Sexual adhesion of Chlamydomonas moewusii; A molecular approach
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CMB1p is the flagellar protein that connects the agglutinin to the sexual signalling pathway in *Chlamydomonas moewusii*
Abstract

The gene CMB1 encodes a Chlamydomonas moewusii protein with a putative extracellular matrix attachment site and a predicted membrane spanning domain. Using antibodies raised against a synthetic peptide in CMB1p, we have confirmed that the native protein is a 125 kDa membrane-bound flagellar glycoprotein. Evidence is presented that CMB1p is associated with the agglutinin complex and influences its activity. First, immunofluorescence studies show that during sexual stimulation the protein comigrates to the flagellar tips (“tipping”) with the agglutinin complex. Second, adding a peptide whose sequence is present in the extracellular domain of CMB1p can block the successful completion of sexual fusion.

Introduction

The sexual cycle of Chlamydomonas moewusii is initiated when the flagella of compatible gametes adhere together. Both mating types contain receptor molecules, called m/r+ and m/r- agglutinins, on the outer surfaces of the respective flagellar membranes. They are responsible for gamete recognition, the mating type and species specificity of sexual agglutination (1-3). The interaction between agglutinins triggers the formation of intracellular signals by which mating responses are induced. The mechanism by which the signal is transmitted over the membrane is unknown, but it must involve an intrinsic membrane protein, which anchors the extra flagellar agglutinin to the membrane surface (4). The redistribution of the agglutinins over the flagellar surface is important for reorientating the gamete for sexual fusion. During redistribution, these glycoproteins migrate along the flagellar surface to the tips, presumably driven by an axonemal motor. Previous publications showed that cross-linking the agglutinins using a monoclonal antibody artificially induced the tipping process together with most of the responses associated with sexual agglutination (5). Kooijman et al. (6) reported that other flagellar glycoproteins co-migrated with the agglutinins to the flagellar tips. After treating gametes with wheat germ agglutinin (WGA), which does not bind to the agglutinin itself but to a 123 kDa protein in the flagellar membrane, WGA was transported to the tips. Also a glycoprotein named PAS 6 was shown to be associated with the agglutinin (7,8). It consists of four components (9), one of which is recognized by an anti-integrin $\beta_1$ antiserum (10). Since in mammalian cells contact between the extracellular matrix and the cytoskeleton can be
mediated by integrins (11), this PAS 6 "integrin" could serve as the connector between the extracellular agglutinins and the motor molecules of the intracellular cytoskeleton.

In this report we describe a 125 kDa membrane protein called CMB1p that is associated with the agglutinin complex on the flagellar membrane of *C. moewusi*. It contains an RGD sequence that is important for agglutination, and is tipped together with agglutinins during sexual adhesion. On the basis of these results we argue that this protein is part of the agglutinin complex.

**Material and Methods**

**Cell cultures**

*Chlamydomonas moewusi*, strains 11/5-10, mating type minus (*mt−*, from the Sammlung von Algenkulturen, Göttingen, Germany) and 17.17.2 (*mt+*, (12)) were cultivated in Petri dishes on agar-containing M1 medium (13) and maintained at 20 °C in a 12 h light/12 h dark regime with an average photon flux of 30 µE m⁻² sec⁻¹ using fluorescent tubes (TL 65W/33, Philips, Eindhoven, The Netherlands). A suspension of swimming gametes was obtained by flooding three-week-old cultures with distilled water at the start of the dark period. Cells were harvested as sexually competent gamete 2-4 h after the onset of the following light period.

**Isolation of flagella and flagellar membranes**

Gametes of the *mt−* mating type were deflagellated by pH shock (14), and the flagella and cell bodies separated by centrifugation on a cushion of 25% sucrose (1000 x g, 10 min). This procedure was carried out twice. The flagella were harvested by centrifugation at 12,000 x g for 20 min. Flagellar membrane vesicles which were formed during deflagellation were collected by centrifugation at 50,000 x g for 60 min.

