Short Communication

Functional Identification of the Glycerol Transport Activity of Chlamydomonas reinhardtii CrMIP1

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By searching a Chlamydomonas expressed sequence tag database and by comparing the retrieved data with homologous sequences from a DNA database, we identified an expressed Chlamydomonas reinhardtii putative major intrinsic protein (MIP) gene. The nucleotide sequence, consisting of 1,631 bp, contains an open reading frame coding for a 300-amino-acid protein named CrMIP1. It possesses conserved NPA motifs, but is not highly homologous to known aquaporins. CrMIP1 was expressed in Saccharomyces cerevisiae and assayed for water and glycerol transport activity. By the stopped-flow spectrophotometric assay, CrMIP1 did not enhance the osmotic water permeability of membrane vesicles of the yeast transformant. However, the transformant cells showed glycerol transport activity in the in vivo assay using [14C]glycerol. This is the first report on the isolation and functional identification of a MIP member from algae.

Keywords: Aquaporin — Chlamydomonas reinhardtii — Glycerol transport — Heterologous expression — MIP — Sequence analysis.

Abbreviations: CrMIP1, Chlamydomonas reinhardtii MIP1; MIP, major membrane intrinsic protein; NPA motif, asparagine-proline-alanine motif; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein; TM, transmembrane domain.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, GenBank and EBI databases under the accession number AY194236.

The membrane channels for water and non-ionic solutes, required for cell-volume regulation and/or osmoregulation in bacteria, plants and animals, belong to the family of major intrinsic proteins (MIPs). In plants, the MIP gene family consists of four subgroups: the tonoplast intrinsic proteins (TIPs), the plasma membrane intrinsic proteins (PIPs), the NOD26-like MIPs (NLMs) and the small and basic intrinsic proteins (SIPs) (Santoni et al. 2000, Chaumont et al. 2001, Johanson and Gustavsson 2002). Functional analyses of the MIP members have revealed three major functional clusters of subfamilies: water-specific channels, solute-specific channels and mixed-type channels. Particular members of the MIP family have been reported to transport glycerol (Ishibashi et al. 1994, Maurel et al. 1994, Luyten et al. 1995, Rivers et al. 1997, Agre et al. 1998, Biela et al. 1999, Gerbeau et al. 1999, Heymann and Engel 1999, Weig and Jakob 2000, Kozono et al. 2003), CO2 (Uehlein et al. 2003), ammonia (Niemietz and Tyerman 2000) and urea (Ishibashi et al. 1994). The structural basis of the substrate specificity, especially specific amino acids distinguishing the substrate, has been under debate; however, a final conclusion has not yet been reached.

MIP family members have been cloned from various organisms including bacteria, yeasts, terrestrial plants and animals. As yet, no gene encoding a MIP has been isolated from algae. It is essential for aquatic creatures such as algae to regulate their osmotic pressure against sharp fluctuations of the environmental osmotic conditions (Pinontoan et al. 2000). The osmoregulation is performed through active and passive transporters and ion channels. So far, however, it still remains to be determined how aquaporins are involved in osmoregulation of algal cells. In this study we cloned a cDNA for a MIP from the single-celled green alga Chlamydomonas reinhardtii. We determined its function by heterologously expressing it in Saccharomyces cerevisiae and found that it belongs to the glycerol facilitator. Here we discuss characteristics of the primary sequences and physiological role of the aquaglyceroporin.

The cloned cDNA encodes a protein (named CrMIP1) of 300 amino acids, which is relatively longer than other MIPs. The deduced sequence of CrMIP1 was aligned with aquaporins from other organisms for comparative analysis of the characteristic domains (Fig. 1). According to Kyte and Doolittle hydrophyt plot, CrMIP1 possesses six putative transmembrane domains (TMs) and a long N-terminal hydrophilic region of 79 amino acid residues. The two asparagine-proline-alanine (NPA) motifs are perfectly conserved and the ExxxTxxF/L

