Differential targeting of closely related ECM glycoproteins: the pherophorin family from *Volvox*

Klaus Godl, Armin Hallmann¹, Stephan Wenzl and Manfred Sumper

Lehrstuhl Biochemie I, Universität Regensburg, D-93053 Regensburg, Germany

¹Corresponding author

K.Godl and A.Hallmann made equal contributions to this work

The alga *Volvox carteri* represents one of the simplest multicellular organisms. Its extracellular matrix (ECM) is modified under developmental control, e.g. under the influence of the sex-inducing pheromone that triggers development of males and females at a concentration below \(10^{-16}\) M. A novel ECM glycoprotein (pherophorin-S) synthesized in response to this pheromone was identified and characterized. Although being a typical member of the pherophorins, which are identified by a C-terminal domain with sequence homology to the sex-inducing pheromone, pherophorin-S exhibits a completely novel set of properties. In contrast to the other members of the family, which are found as part of the insoluble ECM structures of the cellular zone, pherophorin-S is targeted to the cell-free interior of the spherical organism and remains in a soluble state. A main structural difference is the presence of a polyhydroxyproline spacer in pherophorin-S that is linked to a saccharide containing a phosphodiester bridge between two arabinose residues. Sequence comparisons indicate that the self-assembling proteins that create the main parts of the complex *Volvox* ECM have evolved from a common ancestral gene.

**Keywords:** ECM glycoproteins/green algae/ hydroxyproline-rich glycoproteins/pherophorins/ *Volvox*

**Introduction**

Some of the simplest multicellular organisms are found among the green algae of the genus *Volvox*. *Volvox carteri* is composed of only two cell types: 2000–4000 biflagellate *Chlamydomonas*-like somatic cells are arranged in a monolayer at the surface of a hollow sphere and 16 much larger reproductive cells (‘gonidia’) lie just below the somatic cell sheet (Starr, 1969). Due to its simplicity, *Volvox* is an ideal model for a biochemical analysis of developmental processes.

Many developmental responses of cells are mediated by the extracellular matrix (ECM) with which those cells are in contact. In *Volvox*, this is very clearly demonstrated in the fascinating process of sexual differentiation triggered by the sex-inducing pheromone. *Volvox* cells are surrounded and held together by a glycoprotein-rich ECM (for a review see Kirk *et al.*, 1986). The *Volvox* ECM shows a distinct structural architecture. The outermost area, called the boundary zone (BZ; for nomenclature see Figure 1A) (Kirk *et al.*, 1986), contains those components of the ECM that appear to be continuous over the surface of the organism. The area lying internal to the boundary zone, called the cellular zone (CZ), exhibits specializations around individual cells. The deep zone (DZ) of the ECM consists of components that fill the cell-free interior of the spherical organism. This zone contains highly viscous polysaccharide-rich amorphous components (Kirk *et al.*, 1986).

The chemical composition of the *Volvox* ECM is strongly modified under the influence of the sex-inducing pheromone. The pheromone, a glycoprotein (Starr and Jaenicke, 1974; Tschochner *et al.*, 1987; Mages *et al.*, 1988), converts asexually growing males and females to the sexual pathway. This pheromone is among the most potent biological effector molecules known: it exhibits full effectiveness at \(6 \times 10^{-17}\) M (Starr, 1970; Gilles *et al.*, 1984). Many lines of evidence indicate that the ECM plays a key role in this sexual induction process. The earliest biochemical responses to the pheromone detected so far are structural modifications within the ECM (Wenzl and Sumper, 1982, 1986b; Gilles *et al.*, 1983). The CZ of the *Volvox* ECM contains members of the newly described pherophorin family. Pherophorins are glycoproteins that contain a C-terminal domain with homology to the sexual pheromone. Pherophorin I is constitutively expressed and represents a main component of the cellular zone of the ECM. Under the influence of the pheromone, synthesis of pherophorin II is initiated and its C-terminal domain becomes proteolytically liberated from the parent glycoprotein (Sumper *et al.*, 1993). It has been proposed that this modification of the ECM is part of a signal amplification process required to obtain the exquisite sensitivity of this sexual induction system. So far, the DZ has not been investigated in detail for any biochemical changes in response to the sexual pheromone, although some early observations indicate a modification of this ECM region (Gilles *et al.*, 1983).

