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General introduction

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Chapter 1

General Introduction

This thesis focuses on the biflagellate green alga *Chlamydomonas moewusii*. It is used as a model system for studying sexual interactions between mating gametes. In particular the thesis focuses on the molecules that bind gametes together, making sexual fusion possible. Therefore the following text is meant to introduce the reader to the cell system and the sort of molecules involved. Since cell recognition molecules are exposed on the cell surface, they are usually glycoproteins. Because so few of them are present, little is known about them. In contrast, the related cell wall proteins are prevalent and they have been relatively well studied, as related below.

![Fig. 1 An electron microscopic image of C. moewusii vis-à-vis pairs](image)

Vis-à-vis pairs are $mt^+$ and $mt^-$ gametes fused at their anterior ends by a narrow plasma bridge. The photograph illustrates that both mating types are morphologically similar, unicellular and biflagellated. Note that the cell body is surrounded by a cell wall, while the flagella are naked. Bar = 10 µm (Photograph from Dr. D.A.M Mesland).

1 General description

The genus *Chlamydomonas* (Chlorophyceae) represents a group of unicellular algae belonging to the order of the *Volvocales* (1). Under nutrient rich conditions, *Chlamydomonas moewusii* (Figure 1) grows vegetatively as ellipsoid cells (6-10 µm long), and when deprived of ammonium, these cells are transformed into gametes of the same shape (2). *Chlamydomonas* is heterothallic, which means that it occurs either as mating type plus ($mt^+$) or minus ($mt^-$) cells, which are morphologically the same. At the anterior end, two flagella (10-15 µm long) protrude through separate pores in the cell wall and serve to propel the cell...
through the medium. They also act as sensory organelles during the mating process. During gametogenesis, a new flagellar protein, called agglutinin, is incorporated into the flagellar membrane, bestowing the cell with the ability to recognize a sexually compatible partner. *Chlamydomonas* contains a single chloroplast, which partially surrounds the nucleus, has an eye spot which is involved in phototaxis (3), and it possesses contractile vacuoles for regulating its osmotic status (1).

2 Cell cycle

The vegetative cell cycle of *Chlamydomonas moewusii* can be synchronized under specific culture conditions whereby a light/dark regime, aeration and medium composition play an important role. Newly formed daughters start their cell cycle and increase in cell volume during the light period (4). At the end of the light period, cells aggregate due to the secretion of sticky material released by the cells. In the following dark period they synchronously divide (5). Prior to cell division, *Chlamydomonas* cells change from an ellipsoidal to a spherical shape, and become immotile after retracting their flagella. During the dark period, DNA doubling, mitosis and cytokinesis take place (4,5) and, dependent on the size of the mother-cell, an additional number of cell divisions can follow the first one (6,7). About two hours before the onset of the next light period, daughter cells are released from the mother cell wall by the action of a proteolytic enzyme, called sporangial lyasin, which degrades the cell wall of the mother cell but leaves that of the daughter cells intact.

3 Flagella and Flagellar surface motility

The two flagella of *Chlamydomonas* are hair-like protrusions from out of the cell body protoplast (8). They are equal in size, measuring 10-15 μm in length and 0.3 μm in diameter. The flagellar membrane surrounds the complex of microtubules called the axoneme and is structurally continuous with the plasma membrane, although it differs from it in protein composition. The “bracelet”, which is a ring of membrane-spanning proteins at the flagellar basis, acts as a barrier separating both domains and preventing diffusion of cell surface proteins from one to the other. In this way the characteristic complement of glycoproteins is maintained in each domain (9). For example, the flagellar membrane is covered by a “fuzzy coat” of glycoproteins (Figure 2) which is absent from the cell body membrane (1,10,11). It
probably acts as a flexible protective layer around the flagella, which is not around the cell body because it is protected by the rigid cell wall (10).