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Fig. 1 Comparison between amino acid sequences of *C. reinhardtii* MIP1 and representative aquaporin homologs. The alignment of the sequences was obtained using ClustalW (Higgins et al. 1996). Conserved residues within all sequences are marked with asterisks and similar residues are indicated with periods and colons. Thick bars mark putative membrane-spanning domains (TM1–TM6). The signature sequences of NPA motifs for the MIP family are boxed. Residues shown in white on a black background represent the signature residues for aquaglyceroporins and glycerol transporters (CrMIP1, EcGlpF and HsAQP3). In overlined upper-case letters are shown the putative N-glycosylation (double line) and protein kinase II phosphorylation (wavy line on HsAQP3) sites. From top to bottom, sequences are of CrMIP1 (this paper), EcGlpF (P18156), HsAQP1 (P29972), AtPIP1;1 (Quigley et al. 2002), AtTIP1;1 (Quigley et al. 2002), and GmSPCP2 (JQ2288).
motif, which has been proposed as a highly conserved motif in the MIP family (Heymann and Engel 1999), is also conserved in TM1 and TM4. However, CrMIP1 contains neither the Cys-189 residue (numbering of HsAQP1), responsible for mercury sensitivity (Preston et al. 1993), nor Ser-262 (numbering of GmNOD26) responsible for the phosphorylation of NOD26 (Weaver and Roberts 1992). Several interesting features of CrMIP1, including putative N-glycosylation and protein kinase II phosphorylation sites, are further outlined in Fig. 1. With respect to the five amino acid residues (P1–P5) involved in the solute selectivity of aquaporins (Froger et al. 1998, Johansson et al. 2000), only one residue of Ile-270 (P5 site) of CrMIP1 is identical to the corresponding residue of Homo sapiens AQP3. The Ile-270 residue is homologous to the corresponding leucine residues in Escherichia coli GlpF, Glycine max NOD26 and Arabidopsis thaliana NIP1;1, known as aquaglyceroporins (Fig. 1). We found that other residues, Ala-135, Lys-146, Leu-168 and Ser-238 (highlighted residues in Fig. 1), are also conserved in these aquaglyceroporins. We therefore tested the functional ability of CrMIP1 to permeate both water and glycerol.

CrMIP1 was introduced into a yeast mutant strain YSH294, which lacks a glycerol transporter FPS1 (Luyten et al. 1995). As Fig. 2 shows, CrMIP1 tagged with FLAG, a synthetic peptide of 13 residues, was expressed and accumulated in the membranes of transgenic yeast. CrMIP1 was detected at the expected size of 32.2 kDa for the FLAG–CrMIP1 fusion protein (Fig. 2A).

Yeast fps1Δ mutant is sensitive to hypo-osmotic treatment, due to excessive intracellular glycerol retained inside the cells (Tamás et al. 1999). The heterologous expression of Chlamydomonas CrMIP1 could complement the glycerol efflux of fps1Δ in a hypo-osmotic SGd medium (Fig. 2B). Thus, CrMIP1 may be physiologically involved in glycerol transport, probably glycerol export in this case. On high osmotic media (0.7 M NaCl and 1 M sorbitol), the growth rate of fps1Δ cells was slightly lower than that of wild-type cells, but CrMIP1-transformed fps1Δ cells grew as well as the wild-type cells. The results may be related to the observation that overexpression of the FPS1 gene enhances the production of glycerol (Luyten et al. 1995), necessary as the osmoticum for survival on high osmotic media. A similar physiological effect by heterologous expression of CrMIP1 in yeast can be estimated, although we have no experimental evidence.

Next, we investigated the ability of CrMIP1 to facilitate glycerol influx. [14C]Glycerol uptake was measured by a silicon-layer filtering centrifugation method. CrMIP1-transformed

Fig. 2  Expression of CrMIP1 in yeast cells and growth of the transformants. (A) Immunological detection of CrMIP1–FLAG fusion protein expressed in fps1Δ strain of S. cerevisiae. Lane 1, CrMIP1-expressing cells; lane 2, control cells. Membrane fractions isolated by the mechanical method were submitted to SDS–PAGE (2 µg per lane) and immunoblotting with anti-FLAG antibody. (B) Growth of wild-type cells, vector-transformed fps1Δ and CrMIP1-transformed fps1Δ cells under various osmotic conditions. Yeast cells were pre-grown in SGd medium supplemented with 1 M sorbitol. Logarithmic phase cells were harvested, resuspended in the same medium at 1.0 A600 and 5 µl of a 5-fold serial dilution (left to right) of this culture were spotted onto SGd medium plates with or without osmoticum (1 M sorbitol or 0.7 NaCl). Growth at 30°C was monitored after 3 d.

