

# How a sex pheromone might act at a concentration below $10^{-16}$ M

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**The sex-inducing pheromone of *Volvox carteri* is a glycoprotein that triggers development of males and females at a concentration below  $10^{-16}$  M. Evidence is presented for the existence of a novel mechanism of signal amplification operating within the extracellular matrix of this multicellular organism. A family of 70 kDa matrix glycoproteins denoted pherophorins bear a C-terminal domain being homologous to the sex-inducing pheromone. Under the influence of the pheromone, this domain is liberated by highly specific proteolysis.**

**Key words:** extracellular matrix/pheromone/sexual inducer/signal amplification/*Volvox*

## Introduction

The sexual pheromone (sexual inducer) of the multicellular green flagellate *Volvox carteri* is a glycoprotein synthesized and released by sperm cells (Starr, 1969, 1970; Starr and Jaenicke, 1974; Gilles *et al.*, 1981; Tschochner *et al.*, 1987; Mages *et al.*, 1988). It is one of the most potent biological effector molecules known: it exhibits full effectiveness at  $6 \times 10^{-17}$  M and a single sexual male produces enough inducer to convert many millions of asexually growing males and females to the sexual pathway (Starr, 1970; Gilles *et al.*, 1984).

Asexual spheroids of both the male and female strains exhibit 2000–4000 biflagellate somatic cells (similar to *Chlamydomonas* in their morphology) that are arranged in a monolayer at the surface of a hollow sphere (Starr, 1969). Only 16 much larger asexual reproductive cells ('gonidia') lie just below the somatic cells. The cells are held together by a glycoprotein-rich extracellular matrix (ECM) (for review, see Kirk *et al.*, 1986). The ECM sheath shows considerable structural organization in that it exhibits clearly visible boundaries towards the interior of the spheroid as well as honeycomb-like structures around each cell (Ertl *et al.*, 1989).

The sex-inducing pheromone causes a gonidium to cleave in a different pattern to initiate development of a sexual organism (Starr, 1970). In the asexual cycle of both females and males the first unequal cleavage occurs in 16 cells of the 32-cell embryo to generate 16 (large) gonidial initials. In pheromone-treated gonidia of females, however, the first unequal cleavage is delayed one cycle, resulting in 32 (or more) reproductive initials which finally differentiate to egg cells (Figure 1B). In contrast, in sexually induced gonidia of males, the first unequal cleavage takes place at the 256-cell

embryo and affects all cells, so that a 1:1 ratio of somatic and reproductive ('androgonidia') initials is produced (Figure 1C). The androgonidia ultimately develop into packets of sperm cells.

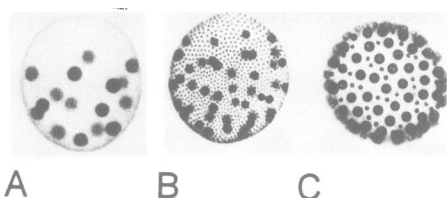
The ultimate target of pheromone action obviously is the gonidium of the asexual organism. Hence it has generally been assumed that the gonidium is also the site of initial inducer action. However, the exquisite sensitivity of the inducing system calls for a potent signal amplification and can hardly be explained in terms of conventional receptor/effector-mediated interactions. The first biochemical response detected so far is a structural modification within the extracellular matrix (Wenzl and Sumper, 1982, 1986; Gilles *et al.*, 1983). In particular, the earliest response, detectable a few minutes after the application of the pheromone is the synthesis of an extracellular sulfated glycoprotein with an apparent molecular mass of 70 kDa (SG 70). Remarkably, this glycoprotein is synthesized by the somatic cells of the organism (Wenzl and Sumper, 1986).

In this paper, we describe the isolation of this extracellular matrix glycoprotein and demonstrate the existence of a gene family for SG 70 glycoproteins. The deduced amino acid sequence reveals a highly unexpected result. The C-terminal third (~190 amino acid residues in length) is homologous to the sex-inducing pheromone and during sexual induction, this domain is liberated by specific proteolysis from the parent molecule. In the light of this finding, a mechanism of signal amplification in this extremely sensitive pheromone system is proposed and supported experimentally.

