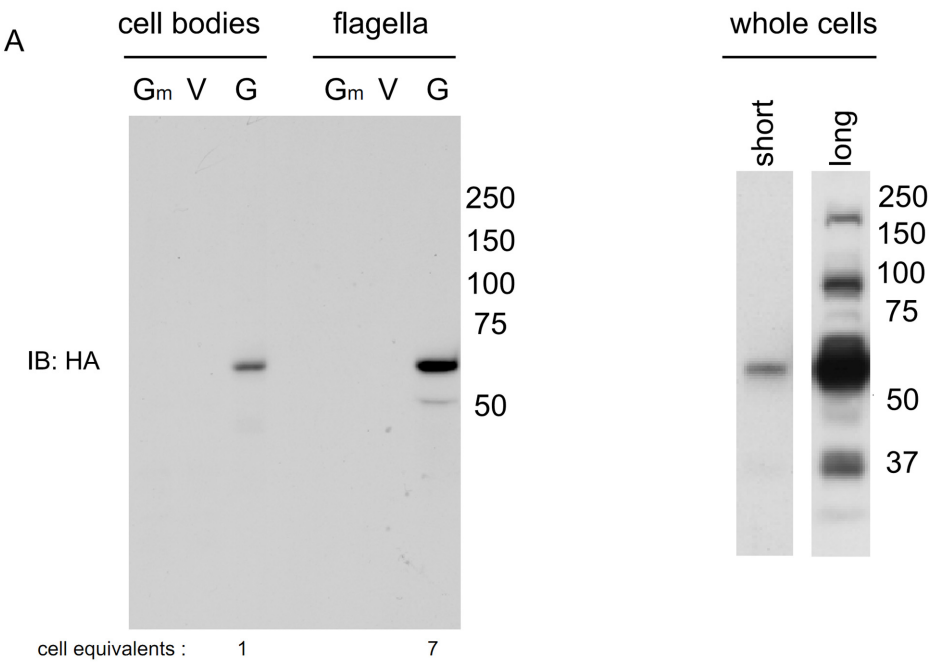


Figure 6

SAG1-HA is expressed as a 65 kDa C-terminal fragment in gametes

A: The SAG1-HA transgene is expressed only in gametes. Immunobloting with an anti-HA antibody demonstrates that expression of SAG1-HA-C65 is present in the flagella and cell bodies gametes but not vegetative SAG1-HA/*sag1-5* cells. Gm: *sag1-5* gametes, V: vegetative SAG1-HA/*sag1-*5 cells, G: gamete SAG1-HA/*sag1-5* cells. 15 μg were loaded per lane, cell equivalents: cell bodies:flagella = 1:7. Right inset: Immunobloting with and anti-HA antibody documents that higher molecular bands can be detected in cell bodies of SAG1-HA/*sag1-5* gametes upon activation with db-c-AMP. Bottom panel: Coomassie blue staining of the PVDF membrane documents equivalent loading. B: Graphical representation of transmembrane domains in SAG1 predicted by the TMHMM. Arrowheads point cleavage sites that could yield an N-terminal soluble fragment and a C-terminal fragment with 3 transmembrane domains (top panel); graphic representation of 3 predicted fragments resulting from the cleavage events of the full length Sag1 (bottom panel).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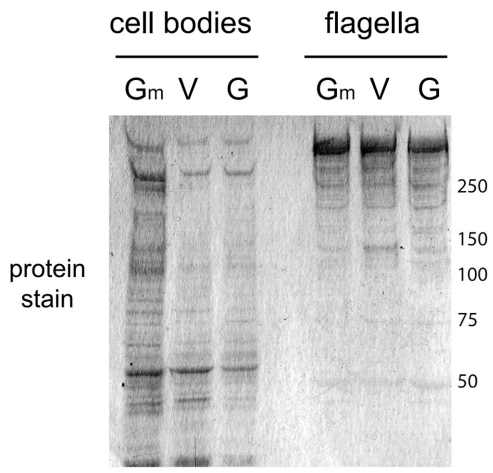
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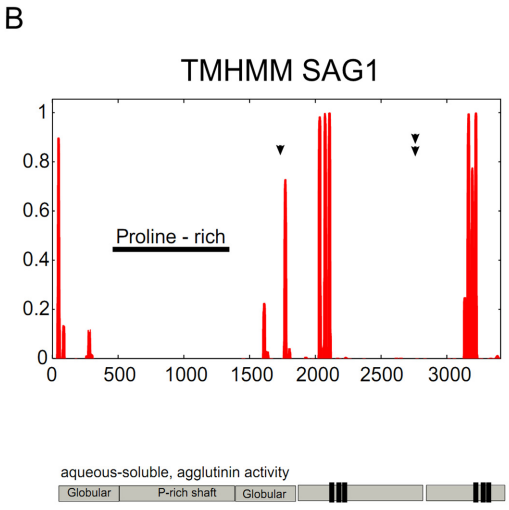
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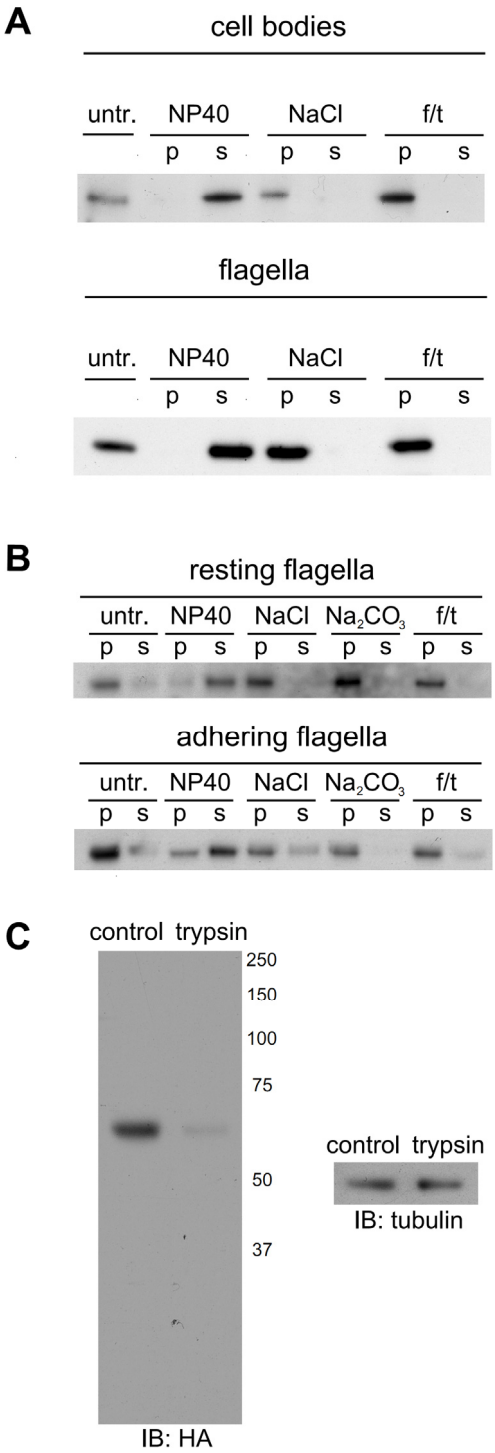
Figure 7