Fig. 3  Glycerol uptake into CrMIP1- (squares) and vacant vector-transformed (closed circles) yeast cells. [14C]Glycerol was incubated with membrane vesicles and then assayed for radioactivity. Vertical bars are standard deviations (n = 5).
A MIP member from *Chlamydomonas* yeast cells took up glycerol at a higher rate than vector-transformed cells (Fig. 3), indicating that CrMIP1 facilitates glycerol uptake. This result mimics the facilitated glycerol influx through *Arabidopsis* *AtNIP1* and *AtNIP2* overexpressed in an FPS1-defective yeast strain (Weig and Jakob 2000). These results suggest that CrMIP1 represents a channel protein that facilitates glycerol transport.

Furthermore, we examined the water channel activity of CrMIP1 expressed in *S. cerevisiae* W303-1A strain. This strain lacks an endogenous aquaporin (Meyrial et al. 2001). Accumulation of CrMIP1 in yeast was confirmed by immunoblotting with the anti-FLAG antibody (Fig. 4A). Crude membrane fractions prepared from the transformants were subjected to stopped-flow spectrophotometric assay. CrMIP1 did not enhance the osmotic water permeability, although low activity was observed fairly similar to that in the membrane vesicles from the control (Fig. 4B, C). This background permeability may be due to the yeast membrane itself, as reported previously (Meyrial et al. 2001). In the same assay system, a radish aquaporin RsTIP1 expressed in yeast gave high water channel activity of more than 8 units, suggesting that this system was working correctly. Therefore, we concluded that CrMIP1 does not function as a water channel.

Structural features of glycerol-conducting channels and aquaglyceroporins have been discussed (Agre et al. 1998, Heymann and Engel 1999, Fu et al. 2000, Zardoya et al. 2002, Kozono et al. 2003). The pore size of human AQP1, a typical aquaporin, is 0.30 nm, which is slightly larger than the 0.28 nm diameter of a water molecule (Murata et al. 2000). Rapid water flow is due to this narrow pore constriction. On the other hand, a glycerol-conducting channel such as *E. coli* GlpF has a wider pore with a diameter of 0.40 nm (Fu et al. 2000). The aquaglyceroporin of *Methanothermobacter marburgensis*, which facilitates transport of water and glycerol, has been estimated by computer-generated homology modeling to have a pore size intermediate between that of AQP1 and GlpF (Kozono et al. 2003). CrMIP1 is estimated to have a wide pore size, although we cannot present the tertiary structural model. We found that Ala-135 located around the NPA motif and Lys-146, Leu-168 and Ser-238 in the TMs of CrMIP1 (Fig. 1) are conserved among glycerol transporters and aquaglyceroporins. Some of the residues may be involved in pore size determination.

The CrMIP1 sequence was phylogenetically analyzed on several typical members of the MIP family, displaying no specific clustering to any of the known aquaporin subfamilies (Fig. 5). The sequence shared the highest homology with soybean GmSPCP2 (28.6% identity) followed by *AtTIP2-1* (28.0%) and *AtTIP1-1* (27.1%). Most members of the plant PIP subfamily have a conserved Pro-rich motif (E/DPPPA/T sequence) in the N-terminal hydrophilic region (Suga et al. 2001); however, the motif is absent in CrMIP1. Judging from the absence of the Pro-rich motif and the relatively high identity of the CrMIP1 sequence with the TIP subfamily, we estimate that CrMIP1 may be localized to the particular endomembrane, although plasma membrane localization is suggested from the yeast complementation assay. The most likely candidate for CrMIP1 intracellular location is the contractile vacuole in *Chlamydomonas* cells. Contractile vacuoles are found in single and multicellular organisms living in fresh water such as *Chlamydomonas*. The contractile vacuole of *C. reinhardtii* has been estimated to play a key role in osmoregulation against the external medium (Luykx et al. 1997a, Luykx et al. 1997b). Heterologous expression of CrMIP1 in yeast dem-