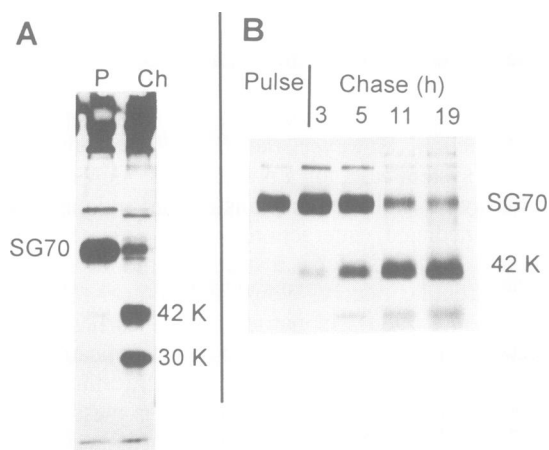
## Results

### Induction of SG 70 synthesis

The earliest biochemical response to the application of the sexual pheromone is the synthesis of a tyrosine sulfate-containing glycoprotein that was denoted SG 70 (Wenzl and Sumper, 1986). SG 70 is synthesized exclusively by somatic cells, rather than by the reproductive cells, the ultimate recipients of the pheromone's message. Shortly after its production, SG 70 becomes deposited in an insoluble form within the extracellular matrix. An important hint for an understanding of its biological function is provided by a pulse-chase labelling experiment (Figure 2). After the application of the sexual pheromone, synchronously developing *Volvox* spheroids were pulse labelled with [ $^{35}$ S]sulfate (60 min), conditions which allow a high incorporation of radioactivity into the SG 70 glycoprotein. Even after its deposition within the extracellular matrix (a process that takes ~60–90 min), SG 70 turned out to be an unstable molecule being proteolytically degraded in a highly specific manner. With a half life of ~6 h, SG 70 decays quantitatively into two defined cleavage products of apparent molecular masses 42 and 30 kDa (Figure 2). These cleavage products were found to be stable for at least 24 h.



**Fig. 1.** Asexual and sexually induced *Volvox* spheroids. (A) the asexual organism (male or female); (B) sexually induced female; (C) sexually induced male.



**Fig. 2.** Specific cleavage of SG 70 *in vivo*. Fluorograms of SDS–polyacrylamide gels loaded with extracts from *Volvox* spheroids pulse- or pulse–chase-labelled with [ $^{35}$ S]sulfate. (A) Pheromone was added to a *Volvox* population ( $\sim 0.1$  pM) followed by the addition of radioactive sulfate. After a pulse labelling period of 60 min, an aliquot was removed and the EDTA extract from 50 *Volvox* ghosts (Materials and methods) was applied to 10% SDS–PAGE (P). After a chase of 18 h, another aliquot of the population was analysed in exactly the same way (Ch). (B) Kinetics of SG 70 cleavage *in vivo*. Pulse–chase labelling as described for (A), except that *Volvox* ghosts were solubilized by HF treatment (Materials and methods) for a quantitative recovery of the cleavage products.

### Purification of SG 70

In a previous article, we have shown that newly synthesized SG 70 becomes converted into a highly insoluble structural element of the ECM, remaining insoluble even in 1% SDS solution at 95°C (Wenzl and Sumper, 1986). This property was used to purify SG 70. Sexually induced *Volvox* spheroids were boiled for 5 min in 1% SDS containing 0.5 M NaCl. This rigorous treatment solubilizes most intracellular proteins but leaves intact the insoluble extracellular matrix structure. Thus colourless *Volvox* ‘ghosts’ are obtained. Extraction of these ghosts with 100 mM EDTA in the presence of 1% detergents like Triton X-100 or Mega-9 highly selectively removes SG 70 as well as the 42 kDa cleavage product. Final purification was achieved by preparative SDS–PAGE. Alternatively, *Volvox* ‘ghosts’ are quantitatively solubilized by treatment with anhydrous hydrogen fluoride at 0°C, a procedure that selectively deglycosylates glycoproteins but does not cleave polypeptides (Mort and Lampert, 1977). After HF treatment of ghosts, only two main polypeptides are detectable on SDS–PAGE. One of these polypeptides was identified as the previously described extracellular matrix glycoprotein SSG 185 (Ertl *et al.*, 1989), whereas the remaining main polypeptide was derived from SG 70.