SAG1-HA-C65 has the biochemical properties of an integral membrane protein exposed at the cell surface

A: SAG1-HA-C65 has biochemical properties of an integral membrane protein. Cell body and flagellar samples from resting SAG1-HA gametes were subjected to the following treatments before centrifugation: 1% NP40 for 30 minutes on ice, 500 mM NaCl for 30 minutes on ice, 4 cycle of rapid freeze-thaw mechanical disruption. Untr: untreated. B: SAG1-HA-C65 has biochemical properties of an integral membrane protein. Flagellar samples from resting or adhering SAG1-HA gametes were subjected to the same treatements as in (A) except that an additional treatment was done: 100 mM Na2CO3 for 30 minutes on ice. P: insoluble fraction (pellet), S: soluble fraction (supernatant). 6μg were loaded per lane. C: SAG1-HA-C65 is exposed at the cell surface. Immunoblots of control SAG1-HA gametes and SAG1-HA gametes incubated for 5 min with 0.01% trypsin. Tubulin was used as a loading control.


A
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NP40
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control
trypsin
250
150
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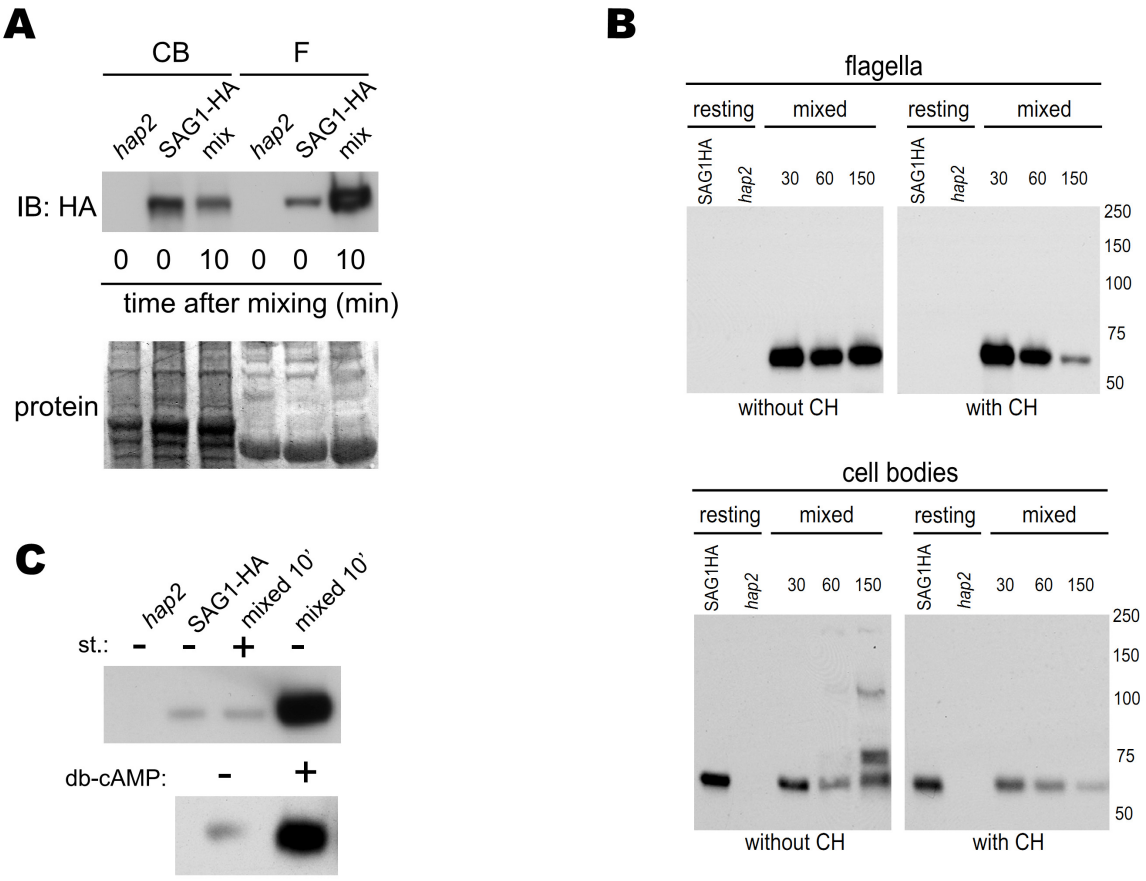
37
IB: HA
IB: tubulin

Figure 8

SAG1-HA-C65 is rapidly mobilized to flagella during adhesion-induced gamete activation.

