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A methionine synthase gene from *Chlamydomonas moewusii*

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Abstract

When $mt^-$ and $mt^+$ gametes of C. moewusii are mixed, they agglutinate. The cells form large clumps by random adhesion, caused by the mutual binding of extrinsic membrane glycoproteins, called agglutinins on the flagella. A differential screen was performed to isolate genes whose products are involved in the agglutination process (see Chapter 4). Two cDNAs, PGA2 and PGA3, were sequenced and analyzed. PGA2 is a novel gene that codes for a protein of 170 amino acids. The PGA3 cDNA is 2800 nucleotides in length with an open reading frame of 799 amino acids coding for methionine synthase. Transcripts of both genes show similar expression patterns during agglutination but also during flagellar regeneration and during the light period of the cell cycle, suggesting that they are involved in protein biosynthesis and flagellar biogenesis.

Introduction

The flagellar membrane of Chlamydomonas (1) is structurally continuous with the plasma membrane, although it differs in protein composition, a characteristic complement of glycoproteins being maintained in each domain (2). For example, the flagellar membrane is covered by a “fuzzy coat” of glycoproteins, which is important for adhesion with the flagella of the opposite mating type (3,4) and is absent from the cell body membrane (5-7). The agglutinin is an extrinsic membrane glycoprotein on the flagella which is essential for sexual reproduction but is one of the minor glycoproteins in Chlamydomonas. To obtain information about other (glyco)proteins involved in sexual recognition, several attempts have been made to clone genes that are induced by gametogenesis (8) or by sexually stimulating gametes (9). These screens produced several extensin-like proteins that are gamete-specific (see Chapter 4) and surprisingly, a gene encoding methionine synthase (9). Why this enzyme is induced during sexual agglutination is unclear, as this is part of a biochemical cycle that is involved in many cellular processes (see Figure 1). Not only proteins but in the case of Chlamydomonas, flagellar glycoproteins (10) can also be methylated. In addition, the methylation of DNA and lipids is important for the control of various processes in animal as well in plant cells (11). During methylation a methyl group derived from the methyl cycle is used as substrate and transferred to different molecules.
Analysis of a methionine synthase gene

Sulphur amino acids

\[ \text{Ado} \rightarrow \text{AdoHeys} \rightarrow \text{Methionine} \rightarrow \text{dcAdoMet} \rightarrow \text{Polyamines} \]

\[ \text{AdoHeys hydrolase} \]

\[ \text{Methyl transferase} \]

Fig. 1 Biochemical pathways, enzymes and metabolites of Cobalamin-independent methionine synthase

dcAdoMet, decarboxylated S-adenosyl-L-methionine; AdoHeys, S-adenosyl-L-homocysteine; Heys, homocysteine; H$_4$(Me)folate, 5-methyl tetrahydrofolate; H$_4$folate, tetrahydrofolate (12)

The enzyme methionine synthase not only operates in this biosynthetic pathway but is also involved in the production of methionine (12). Two isoforms of methionine synthase have been described, the cobalamin vitamin-B$_{12}$-dependent methionine synthases and the cobalamin-independent methionine synthase (13,14). Cobalamin-independent methionine synthase are generally present in lower eukaryotes, possibly helping them to survive under cobalamin-deficient environmental conditions. They are present in organisms that either synthesize vitamin B$_{12}$ (some bacteria) or obtain it from intestinal flora or their diet (animals and some enteric bacteria like E. coli). An analysis of their genes does not show any similarity, suggesting that the two enzymes have arisen independently but essentially catalyze the same overall reaction (15). The cobalamin-independent synthases genes can be distinguished from cobalamin-dependent methionine synthases because they contain a C-terminal region a stretch of conserved amino acids: LWVPNDCGLKTR (16) with a cysteine (cysteine 726) (14), which is essential for its function. Inactivation by alkylation of this residue results in complete loss of enzyme activity (13). The same active site is also present in the cobalamin-independent methionine synthase genes of different species such as Chlamydomonas reinhardtii, Escherichia coli, Coleus blomei and Catharanthus roseus. In
Saccharomyces cerevisiae the amino acid Leucine (L) in this conserved region has been changed to Phenylalanine (F) (Genbank database homology search).