### Protein chemical studies

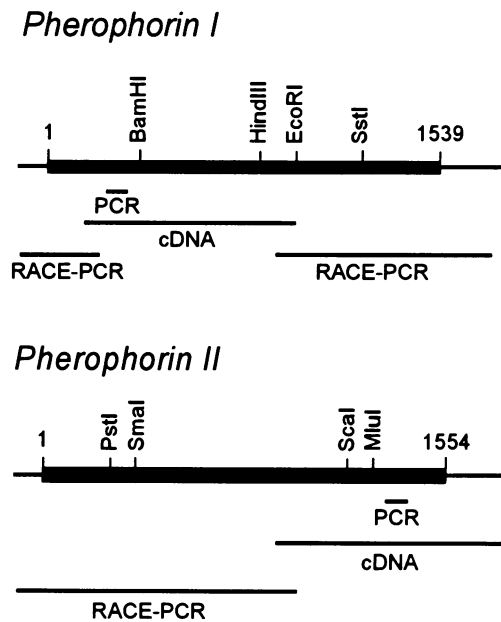
The purified SG 70 polypeptide was readily digested by proteinases like trypsin, chymotrypsin or proteinase Lys-C. The resulting peptide mixtures were separated by reversed phase  $C_{18}$  HPLC. The materials of well-separated peaks were directly subjected to amino acid sequence analysis on an automated gas phase sequencer. All amino acid sequences determined are underlined in Figure 4. A comparison of all amino acid sequences obtained revealed an important fact. Two peptides turned out to be closely related, indicating the existence either of at least two SG 70 genes or of repeating sequences within the same gene product. To avoid any complication introduced by the possibility of mixing information from different genes, we only used the sequence information derived from one and the same peptide for the subsequent cloning procedure.

### Generation of cDNA probes by PCR

The amino acid sequence information from the tryptic peptide KIEINVNQVCDVFGNVV-ATLNGVPTK (peptide 1) was used to synthesize the sense oligonucleotide primer AAG-ATYGAGATYAA corresponding to amino acids 1–5. The antisense primer GTNGGNACKCCGTT was derived from amino acid positions 22–26 of the same peptide. The latter primer served to reverse transcribe mRNA from sexually induced (30 min) *Volvox* spheroids. The resulting cDNA was amplified by the PCR in the presence of both the sense and antisense primers. Thirty cycles of amplification produced a cDNA fragment of 80 bp in length that was cloned by blunt-end ligation into pUC18. Sequencing this insert revealed an open reading frame matching the amino acid sequence of peptide 1. Exactly the same strategy was applied to produce an 83 bp fragment encoding the amino acid positions 3–30 of the peptide (denoted peptide 2) KTIAD-SWGATGTDTLKVNLNWNLQANGGK. The oligonucleotides ATYGCKGAYAGCTGGGG and TTKCCCKC-CGTTKGCTYTG served as sense and antisense primers, respectively. The resulting cDNA fragments were then used as highly specific probes to screen a *Volvox* cDNA library.

### Cloning of the SG 70 genes

A cDNA library in the  $\lambda$ gt11 vector was constructed using poly(A)<sup>+</sup> RNA from sexually induced *Volvox* spheroids. Each of the PCR-generated probes was used separately to screen the library ( $2 \times 10^5$  phages per screening). In both cases a few positive clones could be identified. The corresponding inserts were subcloned into pUC18 and sequenced. As shown schematically in Figure 3, the peptide 1-derived probe detected a 0.9 kb cDNA fragment that terminated at its 3'-end at an *Eco*RI site located within the coding region of the gene. The sequences of the remaining 5'- and 3'-stretches were established by the RACE-PCR technique (Frohman *et al.*, 1988). Figure 3 schematically summarizes the strategy applied to collect the complete cDNA sequence information. The probe derived from peptide 2 allowed the identification of a recombinant  $\lambda$ gt11 clone carrying a 1.8 kb insert. Transcription of this insert had been initiated at the poly(A) tail of the corresponding mRNA without producing, however, a full-length transcript. Therefore the remaining sequence information were again collected by the RACE-PCR technology (Figure 3). The nucleotide sequence analysis of the complete cDNAs confirmed that peptides 1 and 2 are indeed encoded by



**Fig. 3.** Strategy applied to collect the complete nucleotide sequence of pherophorin I and pherophorin II cDNAs. Amino acid sequence information from a peptide was used to synthesize sense and antisense primers for PCR-amplification of a short cDNA fragment. A cDNA library was probed with this fragment. Completion of the cDNA sequences was achieved by RACE-PCR.

different but related genes. The deduced amino acid sequences (Figure 4) share 31% identity and exhibit 73% similarity. Common to both polypeptides is a stretch of proline residues around amino acid position 170, indicating the existence of two well separated domains consisting of ~170 and 300 amino acid residues, respectively. Both polypeptides contain potential N-glycosylation sites (7 and 8, respectively).