In this paper, we describe a novel pherophorin that is synthesized in response to the sex-inducing pheromone. Although highly homologous to other members of the family that are located exclusively within the somatic cell sheets, this novel pherophorin (pherophorin-S) is specifically targeted to the DZ of the ECM. Pherophorin-S exhibits a unique glycosylation pattern among the pherophorins: it contains a phosphodiester bridge between two arabinose residues.

**Results**

**Identification of pherophorin-S**

Pheromone-induced changes in the composition of the ECM have been characterized in detail only within the
Fig. 1. (A) Highly stylized drawing emphasizing the main compartments of the *Volvox* ECM. BZ, boundary zone; CZ, cellular zone; DZ, deep zone, according to the nomenclature of Kirk et al. (1986); S, somatic cells; G, gonidium (reproductive cell). (B) Identification of the soluble pherophorin-S (Phero-S) and comparison with insoluble pherophorin II (Phero II). Fluorogram of an SDS–polyacrylamide gel loaded with different extracts from asexual (veg) or sexually induced *Volvox* spheroids pulse and pulse-chase labelled with $[^{35}S]$sulfate. Sexual induction was initiated by the application of pheromone ($\sim 0.1$ pM) for 40 min. After a pulse labelling period of 30 min, aliquots of *Volvox* spheroids were removed and analysed (ind). After a chase of 18 h, aliquots of the populations were again analysed (chase). First, a DZ extract of the ECM was prepared by carefully disrupting the spheroids. After centrifugation, the somatic cell sheets were further treated with detergent followed by EDTA/detergent (Sumper et al., 1993) to extract pherophorin II from the CZ of the ECM. Extracts from 50 *Volvox* spheroids were applied to a 4–15% SDS–polyacrylamide gel. In contrast to pherophorin II, which is proteolytically processed (42 and 30 kDa; Sumper et al., 1993; Godl et al., 1995), pherophorin-S remains stable during the chase period.

CZ (Figure 1A) of the ECM (Wenzl and Sumper, 1982, 1986b; Ertl et al., 1989; Sumper et al., 1993; Godl et al., 1995). In order to extend this analysis to the DZ (Figure 1A) of the ECM, which may constitute $\geq 90\%$ of the total volume of the organism, the composition of this ECM compartment from asexual and sexually induced organisms was compared after pulse labelling with radioactive sulfate.

Mild mechanical stress as may be exerted by forcing *Volvox* spheroids through a hypodermic needle fragments the spheroids, producing hemispheres or smaller fragments of cellular sheets. The material of the DZ is thereby selectively released. This mild fragmentation of the organism does not affect viability of the cells. After low speed centrifugation, the cell-free supernatant, containing the material from the DZ was subjected to SDS–PAGE. About 30 min after application of the sexual pheromone, synthesis of a previously unobserved $^{35}$S-labelled component, with an apparent molecular mass of 110 kDa, becomes detectable in the fluorogram of the SDS–polyacrylamide gel (Figure 1B, DZ, ind). This component is synthesized only transiently. Maximum expression is found 120 min after application of the pheromone. For reasons explained below, this component of the DZ was named pherophorin-S. Pherophorin-S is only detectable in the DZ of the ECM and is not detectable in asexually growing organisms. As pherophorin-S is quantitively extracted, without any additives (detergents, salt or EDTA), it is a soluble component of the ECM.

In contrast to other members of the pherophorin family which are proteolytically processed (Sumper et al., 1993; Godl et al., 1995), pherophorin-S is a stable protein, as demonstrated by a pulse-chase labelling experiment (Figure 1B).

Pulse labelling experiments using radioactive phosphate revealed that, in contrast to all other known members of the pherophorin family that have been studied previously, pherophorin-S also incorporates phosphate. The chemical nature of the incorporated phosphate will be described below.

**Purification of pherophorin-S**

Mild mechanical disruption of *Volvox* spheroids liberates the material of the DZ, including pherophorin-S. This property was used to purify pherophorin-S; the corresponding extract will be denoted in the following as ‘deep zone extract’. After filtration and centrifugation, the colourless extract containing pherophorin-S was fractionated by anion exchange chromatography (Q-Sepharose followed by Mono Q). Final purification was achieved by preparative SDS–PAGE.

Purified pherophorin-S exhibits different apparent molecular masses on SDS–PAGE, depending on the percentage of the acrylamide used. The observed values range from 90 to 130 kDa ($\sim 100$ kDa on an 8% gel). This is a property of some glycosylated proteins. Treatment of pherophorin-S with anhydrous hydrogen fluoride at 0°C, a procedure that selectively deglycosylates glycoproteins but does not cleave polypeptides (Mort and Lamport, 1977), reduces the apparent molecular mass by $\sim 10$ kDa (8% SDS–PAGE). Therefore, pherophorin-S is a glycoprotein.