Another type of flagellar membrane vesicle, referred to as isoagglutinin, was isolated from a culture liquid of the *C. moewusi* strain 11-5/d, which is an active producer of isoagglutinin. A gamete suspension was centrifuged for 10 min at 1000 x g to remove the cells and then for 20 min at 10,000 x g to remove cell debris. The supernatant was filtered through a Millipore filter type RA with a pore size of 1.2 µm, and the filtrate concentrated to a volume of 100 ml in an Amicon CH4 concentrator fitted with a Diaflo hollow-fiber membrane cartridge (H1P10,
Chapter 3

retention 10,000 Da). A crude isoagglutinin pellet was obtained by centrifugation for 60 min, at 50,000 x g. The pellet was homogenized in 2 ml Hepes buffer (10 mM Hepes, pH 7.4) and purified on a cushion of 2.93 M CsCl in Hepes buffer (20,000 x g 60 min, in a 2 ml Eppendorf tube (15)). The isoagglutinin vesicles present in the upper phase and the interphase were collected. The CsCl centrifugation step was repeated, and the isoagglutinin vesicles were harvested by centrifugation for 60 min at 50,000 x g, washed in Hepes buffer and stored at 20 °C.

Extraction of membrane proteins

Isoagglutinin was incubated with 3M guanidine thiocyanate (GTC) in Hepes buffer for 60 min at 4 °C and centrifuged at 50,000 x g for 60 min. The supernatant was dialyzed against water at 4 °C to remove the GTC, and freeze dried.

Flagella were incubated with 100 mM sodium carbonate (Na₂CO₃) at 0 °C for 30 min. The suspension was centrifuged at room temperature for 5 min at 100,000 x g in a Beckman air driven centrifuge. The supernatant was dialyzed against water at 4 °C and freeze-dried. The membrane extracts were dissolved in 100 µl SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 10% glycerol, bromophenol blue) for polyacrylamide gel electrophoresis (PAGE) according to Laemmli (16).

Immunization

The total cDNA sequence of clone CMB1 isolated from a screen of a cDNA expression library from C. moewusii was scanned with the model of Hopp and Woods (PROSIS, Hitachi) to predict the most immunogenic regions. This program takes into account the secondary structure and solvent accessibility associated with probability values. The following sequences were selected: n-CFEKILALSDKRGEPYA-c and n-MAIDGEAPSVGDTPIKSVLC-c (amino acids 89-105 at the N-terminal part and 459-478 at the C-terminal part, respectively (Chapter 2, Figure 1). The regions were synthesized and coupled by the cysteine to the carrier protein Keyhole Limpet Haemocyanin (KLH). Two rabbits were immunized (Eurogentec, Seraing, Belgium) with a mixture of the two CMB1 gene-product specific peptides to raise antibodies. This rabbit serum (SN104) was used in Western blot and immunofluorescence analyses.
Purification of Anti-CMB1p antibodies

The anti-CMB1p antiserum was raised in rabbits against a mixture of the two CMB1p peptides conjugated to KLH by Eurogentec (Seraing, Belgium) and was purified using affinity chromatography. Synthetic protein representing the N-terminal parts was immobilized on CNBr-activated Sepharose 4B and purified as described by the manufacturers for the ImmunoPure Ag/Ab Immobilization Kit (Pierce Europe, Holland). Finally, the purified antibody solution was concentrated and stored at -20°C.

Gel electrophoresis and Western blot analysis

Electrophoresis was performed on linear gradient (2.2-20%) polyacrylamide gels according to Laemmli (16). Gels were stained with 0.125% Coomassie Brilliant Blue in 40% methanol and 10% acetic acid to visualize the proteins or alternatively, the proteins were transferred to an Immobilon polyvinylidine-difluoride (PVDF, Millipore, Etten-Leur, The Netherlands) membrane for Western blot analysis. The membrane was incubated in block buffer (5% (w/v) non-fat milk powder (Elk, DMV Campina BV, Eindhoven, The Netherlands) in phosphate buffered saline (PBS; 10 mM phosphate buffer, pH 7.3, 150 mM NaCl) for 2 h at room temperature. The membrane was subsequently incubated with a 1:100 dilution of Anti-CMBp serum in block buffer. The blot was washed 3 times in PBS-0.01% Tween 20 and binding of the antibody was detected with goat-anti-rabbit IgG-peroxidase conjugates (GARPO, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and developed using a chemiluminescence detection kit (ECL kit, Amersham Life Science, Great Britain).