Kurvari et al. (9) found in C. reinhardtii that transcript levels of a cobalamin-independent methionine synthase were increased when mt− gametes were activated with flagella from the opposite mating type. Here we describe the characterization of two cDNAs, PGA2 and PGA3 that are induced by agglutination of C. moewusii gametes, which was prolonged by the presence of cysteine. PGA2 codes for a methionine synthase gene similar to that described by Kurvari et al. (9). The second cDNA, PGA3 is expressed in a way similar to PGA2 that codes for a novel protein homologous to one identified in several prokaryotes in Caenorhabditis elegans whose function is presently unknown.

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+, (17)) 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.

Cell cycle analysis

Mr− cells were cultured in Fernbach flasks on a rotary shaker at 20 °C in liquid M1 medium. Synchronization was achieved by alternating 16 h light/8 h dark periods. A high-pressure mercury lamp (Philips HP1/T400W) provided light and the photon flux at the level of the cultures was 120 μE m−2 sec−1 (18). To improve synchronization, the cell suspension was diluted to 3.10^3 cells ml−1 after each dark period for a number of cell. The cell density was maintained below 3.10^5 cells ml−1.
Flagellar regeneration and agglutination

To identify genes coding for flagellar proteins, gametes were deflagellated by pH shock (19) and were allowed to regenerate new flagella. RNA was isolated at different time points during the regeneration process. Deflagellated cells were resuspended in 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. RNA was isolated as described below and separated on a 1.1 % formaldehyde gel, blotted and analyzed using standard techniques (20). RNA gel blots were hybridized with [α³²P]-dATP-labelled DNA probes made by the random prime method (21) from DNA fragments purified from the gels using a standard kit (Geneacean, La Jolla, USA).

Expression of the PGA2 and the PGA3 genes was compared with the expression of ribisco and tubulin whose transcription begins earlier in the agglutination process, to show that expression is specific for such genes during this process.

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, pH 8) 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 10⁸ cells were resuspended in 500μl TE buffer to which 700μl 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, 150μl NaAc (3M, 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 DEPC-treated water. LiCl was added to a final concentration of 2M and the solution was vortexed and put on ice for 16 h. After a second precipitation step, the samples were dissolved in 20 μl H₂O and the RNA concentration and purity estimated by measuring the 260:280 nm ratio.
cDNA library construction

A cDNA expression library was constructed in λZAPII using poly (A)^+ RNA isolated from agglutinating cells, using a Stratagen cDNA kit, and following the manufacture instructions. Equal numbers of \(m^c\) and \(mt^+\) cells were mixed in the presence of 5 mM cysteine, to prolong the agglutination process (22). 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 (Bohringer, 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* DH5α and amplified as a plate lysate on agar plates (20). It contained more than \(10^6\) independent phages.

Differential screen

The phages containing the cDNA library were plated on *E. coli* XL1-blue and grown at 37°C for 16 h. They were then covered with a nitro-cellulose membrane (Schleicher and Schuell) which after 2 min were removed and soaked for 5 min in a denaturation buffer (0.1N NaOH, 0.1N NaCl). They were then incubated for 5 min in renaturation buffer (1.5N Tris, 0.1N NaCl) and washed with SSC solution. The RNA was cross-linked to the membrane using UV light (4 min, 254 nm). The filters were used for hybridization with \([\alpha^{32}\text{P}]\)-dATP-labelled cDNA from swimming gametes and agglutinating gametes. Clones with a gamete specificity were used for further analysis.

Sequencing of cDNA clones

Phage inserts were excised from the λZapII vector and recircularized, in the presence of a helper phage (R408), to the pBluescript plasmid in the *E. coli* host strain JM109. These 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 on the total cDNA clone to generate sub-clones by which it was possible to obtain the overlapping sequences information. Clones were sequenced radioactively with \([^{35}\text{S}]\)-dATP and 7-Deaza-GTP as well as non-radioactively with a fluorescent labeled primer on an Alf automatic sequencer (Pharmacia) by the dideoxy chain-termination method (23), using T7-polymerase. The *PGA3* sequence is present in the EMBL/Genbank database under accession number U77388.
The protein coding sequences of several methionine synthases were aligned using ClustalW 1.7. The results were visualized using SeqVu 1.01 (Garvan Institute, 1994-1995).

Results

Cloning and characterization of the PGA genes

The genes PGA2 and PGA3 were isolated in a differential screen aimed at the identification of C. moewusii genes that are induced by a prolonged period of sexual agglutination (see Chapter 4, Figure 1). Both genes are expressed to a low level in vegetative cells, but are highly transcribed in gametes and induced during prolonged agglutination. The sequence characteristics of these two genes and the regulation of their expression will be described consecutively.