To obtain amino acid sequence data, purified pherophorin-S glycoprotein was digested with trypsin and the resulting peptide mixture was separated by reversed phase C$_2$C$_{18}$ HPLC. The material of well-separated peaks was directly subjected to amino acid sequence analysis on an
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Fig. 2. (A) Strategy applied to collect the complete nucleotide sequence of pherophorin-S cDNA. Amino acid sequence information of tryptic peptide IYPSVGSSSIVTPSWTAIGG was used to design sense and antisense primers for amplification of a cDNA fragment of 56 bp in length (probe). Completion of the cDNA was achieved by 5'- and 3'-RACE-PCR and by sequence analysis of a genomic clone. The positions of known introns are indicated by arrowheads. The EMBL data library accession No. is Y07752. (B) Deduced amino acid sequence of pherophorin-S. The characteristic polyproline stretch is shown in white letters on a black background. Amino acid sequences confirmed from isolated peptides are underlined. An arrowhead marks the potential signal peptidase cleavage site. Potential N-glycosylation sites are boxed. The broken line marks the C-terminal part of the polypeptide found to be homologous to the sex-inducing pheromone.

automated gas phase sequencer. The amino acid sequence data obtained are underlined in Figure 2B.

Cloning of the pherophorin-S gene

The amino acid sequence of the tryptic peptide IYPSVGSSIVTPSWTAIGG was used to synthesize an antisense oligonucleotide primer to reverse transcribe mRNA isolated from sexually induced Volvox algae. A sense primer derived from the same peptide allowed amplification by PCR of a cDNA probe of 56 bp in length (Figure 2A, probe), which was cloned into the SmaI site of pUC18 by blunt ligation. Sequencing of this insert revealed a nucleotide sequence coding for the amino acid sequence of the peptide mentioned above. The RACE-PCR technique (Frohman et al., 1988) was used to obtain additional sequence information. A 3'-RACE-PCR produced the 3'-end of the mRNA, whereas successive 5'-RACE-PCRs did not arrive at the 5'-end of the mRNA (Figure 2A). Similar problems observed with hydroxyproline-rich glycoproteins from Volvox (Ertl et al., 1989, 1992) suggested that the pherophorin-S mRNA might also contain a C-rich stretch that causes premature termination of reverse transcription. Thus a genomic library of V. carteri constructed in the replacement vector λEMBL3 was screened to obtain the missing sequence data from a genomic clone. Nine positive clones were identified out of 60 000 phages screened. The ~17 kb insert of one of these clones was subcloned and sequenced. A GC-rich section coding for a proline-rich domain was indeed identified. The strategy applied to collect the complete cDNA sequence is schematically summarized in Figure 2A. The deduced amino acid sequence for pherophorin-S is given in Figure 2B. A molecular mass of 63.4 kDa was calculated for the polypeptide chain of pherophorin-S.

This is much less than the apparent molecular mass seen on SDS–polyacrylamide gels (Figure 1B, DZ, ind). The amino acid sequence of pherophorin-S exhibits five N-glycosylation sites, but glycosylation only accounts for an increase in molecular mass of ~10 kDa (8% SDS–PAGE). Most likely, the presence of a domain with a high proline/hydroxyproline content explains the difference between the observed and calculated molecular masses of deglycosylated pherophorin-S, because such a domain has a reduced ability to bind SDS (Andres et al., 1993).

Pherophorin-S is a member of the pherophorin family

A striking feature of the deduced amino acid sequence is the central domain, 88 amino acid residues in length, that is composed almost exclusively (89%) of proline residues. Most probably, the secondary structure of this domain is a polyproline II helix that separates the N- and C-terminal domains. A BLASTP search (Altschul et al., 1990) of the SwissProt Protein Sequence Database revealed significant identities of the deduced amino acid sequence to the pherophorin family from Volvox (Figure 3). The region of identity covers nearly the total length of the polypeptide chain. For instance, the N-terminal part of the polypeptide (amino acids 42–207) shows 55.3% identity to the genomic clone. Nine positive clones were identified out of 60 000 phages screened. The ~17 kb insert of one of these clones was subcloned and sequenced. A GC-rich section coding for a proline-rich domain was indeed identified. The strategy applied to collect the complete cDNA sequence is schematically summarized in Figure 2A. The deduced amino acid sequence for pherophorin-S is given in Figure 2B. A molecular mass of 63.4 kDa was calculated for the polypeptide chain of pherophorin-S. This is much less than the apparent molecular mass seen on SDS–polyacrylamide gels (Figure 1B, DZ, ind). The amino acid sequence of pherophorin-S exhibits five N-glycosylation sites, but glycosylation only accounts for an increase in molecular mass of ~10 kDa (8% SDS–PAGE). Most likely, the presence of a domain with a high proline/hydroxyproline content explains the difference between the observed and calculated molecular masses of deglycosylated pherophorin-S, because such a domain has a reduced ability to bind SDS (Andres et al., 1993).