An interesting aspect of the flagella in *Chlamydomonas* is that when cells are subject to changes in salt concentration, temperature or pH, they are shed (12,13), due to the contraction of a Ca\(^{2+}\)-sensitive protein called caltractin at the flagellar basis by which the axoneme is severed (14). After excision, full-length flagella are completely regenerated in a very short time (90 min). During this process of flagellar regeneration, tubulin, dynein and many other structural protein genes coding for flagellar proteins are expressed and translated (15-17). These proteins are added onto the growing axoneme at the distal end (18), which implies that all these components must be transported from the cell body cytoplasm through the flagellar matrix, between the membrane and the axoneme, to the flagellar tip. Most of the structures forming the axoneme are very large multipeptide complexes, and it seems that they do not assemble from single proteins in situ but are pre-assembled in the cytoplasm and then moved as a complex to the flagellar tip during regeneration.

Since gamete agglutinins are extracellular membrane proteins, they must be associated with an integral component of the flagellar membrane to form a complex (Figure 2). A characteristic feature of this agglutinin receptor complex is that it is mobile in the plane of the flagellar membrane. *In vivo* when gametes of opposite mating type are mixed, the initial random contacts between flagellar agglutinins normally develop into tip-to-tip associations due to the migration of the contact sites to the flagellar tips. Flagellar surface components that are not associated with the agglutinins are not redistributed to the flagellar ends (19). Treatment of intact gametes with agglutinin-cross-linking agents such as lectins or monoclonal antibodies (Mab 66.3) directed against the mt\(^{-}\) agglutinin, trigger the responses which result in the redistribution and finally, in the accumulation of the agglutinins at the flagellar tip (19-22). Treatment with monovalent Fab fragments of the same Mab 66.3 that cannot cross-link the agglutinins does not induce any redistribution (21). Therefore the clustering of agglutinins is important for the induction of tipping (Figure 2) (This process is described in more detail in the section gametogenesis).
Transport of agglutinin clusters

Cross-linking

Fuzzy coat

Motor Tubulin

Transport of agglutinin clusters

Appendix:

Transmembrane proteins

Glycoproteins

Axonemal transport mechanism

Agglutinin

Mab 66.3

Fig. 2 Putative model for glycoprotein redistribution in the flagellar membrane of *C. moewusii*

Transport of the agglutinin complex over the flagellar surface is performed after cross-linking the agglutinins. Cross-linking creates clusters of agglutinin complexes, which become attached to the axonemal transport apparatus. These clusters are then transported to the flagellar tips.

Examination of tipping by high-resolution, video-enhanced differential interference contrast (DIC) microscopy has revealed a new flagellar motility: a rapid bi-directional movement of material along the length of the flagellum in the matrix between the membrane and the axoneme (17). Because this motility has been observed in mutants lacking flagellar dynein, this suggests that a motor drive it different from that which drives flagellar swimming movements. Although the exact nature of the moving material cannot be determined by light microscopy, in the electron microscope these structures are seen as “rafts”. They are composed of varying numbers of elements that connect the flagellar membrane and the axonemal microtubules. In cross sections of the axoneme, rafts are seen in specific association with the β-tubules of the outer-doublet microtubules. These structures may play a role in
transporting unassembled flagellar components to the flagellar tip, as suggested by the polarity of axonemal assembly *in vivo* (18). How other proteins (non-axonemal) are transported along the flagella and how they are inserted into the membrane has not yet been elucidated.

Considering that the agglutinins are completely extracellular, a transmembrane anchor protein is essential for sexual agglutination because such a protein is needed to couple the agglutinin to an intramembrane motor. In addition, this membrane anchor must be involved in signal transduction for sexual agglutination invokes the formation of intracellular signals that induce other responses in the gamete cell that are needed to ensure sexual cell fusion. The anchor protein has not yet been identified, but the search for it has revealed several flagellar membrane proteins that are associated with the agglutinin (23). One of them is a wheat germ agglutinin WGA-binding protein (19,24), which co-migrates during tipping with the mt-agglutinin of *C. moewusii*.

