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Differential screening identifies a Chlamydomonas moewusii cDNA encoding a serine / proline-rich glycoprotein

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Abstract

Sexual agglutination of Chlamydomonas moewusii gametes is a dynamic process, which requires the rapid turnover of flagellar membrane proteins. A differential screen was performed to isolate genes that are induced during prolonged gamete agglutination (PGA). PGA1 is described in detail in this chapter. PGA1 cDNA is 2100 base pairs in length with an open reading frame of 573 amino acids. PGA1 contains several Ser/Pro-rich regions suggesting that this cDNA code for a glycoprotein. The N- and C-terminal regions of the PGA1 cDNA display homology to a sulfated surface glycoprotein from Volvox (SSG185). The gene is expressed only in gametes and responds to signals elicited during agglutination. Expression of PGA1 is not affected by deflagellation or flagellar regeneration, suggesting that it is not coding for a structural flagellar protein.

Introduction

In C. moewusii cell fusion is initiated by gametes of opposite mating type adhering together via their flagella. These flagella contain glycoproteins exposed at the exterior, which are involved in the recognition process (1). They are called agglutinins (2-5). Agglutinins are large linear hydroxyproline rich glycoproteins bound as extrinsic membrane proteins on the flagellar surface (6-8) and are presumably the only components responsible for the recognition and adhesion of mating gametes. They generate one or more intracellular signals, which initiate a series of successive events that prepare cells for fusion (6,7,9,10). These include a change in flagellar motility (11) and morphology (12,13), an increase in flagellar adhesiveness (14,15) and transport of agglutinins to the flagellar tip “tipping” (16). During tipping, the more abundant flagellar glycoproteins that are not involved in mating remain evenly distributed over the flagellar surface.

Sugar moieties might be important in sexual recognition. The sensitivity of agglutinability for tunicamycin pre-treatment, which inhibits the transfer of N-glucosamine to glycoproteins (1), or enzymes which degrade the N-glycosidically-linked oligosaccharide chains (17), implies that these side chains are essential for the function of agglutinins. Many hydroxyproline-containing glycoproteins in C. reinhardtii (18) are sulphated.

Here, we report the cloning and characterization of PGA1 from C. moewusii, which is homologous to the sulphated glycoprotein SSG185 from the distantly related species Volvox.
This gene was cloned using an approach whereby an artificially high turnover of flagellar proteins was realized, so those genes coding for these proteins were transcribed more frequently than normal. *PGA1* is expressed in gametes only and is induced upon formation of vis-à-vis pairs. The potential function of this gene will be discussed.

### Material and Methods

#### Cultures

*Chlamydomonas moewusii*, strains 11/5-10, mating type *minus* (*mt* −, from the Sammlung von Algenkulturen, Göttingen, Germany) and 17.17.2 (*mt* +; (19)) were cultivated in Petri dishes on agar-containing M1 medium 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.

#### Flagellar regeneration and agglutination

To identify genes coding for flagellar proteins, gametes were deflagellated by pH shock (20) and were allowed to regenerate new flagella. Deflagellated cells were resuspended in 200 ml cell-free medium and placed under a high-pressure mercury lamp with a photon flux of 200 μE m⁻² sec⁻¹ while maintaining the temperature at 22 °C. During the regeneration process, 20 ml samples were taken at time intervals of 2 h, RNA was isolated and flagellar lengths were measured with an adapted version of NIH-Image 1.60.

For sexual agglutination *mt* + and *mt* − gametes (25 ml each of 2 x 10⁷ cells ml⁻¹) were mixed. Within minutes became aligned along the lengths of their flagella and gradually sorted out into pairs which fused and formed a vis-à-vis pairs (16,21). 10 ml samples of the agglutination mixture were taken at time intervals, RNA was extracted and the number of fused cells determined.
Chapter 4

DNA and RNA analysis

DNA was isolated by extracting cells in lysis buffer (2% CTAB, 100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA) for 1 h at 65 °C. The solution was extracted with an equal volume of phenol/chloroform. DNA was precipitated with 0.7 volume isopropanol and dissolved in water.