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pheromone-dependent transcription purified from the transgenic strain grown in sulfate-containing medium (Hallmann and Sumper, 1994b, 1996; Schiedlmeier et al., 1994). The expression rate was 20- to 30-fold higher in vegetatively grown Volvox transformants than in sexually induced wild-type algae (Figure 5B). The transgenic Volvox strain did not have any visible phenotype.

The overexpression of pherophorin-S in clone PheroS-T1 allowed a much simpler purification protocol. Asexually grown PheroS-T1 algae were disrupted. After centrifugation the supernatant was brought to 10% acetonitrile and passed over a C18 cartridge. The flow-through was concentrated and applied to preparative SDS–PAGE. Pure pherophorin-S could be eluted from the gel. Recombinant pherophorin-S was used for all further chemical characterizations.

The characteristics of pherophorin-S from asexually growing transformant PheroS-T1 were compared with that obtained from sexually induced wild-type algae. Neither targeting into the DZ of the ECM nor post-translational modification causing incorporation of phosphate is affected in the transformant, indicating that these properties are not under the control of the sex-inducing pheromone. The apparent molecular masses of pherophorin-S from wild-type algae and from transformant PheroS-T1 are identical (Figure 5C).

**Identification of a phosphodiester between arabinose residues in pherophorin-S**

The carbohydrate composition of pherophorin-S was determined by radio gas chromatography. Pherophorin-S purified from the transgenic *Volvox* strain grown in the presence of [14C]bicarbonate was hydrolysed and the resulting monosaccharides were converted to the corresponding alditol acetates. Pherophorin-S contains the neutral sugars arabinose and galactose in a 1:1 ratio (Figure 6A).

Pherophorin-S incorporates [33P]phosphate in pulse-labeling experiments. Incorporated radioactivity is quantitatively removed from the polypeptide chain upon treatment with anhydrous HF, indicating that phosphate is not linked to a hydroxyamino acid. In addition, hydrolysis of pherophorin-S in 0.5 M trifluoroacetic acid at 100°C for 2 h quantitatively liberates bound phosphate as a low molecular mass derivative. Analysis of this hydrolysate on polyethyleneimine thin-layer plates resulted in the detection of two radioactive spots. One radioactive product migrated like a phosphodiester (Figure 7A). The latter substance stained with orcinol reagent and was completely hydrolysed after 2 h in 6 M HCl at 100°C. Arylsulfatase activity was determined photometrically by measuring the absorbance of the liberated 4-nitrocatechol. Only transformants treated with the pheromone in the presence of the chromogenic enzyme substrate 4-nitrocatechol sulfate. Arylsulfatase activity was determined photometrically by measuring the absorbance of the liberated 4-nitrocatechol. Only transformants treated with the sex-inducing pheromone exhibited enzyme activity (Figure 4B). Since arylsulfatase is extremely stable, activity could even be assayed in SDS–polyacrylamide gels using the chromogenic substrate 5-bromo-4-chloro-3-indolyl sulfate (Figure 4C).

Thus, the ~1 kb DNA fragment isolated from the upstream region of the pherophorin-S gene mediates transcription of the arylsulfatase reporter gene in response to the sex-inducing pheromone.

**Transgenic Volvox expressing pherophorin-S**

To obtain sufficient amounts of pherophorin-S for structural studies, transgenic *Volvox* were generated that express the pherophorin-S gene under the control of the strong *Volvox* β-tubulin promoter (Figure 5A). Stable transformants were produced as previously described (Hallmann and Sumper, 1994b, 1996; Schiedlmeier et al., 1994). The expression rate was 20- to 30-fold higher in vegetatively grown *Volvox* transformants than in sexually induced wild-type algae (Figure 5B). The transgenic *Volvox* strain did not have any visible phenotype.