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**Fig. 4** Stopped-flow spectrophotometric measurement of the osmotic water permeability of membrane vesicles prepared from CrMIP1-expressing yeast cells. (A) The membrane vesicles were prepared from yeast cells expressing CrMIP1 and vacant vector, and then subjected to SDS–PAGE (2 µg per lane) and subsequent immunoblot analysis with the anti-FLAG antibody. (B) Membrane vesicles (0.5 mg ml⁻¹) were prepared from control (vector) and CrMIP1-expressing cells (CrMIP1), suspended in 0.45 M mannitol, and then mixed with 0.10 M mannitol at 10°C. The reaction curve shows the average trace of five individual shots. (C) Relative water permeability of the control and CrMIP1. Values were calculated from three independent experiments.
A MIP member from *Chlamydomonas*

A member from *Chlamydomonas* demonstrated the following: (i) CrMIP1 complemented FPS1 function in yeast under hypo- and hyper-osmotic conditions and (ii) CrMIP1 gave glycerol uptake activity in a yeast expression system. These observations can be explained by our hypothesis: CrMIP1 may facilitate glycerol transport across plasma membranes and the membranes of contractile vacuole of *Chlamydomonas* cells. Currently, we are trying to determine the intracellular location of CrMIP1 in *Chlamydomonas* cells.

A partial nucleotide sequence of *C. reinhardtii* aquaporin was found as an expressed sequence tag (EST) sequence (BE056501) in the KDRI database (http://www.kazusa.or.jp/en/plant/chlamy/EST), with the amino acid sequences of several plant aquaporins as queries. The tentative 1,631-bp cDNA sequence encoding a full-length transcript was obtained with BLAST searches on the NCBI server (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST) and by assembling the retrieved nucleotide sequences. The estimated coding region, temporarily named CrMIP1, was amplified by PCR using two synthetic primers: CrMIP1fwd [5'-GGT(ATGGATTACAAGGATGAC-GAGCTAAG)TCTCTGGAGGTGCAGTCCAAG-3'] designed to add a FLAG tag (internal brackets) and CrAQP1rev (5'-GTTTAAATCGCCGGCGCGACGCTGTC-3'). An oligo(dT) primed *C. reinhardtii* cDNA library in λZAP was diluted 1:20 and used as the PCR template with a GC-rich PCR system (Roche Diagnostics, Mannheim, Germany). A cDNA fragment encompassing the entire open reading frame was inserted into an engineered pYTF1Trp plasmid, which contained the promoter of the *S. cerevisiae* GAL1 gene and TRP1 as the selection marker. DNA sequencing was performed on an ABI PRISM 310 sequencer (PE Applied Biosystems, Foster, U.S.A.) (Furuichi et al. 2001). Protein translation, molecular mass computation and hydropathy analysis were performed with DNASIS v3.1 software (Hitachi Software Engineering, Tokyo, Japan).

The versatile *S. cerevisiae* expression system was used for transport tests of water and glycerol. *S. cerevisiae* W303-1A strain (Thomas et al. 2002) and its FPS1-defective mutant YSH294, sensitive to hypo-osmotic treatment (Tamás et al. 1999), were used.

Membrane fraction was prepared from transformed yeast cells either by a mechanical or an enzymic method. For the mechanical method, cells were grown in yeast nitrogen base minimal medium containing appropriate amino acids and 2% galactose (SGd medium). Exponentially growing cells were transferred into fresh SGd medium, grown to 1.5–2.0 A600, harvested and suspended in 90 mM KCl, 1 mM EDTA, 600 mM mannitol, 1 mM DTT, protease inhibitor cocktail (Complete EDTA-free, Roche Diagnostics) and 20 mM Tris–HCl, pH 7.2. The cells were disrupted by mixing with acid-washed glass beads. The resultant lysate was centrifuged at 100,000 x g for 15 min at 4°C and the microsome membrane pellet was suspended at 0.2 mg ml−1 in an isotonic buffer with a handheld sonicator. The membranes obtained were suspended in 0.45 M mannitol, 90 mM KCl, 1 mM EDTA and 20 mM Tris–HCl, pH 7.2 and used for immunological detection of CrMIP1.