RNA was isolated as follows: cells were harvested by centrifugation for 2 min at 2000 g. Portions of 10^8 cells were resuspended in 500 μl TE buffer to which 700 μl hot SDS buffer (100 mM Tris-HCl, 600 mM NaCl, 10 mM EDTA, 4% SDS, 65°C) was added. The samples were quickly frozen in liquid nitrogen and held at -70°C. After thawing, 150 μl 3M NaAc pH 4.3 was added. The mixture was vortexed for 10 sec and put on ice for 5 min. After centrifugation for 5 min in an Eppendorf centrifuge at 4°C, the solution was extracted twice with phenol and once with chloroform. The nucleotides were precipitated with 96% ethanol and dissolved in 400 μl diethyl pyrocarbonate DEPC-treated water. LiCl was added to a final concentration of 2M and the solution was vortexed and put on ice for 16 h. The precipitated RNA was pelleted by centrifugation for 10 min. The pellet was resuspended in TE and after a second precipitation step, the samples were dissolved in 20 μl TE and the RNA concentration and purity estimated by measuring the 260:280 nm ratio. RNA was separated on a 1.1 % formaldehyde gel, blotted and analyzed using standard techniques (22). RNA gel blots were hybridized with [α-32P]-dATP-labelled DNA probes made by the random prime method (23) from DNA fragments purified from the gels using a standard kit (GeneClean, La Jolla, USA).

cDNA library construction

A cDNA expression library was constructed in λZAPII using poly (A)^+ RNA isolated from agglutinating cells, using a cDNA kit (Stratagen, Pharmacia, The Netherlands) and following the manufacture’s instructions. Equal numbers of mt^+ and mt^- cells, 100 ml each, were mixed in the presence of 5 mM cysteine, to prolong the agglutination process by inhibiting cell fusion (24). After 180 min, the cells were harvested. Total RNA was extracted from Chlamydomonas as described above. The poly (A)^+ fraction was purified by oligo(dT) cellulose chromatography (Boehringer, Mannheim). Double stranded cDNA was synthesized from 5 to 10 μg of poly (A)^+ RNA template, ligated to EcoRI-XhoI digested λZAPII DNA and packaged. The packaged library was plated on E. coli XL1-blue-MRF^+ and amplified as a plate lysate on agar plates (22). The initial library contained 5x10^6 independent phages.
Differential screen

The cDNA library was plated on *E. coli* XL1-blue and grown at 37°C for 16 h. Plaques were then covered with a nitro-cellulose membrane (Schleicher and Schuell) which after 2 min was removed, soaked for 5 min in a denaturation buffer (0.1N NaOH, 0.1N NaCl), for 5 min in renaturation buffer (1.5N Tris, 0.1N NaCl) and washed with SSC solution. The DNA was cross-linked to the membrane using UV light (4 min, 254 nm). The filters were used for hybridization with [α-32P]-dATP-labelled cDNA from swimming gametes and agglutinating gametes. Clones identified only by the cDNA probe made from RNA of agglutinating gametes were purified.

Sequencing of cDNA clones

Phage inserts were excised from the λZapII vector and recircularized in the presence of a helper phage (R408). The pBluescript plasmid was transferred to the *E. coli* host strain JM109. Transformants were grown in liquid LB-medium at 37 °C to isolate enough plasmid (Qiagen) DNA to make sub-clones for further analysis. An Exonuclease III digest was used to generate sub-clones for sequencing. Clones were sequenced radioactively with [35S]-dATP and 7-Deaza-GTP as well as non-radioactively with a fluorescently labeled primer on an ALF express automatic sequencer (Pharmacia, The Netherlands) by the dideoxy chain-termination method (25), using T7-polymerase.

Results

Differential screen

cDNA libraries were constructed using mRNA enriched for transcripts induced during agglutination after mixing gametes of both mating types of *C. moewusii*. They were mixed in the presence of cysteine to prevent fusion and to maintain flagellar protein transport during the agglutination process. An unamplified gamete cDNA library was plated and blotted on membranes. Identical plaque lifts were screened using cDNA made from RNA of a mixture of swimming gametes and with a cDNA probe from agglutinating gametes. In the resulting differential screen, twenty-six clones out of 1.6x10^5 plaque forming units (pfu’s) were identified. The expression pattern of seven purified cDNA fragments was analyzed (Figure 1) using RNA samples from swimming gametes (lanes 1 and 2), a-synchronously grown
vegetative cells (lane 3), agglutinating gametes 30 and 60 min after mixing (lanes 4 and 5) and agglutinating gametes that were incubated for 3 h in the presence of cysteine (lane 6). The \( PGA \) (Prolonged Gamete Agglutination) genes could be divided into two groups: genes \( PGA\) 1-4 were expressed at low levels in vegetative cells (Figure 1, lane3) and were strongly induced by prolonged agglutination (lane 6), genes \( PGA\) 5-7 were highly expressed in vegetative cells and responded only marginally to agglutination. In this chapter we shall describe the characteristics of gene \( PGA1\) and its regulation upon sexual stimulation.