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![Diagram](image)

**A**

**pheroorphorin-S**

5'-sequence

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Volvox

aryl sulphatase

genoamic sequence

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pUC18

KpnI

EcoRI

BamHI

SalI

ATG

TA

1 kb

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pUC18

**B**

![Activity assay graph](image)

**C**

![Activity stain graph](image)

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with trace amounts of labelled material) was hydrolysed and reduced. The resulting products were separated by high performance anion exchange chromatography (HPAEC) (Figure 7B). Radioactive fractions were subjected to thin-layer chromatography (data not shown) and to mass spectrometry. After reduction of the anomic carbon atoms with NaBH₄, the phosphodiester produced a mass signal at 365.0 (Figure 7C). This exactly corresponds to the calculated mass for the reduced phosphodiester.

The polyproline domain carries the phosphodiester

Pherophorin-S is not completely digested if treated with proteases like pronase, proteinase K or subtilisin. Rather, a resistant core with an apparent molecular mass of ~50 kDa (8% SDS–PAGE) remains (Figure 8A). Proteolytic degradation of ³²P-labelled pherophorin-S results in a core material that still contains all of the originally incorporated radioactivity. Consequently, the phosphodiester is located within this protease-resistant core material. In order to define this core material, purified pherophorin-S was digested with subtilisin. The 50 kDa core material was eluted from a preparative SDS–PAGE, deglycosylated with anhydrous HF (because the glycosylated material could not be analysed by Edman degradation) and purified by reversed phase HPLC (C₂₁/C₁₈). A single peptide eluted at 20% acetonitrile. Edman degradation of this material resulted in the sequence shown in Figure 8B. As expected, the resistant core material represents the proline-rich domain of pherophorin-S. The amino acid sequence analysis also confirms that the prolines at the very beginning of this domain (residues 211 and 213) remain unmodified, whereas prolines 215, 218, 219, 221 and 223 (and probably all the following) become hydroxylated and can serve as saccharide attachment sites.

The carbohydrate composition of the protease-resistant 50 kDa core material was determined by radio gas chromatography. The core material contains the neutral sugars arabinose and galactose in a 1:1 ratio (Figure 6B), exactly as found for intact pherophorin-S.

**Discussion**

Sequence homology proves that pherophorin-S is a member of the pherophorin family of Volvox ECM proteins. Like pherophorin II, it is synthesized in response to the sex-inducing pheromone. However, pherophorin-S
exhibits unique properties: it is accumulated in the DZ of the 
Volvox ECM in a completely soluble state, in contrast to all the other members known so far, which are insoluble 
and restricted to the CZ. Biogenesis of ECMs occurs by 
self-assembly, which means that each component contains 
within its structure the information necessary for this 
fascinating process. In pherophorins I–III the N- and 
C-terminal domains are separated by a short polyhydroxy-
proline spacer. It is this spacer element that is strikingly 
different in pherophorin-S: a stretch of ~90 amino acid 
residues that are almost exclusively hydroxyproline 
residues separates the terminal domains. This spacer 
is glycosylated and, of particular interest, contains a 
phosphodiester bridge between two arabinose residues. 
This type of modification was originally discovered in 
another ECM glycoprotein, namely SSG 185 from 
Volvox (Ertl et al., 1989). SSG 185 is the monomeric precursor 
of a polymeric substructure within the CZ of the ECM 
that surrounds individual cells, creating honeycomb-like 
chambers. Remarkably, SSG 185 contains exactly the 
same type of polyhydroxyproline spacer. Since SSG 185 
and pherophorin-S are found in completely different 
regions of the ECM, it is unlikely that this particular 
spacers provides the signal for targeting pherophorin-S to 
the DZ. Using the newly established system of 
Volvox transformation (Schiedlmeier et al., 1994), chimeras of 
pherophorin domains should allow identification of struc-
tural elements that are responsible for specific targeting 
within the ECM.