#### SG 70 shares homology with the sexual inducer glycoprotein

The result of a homology search was highly unexpected: the C-terminally located domains of both SG 70 polypeptide chains share 30% identity (71% similarity) with the sex-inducing pheromone glycoprotein. This is documented in Figure 5. The pheromone glycoprotein that is synthesized and secreted into the medium by sperm cells consists of a polypeptide with 197 amino acid residues (Mages *et al.*, 1988). The region of homology within the SG 70 molecules covers nearly the total length of the pheromone polypeptide chain. Particularly, all of the cysteine residues of the pheromone remain conserved in the SG 70 domains indicating conservation of the three-dimensional structure. Since both the SG 70 molecules carry a homologue of the pheromone as a C-terminally located domain we would like to denote these novel extracellular matrix glycoproteins as pherophorins I and II.

#### Transcriptional control of pherophorin synthesis

PCR was used to determine whether synthesis of both pherophorins is influenced by the presence of the sexual pheromone at the level of transcription. RNA was extracted from 10 asexual spheroids (1 h after release from the mother spheroid). Spheroids from the same population were treated with the sexual pheromone and after 20 min, RNA was again

#### Pherophorin I:

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QAQNVAYPNF PYCQCIKSPS PYSLEPVVKS NRTGQYCFTL
RVTKPSPSAT GYCATAKADIK KIEINVNQVC DVFGNVVNAT
LNGVPTKVGP AFDTPPDGPN TSRILRFTQL NLGLSDSGAM
LCITLGQNDK GKGCITLEDL CAPPAGAPKG TCSLALEDISK
PDCCPISRVS PPAPPPPPPP PPPEPVAVPI TPPCKTCVYA
TITALPPLL FPFQLTPSICQ SVADKIAGDL EMIVTSYSIG
YSGATITCSG NVIKVCASF S LPPGAPYTGL QADISNALTF
WLSLLAPSTG CPAYFANHQV TVTVGGDGD NSVTCLEGTA
TTTCKPGNPD FPKCECTKP AATRFALPT LQEPGRPSN
RTNSTLYCFT LQVVAPINEN GLCNTTLL KAEWGNIDIP
TQRRKILALA FKAAGASSPL RYLSPSWGSA GEOTLKVSGI
NWDASQADGA KICMELSNDT NLKTFCNTGQ DTCWINLFSP
DKQCCPLFAA SLTP -494
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#### Pherophorin II:

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AVTPGSIFNF PFRDQNTTNG AYRLAPVWRP SGSNTYCFKI
QVSQDALSC T GACCSADLHK IEFNVCSCL VAGASVDATV
NGVTRTVGAT LDKAPAGVPG SAVLRLTQLG LDTTTAQDAE
VCLTLKTNRG GQGCTTLDQL CSSPGFPAGT CTAAMFDVAC
DCCFVSQAGQ AIPPPPVPI PVLRPCDVCI AATIVPPAND
VRPYRYSAT CAAIQONIAN AMNSLLGGAN INVSPFAEN
ASQCFDTQII TCGRENGSDT DALANLJEAV QQQLSAFIGV
ASGNGVCNPK LEKYTVTVST INNVADQCLD LSQSASCFLP
GVPPFNCTON TTQGVMPFTV SPTWYAQPAN VRWGRNVJEY
CFTVNTLQPS QVVPSTCYNA NDALAKIEWY ASDAFRSAVK
GFTVYPAGGS NKTIADSWGA TGTDTLKVNL NWNLLQANGG
KVCVAIQNPF TMGDICKGAL GQCYASIFNR DNSDYCCPIY
RTGP -484
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**Fig. 4.** Deduced amino acid sequences of pherophorins I and II. A polyproline stretch around amino acid position 170 is denoted in white letters. Amino acid sequences confirmed from isolated peptides are underlined. 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.

extracted from 10 *Volvox* spheroids. Two antisense oligonucleotides specific for pherophorins I and II, respectively were applied simultaneously to reverse transcribe the RNA populations. Subsequent PCR amplification of pherophorin I and II cDNA fragments yielded the results documented in Figure 6. Pherophorin I mRNA levels remain essentially unaffected by the pheromone treatment. In sharp contrast, transcription of the pherophorin II gene is strongly induced in the presence of the sexual pheromone. Thus, pherophorin I appears to be constitutively expressed, whereas pherophorin II synthesis is strongly stimulated in response to the application of the sexual pheromone.