One can draw parallels between an agglutinin anchor component in the *Chlamydomonas* flagellar membrane and integrin molecules in animal cells. Contact between mammalian cells and their extracellular matrix is often mediated by members of the integrin super family of adhesion receptors, which are heterodimeric membrane glycoproteins (25). While binding to the extracellular cell matrix and the intracellular cytoskeleton, they may transfer information across the membrane in both directions. If one envisions the *Chlamydomonas* agglutinin as part of an extracellular matrix and the anchor protein as an integrin, the latter could stimulate second messenger production, and also connected to, and be distributed by, the cytoskeleton to which it is bound.

The mechanism of glycoprotein-redistribution is unknown, but it is hypothesized that transmembrane proteins interact with a submembrane energy transducing motor, which may be associated with the outer microtubules of the axoneme (26).

4 Cell wall: a glycoprotein complex

Structural cell wall proteins are classified, mainly on the basis of their characteristic amino acid composition and their carbohydrate moiety, into five major categories: a family of hydroxyproline-rich glycoproteins (HRGPs), glycine-rich proteins (GRPs), proline-rich proteins (PRPs), arabinogalactan proteins (AGPs) and solanaceous lectins. The latter represent a unique class of plant lectins that can be distinguished from other lectins by their restricted occurrence in solanaceous plants, their ability to agglutinate oligomers of N-
acetylglucosamine, their predominantly extracellular location and their unusual amino acid and carbohydrate composition, in which hydroxyproline and arabinose are major constituents (27,28) of the other categories that could occur in Chlamydomonas, only the HRGP's are as yet well represented.

In higher plants, HRGPs have a polyproline II helix conformation. A high content of hydroxyproline residues in a Ser-(Hyp)_4 repetitive motive gives these proteins a rod-like structure (29). In the cell wall of Chlamydomonas, these rod-like structures have more Ser-Pro repeats than usual (30).

In Volvox carteri, a colonial form of Chlamydomonas that is composed of only two cell types, 2000-4000 biflagellated Chlamydomonas-like somatic cells are arranged in a monolayer at the surface of a hollow sphere that encloses a small number of much larger reproductive cells ('gonidia') that lie just below the somatic cell sheet. The individual cells, surrounded by insoluble fibrous layers are held at a distance from each other to form connected cellular compartments. Some extracellular matrix (ECM) proteins from Volvox have proline-rich spacers so that they are very similar to HRGPs in plant cell walls (31). The outer cell wall layer is made up of HRGPs that can be divided into two different peptide motives. One contains (Hyp)_x, the other one is the abundant Ser(Hyp)_4-7Arg peptide motif, but all these regions are heavily O-glycosylated.

Using immunological techniques, a sulfated surface glycoprotein (SSG 185) has been identified within the ECM of Volvox carteri (32). The primary structure of the SSG 185 polypeptide chain has been derived from cDNA and genomic DNA sequences, and it appears that a central domain of the protein, 80 amino acid residues long, consists almost exclusively of hydroxyproline residues. Under the electron microscope, SSG 185 is seen as a rod-shaped molecule with a long polysaccharide strand protruding from its central region. Remarkably, pherophorins, which are a family of ECM glycoproteins also from Volvox, contain exactly the same type of polyhydroxyproline spacer (31,33). This spacer is glycosylated and, interestingly, contains a phosphodiester bridge between two arabinose residues, which is the same type of modification that was originally discovered in SSG185 (31). These two Volvox glycoproteins represent the main components of the cellular zone within the ECM (33).

In Chlamydomonas, the cell wall is composed of a complex glycoprotein-rich structure consisting of approximately 15 glycoproteins (24,34). By using the quick-freeze deep etch technique, the wall was shown to be organized into two domains (Figure 3) (35). An outer crystalline layer composed of hydroxyproline-rich glycoproteins (HRGPs) and an inner
glycoprotein framework (36,37). The chaotrope-soluble outer layer contains HRGPs, which assemble through ionic interactions to form a crystalline matrix (36). The chaotrope-insoluble inner layer provides a loose fibrillar network that interacts with, and extends from, the plasma membrane (Figure 3) (35,37).