![Fig. 1 Northern blot analysis of total RNA (10 \( \mu \)g/lane) from \( C. moewusii \)](image)

RNA samples were taken from cells during several physiological conditions and RNA gel blot was probed with fragments from the cDNA clones picked up with the differential screen.

Lane 1: Represents RNA isolated from an a-synchronous culture of \( mt^-\) cells.
Lane 2: Represents RNA isolated from an a-synchronous culture of \( mt^-\) cells.
Lane 3: Represents RNA from a vegetative culture of \( mt^-\) cells in the period they exist without flagella.
Lane 4: Represents RNA isolated 30 min after \( mt^-\) gametes started regenerating flagella.
Lane 5: Represents RNA isolated 60 min after \( mt^-\) gametes started regenerating flagella.
Lane 6: Represents RNA isolated from agglutinating gametes. The presence of 2 mM cysteine in the medium prolonged the agglutination process for a period of 180 minutes.
cDNA sequence analysis of \textit{PGA1}

To unravel the possible function of \textit{PGA1}, we determined the cDNA sequence of the cloned fragment. Apart from the poly(A)$^+$ tail, it was approximately 2100 bases in length. The exact sequence of a 238 base-pair-region in the middle of the fragment could not be obtained. This gap has a very high G/C content and therefore it was impossible to sequence with T7 or Taq polymerase, even for commercial firms like Eurogentec and Base Clear. Nevertheless, we deduced an open reading frame of 1719 bases predicting a protein of 573 amino acids with a Mw of 63 kDa (Figure 2). The cDNA contains a polyadenylation signal TGTAA, 18 bases upstream from the poly(A)$^+$ tail common to most mRNAs of \textit{Chlamydomonas} (26,27). The predicted ORF has all the characteristics of an extracellular matrix glycoprotein. It contains a 24-amino acid-long signal sequence suitable for entering the secretion pathway. \textit{PGA1} also contains two potential N-glycosylation sites (boxed, Figure 2A) and a proline / serine-rich stretch that might be suitable for attaching O-glycosidic side chains. BLAST searches identified a region that has homology (48% identity in 37 amino acids) with the \textit{Volvox carteri} extracellular matrix protein SSG 185 (Figure 2B).

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**Fig. 2** cDNA sequence and homology plot of \textit{PGA1} (see next page)

A. The nucleotide sequence and the predicted amino acid sequence of the \textit{PGA1} gene. Numbering is related to the first base of the ATG translation start codon at position +1. After the “G/C-gap” the numbering is arbitrarily chosen to end finally with 2100 bp. The signal sequence is depicted in gray, the Ser/Pro-rich region is shown in a gray block, while the homology with SSG185 is depicted in a gray dashed block. The polyadenylation site TGTAA is underlined, while the stop codon is shown as an asterisk. Putative N-glycosylation sites are boxed.

B. Alignment of homologous regions from \textit{PGA1} and SSG185. Identical residues are indicated.
A

-42  AATTCGCGGAGATAGAACACACGAGGCCTCATGTTGTGAGCCTGCCGCTTTGACCGGAGTTGCCCAGCTGCTATACCACTTACACATCTCCACGCTCTA
-1  
1  AAATTCGCGGAGATAGAACACACGAGGCCTCATGTTGTGAGCCTGCCGCTTTGACCGGAGTTGCCCAGCTGCTATACCACTTACACATCTCCACGCTCTA  99
100  CTACGCTTACTGGCTGCGTGAACTGACCCATTTACGCACGAGCTCGCTCCACACATCTCATCCTACATCTACACACGCTCTACACATCTCCACGCTCT  198
199  ATGGCGTCGAGGTTGTGTTCCTCCCTGTTGCGGGCACTGCTGGCCGCCGCCTTGCTGGGCGTCGCGACCGCGGGCCAAGGACAATGGTGGTCTGTGCAC  297
298  MASRLCSSLLRALLAAALLGVATAGQGQWWSVH  33
349  CTACCCTTACTGCGGCTGCTGGACCGACTTCCCCAGCCAGTGACCTCACGGTCACCCCTCCCACCATCATCGGCCAGTCCTACTCCTTCACCATCTCCA  396
397  LPLLRLLDRLPQPVTSRSFLPPSSASPTPSPSP  66
458  ACCGCTGCGCCACGCCCTTGGGCCACGACTGCACCCAGGACCTGCGCAAGATCGAATTCAACACCTCCCCACGTGCCGCGGCAGCGGCGTAACCGCCAC  495
496  TAAPRPWATTAPRTCARSNSTPPHVPRQRRNRH  99
594  CCTCCTGGAGCGAGCTGTTCAACACCACCGCCACCAACAGCCCCTACCCCATGTCCCTCTTCTCCTCCGACAACCGGTGCTGCGCCCTGCCCCCCCTCG  594
595  PPGASCSTPPPP  132
693  G/C rich, impossible to sequence due to secondary structures