The CMB1 sense/anti-sense constructs in plasmid pBluescript (pSK) were located behind the β-lactamase gene for inducing transcription/translation with IPTG. The E. coli culture was induced when the cells were in the exponential phase. After 4 h the cells were collected and the total protein extract was used for Western blot analysis.

Immunofluorescence

Cells fixed in 1.25% glutaraldehyde were washed 3 times in phosphate buffered saline (PBS) and incubated for 1 h with the appropriate antibody (anti-CMB1p or Mab 66.3) diluted 100 times in PBS. Non-bound antibodies were removed by washing twice in PBS. Bound antibodies were labeled with goat-anti-rabbit-fluorescein isothiocyanate (GAR-FITC, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) or goat-anti-mouse-FITC respectively. A droplet of the cell suspension was applied to a siliconized slide that was previously coated
with 2% agarose and placed on a hot plate to dry. When the liquid had evaporated, the cells were covered with 20 μl glycerol/PBS solution (1:1, v/v, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) to protect the FITC-label for quenching and covered with a coverslip. As a control, a monoclonal antibody (Mab 44.2) was used that recognizes epitopes on several glycoproteins that are not redistributed (17).

The goat-anti-mouse-FITC (GAM-FITC) antibody did not cross-react with the antibodies raised in rabbits (the SN104 serum), and vice versa the goat-anti-rabbit-FITC (GAR-FITC) antibody did not recognize the mouse monoclonal antibody (Mab 66.3).

Confocal microscopy and image analysis
Images were recorded using a vertical Leica confocal laser-scanning microscope (Leica CLSM, Heidelberg, Germany). The fluorochrome FITC was excited using the 488 nm line of an argon laser in combination with a 510 nm dichroic mirror. Dual detection was performed by using a 615 nm longpass filter to detect the signal from the autofluorescence of the cell body and simultaneously a 525 DF 10 bandpass filter to detect the FITC signal. The gametes were scanned using either a 40x or a 100x objective (pixel size 0.245μm lateral / 0.417μm axial), and each Z-scan was the result of 8 times line sampling to produce a high signal to noise ratio. Before the actual measurements took place, images were separated into an A-file (autofluorescence signal) and a B-file (FITC signal). All separated file images of the Z-scan were compressed to a 2D image and the processing was performed with Scilimage software (18). Digital images were made with the program Object-Image (19) (http://www.bio.uva.nl/norbert/object-image.html) which is an improved version of NIH-Image (ftp://zippy.nih.gov). A Gaussian filter (20) was applied to the A- and B-files to remove noise components in the images and finally, both files were combined into the complete picture.

Effects of antibodies on flagellar membrane protein redistribution
During agglutination, two of the most prominent responses are a change in morphology of the flagellar tip from a pointed to a bulbous form due to the extension of the peripheral microtubuli in the axoneme, described as “flagellar tip activation” (21,22), and the transport of agglutinins involved in adhesion to the flagellar tip, described as “tipping” (23). Flagellar tip activation was induced by treating mt- gametes for one hour with Mab66.3, a mouse monoclonal antibody that is specifically directed towards the mt- agglutinin. The properties of this antibody have been described previously by Homan et al. (5). After this treatment, cells
were fixed in glutaraldehyde and used for indirect immunofluorescence. To visualize tipping, living gametes were incubated with Mab66.3 for various times. Gametes were then fixed in glutaraldehyde (final conc. 1.25%) and the redistribution of antigenic glycoproteins to the flagellar tips was assessed by indirect immunofluorescence.

Effect of RGD peptides on gamete interaction
The ARGDQV peptide and the control peptide AGDRQV were tested for their ability to inhibit the interaction between rat embryo fibroblasts and vitronectin. Peptides from Eurogentec were lyophilized and dissolved in 100μl PBS (pH 7.3). The peptide ARGDQV inhibited this reaction in a concentration dependent manner.

The effect of the same peptides on sexual agglutination between C. moewusii gametes was studied by pre-incubating them with the synthetic peptides at different concentrations (0.25 mM - 5 mM) for 10 minutes. After mixing the cells they were allowed to mate for 30 min, after which they were fixed and the number of vis-à-vis pairs formed was calculated.
Results

Western blot analysis of CMB1p.