To obtain intact membrane vesicles without mechanical damage, samples were prepared by the enzymic method (Nakanishi et al. 2001). The obtained membrane vesicles were assayed for the osmotic water permeability with a stopped-flow light scattering spectrophotometer (model SX.18MV, Applied Photophysics Ltd., Surrey, U.K.) (Ohshima et al. 2001). Yeast cells were precultured at 30°C in SGd medium, 0.01% uracil, 2% galactose, 1% casamino acids and 0.67% yeast nitrogen base without amino acids. The cell culture was diluted and then grown for 13 h to reach exponential phase. After incubation in 0.1 M Tris–HCl, pH 9.4, 50 mM 2-mercaptoethanol and 0.1 M glucose at 30°C for 10 min, cells were treated with 0.05% zymolase 20T, 0.9 M sorbitol, 0.1 M glucose, 5 mM DTT, 0.043% yeast nitrogen base without amino acids and ammonium sulfate, 0.25× dropout solution composed of all amino acids and adenine, and 50 mM Tris–MES, pH 7.6, at 30°C for 1 h with gentle agitation. Spheroplasts were collected by cen-
trifugation at 3,000×g for 5 min and washed with 1 M sorbitol. The spheroplasts were resuspended in 10% glycerol, 1.5% polyvinylpyrrolidone (M, 40,000), 5 mM EGTA–Tris, 1 mM DTT, 0.2% bovine serum albumin, 1 mM PMSF, 1 mg liter−1 leupeptin, 2 mg liter−1 pepstatin and 50 mM Tris–HCl, pH 7.5, with a motor-driven Teflon homogenizer. After centrifugation at 2,000×g for 10 min, the precipitate was suspended in the same buffer and centrifuged again. The supernatant was centrifuged at 120,000×g for 40 min. The precipitate (crude membranes) was washed with 5 mM Tris–HCl, pH 7.6, 0.3 M sorbitol, 1 mM DTT, 5 mM MgCl2, 1 mM PMSF, 1 mg l−1 leupeptin and 2 mg liter−1 pepstatin.

Proteins in yeast membrane fractions were separated by SDS–PAGE. Immunoblotting was carried out with an anti-FLAG antibody (Sigma, St. Louis, U.S.A.) and ECL. Western blot detection reagent system (Amersham Pharmacia Biotech, Buckinghamshire, England) (Yuasa and Muto 1996).

Glycerol uptake by yeast cells was measured by the method of silicon layer filtering centrifugation according to Heldt and Sauer (1971) with some modifications. Cells (106 cells ml−1) were grown in SD medium. Glycerol uptake was initiated by adding [1,3-14C]glycerol solution (37 kBq mmol−1; ICN Pharmaceuticals, Aurora, U.S.A.) at a final concentration of 450 mM mannitol containing 90 mM KCl, 1 mM EDTA and 50 mM Tris–HCl, pH 7.2. After 20 min of incubation, the cells were centrifuged for 1 min at full power with a microcentrifuge. At the desired times the tubes were centrifuged for 10 min, the precipitate was suspended in the same buffer and centrifuged again. The supernatant was centrifuged at 3,000×g for 40 min. The precipitate (crude membranes) was layered onto a layer of silicon oil (SH556 : SH 550 = 2 : 3 mixture; Torey, Tokyo, Japan) on a sucrose cushion (25 ml of 35%) in 500-ml plastic microcentrifuge tubes. At the desired times the tubes were centrifuged for 1 min at full power with a microcentrifuge (Beckman, type B) to separate the cells from the medium and terminate glyceral uptake. Radioactivity in the precipitated cells was counted with a scintillation counter.

The osmotic water permeability of membrane vesicles was measured as described previously (Ohshima et al. 2001). Yeast membrane vesicles (0.5 µg µl−1) containing CrMIP1 in 450 mM mannitol containing 90 mM KCl, 1 mM EDTA and 20 mM Tris–HCl (pH 7.2) were mixed with an equal volume of hypotonic 100 mM mannitol containing the same constituents as above.

Acknowledgements

We are highly grateful to Prof. Stefan Hohmann (Göttingen University, Sweden) for the generous gift of S. cerevisiae W303-1A and its mutant YSH294. We would like to dedicate this report to the late Prof. Shoshi Muto.

References


(Received February 20, 2004; Accepted June 3, 2004)