SSG 185 and pherophorin I represent the main components 
of the cellular zone of the ECM in asexually 
growing Volvox (Godl et al., 1995). Under the influence 
of the sex-inducing pheromone, pherophorin II is deposited 
within the CZ of the ECM and newly synthesized phero-
phin-S modifies the composition of the DZ. As the 
primary structures of all these ECM glycoproteins are 
known, it is possible to compare their modular composition

and to search for structural homologies among the 
modules. Figure 9A presents the domain structure of these 
glycoproteins in diagrammatic fashion. The C-terminal 
domains of all pherophorins (B-type domain) are character-
ized by sequence homology with the sex-inducing phero-
mone. The N-terminal domains of all pherophorins (A-type 
domain) are related to both the N- and C-terminal domains 
of SSG 185 (Figure 9B) (Godl et al., 1995). Moreover, a 
sequence comparison of the A- and B-type domains of 
pherophorin II reveals that even these two regions exhibit 
24% sequence identity over a stretch of 179 amino acid 
residues. Thus, the main parts of the complex ECM of 
Volvox, and even the species-specific signalling molecule 
(sex-inducing pheromone), appear to have been derived 
from the same ancestral gene (Figure 9B). Differently 
modulated domains have been linked together via spacer 
elements that are composed of polyhydroxyproline

Fig. 5. Transgenic 
Volvox constitutively expressing pherophorin-S. (A) Structure of the chimeric gene containing the Volvox β-tubulin promoter region and the pherophorin-S coding region (genomic clone). (B) Constitutive expression of pherophorin-S (Phero-S) in transgenic Volvox. Silver 
stain of a SDS–PAGE gel (8%) loaded with DZ extracts from vegetatively grown wild-type Volvox (lane 1) and transgenic Volvox (clone PheroS-T1) (lane 2). (C) [33P]Phosphate incorporation into pherophorin-S (Phero-S). Purified [33P]labelled pherophorin-S from sexually induced wild-type Volvox (lane 1) and DZ extracts from asexual wild-type Volvox (lane 2) and the transformant (lane 3), prepared after pulse labelling (30 min) with 
[33P]phosphate. Autoradiogram of a SDS–PAGE gel (8%). X-Ray film after 0.5 days’ exposure.

Fig. 6. Sugar analysis by radio gas chromatography. The scan at the 
top of each chromatogram records the mass signal of internal 
standards. (A) 14C-labelled alditol acetates derived from pherophorin-S after acid hydrolysis. (B) 14C-labelled alditol acetates derived from the subtilisin-resistant core peptide of pherophorin-S. (C) 14C-labelled 
alditol acetates derived from the purified phosphodiester that is a 
structural element of pherophorin-S.
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Fig. 7. Identification of a $^{33}$P-labelled phosphomono- and phosphodiester of arabinose in acid hydrolysates of pherophorin-S. (A) Autoradiogram of a polyethyleneimine thin-layer chromatogram. Lane 1, phosphodiester and phosphomonoester obtained from the ECM-protein SSG 185 (Holst et al., 1989) plus inorganic phosphate; lane 2, hydrolysed pherophorin-S; lane 3, inorganic phosphate. (B) The compounds of pherophorin-S released by acid hydrolysis were purified by HPAEC under strong alkaline conditions. The eluent was monitored by pulsed amperometric detection (PAD). For details see Materials and methods. D, phosphodiester; M, phosphomonoester; P, inorganic phosphate. (C) Characterization of the phosphodiester derived from pherophorin-S by electrospray mass spectrometry.

Fig. 8. The polyproline domain of pherophorin-S carries the phosphodiester of arabinose. (A) Purified $^{33}$P-labelled pherophorin-S (Phero-S) subjected to 8% SDS–PAGE. Silver stain (lane 1) and fluorogram of the same sample before (lane 2) and after digestion with subtilisin (lane 3). (B) Sequence analysis of the subtilisin-resistant fragment (50 kDa) from pherophorin-S. Non-hydroxylated prolines are indicated by arrowheads. The section containing no prolines, but only hydroxyprolines, is marked by a horizontal arrow.

sequences and it is only the introduction of these spacers that qualifies these Volvox ECM proteins for membership in the class of hydroxyproline-rich glycoproteins (HRPGs) typical of plant cell walls. These molecular data offer strong support for the idea of a gene superfamily of hydroxyproline-rich glycoproteins (for reviews see Kieliszewski and Lamport, 1994; Woessner and Goodenough, 1994) from which new ECM proteins could
Purification of pherophorin-S

Cloning of the pherophorin-S gene

Radioactive labelling of pherophorin-S with [35S]sulfate or [33P]phosphate, incubation under standard conditions Ci [33P]phosphate, and the degenerate antisense oligonucleotide primer CCKATNGCNGTCCA

Materials and methods

Volvox strains

Wild-type *V. carteri* strain HK10 (female) was obtained from the Collection of Algae at the University of Texas (R.C.Starr). Mutant strain 153-48 of *V. carteri* (Adams et al., 1990), obtained from D.L.Kirk (Washington University, St Louis, MO) was used as the DNA recipient in transformation experiments. This strain with wild-type morphology carries a stable loss-of-function mutation in nitA, the structural gene encoding nitrate reductase (Gruber et al., 1996).