A: Rapid mobilization to flagella. Immunoblot showing that after 10 minutes of mixing SAG1-HA/*sag1-5* gametes with gametes of a fusion defective mutant of the opposite mating type (*hap2*), SAG1-HA-C65 undergoes a massive increase in flagella. Lanes were loaded with 9μg protein. Bottom panel is a Coomassie stain of the PVDF membrane that documents equivalent loading. B: SAG1-HA-C65 translocated to flagella derives from pre-existing cell body pool. Effect of protein synthesis inhibitor cycloheximide (CH) on levels of SAG1-HA-C65 in resting and adhering gamete cell bodies and flagella. Cell equivalent loaded: cell bodies: 7.3 x 10^5, flagella: 2.4 x 10^7. C: Signaling is necessary and sufficient for rapid SAG1-HA-C65 flagellar translocation. Immunoblot of flagellar samples showing that the movement observed in *(A)* is blocked by 1 μm of the protein kinase inhibitor staurosporine (cell equivalents loaded: 10^7); and that translocation to flagella can be induced by di-butyryl cyclic AMP (15mM) in resting gametes (cell equivalents loaded: 9 x 10^7).


A
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SAG1HA
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cell bodies
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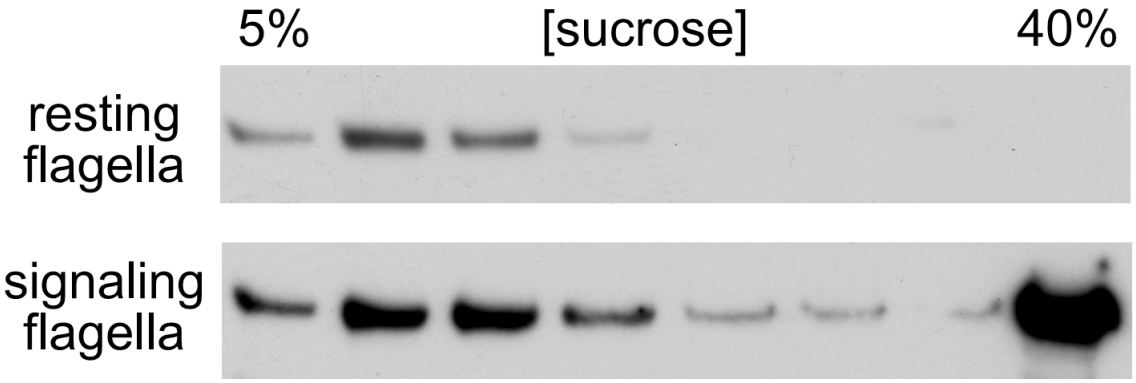
Figure 9

SAG1-HA-C65 that undergoes signaling-induced translocation into flagella forms large detergent-resistant complexes.

Flagellar samples from unactivated and activated flagella were incubated in 2% NP-40 in HMDEK buffer for 30 minutes on ice and centrifuged at 13,000 RPM for 30 minutes. The supernatants were loaded onto a 5-40% sucrose gradient containing 0.5% NP-40 and then subjected to centrifugation at 40,000 rpm (137,000 g) at 4°C for 4 hours. 256 μM fractions were collected from the top and analyzed by SDS-PAGE and immunoblotting.


5%
[sucrose]
40%
resting flagella
signaling flagella

C-terminal SAG1 is membrane associated and on the cell surface

Biochemical analysis confirmed that the 65 kDa fragment (SAG1-HA-C65) had the biochemical properties of an integral membrane protein. Both cell body and flagellar forms of SAG1-HA-C65 were soluble in the detergent NP-40 after high-speed centrifugation, whereas treatment with high salt, sodium bicarbonate, or freezing and thawing failed to release the protein in a soluble form (Fig. 7 A and B). Confirming these fractionation results, protease sensitivity experiments also showed that the SAG1-HA-C65 is a membrane protein. SAG1-HA-C65 no longer was detectable in live gametes that were treated briefly with low concentrations of trypsin. Furthermore, I failed to detect low molecular weight HA-staining bands in the immunoblots of the trypsin treated cells, suggesting that the C-terminus *per se* was on the external side of the plasma membrane.

Flagella isolated from adhering gametes are many-fold enriched in SAG1-HA-C65 compared to resting gametes

I used cell fractionation and immunoblotting as an independent approach to test for flagellar adhesion-induced translocation of SAG1-HA-C65

from the cell body to the flagella. SAG1-HA-C65 gametes were mixed with *hap2 minus* gametes for 5 min, followed by cell fractionation and immunoblotting. Confirming the immunofluorescence results, I found that SAG1-HA-C65 underwent adhesion-induced translocation from the cell body to the flagella upon mixing with gametes of the opposite mating type (Fig. 8 A). The increase in flagellar levels of SAG1-HA-C65 was striking, and cell bodies showed a corresponding decrease in SAG1-HA-C65 levels.

Flagellar adhesion alone is neither necessary nor sufficient to trigger translocation, but translocation requires signaling

To test whether the interaction between the *plus* and *minus* agglutinins *per se* was sufficient for translocation of SAG1-HA-C65 to the flagella, I allowed the cells to undergo flagellar adhesion in the presence of the protein kinase inhibitor staurosporine, which we have shown previously blocks adhesion-induced signaling (Wang and Snell, 2003; Zhang and Snell, 1994). As shown in Figure 8 B, whereas SAG1-HA-C65 rapidly translocated to the flagella in the control cells mixed with *minus* gametes, translocation was completely abrogated in cell mixed in the presence of the inhibitor. Given that signaling was necessary for translocation, I then tested whether signaling alone was sufficient to trigger translocation by incubating SAG1-HA gametes in the absence of *minus* gametes

with the cell permeable cAMP analogue, db-cAMP. As shown in Fig 8 C, db-c-AMP treatment alone triggered translocation. Taken together, these results indicated that gamete activation was necessary and sufficient to trigger SAG1-HA-C65 translocation; flagellar adhesion itself was not required.