B

PGA1  460  NAACNRLGSGSRNCCTACACAGATCGTGCCTCTGCAGACAACAAGGAAGGAGGCCTTATCATGCTCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCT  496
SSG185  373  NATCNGLGG---CCSDGELKVELFNGKCGSILPFT  406

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Fig 2 Legend see previous page
The cDNA PGA1 was used for Southern analysis to see whether there were more copies in the *Chlamydomonas* genome. The entire coding region of 2100 bp of this cDNA was used under high stringency hybridization and wash conditions. The results are presented in Figure 3. Considering the restriction enzymes used and the number of bands detected, we conclude that a single copy of this *PGA1* gene is present in the genome of *C. moewusii*. Hybridization with the same fragment under low stringency conditions did not reveal any hybridization with genomic DNA of *C. reinhardtii* (results not shown), suggesting that there was no homologue of this gene present in *C. reinhardtii*.

**Analysis of *PGA1* gene expression**

Different physiological conditions were analyzed to define the role that *PGA1* plays in *C. moewusii* gametes. A well defined process for *C. moewusii* is flagellar regeneration. Therefore gametes were deflagellated by pH shock (22) and after neutralizing the medium, resuspended in cell-free medium while illuminated. Under these conditions, the cells started to regenerate their flagella immediately in a synchronous manner (Figure 4). The mRNA was isolated at different time points, until the flagella had reached their full length. The expression of β tubulin, a flagellar protein, was induced with a maximum at 15-30 min, after which the mRNA levels decreased again to basal level. In contrast, there was only a minor increase in the transcript level off the *PGA1* gene at a late stage of flagellar regeneration (30-90 min) and transcript levels were decreasing again when the flagella reached their full length. This
suggests that this gene does not code for a protein important for the assembly of flagella and does not belong to the family of axonemal protein genes such as tubulin.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Expression of PGA1 after deflagellating \textit{C. moewusii} cells}
\begin{enumerate}
\item[A.] At the indicated times after deflagellation, cell samples were fixed and flagellar lengths measured. The average lengths with the standard errors are presented. C = control = flagellar length before deflagellation (N=50).
\item[B.] RNA gelblot analyses. Total RNA (10 \(\mu\)g/lane) isolated from mt\(^+\) cells during flagellar regeneration was probed with PGA1 cDNA, a \(\beta\)-tubulin and a rubisco SSU \(\approx rbcS2\) cDNA (28) fragment from \textit{C. reinhardtii}. M = marker = an RNA sample loaded together with ethidium bromide to detect the ribosomal bands under fluorescent light. C = control = the RNA isolated from cells harvested before deflagellation. Hybridization occurred under stringent conditions (42 °C, in the presence of formaldehyde).
\end{enumerate}
\end{figure}

\textit{PGA1} was isolated in a differential screen using a cDNA probe made from mRNA of gametes during prolonged agglutination. Therefore the expression of this gene was analyzed during mating. Agglutination starts when \(mt^+\) and \(mt^-\) gametes are mixed. They form large
clumps of agglutinating cells by random adhesion (11) and within minutes the cells become aligned over the length of their whole flagella. During this alignment, agglutinin molecules involved in the adhesion are transported to the flagellar tips (16,29), gametes are sorted into pairs of opposite mating type and a specific protoplasm bridge is formed by which two gametes fuse and form a vis-à-vis pair (16,21). In this experiment 50% of the cells had fused within 30 min (Figure 5A). The expression of the PGA1 gene started to increase from 30 min onward with maximum expression after 90 min. The level of PGA1 transcripts was about 10-fold higher than the basal level (Figure 5B). The genes coding for the photosynthetic gene rubisco and the flagellar β-tubulin show different expression patterns indicating that PGA1 gene expression is responding to changes in the mating process. Apparently the gene is expressed as more and more cells became fused, suggesting there might be a function in this process.