PGA2 was cloned as a 718 bp cDNA fragment, with an open reading frame of 170 amino acids, coding for a protein of approximately 20 kDa (Figure 2). Although the cDNA lacks the characteristic TGTAA Chlamydomonas polyadenylation signal, there is a TTTAA sequence 15 bp upstream of the poly(A) tail that could be used as an alternative (24,25). A search in a database identified proteins from Caenorhabditis elegans (U13875), Bacillus subtilis (Z99120), Pyrococcus horikoshii (AB009487) and Clostridium parsteuianum (AF006034) with an average of 66 % homology to PGA2. Unfortunately none of these homologues has a known function. A protein sequence analysis with PSORT resulted in the prediction (with a certainty of 0.9) that the PGA2 protein is located in the mitochondrial matrix space.
The cDNA sequence of *PGA3* (Figure 3; Genbank Accession no. U77388) contains the polyadenylation signal TGTAA 13 bp upstream of the poly(A)^+ tail. The cDNA is 2820 bases in length and has an open reading frame of 2397 bases predicting a protein of 799 amino acids with a molecular mass of approximately 96 kDa. The ATG codon was selected as the start via sequence alignment. The sequence exhibits a GC content of about 65%, with the codon bias typical for *Chlamydomonas* (5,25).

Fig. 3 Physical map and the cDNA sequence of *PGA3* (see next page)
A. Physical map of the complete cDNA showing the restriction sites used for sub-clone construction to obtain sequence data. The thick line represents the open reading frame flanked by the 5' and 3' untranslated regions.
B. The nucleotide sequence and the predicted amino acid sequence of the *PGA3* gene. Numbering is related to the first base of the ATG translation start codon at position +1. The polyadenylation site TGTAA is underlined, the active site is depicted in bold while the stop codon is shown as an asterisk.
Analysis of a methionine synthase gene
The nature of the gene encoded by PGA3 was more obvious. The cDNA fragment encoded for an open reading frame of 799 amino acids that would result in a polypeptide of approximately 86 kDa. (Figure 3). A search in the databases revealed that we had cloned a methionine synthase gene. The predicted protein sequence encoded by the C. moewusii gene PGA3 nicely aligned with the cobalamin-independent methionine synthases from C. reinhardtii (GAR1, U36197), E. coli (M87625), Catharanthus roseus (X83499) and S. cerevisiae (U15099). This alignment is presented in Figure 4. Several conserved stretches of amino acids are present, predominantly in the C-terminal part of the proteins. They include the predicted active site LWVNPDCGLKTR, in the PGA3 sequence residues 725-736, which is typical for cobalamin-independent methionine synthases. The overall homology of PGA3 with the other cobalamin-independent methionine synthases varies from 44% identity with the C. reinhardtii gene to 35% identity with the C. roseus protein.

Fig. 4 The alignment of clone PGA3 with different methionine synthase genes (see next page)
The amino acid sequences from genes in the EMBL/Genbank and their Accession numbers: C. moewusii (U77388) C. reinhardtii (U36197), E. coli (M87625), C. roseus (X83499) C. blumei (Z49150) and S. cerevisiae (U15099) were compared using the Clustal W program and homologous part are depicted in boxes. The underlined amino acids represent the active site.
Analysis of a methionine synthase gene
Genomic organization of *PGA2* and *PGA3*

The cDNAs of *PGA2* and *PGA3* were used in DNA gelblots to determine whether there were more copies in the *C. moewusii* genome. The results indicate that *PGA2* is present in *C. moewusii* as a single-copy gene. The *PGA3* probe recognized a few strong hybridizing DNA fragments as well as some faint hybridizing DNA fragments, indicating that there are more homologues of *PGA3* in the genome of *C. moewusii* and *C. reinhardtii* (Figure 5).

![Figure 5 DNA gelblot analysis of genomic DNA](image)

Expression of *PGA2* and *PGA3* during sexual agglutination

Since *PGA3* is homologous to a cobalamin-independent methionine synthase gene from *C. reinhardtii*, we wondered if the expression patterns would be comparable. The methionine synthase in *C. reinhardtii* was expressed during gamete activation. In this paper Kurvari et al. (9) treated *mt*+ gametes with flagella from *mt*− gametes to maintain the cells in a state of prolonged sexual agglutination (9). For *C. moewusii* the expression of *PGA2* and *PGA3* was followed during different physiological circumstances to develop a better understanding of
when and where these genes are transcriptionally active. First, expression during agglutination, followed by the formation of vis-à-vis pairs was studied. This was done by mixing equal amount of gametes of both mating types of *C. moewusii*. Samples were taken during a two-hour period and used for RNA gelblot analysis as shown in Figure 6.