Cells regulate SAG1 levels at rest and during adhesion-induced signaling

Even though the result that SAG1-HA-C65 appeared in flagella within 10 min after mixing strongly suggested that pre-existing SAG1-HA-C65 moved from the cell body to the flagella, it was important to carry out the experiment in cells in which protein synthesis was blocked. To do this I took advantage of the *minus hap2* fusion mutant. By mixing these cells with SAG1-HA/*sag1-5* they interact together for several hours without fusing. As expected, flagellar levels of SAG1-HA-C65 increased similarly both in the control cells and in the cells in cycloheximide (a protein synthesis inhibitor), thereby demonstrating that pre-existing SAG1-HA-C65 moved from the cell body to the flagella. The impressive enrichment of SAG1-HA-C65 in the flagella persisted for up to 150 minutes but was gradually lost when protein synthesis was inhibited (Fig. 8 C).

Figure 8C also shows that the cell bodies of the control samples at 150 minutes after mixing contained new high molecular weight bands (Fig 6A right panel) reflecting newly synthesized SAG1-HA that had not yet been cleaved (Fig.

8 B and C). In the cell bodies of samples incubated in cycloheximide, as expected, failed to express the higher molecular mass proteins and by 150 min, SAG1-HA-C65 levels were greatly diminished.

The SAG1 that undergoes signaling-induced translocation into flagella forms large, detergent-resistant complexes.

To further characterize SAG1 during flagellar adhesion, I designed an experiment to ask whether SAG1-HA-C65 changed its properties during gamete activation *vs* resting gametes. To do so, detergent extracts from flagella isolated from resting and adhering (with *hap2)* SAG1-HA gametes were fractionated by sucrose velocity sedimentation followed by SDS-PAGE and immunoblotting. As shown in figure 9, SAG1-HA-C65 from unactivated flagella remained in the upper fractions of the gradient. In contrast, a large proportion of the SAG1-HA-C65 extracted from flagella isolated from adhering cells was found near thebottom of the gradient, indicating that large SAG1-HA-C65-containing complexes were formed during flagellar adhesion.

DISCUSSION

In my study of the *Chlamydomonas SAG1* agglutinin gene, I used several experimental methods to directly detect a tagged protein encoded by the *SAG1* gene. The *SAG1* gene product was cleaved soon after synthesis, to yield a 65 kDa C-terminal fragmentI showed that SAG1-HA-C65 localizes mostly at the cell body in resting gametes, and that upon cell activation (resulting from cell-cell adhesion or signaling stimulation) a large amount of SAG1-HA-C65 is rapidly translocated to flagella. My work is the first to biochemically document a rapid signaling-triggered movement of a membrane protein from the cell body to the flagella. This work is also the first report showing that SAG1 is cleaved after its synthesis and that its C-terminal fragment of around 65 kDa is a transmembrane polypeptide. Finally, I show that during signaling, this fragment forms large detergent-resistant complexes within flagella.

A new model for the *Chlamydomonas* agglutinins

A construct to tag SAG1 at the C-terminus of the protein with an HA epitope was made and rescued a *SAG1* mutant (*sag1-5*); my transformant regained its ability to adhere and fuse with *minus* gametes. I showed that the product of this

construct displayed gamete-specific expression. These results indicate that the SAG1 gene is responsible for the *plus* agglutinin activity.

Interestingly, the band I observed by immunoblotting was much shorter than the predicted full length SAG1 protein, indicating that it had been cleaved from the N-terminal part. The observation that SAG1 is being cleaved force us to re-interpret all previous work on the agglutinins and design a new model that considers both previous and new results. Because two regions of Sag1 contain 3 strongly predicted transmembrane domains, and because of the fact that the HA-tagged C-Terminal fragment is long enough to include one of these clusters of 3 transmembrane helices but too short too include the other cluster, I propose that a cleavage event occurs to create the observed 65 kDa polypeptide and that this polypeptide contains 3 transmembrane domains. In addition, I propose that at least one other cleavage event occurs to generate another polypeptide containing the other cluster of 3 transmembrane helices. This second cleavage also produces a larger N-terminal polypeptide devoid of transmembrane domains.

I believe that the previous work in which investigators used EDTA or mechanical disruption to solubilize polypeptides with agglutinin activity characterized, not the full length protein, but only the transmembrane-domain-free, N-terminal polypeptide that I estimate accounts for roughly half of the Sag1

coding sequence and that would possess the flagellar adhesion domain of SAG1. Electron microscopic images of the agglutinins (Goodenough et al., 1985), showed them as long fibrous molecules composed of 3 parts: the head, the shaft, and the hook. I think that these images fit my model more than the model proposed by Ferris *et al.* (Ferris et al., 2005) in which the head domain is predicted to be quite large (2006 amino acids or 59% of the entire protein). In my new model the predicted head domain of the agglutinin would be more similar in size to the N-terminal hook domain (469 amino acids). The model of Goodenough *et al.* (Goodenough et al., 1985) show the head domain as containing more than half of the amino acids of the protein, but in the EM images, the head domains appear more modest in size.

I propose that this N-terminal polypeptide is extracelluar and associated with transmembrane protein(s), possibly with the 65 kDa C-terminal fragment of SAG1. My discovery that the C-terminal fragment translocates to the flagella during cell adhesion or activation by db-cAMP is consistent with the possibility that it functions in cell adhesion, possibly interacting with the N-terminal fragment. According to my model, at least a third polypeptide would result from 2 cleavage events. I have no data on this predicted polypeptide but I predict it to be a transmembrane polypeptide that might also participate in the agglutinin activity. Epitope-tagging these 2 other polypeptides will help clarify their respective role in the adhesion process. Although it wasn't proposed until

now, the idea that SAG1 undergoes cleavage events is realistic since other ciliary signaling membrane proteins also are cleaved, including polycystin 1 (Qian et al., 2002; Woodward et al., 2010) and 2 (Huang et al., 2007) and fibrocystin (Kaimori et al., 2007). In the case of polycystin 1 and fibrocystin, a large ectodomain is produced and remains tethered to the remaining transmembrane fragment (Kaimori et al., 2007; Qian et al., 2002).

SAG1-HA-C65 behaves in a similar fashion as the agglutinin activity, suggesting it plays a role in this activity.