As described in the previous chapter, we isolated the CMB1 gene by screening a cDNA expression library with an anti-integrin antibody (RM12). From its sequence, two synthetic oligopeptides were designed and used to raise a polyclonal antiserum (SN104) in order to locate CMB1p in the C. moewusii gamete.

![Western blot analysis](image)

Fig. 1 Western blot analysis of CMB1p in *E. coli* and flagellar extracts

A. Protein extract from *E. coli* JM109 cells with the CMB1 cDNA in the sense (lanes 1 and 2) or anti-sense (lanes 3 and 4) orientation after 4 hr induction with IPTG. The anti-serum (SN104) raised against a synthetic peptide of CMB1p was used in lanes 1 and 4 in a 1:250 dilution, while a SN104 affinity purified antibody was used in lanes 2 and 3 in a 1:500 dilution.

B. Protein extracts from *C. moewusii* flagella. Lane 1: the anti-serum (SN104) against a synthetic peptide of CMB1p was used in a 1:250 dilution. Lane 2: the anti-integrin antibody (RM12) raised against a synthetic peptide representing the cytoplasmic region of β-integrins was used in a 1:500 dilution.
Using SN104 antibodies, several proteins were detected in Western blots of an extract of *E. coli* expressing the *CMB1* gene (Figure 1A), including a protein with a Mr of 85 kDa, which corresponds with the calculated mass based on the cDNA sequence of *CMB1* plus the in-frame part of the β-lactamase gene. When SN104 was affinity purified, only an 85 kDa and an 18 kDa protein were detected. It seems probable, therefore, that the 85 kDa protein was the gene product of *CMB1*, whereas the other was a breakdown product. In protein extracts of *E. coli* cells harbouring the antisense *CMB1*, no proteins of this size were detected with SN104, indicating that the antibodies recognized specific *CMB1* gene products.

The crude SN104 antiserum was also used to detect the native *CMB1* product in *C. moewusi*. Swimming gametes (*nuT*) were subjected to pH shock to dissociate their flagella (14). Cell bodies and flagella were then separated by centrifugation on a cushion of 25% sucrose. As shown in panel B of Figure 1, the SN104 antiserum bound only to proteins of 125, 32 and 18 kDa in blots of flagellar proteins. The anti-integrin antibody (RM12) which was originally used to select this gene, did not cross react with the 125 kDa protein as even through another anti-integrin antibody (9) has been reported to recognize a flagellar protein of such size. In Figure 2, a more detailed immuno-analysis of *C. moewusi* protein is presented. A comparison of flagellar and cell body proteins (Panel A) showed that the 125 kDa protein and the two smaller proteins, which we suggest are break-down products of the larger protein, were not detectable in cell bodies. The 125 kDa protein was also detected in flagellar membrane vesicles formed during deflagellation and also in isoagglutinin, which also consists of flagellar membrane material shed from mating competent gametes into their culture medium. ((24); Panel B). When these membrane preparations were extracted with guanidine thiocyanate or with Na₂CO₃ (data not shown), the 125 kDa protein recognized by the SN104 serum remained in the pellet. The presence of this immuno-reactive protein in the purified flagellar membranes and the lack of extraction by chaotropic agents, suggests that it is an integral membrane protein, supporting the predictions made on the basis of the amino acid sequence (see Chapter 2).
Fig. 2 Localization of the putative CMB1 protein in C. moewusii mf− gametes
Protein extracts from cell bodies and flagella were subjected to Western blotting and immuno-reactive bands detected with SN104 anti serum and visualized with a secondary fluorescent antibody.
A. Isolated flagellar (Fl) or cell body (Cb) protein from an equal number of cells, immunoblotted with SN104.
B. Flagellar membranes (Fl), membrane vesicles (Vs) and isoagglutinin (Iso) proteins immunoblotted with SN104.
C. Association of the putative CMB1 protein with flagellar membranes. GTC extracted flagella were pelleted and both fractions separated and immunoblotted with SN104.