#### Site of pherophorin II cleavage

Originally, the 42 kDa cleavage product was found to be derived from a pherophorin species produced under the

Sex-Ind	SLLAVCLVFMVGLCTGQIVDVKFPSCRC--RELTPFAIKSA--ATQLTS
Phero I	PNSVTCLGTAATTC--KPGNPDFPKCECTKPAATRFAALPT--LTQEPG
Phero II	NVADQCLDLSQSASC--LPGVPEPNCNTTQGVMPFTVSPTWYAQPANV
Sex-Ind	RNPGVVN--LYCFEIGIVNSG---SGACYTEPASQNLKSVSVYAQAQRDR
Phero I	RPSNRTNSTLYCFTLQVVAPL---NPNGLCGNTT-TLLKAEWGNDIPTOR
Phero II	RNGRNVN--EYCFEVTNLQPSQVVPSTCY--NANDALAKIEWYASDAERSA
Sex-Ind	LSAFGVLL-AGAPVSNMITYLTPRWDSL--NMTTIS-NLNFSKTQANGTRIC
Phero I	RKILALAFKAAGASSPLRYLSPSWGSAGEQTLKVS-GLNWDASQADGAKIC
Phero II	VKGETVYP-AGG--SNKT-IADSWGAT--GDTTLKVNLNWNLLQANGGKVC
Sex-Ind	LGLFKPTTINEFCEREGASGSFCWVALFN-DNNCVPP -176
Phero I	MELSDNTNLKTFC---NTGQDTCWINLSPDKQCPL -491
Phero II	VAIQNPFTMGDTC--KGALGQ-CYASIFNRDNDYCC -477

Fig. 5. Alignment and comparison of the sex-inducing pheromone (Sex-Ind) with the C-terminally located domains of pherophorins I and II. Only identities are indicated. A comparison of the pheromone and the pherophorins indicates 30% identity and 71% similarity.

influence of the sexual pheromone. Therefore, the PCR results presented so far suggest that the 42 kDa cleavage product may be derived only from pherophorin II. The purification procedure for pherophorins described above also allows the purification of the 42 kDa cleavage product. Automated Edman degradation of the purified fragment showed that its N-terminal sequence was VLRPCDVCIA-ATI. This sequence is indeed derived from pherophorin II, matching amino acid positions 182–194. Thus, the cleavage site is exactly adjacent to the polyproline stretch supposed to operate as a linker between two protein domains. Out of four tryptic peptides isolated from the 42 kDa cleavage fragment, none was derived from pherophorin I, substantiating the idea that only pherophorin II is proteolytically processed. It is interesting to note that pherophorin I exhibits an insertion of seven amino acid residues (EPVAVPI) at the position corresponding to the pherophorin II cleavage site and this might cause the resistance of pherophorin I to proteolytic cleavage.

#### Pherophorin II synthesis in sterile mutants

In the literature, a number of mutants are described which are affected in the sexual induction process (Zeikus and Starr, 1980). Sterile mutants are particularly easy to detect, since they continue asexual growth in the presence of the sex-inducing pheromone. If expression and proteolytic cleavage of pherophorin II are essential processes in the sexual induction pathway, at least some of these sterile mutants should be affected in these particular steps. For this reason, the expression of pherophorin I and II genes in sterile mutants was studied either in the absence or in the presence of the sexual pheromone. Again, the PCR based technology mentioned above was used for this intention. A total of three independently isolated sterile mutants were investigated (ster gone 12 from the culture collection of algae at Austin and another two sterile mutants which had spontaneously originated in our laboratory). The results of this PCR experiment are documented in Figure 7. All of the sterile mutants investigated are indeed unable to increase the transcription rate of the pherophorin II gene in response to the application of the pheromone, whereas the pherophorin I gene remains constitutively expressed as it is found in the wild type strain.