The immunofluorescence and biochemical data show that Sag1-HA-C65 behaves quite like the agglutinin activity studied by. Hunnicutt *et al.* (Hunnicutt et al., 1990). My results resemble their findings about the agglutinin activity: in resting cells, most SAG1-HA-C65 is located at the cell body; but that in activated cells, SAG1-HA-C65 rapidly and massively translocates to flagella. This suggests that SAG1-HA-C65 could play a role in the agglutinin activity, possibly by interacting with the putative N-terminal polypeptide and anchoring it to the membrane: both the adhesion domain and SAG1-HA-C65 are likely associated with each other in the cell body in resting gametes and move together to the flagella during activation.

Other proteins seem to associate with SAG1-HA-C65 as evidenced by the large SAG1-HA-C65-containing complexes that form during flagellar adhesion. It will be important will be to determine the protein composition of these complexes. Possibly, other proteins associate with SAG1 fragments during activation and participate to the adhesion activity. It is also possible that during activation, Sag1 fragment bind to each other to form large clusters. I suspect that some IFT components could associate to SAG1-HA-C65 and further work is required to dissect the role of IFT in regulated movement of SAG1-HA-C65. It will be important to discover the mechanisms of how cells can dynamically regulate the membrane composition of their cilia and flagella because this seem to be critical in several cilium-generated signaling cascades such as the sonic hedgehog pathway in mammals in which smoothened rapidly localizes to the primary cilium in presence of the hedgehog ligand.

What are the mechanisms for SAG1-HA-C65 accumulation in flagella?

When gametes are activated, pre-existing SAG1-HA-C65 rapidly becomes enriched in flagella. At least two mechanisms could explain this enrichment.

First, it could be that a small amount of SAG1-HA-C65 is constantly trafficking through the flagella and signaling causes it to be trapped there, similar to what Kim *et al.* propose for Smoothened in mammalian cells (Kim et al., 2009). In the absence of signaling, SAG1-HA-C65 would not be retained in the flagella and would either be shed from the flagella or returned to the cell body. The nature of the flagellar trap would be another interesting biological question. Possibly the large detergent-resistant complexes that form during flagellar adhesion and signaling somehow prevent SAG1-HA-C65 from leaving the organelle.

Second, there could be an active process that translocates SAG1-HA-C65 from the cell body membrane to the flagella upon signaling. This could take form by release of a membrane diffusion barrier or by stimulating endocytosis of SAG1-HA-C65 from the cell body membrane into vesicles that would be targeted to the base of the flagella for integration into the flagellar membrane, similar to the model for ciliary accumulation of Smo proposed by Wang *et al.* (Wang et al., 2009). The fact that the translocation is rapid seems to favor the simplest model: weakening of a diffusion barrier to allow lateral transport from the cell body membrane to the flagellar membrane. Such lateral transport would be consistent with the smoothened lateral movement model proposed by Milenkovic *et al.* that

allows Smoothened to become enriched in primary cilia after patched binds it ligand (Milenkovic et al., 2009).

Chapter 3

GENERAL CONCLUSIONS, MODELS, AND FUTURE DIRECTIONS

I- Introduction

Why are cilia and flagella are so widely used as signaling hubs is currently a major question in cell biology. Studies like the one I presented in the previous chapter provide part of the answer. My work has shown that in *Chlamydomonas*, the flagellar membrane composition is dynamically regulated during sexual signaling. Contact between the flagella of *plus* and *minus* gametes (physiological activation) or addition of di-butyryl cyclic AMP to *plus* gametes rapidly induces a striking enrichment in the transmembrane domain-containing polypeptide SAG1-HA-C65, a product of the *SAG1* gene also encoding the polypeptide possessing the agglutinin activity. This demonstrates that this organism has a mechanism to alter its flagellar membrane composition by rapidly increasing (and possibly decreasing) flagellar concentration of one (and possibly more than one) membrane protein.

This ability to be able to quickly change the concentration of molecules in flagellar membranes and in the flagellar matrix is a reason why they are suitable structures to be a signaling center because it allows to control steps in signaling cascades (Rohatgi and Snell, 2010), along with other reasons such as

their unique location on the cell that allow them to protrude from the cell body and sense signals in the extracellular environment, antenna-style.

Although the mechanism by which this rapid enrichment of SAG1-HA-C65 occurs remain to be fully understood, it is very likely that the same mechanism is present in mammalian cells to control the ciliary membrane protein composition. Considering the breath of knowledge that *Chlamydomonas* taught us about structures and processes occurring in other organisms (including humans), it is likely that studying *Chlamydomonas* will continue to help us increase our knowledge of how signaling works in other organisms, because of the advantages it has to offer to biologist, both biochemically and genetically.

In the following pages, I will present models about 1-) the functional properties of SAG1, including its intra molecular interactions and 2-) the regulation of SAG1 localization and fate.

II- Functional properties of SAG1

Questioning the role of the other gene products of Sag1

The first thing I did to start this project was to generate a C-terminal HA-tagged construct of *SAG1* that I used to rescue the *sag1-5* agglutination mutant. Somewhat unexpectedly, I found out that the tagged polypeptide appeared as a smaller than expected 65 kDa band on immunoblots. As explained in the discussion of chapter 2, this result, along with re-evaluation of older experiments, lead to a new model proposing that the *SAG1* gene product is cleaved at least twice shortly after synthesis. According to this model, two other resulting polypeptides would result from these cleavage events. One, the most N-terminal, would be a large membrane associated polypeptide possessing the agglutinin activity and that can be solubilized by EDTA and mechanical disruption; I think that this fragment was what biologists studied in the past, thinking this "agglutinin" was a full gene product. I will refer to this polypeptide as the "*plus* agglutinin domain" from now on. The new model also implies that a third polypeptide is generated and that it would contain 3 transmembrane helices, I will refer to it as the "*plus* fragment #2". It would be interesting to tag these 2 other fragment to follow them biochemically and by immunofluorescence microscopy, to establish how they behave compared to SAG1-HA-C65. Although it's possible that they behave differently from SAG1-HA-C65, I am tempted to speculate that they would behave like SAG1-HA-C65. I propose the hypothesis that the two shorter fragments containing transmembrane domains act together as an anchor

for the *plus* agglutinin fragment. If the *plus* agglutinin domain fragment, which is responsible for the agglutinin activity, would associate to other molecules instead of the remaining SAG1 fragments, SAG1-HA-C65 (or the *plus* fragment #2 for that matter) would not be expected to enter the flagella during gamete activation.