*In vivo* localization of CMB1p by immunofluorescence

If CMB1p is a flagellar protein, it should be possible to detect it in the flagella using the antiseraum SN104 in an indirect immunofluorescence assay. Since one of the synthetic peptides used to raise SN104 is predicted to have an extracellular location in CMB1p, it should be possible to detect it using intact non-permeabilized cells. Indeed, that was the case, as shown is the experiment presented below. However, in general the labelling intensity was improved if the cells were first permeabilized by 1% Triton X-100, as if this exposed the second intracellular antigenic site of the peptide. These results support the contention that CMB1p is a transmembrane protein in flagella.

A characteristic feature of some flagellar glycoproteins in Chlamydomonas is that they exhibit lateral mobility, induced by interaction with flagellar components of the opposite mating type during sexual adhesion (agglutinin-agglutinin interaction), or by lateral cross-
linking with antibodies. These treatments result in redistribution, culminating in the concentration of the glycoproteins at the flagellar tips. In *C. reinhardtii*, antibody treatment has sometimes been seen to result in the concentration of antigen at the flagellar base (25). In *C. moewusii*, lateral mobility is thought to be restricted to the agglutinins and associated membrane proteins and is best induced by treating gametes with low concentrations of specific antibodies (26). One of the most effective antibodies in this respect is the monoclonal antibody Mab 66.3, raised against, and reacting specifically with, a purified agglutinin preparation (5). Another monoclonal antibody, Mab 44.2, raised against a more predominant class of flagellar glycoproteins in this species and not cross-reacting with the agglutinins, did not evoke redistribution nor were they redistributed with the tipped agglutinins. The fact that redistribution seems specific for the agglutinins can be used to designate which glycoproteins are associated with them, because they will be co-distributed. The possibility that CMB1p is associated with agglutinins is of considerable interest, since the agglutinins are extrinsic proteins, and one expects them to be associated with intrinsic proteins, by which they are anchored to the flagellar membrane. We therefore tested whether CMB1p co-distributes with the agglutinin.
Fig. 3 CMB1p co-migrates with flagellar agglutinin during sexual stimulation

Gametes (mt) were incubated with various antibodies, fixed and the antibodies detected by indirect immunofluorescence using a confocal laser scanning microscope.

A. Gametes were sexually activated by incubating them with Mab 66.3 for 0, 2.5, 5 and 15 min and the primary antibody visualized with GAM-FITC. C depicts a cell stained with Mab 44.2 to show that not all flagellar proteins were redistributed.

B. Gametes were incubated for 30 min with Mab 66.3, fixed, pretreated with 1% Triton-X100 (B-1 and B-2) or water (B-3) and incubated with SN104 serum. The binding of SN104 was visualized with GAR-FITC.

C. Gametes were incubated for 30 min with SN104 serum, fixed and incubated with Mab 66.3. Binding of the antibody was visualized with GAM-FITC. The control (C) represents cells treated in the same manner but without Mab 66.3 to show that GAM-FITC does not label bound SN104.
Gametes were treated with Mab 66.3, fixed with glutaraldehyde at different time points, and incubated with a secondary FITC-labeled anti-mouse antibody (GAM-FITC) to visualize the location of Mab 66.3. The results are shown in Figure 3A. These photographs are compound pictures representing the red autofluorescence of chlorophyll in the cell body and the yellow-green fluorescence of the flagellar agents. The images are combined to give a realistic picture of *C. moewusi* cells, but we emphasize that immunolabelling was restricted to the flagella. At time 0, antibody binding was not visible, because the agglutinins constitute only a minor portion of the total set of flagellar surface proteins that are evenly dispersed over the flagella surface. However, within 1 minute of adding Mab 66.3, fluorescent spots became visible, reflecting the clustering of agglutinins into concentrated spots that were readily detected by indirect immunofluorescence. Extensive redistribution to the tips was visible after 5 - 15 min and sometimes even after 2.5 min. As expected, antigen redistribution was not seen after incubating the cells with Mab 44.2 (Figure 3A, control).