#### Discussion

The sex-inducing pheromone of *V. carteri* is one of the most powerful regulatory proteins known: it triggers the biological

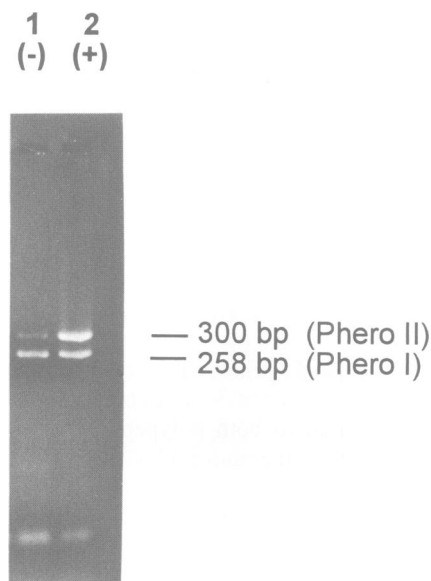
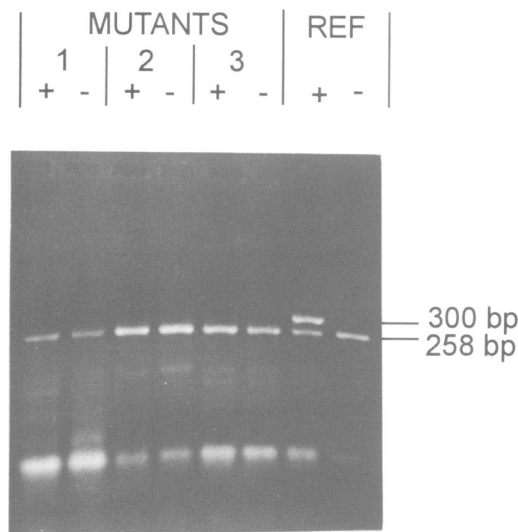


Fig. 6. Transcriptional control of pherophorin II synthesis. RNA was extracted from 10 asexual *Volvox* spheroids (–) or from 10 sexually induced spheroids (+). After reverse transcription in the presence of both a pherophorin I- and a pherophorin II-specific antisense oligonucleotide, a 258 bp fragment derived from pherophorin I and a 300 bp fragment derived from pherophorin II were amplified by PCR. For details see Materials and methods.

response at a concentration below  $10^{-16}$  M. This exquisite sensitivity allows quantitative conversion to the sexual pathway of a *Volvox* population living in a pond or even in a small lake. For this reason, attempts to elucidate the mechanism of this signal transduction have constituted one of the intensive areas of *Volvox* research for more than two decades. However, none of the models proposed so far have been verified experimentally and the mechanism remained highly enigmatic. In particular, a role of cAMP claimed many times as an extracellularly acting second messenger (Gilles *et al.*, 1985) could be excluded experimentally (Wenzl and Sumper, 1986, 1987). Any mechanism proposed for the sexual induction process has to explain at least the three following paradoxes observed for the action of the pheromone. (i) Despite its extreme sensitivity, a rather prolonged exposure to the pheromone is required to trigger sexual induction (Gilles *et al.*, 1984). Independent of the pheromone concentration applied, an exposure of at least 6 h to the pheromone is required before uncleaved gonidia of a *Volvox* spheroid become committed to initiating sexual



**Fig. 7.** Pherophorin I and II transcription in sterile *Volvox* mutants. RNA was extracted from 10 spheroids incubated in the absence (–) or presence (+) of the sex-inducing pheromone. The following strains were tested: wild type HK 10 (REF) and the sterile mutants gone 12 (1), HK 10-N2 (2) and HK 10-N3 (3). Reverse transcription and PCR amplification of pherophorin I and II mRNA were performed as described in the legend to Figure 6.

development. These observations suggest that some slowly accumulated product of pheromone action must reach a critical threshold level.

(ii) Obviously, the ultimate target of the pheromone's signal is the uncleaved reproductive cell (gonidium) that will eventually divide to produce either an asexual or a sexual offspring. However, all of the early biochemical events that have been detected in response to the pheromone take place in either the somatic cells or the extracellular matrix. This is all the more puzzling since gonidia can actually be induced by the pheromone in the absence of either somatic cells or ECM (Wenzl and Sumper, 1986).