Does the N-terminal part of SAG1-HA-C65 play an active role in signaling?

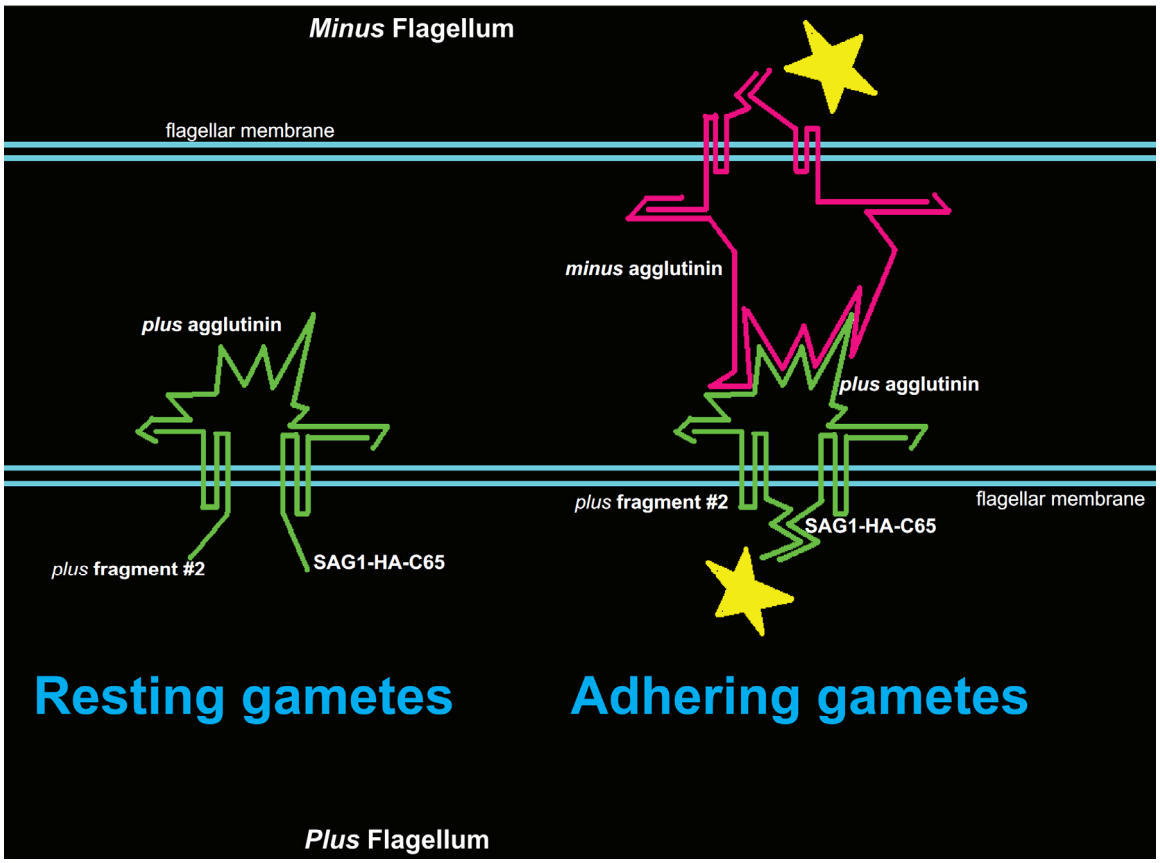
Analyzing the predicted amino acid sequence of the full length Sag1 by different transmembrane domain predictors yield different predictions. Although both TMHMM and Phobius agree that Sag1 should contain several transmembrane helices, Phobius predicts 6 of them while TMHMM predicts 7 transmembrane domains (and the extra domain is not predicted as strongly as the 6 others on which both predictors agree). If the 6 transmembrane domains model is correct, the C-terminus and the HA tag of SAG1-HA-C65 are expected to be extracellular, trypsin digestion experiments support this model because exposure to the protease destroys the signal on immunoblots. A consequence of this is that SAG1-HA-C65 would likely interact with its C-terminal end to anchor the *plus* agglutinin domain fragment to the extracellular side of the flagellum, leaving its N-terminal tail intraflagellar, available for interactions with other molecules inside the flagellum. It is therefore possible that upon binding with the *minus* agglutinin, the *plus* agglutinin domain could induce a conformation change and/or

post-translational modifications in SAG1-HA-C65's intraflagellar amino acid chain that could play a direct role in signaling. A similar role for the C-terminal amino acid chain of *plus* fragment #2 is also possible. These polypeptides could play a role into activating the protein-tyrosine kinase which is being activated by flagellar adhesion *per se* and not by cyclic AMP increase or cell fusion (Wang and Snell, 2003). Figure 10 illustrates an interpretation of such a model as a cartoon form.

Figure 10

Possible model of how SAG1 and SAD1 polypeptides interact

In this model the *plus* agglutinin domain fragment is anchored to the *plus* fragment #2 and SAG1-HA-C65; and does not activate a signaling cascade in absence of mating type *minus* agglutinin (left side). In presence of *minus* agglutinin (right side of the image), an interaction would occur between *plus* and *minus* agglutinin domains, leading to conformational changes and/or post-translational modifications of intraflagellar amino acid tails of the transmembrane SAG1 (and SAD 1) fragments; therefore activating a signaling cascade (represented by yellow stars).