In order to examine whether CMB1p was co-distributed with agglutinin, cells were treated with Mab 66.3, fixed after 30 min and consecutively incubated with SN104 and a secondary FITC-labeled anti rabbit (GAR-FITC) antibody. Cells were found to have SN104-reactive proteins at the tips and at the bases of the flagella (Figure 3B, panel 3). When fixed cells were permeabilized with Triton X-100, a stronger fluorescent signal was obtained (panel 1 and 2) which revealed a speckled staining along the flagellar surface. Presumably, permeabilization resulted in increased accessibility of the (intracellular) epitopes. The result of this experiment suggests that CMB1p, to which SN104 was directed, is associated with the agglutinin and on cross-linking the agglutinin, be co-distributed to the flagellar tips. It should be noted that SN104 did not cross-react with the agglutinin or any other flagellar glycoprotein. Neither did the GAR-FITC cross-react with Mab 66.3.

Panel 3C shows that it was also possible to induce the redistribution of agglutinin by treating gametes with SN104. Cells were incubated with SN104 for 60 min, fixed, incubated with Mab 66.3 and the Mab 66.3 visualized with GAM-FITC. The fluorescent spots representing the agglutinin molecules were clearly visible along the whole length of the flagella, with a tendency to become concentrated at the tips indicating that cross-linking CMB1p molecules with SN104 induced both the clustering and tipping of agglutinin molecules. These results strongly suggest that CMB1p is co-distributed with the agglutinins and therefore may be part of the mating receptor complex. In these experiments the overall pattern of fluorescence was more or less the same whether Mab 66.3 or SN104 induced
redistribution. However, the kinetics of redistribution are slower with SN104 compared Mab 66.3 (3A) and the degree of tipping was less pronounced. This may simply reflect the fact that different types of antibodies to different antigens were used, for their affinity and/or accessibility to the antigenic site(s) could be very different.

Inhibition of gamete interaction by RGD peptides

Since CMB1 contains an RGD sequence, as mentioned in chapter 2, that could be involved in protein-protein interactions (27-30), we raised the question as to whether this sequence was important in the sexual adhesion process. The biological activity of RGD-containing peptides is usually assayed in mammalian systems by their capacity to inhibit adhesion of cells to a particular extracellular matrix in which the RGD sequence is present. The biological activity of the synthetic peptide ARGDQV, present in the CMB1 gene, and its control peptide AGDRQV were therefore tested in a mammalian system. This experiment demonstrated that the ARGDQV peptide inhibited the interaction between rat embryo fibroblasts and vitronectin in a concentration dependent manner, with a maximum of 87% inhibition when tested at 5mM. We therefore tested whether RGD containing peptides could also influence sexual agglutination and the ensuing level of sexual fusion between C. moewusii gametes. Mixed gametes first agglutinate at random but gradually sort themselves out into pairs, which are correctly aligned for fusion, resulting in the formation of vis-à-vis pairs. When m+ and m− gametes were pre-incubated for 10 min with different concentrations of RGD-containing peptides, mixed and allowed to mate for 30 min, vis-à-vis pair formation was inhibited in a concentration-dependent manner, as shown in Figure 4. The control treatments using peptides consisting of the same amino acids in different orders gave significantly less inhibition. The ARGDQV peptide that completely matches the motif in CMB1p completely blocked the formation of vis-à-vis pairs when used in a concentration of 5mM. In a control experiment (data not shown) cells were incubated with the same RGD-containing peptides and then washed and resuspended in conditioned medium. These cells recovered their agglutinability and fusion competence, illustrating that the protein was biological active without affecting cell viability.
Fig. 4 Effect of RGD and related peptides on gamete fusion
C. moewusi cells (mi- and mi+) were incubated for 10 min with different concentrations of the peptides indicated. They were then mixed, allowed to mate for 30 min and fixed. Samples were collected and the percentage of vis-à-vis pairs at that moment was determined. All values are normalized to 100% vis-à-vis pair formation compared with the untreated controls.

The inhibition of vis-à-vis pair formation could be due to the disturbance of sexual agglutination (and the ensuing signaling) or to the prevention of cell fusion or both. Therefore the agglutinability of gametes treated with 5 mM peptide was tested in triplicate by incubating them with a serially diluted suspension of untreated gametes of the opposite mating type. The strongest dilution that still showed agglutination was then taken as a measure of the agglutinability of the treated cells. As shown in Table 1, incubating gametes overnight with an RGD-containing peptide significantly reduced agglutinability. Interestingly, only the mi− mating type was susceptible, suggesting that this sequence be involved in maintaining the integrity of a functional mi+ mating receptor complex in the flagellar membrane, in which CMB1p might be a component. Cells with reduced agglutinability re-established their agglutinability when washed several times in conditioned culture medium, re-confirming that treatment did not affect cell vitality.
Table 1 Effect of 5mM peptide treatment on the agglutinability of \( ml^- \) and \( mt^+ \) gametes.