Fig. 3 An electron microscopic image of the cell wall of *C. moewusii* (36)

*Chlamydomonas moewusii* gametes are specifically characterized by the presence of mating type specific agglutinins on the flagellar surface (2,38,39). They are hydroxyproline-rich glycoproteins (HRGPs) and almost 50% of the molecular mass is due to sulfated oligosaccharides (Figure 4). The agglutinins have a rod-like shape and have been characterized as long (210-336 nm) glycoprotein molecules with a molecular mass in the order of 1200-1300 kDa (40-43). In the sulfated oligosaccharides, the sugar that is O-linked to hydroxyproline is almost always arabinose or galactose (44,45). The agglutinin is one of the minor glycoproteins in *Chlamydomonas*. There are several other glycoproteins present at the flagellar surface (24,34), most of which contain sugars that are methylated to some extent (46). A particular phenotype contains either the so-called A-sugars (4-O-methyl xylose, 2-O-methyl arabinose and 3-O-methyl galactose), or B-sugars (6-O-methyl mannose and 3-O-methyl glucose) (47). The protein-sugar content is similar to that of the HRGPs in the cell walls of dicotyledons (27).
Gametogenesis

The differentiation of vegetatively dividing cells into sexually active non-dividing gametes is called gametogenesis (Figure 5). Normally in higher plants and animals, haploid gametes arise from diploid cells in the germinal tissues, but *Chlamydomonas* is haploid so that gametogenesis does not require meiotic cell division. *Chlamydomonas* spends most of its life as a haploid vegetative cell, which undergoes gametogenesis only under particular circumstances. For *C. reinhardtii*, nitrogen starvation is the trigger to undergo this differentiation process (48,49), while for *C. moewusii*, other nutrient limitations are also effective in producing gametes (2), although this is less well understood. Light is a signal that can trigger *Chlamydomonas* gametogenesis and in *C. reinhardtii*, the role of a blue-light receptor in inducing mating competence has been investigated (50,51). Nitrogen starvation induces vegetative cells to differentiate into pregametes, while further progression of the program to form gametes requires the action of light (51). Although the results and the model the authors propose for the induction of gametogenesis in *C. reinhardtii*, light is also important for gametogenesis in *C. moewusii* (51).

Routinely, gametogenesis of *C. moewusii* is achieved on solid agar media. When 2- to 3-week-old cultures are flooded with water, the cells become motile and are mating competent for several days. Tomson et al. (2) reported that gametogenesis in *C. moewusii* can occur at the end of the exponential phase of vegetative growth without nitrogen deficiency and may be triggered by other nutrient stresses. In contrast, Zachleder et al. (52) reported that in synchronized high-density cultures, a gametic stage exists as a regular part of the cell cycle. This gametic phase extends from the release of daughter cells until about two hours prior to
the point where the cells are committed for the next cell division. Molendijk et al. (4) however, grew the cells at a lower cell density and did not observe gametogenesis, and so they concluded that gametogenesis in *C. moewusii* is not a normal phase of the cell cycle.

In some strains of *C. moewusii*, light influences flagellar adhesiveness of gametes (53,54). Light-sensitive strains lose their flagellar agglutinability in the dark as a consequence of the inactivation of flagellar agglutinins. When illuminated they quickly become agglutinable again. Sexual reproduction starts when *mt*⁺ and *mt*⁻ gametes are mixed in the light (Figure 5). The cells instantaneously form large clumps of agglutinating cells by random contact and adhesion.

![Gametogenesis](image)