Minus Flagellum
flagellar membrane
minus agglutinin
plus agglutinin
plus agglutinin
plus fragment #2
SAG1-HA-C65
flagellar membrane
plus fragment #2
SAG1-HA-C65
Resting gametes
Adhering gametes
Plus Flagellum

III- Regulation of SAG1 localization and fate

Possible binding partners of SAG1-HA-C65

An interesting finding of my experiments was that SAG1-HA-C65 from isolated adhering flagella took part in very large detergent-resistant complexes while SAG1-HA-C65 from non-adhering flagella did not. Discovering the protein composition of those large complexes could yield invaluable information about how the flagellum-generated signaling cascade is initiated. Immunoprecipitations followed by mass spectrometry analysis should give us a list of proteins that interact with SAG1-HA-C65. In non adhering flagella, where no large complexes form, it is reasonable to think that I would find the *plus* agglutinin domain and the *plus* fragment #2 bound with SAG1-HA-C65, and possibly several other membrane proteins that could serve as extra anchors for the *plus* agglutinin domain fragment. On the other hand, in adhering flagella I would expect to find, in addition of the polypeptides mentioned above, polypeptides from the gene *SAD1* (probably cleaved fragment analogous to those from *SAG1*), and most likely signaling proteins that are being recruited to these complexes during adhesion. In addition I would not be surprised to discover proteins

involved in intraflagellar transport in both conditions, but no necessarily the same set of IFT proteins for adhering versus resting flagella. I think this is possible that some signaling proteins might bind to SAG1-HA-C65, such as the PKD2 or the protein tyrosine kinase acting early in the signaling cascade. Finally I suspect that in adhering flagella SAG1-HA-C65 and their binding partners cluster in large numbers to generate the very large complex that I saw in sucrose gradients.

What are the mechanisms of the rapid translocation of SAG1-HA-C65 from the cell bodies to the flagella?

How a ciliary membrane protein rapidly becomes enriched in a cilium or flagellum is a major question in the field. I feel that our laboratory will be able to shed some light on this issue because of the advantages of *Chlamydomonas* and because we have a unique mutant that will allow to address the role of IFT in this translocation. The role of IFT in transporting structural elements of the axoneme is well known, but for ciliary membrane proteins, its less clear. Although some membrane protein show IFT-like movement (Huang et al., 2007; Qin et al., 2005), the significance of this transport for membrane protein is not fully understood (Ishikawa and Marshall, 2011). By crossing my SAG1-HA/*sag1-5* cells with *fla10* cells, a temperature sensitive, conditional IFT mutant I will be able to answer several questions. For example I can look for the rapid SAG1-HA-C65

translocation to flagella at the permissive vs restrictive temperature. If no significant translocation is noticed at the restrictive temperature, it will indicate that IFT is involved in transporting SAG1-HA-C65 in the flagella. The mutant *fla10* has an anterograde IFT defect and would not be useful to address the retrograde transport of SAG1-HA-C65 (if any). I believe that SAG1-HA-C65 does not undergo retrograde transport because it appears to accumulate at the tip of the flagella by immunofluorescence (possibly part of the flagellar tip-activation phenomenon described by Mesland *et al.* (Mesland et al., 1980)) and it is probably shed from there as vesicles instead of being carried back to the cell body. No proteasome are present in flagella to degrade it there.

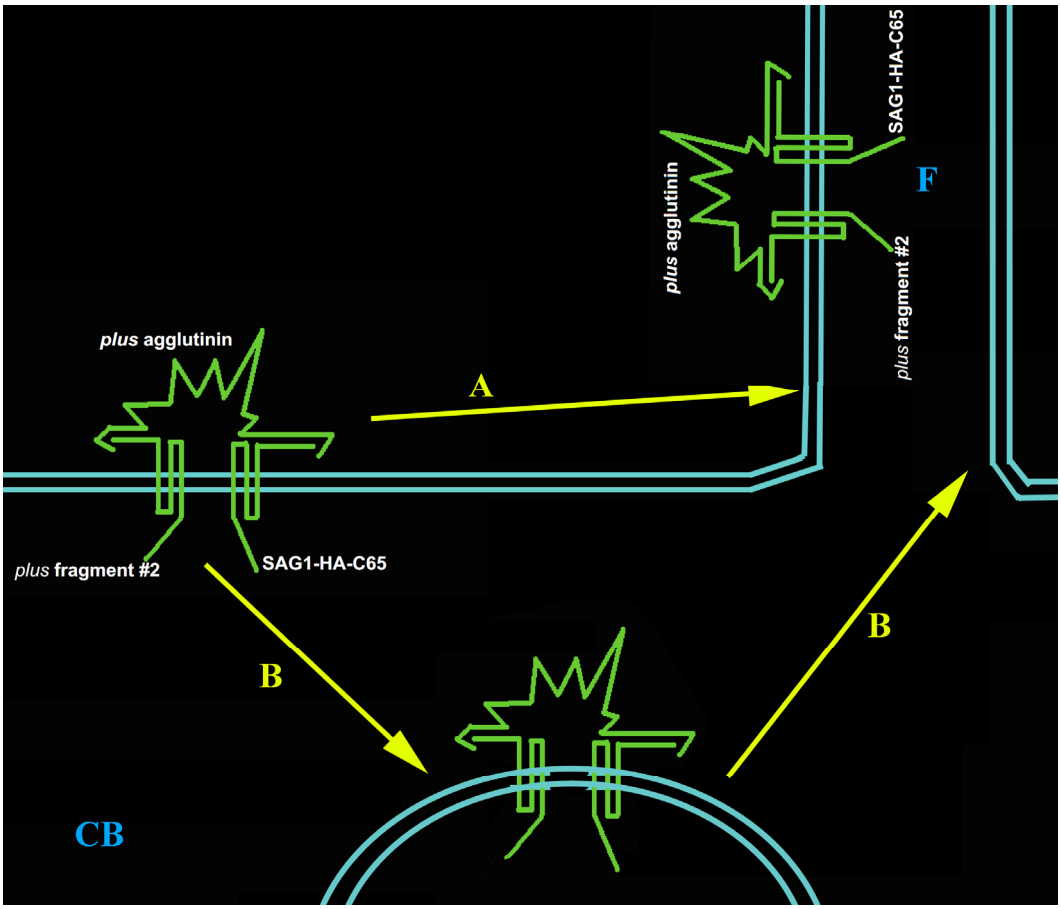
However, IFT cannot explain the whole process by itself, because IFT is constantly in motion, before and during adhesion. Some unknown mechanism blocks this massive influx of SAG1-HA-C65 to the flagella until two gametes adhere to each other. More precisely, this barrier does not let SAG1-HA-C65 enter the flagella until signaling caused an increase in cyclic AMP. Presumably the increase in cyclic AMP or a downstream effect of this increase cause this barrier to lose its effectiveness and allows SAG1-HA-C65 to easily access the flagellar membrane. The mechanisms causing this are still obscure.