Culture conditions

Synchronous cultures were grown in *Volvox* medium (Provasoli and Pintner, 1959) at 28°C in a 8 h dark/16 h light (10 000 lux) cycle (Starr and Jaenicke, 1974). The non-selective medium used in transformation experiments was *Volvox* medium supplemented with 1 mM NH₄Cl; selective medium was *Volvox* medium lacking NH₄Cl and containing only nitrate as a nitrogen source.

Radioactive labelling of pherophorin-S with [35S]sulfate or [33P]phosphate

Pulse labelling with [35S]sulfate was performed as described by Wenzl and Sumper (1981). In pulse and pulse-chase experiments with [33P]phosphate, *Volvox* spheroids were washed thoroughly with and then suspended in 1 ml glycophosphate-free *Volvox* medium. After the addition of 50 μCi [33P]phosphate, incubation under standard conditions was continued for 0.5 or 1 h.

Purification of pherophorin-S

Sexually induced wild-type *Volvox* spheroids from six 20 l cultures were harvested by filtration on a 100 μm mesh nylon screen. The spheroids were broken up by forcing them through a 0.5 mm hypodermic needle. The disrupted spheroids were centrifuged at 20 000 g for 30 min. In order to remove any remaining insoluble components, the supernatant was brought to 25 mM Tris–HCl, pH 7.5, 0.9 M NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and applied to a QAE-Sephadex A-25 column (Pharmacia) equilibrated with the same buffer. Pherophorin-S does not bind and was therefore detected in the flow-through. After this filtration step, the material containing pherophorin-S was diluted to 0.3 M NaCl and applied to a Q-Sepharose FF anion exchange column (Pharmacia). Elution was performed with a linear gradient of 0.3–0.9 M NaCl in 25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 mM PMSF. Pherophorin-S elutes at ~0.7 M NaCl. The fraction containing pherophorin-S was diluted to 0.35 M NaCl and applied to a MonoQ HR 5/5 FPLC anion exchange column (Pharmacia). Again, elution was performed with a linear gradient of 0.35–1.5 M NaCl in 25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 mM PMSF. Pherophorin-S was recovered at ~0.80–0.85 M NaCl. Fractions containing pherophorin-S were concentrated by precipitation with deoxycholate and trichloroacetic acid (Mahan et al., 1983). Final purification was achieved by preparative SDS–PAGE (7%).

Proteolytic digestion and separation of peptides

Aliquots of 20–30 μg pherophorin-S were applied to a 7% SDS–PAGE gel and stained with Coomassie brilliant blue. The gel slice containing pherophorin-S was cut into small pieces. Further treatment and digestion with trypsin was performed as described by Selmer et al. (1996). The resulting peptides were eluted from the gel by diffusion in 0.2 M (NH₄)HCO₃/50% acetonitrile. The eluate was passed through a 0.22 μm filter (Millipore), brought to 0.1% trifluoroacetic acid with a flow rate of 200 μl/min. Peptides only nitrate as a nitrogen source. The eluates were dissolved in 6 M guanidine–HCl/0.5% acetonitrile. The eluate was passed through a 0.22 μm filter (Millipore), brought to 0.1% trifluoroacetic acid and dried by lyophilization. The peptides were dissolved in 6 M guanidine–HCl/0.1% trifluoroacetic acid and fractionated by reversed phase HPLC (SMART and Jaenicke, 1974). The peptides were eluted with a 30 min linear gradient of 5–40% acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 200 μl/min. Peptides were sequenced by Edman degradation using an automated gas phase peptide sequencer (Applied Biosystems, Foster City, CA).

Generation of a cDNA probe by PCR

Generation of a cDNA probe for pherophorin-S was performed using the degenerate antisense oligonucleotide primer CCKATNGCNGTCCA and the degenerate sense oligonucleotide primer ATHTAYCNAAGYGT. The resulting 56 bp cDNA fragment was ligated into the Smal site of vector pUC18 by blunt ligation and sequenced.

Cloning of the pherophorin-S gene

The RACE-PCRs were performed as described by Frohman et al. (1988). The *V. carteri* genomic library in λEMBL3 (Frischauf et al., 1983)
described by Ertl et al. (1989) was used to clone the pherophorin-S gene. The screening procedure followed standard techniques (Sambrook et al., 1989). DNA sequencing was performed by the chain termination method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia).