![Graph](image)

**Fig. 6 Expression of PGA2 and PGA3 during sexual agglutination**
A. The percentage vis-à-vis pairs formed within two hours of mixing *mt*<sup>+</sup> and *mt*<sup>−</sup> *C. moewusii* gametes.
B. RNA gelblot analyses. Total RNA (10 μg/lane) isolated from the cells in 'A', was probed with PGA2 and PGA3 cDNA. A fragment of the rubisco SSU (= *rbcS2*) and tubulin cDNA from *C. reinhardtii* (26) were used as control genes. Ribosomal RNA was used as a loading control. Hybridization was under stringent conditions (42 °C, in the presence of formaldehyde).

The formation of vis-à-vis pairs is an indication for completion of the agglutination process. The first pairs were formed within 5 min and a maximum level of 73% was reached at 90 minutes. For both PGA2 and PGA3 the mRNA increased strongly from 30 to 60 min and declined again within the next 60 min (Figure 6B). This expression pattern might be related to
the formation of vis-à-vis pairs, because when the number of vis-à-vis pairs reached their maximum level (Figure 6A), expression of the genes decreased. To check whether these expression patterns were specific we hybridized the same RNA gelblot with β-tubulin and rubisco (rbcS2). β-tubulin expression increased rapidly after the gametes were mixed and then decreased again to basic levels. RbcS2 was only expressed within the first 30 min, after which it decreased rapidly.

**Expression of PGA2 and PGA3 during flagellar regeneration**

The result of the last experiment pointed to the possibility that these genes could be involved in the biosynthesis of flagellar proteins. Therefore we examined the expression patterns of the PGA2 and PGA3 genes during flagellar regeneration. *C. moewusii* gametes were deflagellated and resuspended into fresh medium and stirred gently under a strong light source, where they immediately started synchronously to regenerate their flagella. Within two hours the flagella reached their full length again. For Northern blot analysis and flagellar length measurements, samples were taken at different time points. The total RNA was isolated, separated and analyzed with different cDNA probes (Figure 7B). The expression of β tubulin, an axonemal protein, was induced directly with a maximum at 15-30 min, after which the mRNA levels decreased again to basal level. In contrast, the expression of the PGA2 and PGA3 genes started to increase when the flagellar length was ¼ of the total length and gene expression reached a maximum when the flagella had reached their final length (Figure 7A). This suggests that the proteins encoded by these genes are not structural axonemal proteins but are involved in a final step in flagellar biogenesis. This expression pattern is comparable with PGA1 described in Chapter 4.
Analysis of a methionine synthase gene

Fig. 7 Expression of *PGA2* and *PGA3* after deflagellating *C. moewusii* cells
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.
B. RNA gelblot analyses. Total RNA (10 μg/lane) isolated from mt" cells during flagellar regeneration was probed with *PGA2* and *PGA3* cDNA, a β-tubulin and a rubisco SSU (= *rbcS2*) cDNA (26) fragment from *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 was under stringent conditions (42 °C, in the presence of formaldehyde).

Cell cycle regulated expression of *PGA2* and *PGA3*

To understand more about the function of these genes it is necessary to test them in different biological processes. As both genes were expressed at low levels in an a-synchronous vegetative culture, their expression during the cell cycle in a synchronous culture was investigated. Northern blots of RNA isolated at 2 h intervals were hybridized with the cDNA of clones *PGA2* and *PGA3* using *rbcS2* (26) as a control (Figure 8).
Both clones showed comparable expression patterns, a sudden increase at the beginning of the light period (G1 phase) and a decline over a time period of 10 hours, until the beginning of the dark period where the transcripts were no longer detectable. There was one slight difference between both genes. The transcripts of the PGA3 gene were detectable two hours after the light was switched on, whereas the gene coding for clone PGA2 was already expressed when the light period started.

Discussion

We have described two C. moewusii cDNAs isolated by using a differential screen. One is a cobalamin-independent methionine synthase (PGA3) and the other is a new gene of
unknown function (PGA2). Their transcript levels were regulated in an almost identical fashion. Therefore, we propose that both genes are involved in the same biological processes. The discussion will focus on the gene coding for the cobalamin independent methionine synthase (PGA3).