(iii) Isolated gonidia, however, require much higher concentrations (at least two orders of magnitude) of pheromone in order to enter the sexual pathway. This had been interpreted to indicate the existence of a signal amplification system provided by the somatic cells and the surrounding ECM (Wenzl and Sumper, 1986).

The discovery of the pherophorins and the characterization of their biochemical properties offers a new approach to solve the puzzles mentioned above. The answer emerging for the question 'how might a pheromone trigger a biological response at or below  $10^{-16}$  M', is as follows.

(i) The highly negatively charged ECM *per se* has the property to bind in a non-specific manner positively charged molecules, including the positively charged pheromone molecule (Gilles *et al.*, 1984). This leads to an accumulation of pheromone within the ECM (a 300-fold accumulation was estimated experimentally). If this electrostatic binding is restricted to the surface of the spheroid, the transition from three- to two-dimensional diffusion would further increase the probability of encountering a somatic cell.

(ii) A *Volvox* spheroid contains ~4000 somatic cells at the surface and only 16 gonidia lying much deeper. Therefore, the somatic cell population has a probability at least two orders of magnitude higher than the gonidia of encountering a pheromone molecule or another signal

molecule produced within the ECM in response to the pheromone. It is the somatic cell population that synthesizes pherophorin II in response to the application of the pheromone. This glycoprotein is secreted and deposited within the ECM and within a few hours, a 42 kDa domain that is a homologue of the sex-inducing pheromone is proteolytically cleaved from pherophorin II. If this cleavage product also has the biological property to induce a gonidium, the massive production of pherophorin II would raise the inducing principle within the ECM by orders of magnitude. Subsequently, a classical receptor/effector-mediated interaction would be sufficient to trigger the gonidium.

A number of experimental observations support the idea that the 42 kDa cleavage product of pherophorin II indeed has sex-inducing activity. In particular, all sterile mutants tested lack the ability to produce this fragment. Unfortunately, we were not able to demonstrate biological activity for a corresponding polypeptide expressed in *Escherichia coli* by recombinant DNA technology. However, authentic pherophorin II is a highly glycosylated and tyrosine-sulfated polypeptide (Wenzl and Sumper, 1986) and thus, the recombinant product may indeed be expected to lack biological activity.

Interestingly, pherophorin I which also bears a pheromone-homologous domain is constitutively expressed and is not cleaved under any circumstances. This glycoprotein represents one of the most abundant components of the insoluble fraction of the extracellular matrix. It is tempting to speculate that the highly potent sex-inducing pheromone is evolutionarily derived as a cleavage product from a member of the pherophorin family that originally served a structural function within the ECM of multicellular green algae.

## Materials and methods

### Culture conditions

*Volvox carteri f. nagariensis*, strains HK 10 (female), 69-1b (male) and the sterile mutants ster gone 12 and ster gone 78 were obtained from the culture collection of algae at the University of Texas (Dr R.C. Starr). Synchronous cultures were grown in *Volvox* medium (Provasoli and Pintner, 1959) at 28°C in an 8 h dark/16 h light (10 000 lux) cycle (Starr and Jaenicke, 1974).

### Pulse labelling of pherophorins

Pulse labelling with [ $^{35}$ S]sulfate was performed as described by Wenzl and Sumper (1986).

### Purification of pherophorins

Sexually induced *Volvox* spheroids (HK 10) from three 20 l cultures containing gonidia initiating embryogenesis were harvested by filtration through a nylon screen. The spheroids were sliced by forcing them through a 0.6 mm hypodermic needle. The suspension of spheroid fragments (500 ml) was adjusted to 1% SDS and 0.5 M NaCl and incubated at 95°C for 5 min. The ruptured *Volvox* 'ghosts' were collected by centrifugation (16 000 g, 10 min) and washed four times with water. This material was used for the isolation of intact pherophorins as well as deglycosylated pherophorins and the 42 kDa cleavage product. For an easy detection of SG 70, a small amount of pulse-labelled ghosts ([ $^{35}$ S]sulfate) were added at this stage.

Intact pherophorin was extracted from the 'ghosts' with 250 ml 1% Mega-9 detergent (Sigma) and 100 mM EDTA pH 8.0 at 65°C for 20 min. This extract contains the pherophorins as the main protein component, but is still contaminated by large quantities of polysaccharides. These were removed by a phenol extraction (80 ml phenol at 65°C, 10 min). Addition of 3 vol of acetone (–20°C) to the phenol phase precipitated the pherophorins, which were collected by centrifugation. Final purification was achieved by SDS–PAGE (10% gel). The pherophorins were localized by staining with

KCl (Hager and Burgess, 1980), and eluted from the gel by diffusion. This procedure yielded ~100 µg pherophorin.