**Fig. 5** Schematic presentation of the sexual life cycle of *C. moewusii*

Under the microscope, these cell clumps have a characteristic vibrating appearance (55). The adhesion of gametes with those of the opposite mating type is caused by extrinsic glycoproteins on their flagella called agglutinins (41,56-59). Within minutes, the cells in the clumps become aligned, sticking to each other along the whole length of their flagella. During this alignment, the agglutinin molecules involved in adhesion are transported to the flagellar tip, a process called tipping (22,60). As a consequence of this process and the fact that *mt*⁻ gametes bend their flagella back around their cell bodies, gametes are sorted into pairs of opposite mating type whereby the two anterior ends of the interacting cells are brought into close proximity, the so-called vis-à-vis orientation (60,61). During this alignment process in *C. reinhardtii*, a gamete lytic enzyme (GLE) (62,63) dissolves the cell wall. Two proposals for activation of this enzyme have been presented: one whereby the secretion of a specific serine protease activates GLE in the extracellular matrix (62), while in the other, in analogy with collagenase family members, GLE is proposed to have an autocatalytic activation site, which results in the conversion of latent proenzyme into an active enzyme (63). The aligned naked *C. reinhardtii* gametes then fuse and form a quadriflagellate cell. Presumably *C.*
moewusii produces a similar lysin but its activity is restricted to a specific part of the cell wall between the flagellar bases. During this degradation of the cell wall, a protoplasmic protrusion, called the mating structure or papilla, is formed at the anterior end that protrudes through the wall. It is by means of their mating structures that C. moewusii gametes fuse together and form a vis-à-vis pair. The two cells do not fuse quickly together as in C. reinhardti, but remain joined for hours by the simply plasma bridge that results from mating structure fusion. The fusion process can be inhibited in C. moewusii by 5 mM cysteine, which is thought to interfere with lysin activity. The agglutination process is then prolonged for many hours (64). After fusion, the flagella lose much of their adhesiveness, stop agglutinating and the $mt^+$ flagella regain their swimming activity, while the $mt^-$ flagella are held immotile around the $mt^+$ body (65,66). About 6-8 hours after vis-à-vis pair formation, the pair retracts its flagella, the plasma bridge widens and a stage is reached which allows the fusion of the nuclei (67,68). The resulting diploid zygote matures during the next 24-48 hours, after which it can undergo meiosis under favorable environmental conditions, producing two $mt^+$ and two $mt^-$ vegetative haploid progeny.

Although C. reinhardti and C. moewusii belong to the same genus, they are distantly related to each other (69,70) and consequently differ in many aspects of sexual reproduction and are not sexually compatible (71).

The mating type of each new cell is determined by the mating-type locus. The locus of C. reinhardti, which resides in the left arm of linkage group VI, has recently been cloned (72) and appears to be a large (~1 Mb) region under recombinational suppression. It is composed of three domains: a centromere-proximal domain, a telomere-proximal domain in which the $mt^+$ and $mt^-$ sequences are homologous and a central region in which the $mt^+$ and $mt^-$ sequences show inversions, deletions/insertions and nonhomologous regions within a 190 kb sector. The entire locus is likely to be a mosaic of housekeeping and life-cycle-related genes, with genes restricted to one or the other mating type possibly embedded within the rearranged domain that governs the mating type (73). Some of the nonhomologous regions contain genes coding for structural and regulatory proteins unique to either $mt^+$ or $mt^-$ gametes (74). There is switching of mating type in homothallic Chlamydomonas species.
Chapter 1

6 Cell-cell interaction and cell fusion in other organisms

In studying cell-cell interaction between Chlamydomonas gametes, the concept of an agglutinin complex composed of the agglutinin and its membrane anchor had similarities to adhesion complexes described for mammalian and other cells. Because the potential homology between these molecule complexes has played an important role in this research, a brief description of these mammalian counterparts will be given.

Cell recognition and cell adhesion have been more widely studied in animal systems (75,76) than in plants, and membrane fusion is an important consequence in many different cellular interactions, e.g. leukocyte adhesion, fertilization etc. Two glycoprotein families have been implicated in these processes and are relevant to Chlamydomonas:

1. A family of cell adhesion molecules that is involved in Ca\(^{2+}\)-dependent binding processes are the integrins (76). They occur in many different species as highly conserved \(\alpha \beta\)-heterodimeric cell surface glycoproteins with a large extracellular domain, a single membrane spanning region, and usually a short cytoplasmic domain (77). Signal transduction to the cell interior via integrins in mammalian, yeast and plant cells (78-81) is mediated in at least two ways. One is by direct association with the F-actin cytoskeleton through linking proteins such as \(\alpha\)-actinin and talin. This pathway regulates cell shape, differentiation and cytoplasmic organization (82-85). The other occurs indirectly through biochemical processes where transmission of extracellular signals into the cell takes place via modulation of, for instance, tyrosine kinase activity, cell alkalinization and/or, transient Ca\(^{2+}\) increases in the cytoplasm (25,86-89). Cell-cell recognition whereby integrins are involved is restricted to the globular head of the integrin molecule (25,90). This extracellular domain recognizes specifically the tripeptide Arg-Gly-Asp (RGD) motif of the counter peptide (91-93). Antibodies against that specific integrin domain or a tripeptide containing this RGD-sequence could bind to and mask the external integrin domain and thereby inactivate all the functions of such protein (94).