![Fig. 5 PGA1 expression during sexual agglutination](image)

A. The percentage vis-à-vis pairs formed after mixing mt+ and mt− C. moewusii gametes.
B. RNA gelblot analyses. Total RNA (10 μg/lane) isolated from the cells in ‘A’, was probed with PGA1 cDNA and a fragment of the rubisco SSU = rbcS2 and β-tubulin probe from C. reinhardtii (28). Ribosomal RNA was used as a loading control. Hybridization occurred under stringent conditions (42 °C, in the presence of formaldehyde).
**Discussion**

In this chapter we describe the cloning and characterization of *PGA1*, a gene that is expressed only in gametes and whose expression is induced by sexual fusion. It is one of several cDNAs that were obtained from a differential screen using a cDNA probe made from RNA of agglutinating gametes. Two other clones identified in this screen will be described in the next chapter. Although we aimed at the identification of genes that are associated with the flagellar membrane and are induced by prolonged adhesion of gametes, the gene described here is apparently not associated with the flagella, as its expression is not upregulated upon deflagellation and subsequent regeneration (Figure 4). However, this is a characteristic of many proteins associated with the axoneme (30) and could be expected to hold true for flagellar surface proteins as well. From the results in previous chapters it is clear that not all flagellar proteins are upregulated by deflagellation (Chapter 2, Figure 5), therefore we have to leave open the possibility that *PGA1* codes for a flagellar protein.

The DNA sequence of *PGA1* could not be completed because of a peculiar CG-rich stretch in the center of the cDNA. Two commercial sequencing firms and us made several attempts with different subclones. Although we obtained additional sequence information, the reliability was very low and the sequence was exclusively made up of Gs and Cs. This suggests that there is a proline-rich spacer (approx. 80 amino acids) in the center of the protein. Up till now we have not been able to generate the complete DNA sequence. However, the remaining part of the cDNA contains valuable information. Its structure suggests that *PGA1p* is an extracellular protein with homology to *Volvocalean* cell wall proteins. As described by Godl et al. (31), *Volvox carteri* contains a large family of matrix proteins that have been named pherophorins. Two of these proteins (phe-S and SSG185) have globular domains separated by a proline-rich spacer, a structure similar to that of *PGA1*. To be able to compare *PGA1* with the available HRGP genes from *Volvocalean* species, we collected protein sequences of several *Volvocalean* HRGPs from databases and from colleagues that had unpublished sequences available. In total we used 12 protein sequences two of which are unpublished: ZSP6 (*C. reinhardtii*, zygote specific) and ATVC13 (*C. reinhardtii*, vegetative cells specific) were made available by J. Woessner, P. Ferris and U. Goodenough. GAS28 and GAS29 are gamete-specific HRGP genes from *C. reinhardtii* (32). Other proteins are the zygote specific ZSP1 (33) vegetative cells specific VSP1 (34) and VSP3 (34) from *C. reinhardtii*, the vegetative cells-specific WP6 from *C. moewusii* (34), and finally the *Volvox*
carteri ISG (35), Phe-S (31) and SSG185 (36) proteins. These proteins were aligned using ClustalW 1.7 and the aligned data were converted into a dendrogram using bootstrap analysis of the PAUP 3.1.1 program, with ZSP6 as outgroup. The resulting Figure 6 illustrates that the structural cell wall proteins are grouped together while the pherophorin-like proteins form the second branch. The relatedness of the PGA1 protein with the previously mentioned pherophorins is confirmed, but the homology with the gamete-specific genes GAS28 and 29 from *C. reinhardtii* is apparent. Keeping in mind that the PGA1 protein sequence is incomplete, these results suggest that there are similar proteins in the distantly related species *C. moewusii* and *C. reinhardtii* that are associated with gamete specific processes. It remains to be seen whether these proteins are involved in the process of agglutination or whether they are somehow associated with the sexual fusion process or the formation of a new zygote wall.

The data presented in Figure 5 show that expression of the *PGA1* gene is upregulated as more and more cells fused, suggesting that it may have a function in the completion of this process or the processes that immediately follows sexual cell fusion.

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**Fig. 6** A dendrogram of the alignment of several Volvocalean HRGPs
Homology is based on the global alignment of protein sequences using the ClustalW 1.7 computer program.
References