Deglycosylated pherophorins as well as the C-terminal cleavage product were isolated as follows. The suspension of 'ghosts' was dried (eight aliquots) *in vacuo*. The dry material was dissolved in 1 ml anhydrous hydrogen fluoride and incubated at 0°C for 90 min. HF was removed in a stream of nitrogen and the remaining material was dissolved in SDS sample buffer and immediately adjusted to pH 8 with 1 M Tris base. Final purification was achieved by SDS-PAGE (12% gel) as described above.

#### Proteolytic digestion and separation of peptides

100 µg pherophorin were dissolved in 400 µl 50 mM Tris-HCl (pH 8.0) and digested with 2.5 µg of endoproteinase Lys C (Boehringer, Mannheim) at 37°C. After 4 h, an additional 2.5 µg of endoproteinase Lys C was added and digestion was continued for 6 h. Peptides were separated by reversed phase HPLC on a Lichrospher 100 CH18/2 (10 µm) column (E. Merck, Darmstadt, Germany) as described by Ertl *et al.* (1989).

#### Generation of cDNA probes by PCR

RNA and poly(A)<sup>+</sup> RNA was extracted from sexually induced *Volvox* spheroids as described by Kirk and Kirk (1985). Reverse transcription was performed in a final volume of 20 µl containing the following reagents: 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 20 U RNAGuard (Pharmacia), 200 pmol degenerate antisense oligonucleotide, 1 mM each dNTP, 250 ng poly(A)<sup>+</sup> RNA, and 200 U of MoMuLV reverse transcriptase. Incubation was at 42°C for 60 min. After addition of 80 µl of 1× PCR buffer containing 500 pmol of degenerate sense oligonucleotide and 2.5 U of Taq polymerase thermal cycling (Perkin-Elmer cyclor 9600) was initiated (30 cycles: denaturation at 94°C for 45 s, annealing at 50°C for 30 s and extending at 72°C for 15 s). The resulting 80 bp (or 83 bp) cDNA fragment was ligated into the *Sma*I site of vector pUC18 and sequenced.

#### Cloning of the pherophorin cDNA

A *V. carteri* cDNA library constructed in λgt11 using poly(A)<sup>+</sup> RNA from sexually induced (30 min) female spheroids was screened to clone cDNA fragments of pherophorins. The screening and cloning procedures 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). Synthetic oligonucleotides were used to sequence the pherophorin cDNAs in both directions.

#### PCR amplification of pherophorin I and II mRNA

Ten *Volvox* spheroids immediately after their release from the mother spheroid were selected under the stereo microscope and transferred into 20 µl sterile lysis buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl, 5 mM EGTA and 2% SDS), containing 1 µg *E. coli* 5S RNA as carrier. After 15 min at 30°C, *Volvox* spheroids were removed under the stereo microscope and RNA was precipitated with 60 µl ethanol. The washed precipitate (70% ethanol) was dissolved in 20 µl of reverse transcription buffer, containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 20 U RNAGuard (Pharmacia), 1 mM each dNTP and 50 pmol each antisense primer (ATCGGCCTTAGTAGCGCAGTACCC for pherophorin I, amino acid positions 51–58 and CAGCAGGTTCCAGT-TGAGATT for pherophorin II, amino acid positions 429–435). Reverse transcription was initiated with 200 U of MoMuLV reverse transcriptase and continued for 60 min at 40°C. 90 µl of 1× PCR buffer containing the sense primers (50 pmol each) as well as the antisense primers (25 pmol each) were added to a 10 µl aliquot of the reverse transcription sample. The sense primers used were TAGTGTGTTTCATTGGATAC for pherophorin I, 5'-non-coding sequence and ATGCCGTTTCACAGTCTCC for pherophorin II, amino acid positions 336–341). 29 cycles of PCR amplification were performed (94°C, 45 s; 60°C, 30 s; 72°C, 30 s).

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## Note added in proof

The sequence data reported here have been deposited in the EMBL Sequence Data Library under the accession numbers X69801 (pherophorin I) and X69802 (pherophorin II).