Construction of the chimeric pherophorin-S–arylsulfatase gene

The Volvox arylsulfatase reporter gene (Hallmann and Sumper, 1994b) was placed under the control of the pherophorin-S gene 5’-region. Additional restriction sites were introduced by PCR to facilitate ligation of the parent DNAs. An EcoRV site was generated directly in front of the start codons of both the pherophorin-S gene and the Volvox arylsulfatase gene. A KpnI site was introduced into the pherophorin-S 5’-region ~1 kb upstream of the start codon. Then, a KpnI–EcoRV fragment bearing ~1 kb upstream sequence of the pherophorin-S gene was ligated to an ~10 kb EcoRV–SalI fragment containing the Volvox arylsulfatase gene (Hallmann and Sumper, 1994a) with its 15 introns. The complete construct was confirmed by sequencing.

Construction of the chimeric β-tubulin–pherophorin-S gene

To achieve high pherophorin-S production, the pherophorin-S gene was placed under the control of the Volvox β-tubulin promoter. For construction of the chimeric gene, genomic clones of Volvox β-tubulin (Harper and Mages, 1988) and pherophorin-S were used. An additional EcoRV site was generated by PCR directly in front of the start codons of both the pherophorin-S gene and the Volvox β-tubulin gene to facilitate ligation of the parental DNAs. A KpnI site was introduced into the β-tubulin promoter region ~0.5 kb upstream of the start codon. Then, a KpnI–EcoRV fragment bearing ~0.5 kb Volvox β-tubulin promoter region was ligated to an ~9 kb EcoRV–SalI fragment containing the pherophorin-S gene. During cloning a T nucleotide from the EcoRV site in front of the start ATG was deleted for unknown reasons, destroying the EcoRV site. The complete construct was confirmed by sequencing.

Stable nuclear transformation of Volvox carteri

Volvox carteri strain 153-48 was transformed by using a particle gun to bombard cells with DNA-coated gold particles as described previously (Schiedmeier et al., 1994). Plasmids carrying the artificial gene constructs were introduced into Vcarteri nttA strain 153-48 by co-transformation with plasmid pVcNR1 (Gruber et al., 1992; Schiedmeier et al., 1994), containing the coding region of the Vcarteri nttA gene plus downstream and upstream DNA. Bombarded cultures were cultivated in selective Volvox medium containing only nitrate as a nitrogen source.

Reverse transcription–PCR amplification and sequencing of chimeric transcripts

For reverse transcription–PCR the antisense primer 5’-TTTGGAGGCCT-AATTCGG (pherophorin-S) was used for transformations containing the β-tubulin promoter-pherophorin-S chimeric gene. The sense primer was 5’-GATACAAGA ACCA GACTAC (β-tubulin). Products of PCR amplification were ligated into the Smal site of pUC18 and sequenced.

Preparation of recombinant pherophorin-S

Volvox transformants (clone Pheno-S-T1), constitutively expressing the pherophorin-S gene under the control of the β-tubulin promoter, were grown in 20 l glass flasks under standard conditions. Spheroids were disrupted and centrifuged as described before. After centrifugation 33P-labelled pherophorin-S was added to facilitate identification. The supernatant was brought to 10% acetonitrile and passed over a C18 (octadecylsilane) cartridge (Millipore). The flow-through was concentrated by lyophilization and applied to a preparative 8% SDS–PAGE gel. After autoradiography pherophorin-S was eluted with water by diffusion, dialysed and lyophilized.

Preparation of the 50 kDa subtilisin fragment

Purified and 33P-labelled pherophorin-S was digested with 0.6 µg/ml subtilisin (Carlsberg, type VIII, Sigma) in 50 mM Tris–HCl, pH 8.0, 0.5% SDS for 1 h at 30°C and applied to 10% SDS-PAGE. After autoradiography the 50 kDa subtilisin fragment was eluted with water, dialysed and lyophilized. Deglycosylation of the 50 kDa subtilisin fragment was performed with anhydroxy glucose hydrofluoric acid at 0°C as described by Mort and Lampori (1977). The deglycosylated peptide was purified by reversed phase HPLC (SMART system; Pharmacia) on a 3 µm µRPC C8/C18 column (Pharmacia) by applying a 30 min linear gradient of 5–40% acetonitrile in 0.1% CF3CO2H at a flow rate of 200 µl/min.