2. A family of membrane-anchored, disintegrin-domain-containing proteins may also be involved in cell-cell interactions (95). Disintegrins themselves are a family of soluble peptides from snake venoms that contain an amino acid sequence that resembles the RGD containing sequence present in several extracellular matrix ligands that bind integrins (92,96-98). In addition, it is now realized that snake venoms are derived from larger multifunctional protein (95). Examples of such large snake venoms are cyritestin, metargidin and fertilin (99-102), the last one being a popular protein for research because of its involvement in sperm-egg fusion. They are related to a family of soluble snake venom proteins because they contain a
metalloprotease domain as well as an integrin-binding (disintegrin) domain (103,104). This family is named ADAM, for membrane proteins containing A Disintegrin And Metalloprotease domain (105). They have now been found in a variety of tissues and species. These proteins are believed to be important for cell-cell and cell-matrix interactions and may also participate in membrane-fusion events (76,106).

Here one can hypothesize similar modes of activity for *Chlamydomonas* agglutinins and animal cell adhesion molecules. Besides serving as transmembrane linkers between the ECM and the cytoskeleton, integrins are capable of transducing biochemical signals across the plasma membrane to regulate various cellular functions. When ECM ligands bind integrins, they are aggregated into protein clusters, designated as focal adhesions or focal contacts. Formation of focal adhesions increases intracellular tyrosine phosphorylation, even though integrins are not themselves tyrosine kinases (89). The actual linkage between the cytoplasmic domains of integrins and the cytoskeleton appears to be mediated by a complex structure of focal adhesion-associated proteins. Through protein interactions, multiple focal adhesion tyrosine kinase molecules (pp125FAK) are also aggregated within the focal contacts, whereby transphosphorylation presumably occurs leading to activation of other signaling molecules (107). Although it is not known whether this analogy is applicable to *Chlamydomonas* agglutinins, it does provide a model of downstream events that could result from agglutinin clustering in the flagellar membrane.

7 Outline of this thesis

As discussed above, *Chlamydomonas* flagellar agglutinins are the molecules involved in the initial recognition between two compatible gametes. The intracellular events following this interaction are very complex. Signals are transduced and different genes are activated whose translation products are needed to maintain agglutination, promote steps leading to sexual cell fusion and prepare the cell for later differentiation into a zygote. The major aim of this thesis was to discover genes whose expression is increased during sexual agglutination. We expected to find proteins involved in flagellar assembly as well as proteins responsible for flagellar protein modification, for example the agglutinins and agglutinin associated proteins that are needed to promote and maintain agglutinability. Because of the parallels that have been drawn between the agglutinin anchor and integrins, a cDNA library from *C. moewusii* gametes was screened for proteins similar to the classic mammalian integrins. The result of that screen is described in Chapter 2, in which a gene is characterized that codes for a putative
transmembrane protein with a RGD binding motif that is possibly involved in intracellular signalling transduction. This study is extended in Chapter 3 by using antibodies against this protein to show that it could be the membrane anchor protein for the agglutinin, or at least part of the agglutinin-complex involved in the redistribution of agglutinin molecules during sexual agglutination.

The flagellar surface of *C. moewusii* is occupied by several hydroxyproline-rich glycoproteins and in Chapter 4, the partial characterization of a cDNA encoding a flagellar glycoprotein with homology to a *Volvox* extracellular matrix protein is described. A characteristic of many glycoproteins on the flagellar surface is that they contain methylated sugars. In this respect, two other cDNA clones that have been characterized are interesting, because one of them codes for a methionine synthase. They are described in Chapter 5.

References