The sequence data depicted in Figure 3 represents the PGA3 gene with an open reading frame that encodes a protein of 799 amino acids with a predicted molecular weight of 86 kDa. This is in good agreement with the values described in literature, for the native 5-methyltetrahydropteroyl triglytamate-homocysteine transmethylase (Met E) from E. coli, has a molecular mass of 84 kDa as calculated from ultracentrifugation data (27). On the basis of mobility in electrophoresis gels a molecular mass of 88 kDa was calculated (28). The methionine synthase from C. moewusii is highly homologous at the amino acid level with those from C. reinhardtii (U36197), Escherichia coli (M87625), Catharanthus roseus (X83499) and Saccharomyces cerevisiae (U15099). Deduced amino acid sequences for these methionine synthases exhibited high percentages of similarity and identity over their whole lengths (Figure 4). This similarity is not in accordance with the result obtained by immunological methods used by Eichel et al. (16). They used an antibody raised against the C-terminal part (35 kDa) of C. roseus methionine synthase that was fused to a maltose-binding protein (42 kDa). This antibody detected a protein of about 85 kDa in different plant species, but not in C. reinhardtii. This is difficult to understand in view of the amino acid alignment of the different species shown in Figure 4. The cDNA from C. reinhardtii contains a single open reading frame of 815 amino acids encoding a polypeptide of a calculated relative mass of 87 kDa and compared with the cDNA of C. moewusii, there is 64% similarity and 44% identity. The largest homologous region, as shown in Figure 3, contains the cysteine from the active site flanked by the conserved sites. For C. moewusii the consensus sequence is 100% identical to the active site of the methionine synthase gene described for the E. coli (Met E (13)) and C. roseus (16). The functions of the other homologous regions are unknown.

We found with Southern blot analyses that under stringent conditions PGA3 hybridized with DNA from the distantly related species C. reinhardtii, confirming the sequence homology found between the two cloned genes. We have to mention that, based on the amount of hybridizing DNA fragments, the PGA3 gene is probably a member of a gene family. Expression of the methionine synthase gene GAR1 in C. reinhardtii was induced during gamete activation (9), a feature that is shared with the PGA3 gene. Kurvari et al. (9) explained the enhanced expression levels during gamete activation as a result of the starvation
of AdoMet, because in active gametes AdoMet may be involved in signal transduction or in cellular responses to fertilization-related signals. We, however, propose that the PGA3 gene products from C. moewusii are not involved in signaling. Such responses take place in a time range of seconds or sometimes minutes. The PGA3 gene expression studied here during flagellar regeneration and agglutination was induced one hour after stimulation, which is a much longer time scale than that expected of signaling processes.

We prefer the possibility that AdoMet is mainly used for the methylation of flagellar surface glycoproteins and for protein biosynthesis. The surface of the flagellar membrane is covered by a number of glycoproteins (29,30), which are partially methylated (10). We speculate that this results in an increased hydrophobicity of the flagellar surface that might be instrumental in decreasing the viscous drag during flagellar beating. This is based on the fact that during flagellar regeneration, methionine synthase genes are expressed at the end of flagellar biogenesis, as if to provide the finishing touch to the flagella. One may envisage the formation of methylated glycoproteins (or the methylation of glycoproteins) taking place at the end of flagellar biogenesis, when the structural part of the flagella (the axoneme and the membrane) has been completed. The expression of the genes during prolonged agglutination is consistent with this interpretation. It is well known that sexual agglutination is accompanied by increased turnover of surface glycoproteins (31). Alternatively, we can imagine that when cells are fused, the expression of PGA3 is increased because at that moment the formation of the zygote cell wall must be realized whereby the synthesis of new glycoproteins is involved. Again, this calls for increased levels of enzymatic activity involved in the methylation and biosynthesis of glycoproteins.

During the cell cycle the expression of PGA3 is limited to the G1/S phase, where cell growth is a major process, and the cells still have flagella. It should be noted that this experiment was done with vegetative cells while all the other experiments was performed with gametes.

The expression of PGA2 exhibited the same pattern as discussed previously for PGA3, which allowed the suggestion that these genes are involved in the same or related processes. The fact PGA2 is conserved in C. reinhardtii (data not shown) and that this gene is homologous to an unknown protein from C. elegans and several other microorganisms, suggests that the protein encoded by PGA2 is involved in a biological process that is conserved among different species. Flagellar regeneration and agglutination are energy consuming processes and therefore the predicted location of this molecule in the
mitochondrion, based on analysis of the PGA2 protein sequence, might to be an explanation for its function.

References