<table>
<thead>
<tr>
<th>Biological activity (titer)</th>
<th>Mating type plus</th>
<th>Mating type minus</th>
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<tbody>
<tr>
<td>CONTROL</td>
<td>( 2^6 )</td>
<td>( 2^6 )</td>
</tr>
<tr>
<td>ARGDQV</td>
<td>( 2^4 )</td>
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<tr>
<td>AGDRQV</td>
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A gamete suspension was incubated overnight with the different synthetic peptides (5 mM). The highest dilution that still agglutinated the test cells is referred to as the agglutination titer. Cells were then tested for agglutination with several dilutions of gametes of the opposite mating type, diluted in conditioned medium.

**Discussion**

Using an antibody raised against predicted sequences of a flagella-specific gene product CMB1p, we have detected a 125 kDa protein, which may be associated with agglutinins on the flagellar surface, as can be deduced from their common redistribution pattern during sexual stimulation. The antibody also recognize 32 kDa and 18 proteins which were probably breakdown products. Completely absence of these proteins in vesicles is perhaps related to the extended process of purification, removing possible breakdown products.

Because of the discrepancy between the mass of the \( E. coli \) protein (85 kDa) and the presence of N-glycosylation sites in the deduced protein sequence, we assume that the CMB1p is a glycosylated protein. A 125 kDa protein (PAS6) in \( C. moewusii \) flagella was also described by Kalshoven et al. (9), which was shown to be associated with the agglutinins in a manner similar to that described above. This protein was shown to bind wheat germ agglutinin (WGA) (9). Gametes were also shown to exhibit sexual reactions when incubated with this lectin. Whether CMB1p binds WGA, has not been established, but is feasible since the considerable increase in molecular mass is presumably caused by extensive N-glycosylation, which could confer affinity for WGA.
In the immuno screen (Chapter 2) an antibody (RM12) was used which was raised against a synthetic peptide that codes for the cytoplasmic part of the $\beta_1$-subunit of a human $\beta$-integrin. The same amino acid sequence was utilized for raising antibodies by Marcantonio et al (10), and these were used by Kalshoven and co-workers (9) to detect a 125 kDa protein in flagellar extracts. However, we were not able to detect a 125 kDa protein with the RM12 anti integrin antibody possibly due to the dissimilarity of both sera. Therefore we decided to raise antibodies (SN104) against synthetic proteins representing our CMB1p, to make sure that in all the protein characterization experiments we were dealing with the protein coded by the CMB1 gene. The protein we have characterized exhibited a number of interesting features.

In the first place, it is an integral membrane protein present only in the flagella of *Chlamydomonas*. This is suggested by the CMB1p sequence, but also based upon the tight association of CMB1p with flagellar membrane preparations, including isoagglutinin that contains essentially only membrane bound proteins. Even treatment with chaotropic agents like GTC could not release all the CMB1p into solution.

In the second place, CMB1p is directly or indirectly associated with the agglutinin. This is based on the fact that both molecules were shown to migrate together over the flagellar membrane when cross-linked by either anti agglutinin antibodies or anti CMB1p (see Figure 5 on the next page).

The labelling of CMB1p using polyclonal antibody SN104 was considerably more intensive when the flagellar membrane was permeabilized with Triton X100 after fixation. After Triton treatment the SN104 antibodies could well able to reach the intracellular parts of flagella membrane proteins, and therefore more epitopes would be detected increasing the sensitivity. Another reason for the higher fluorescent level could be that Triton X100 the removes the flagella surface glycoproteins. A “fuzzy coat” of glycoproteins (31,32) which may prevent SN104 antibodies reaching the epitopes close to the flagellar surface covers the flagellar membrane. In comparison, the antibody Mab 66.3 is directed against the agglutinin which is exposed on the outside this glycocalyx, and labelling with the antibody is negatively influenced by Triton X100 treatment (data not shown).
Fig. 5 A model for antibody-induced redistribution of glycoproteins in the flagella of C. moewusi

Transport of glycoproteins (in particular the agglutinin-complex) over the flagellar surface is stimulated by cross-linking. The agglutinin complex contains epitopes for Mab 66.3 and SN104. Both antibodies cross-link the agglutinin-complex and induce their redistribution. Mab 66.3 recognizes the agglutinin molecule while SN104 cross-links the transmembrane protein part of the agglutinin-complex.