Two routes are possible for the pre-existing SAG1-HA-C65 to translocate from the cell bodies to the flagella as mentioned in the chapter 1. It could be by lateral transport (going directly from the plasma membrane to the flagellar membrane) or by the recycling pathway (undergoing endocytosis into vesicles that would deliver the protein at the base of the flagellar membrane). These two routes are illustrated in figure 11. Because the movement is rapid, I think that lateral movement is more likely because it would not require more complex phenomena such as endocytosis and vesicle transport and fusion at the base of the flagellum.

Figure 11

Possible routes by which SAG1-HA-C65 translocates from the cell body to the flagella

In the lateral transport route (A), the polypeptide would move directly from the cell body plasma membrane to the flagellar membrane. In the recycling transport model (B), the polypeptide would first be endocytosed into a cytoplasmic vesicle that would then be transported to the base of the flagellum for fusion and integration into the flagellar membrane. CB: cell body, F: flagellum.


SAG1-HA-C65
F
plus agglutinin
plus fragment #2
plus agglutinin
A
plus fragment #2
SAG1-HA-C65
B
B
CB

What is the fate of the SAG1 fragments after adhesion?

I have shown that upon binding with *minus* gametes, large amounts of SAG1-HA-C65 become enriched in flagella of *plus* gametes. These high levels are maintained by protein synthesis. In experiments in which I blocked protein synthesis with cycloheximide, SAG1-HA-C65 eventually was depleted from flagella during prolonged adhesion. What is the nature of this turnover? One possibility is that some of the flagellar SAG1-HA-C65 is returned to the cell body for degradation. Another possibility is that the polypeptides are shed from the flagella into the environment of the adhering cells. We are currently testing the model that SAG1-HA-C65 is indeed being shed into the medium during gamete adhesion. One idea would be that the proteins are being released as vesicles, possibly from the tips of the flagella. Such shedding of SAG1-HA-C65 might finally explain the nature of the mysterious ~ 70 kDa "U band" released and found only in the media of plus gametes only 37 years ago by Snell (Snell, 1976); it might have been a fragment of SAG1.

If shedding indeed occurs, it could be a common biological way for cilia/flagella to remove a specific signaling protein from the organelle instead of

relying on retrograde transport to return this protein to the cell body for degradation. For example, in the sonic hedgehog pathway, the receptor patched could be shed from the flagella after it binds the hedgehog ligand, or it could be returned to the cell body. Both possibilities are illustrated on figure 12. This shedding route could also be used to remove Smoothened from the flagella once signaling is turned off again.

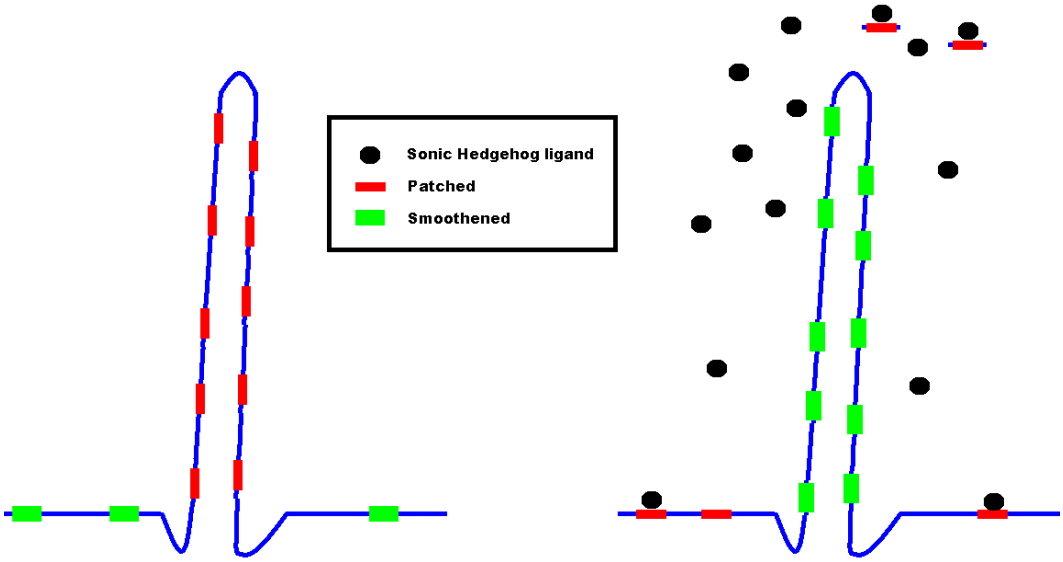
Formation of large complexes in flagella could be a way to promote accumulation of membrane proteins in cilia and flagella

The observation of large detergent-resistant complexes of SAG1-HA-C65 during signaling might correspond to a mechanism of flagellar enrichment of the polypeptide. It is interesting to speculate that such a mechanism has been conserved during evolution and that, for example, Smoothened could also form large complexes in primary cilia during signaling and it being trapped in these complexes would be necessary for ciliary enrichment of Smoothened when the hedgehog ligand is present. Formation of large complexes could also be required to maintain high level of Patched in primary cilia prior to hedgehog ligand binding.

Figure 12

Model illustrating the early events in sonic hedgehog signaling

Left: In absence of hedgehog ligand, the receptor Patched is enriched in the primary cilium and Smoothened is excluded from it. Right: In presence of hedgehog ligand, Patched is removed from the primary cilium (either by shedding into the extracellular environment or by return to the cell body membrane) and Smoothened become enriched at the ciliary membrane.


Sonic Hedgehog ligand
Patched
Smoothened

APPENDIX A
DNA sequence of pBSAG1-HA

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