Tipping induced by Mab 66.3 or SN104 have different kinetics (compare Figure 3A and C). This can be explained by assuming that the SN104 antibodies have difficulty reaching their epitope. Moreover, this would mean that cross-linking epitopes on the membrane is also more difficult. The epitope for Mab 66.3 is readily available for recognition and cross-linking, therefore the tipping response can be expected to be much faster. Finally, it appears that not all SN104 epitopes were cross-linked and transported upon stimulation with Mab66.3 (Figure 3 panel B). This suggests that some of the CMB1 membrane proteins are not in the agglutinin complex and do not contribute to the transport of agglutinins to the flagellar tips. Taken
together these results strongly suggest that CMB1p is often part of a protein complex that includes the flagellar agglutinin. The presence of such a complex has also been inferred by reconstitution experiments, in which isolated agglutinin was shown to bind to agglutinin-free flagellar membrane vesicle (4). Interestingly, these experiments indicated that a membrane-bound glycoprotein of 125 kDa was necessary for reconstituting agglutinin activity. This protein could be CMB1p.

In view of the possibility that CMB1p has an agglutinin anchoring function, it is interesting to note that it has a histidine-rich domain, presumed to be intracellular, which could be associated with cytoskeletal actin. It has been argued by Bloodgood et al. (33) that cytoskelon-based motor proteins drive redistribution of flagellar membrane components. CMB1p might link the mating receptor complex to such proteins. Still, part of the CMB1p population is not tipped by treatment with Mab 66.3, but is concentrated at the basis of the flagella (Figure 3, panel B). This can only be seen in CSLM recordings, where the red autofluorescence of the chloroplast is filtered out and recorded in a different channel. Kalshoven et al. (6) reported a similar picture for Wheat Germ Agglutinin (WGA) induced tipping. This suggests that alignment of gametes for a bi-directional movement of flagellar proteins coordinate fusion. The presence of a CMB1 homologue in C. reinhardtii merits analysing the distribution of similar proteins in this species, where mutants are available with paralyzed flagella that are still capable of tipping. These strains could be used for a spatio-temporal analysis of agglutinin complex movement during sexual stimulation.

A striking feature of the redistribution of the glycoproteins recognized by Mab 66.3 on the flagellar surface was the fluorescence at the base of the flagellum which was never previously observed so well because of the autofluorescence of the chloroplast. The use of CSLM overcame this detection problem such that we could simultaneously observe the fluorescence label at the flagellar tips and base, which suggests that the CMB1p has the same properties as the WGA-binding protein. Kooiman et al. (6) reported that incubation of gametes with the lectin wheat germ agglutinin (WGA), which does not bind to the agglutinins, resulted in tipping of the agglutinin, but also in its accumulation at the flagellar base. So this WGA-induced redistribution was very similar to the effect induced by the SN104 serum.

Another interesting characteristic of CMB1p is that it contains a RGD sequence. In all eukaryotic systems this sequence is recognized by integrins, which play a role in transmembrane signaling (34,35). It was possible that CMB1p is involved in binding to other proteins, including an integrin-like constituent of the mating-receptor complex.
with ARGDQV, the RGD-containing oligopeptide representing CMB1p, perhaps dissociates the mating receptor complex and so prevents the effective agglutination. This detachment was not absolute because after washing the cells with normal medium full sexual competence returned and the gametes agglutinates as usual. This effect was particularly clear in the mt− mating strain, even though CMB1 is present in the genome of both strains. However, another, more direct role of CMB1p in sexual interaction is not excluded.

The characteristics of CMB1p but need further research to test them in more detail.

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References


Analysis of a flagellar protein


