A novel extensin that may organize extracellular matrix biogenesis in *Volvox carteri*

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Communicated by J. Schell

ISG is a sulphated, extracellular glycoprotein synthesized for only a few minutes in inverting *Volvox* embryos and inverting sperm cell packets. This control operates at the level of transcription. ISG has been characterized by studies of protein chemistry and electron microscopy. The primary structure of ISG has been derived from genomic DNA and cDNA. ISG is composed of a globular and a rod-shaped domain. The rod-shaped domain represents a member of the extensin family with numerous repeats of Ser-(Hyp)4-6 motifs. A synthetic decapptide matching the C-terminal sequence is able to disaggregate the organism into individual cells. Immunofluorescence microscopy localizes ISG within the boundary zone of the ECM.

**Key words:** extensin/extracellular matrix/ISG/Volvox

**Introduction**

Members of the genus *Volvox* are among the simplest multicellular organisms. They are composed of only two cell types: somatic and reproductive cells. The reproductive cycle of *Volvox carteri* has been described in detail (Starr, 1969, 1970). The asexual organism exhibits 2000–4000 biflagellated somatic cells (similar to *Chlamydomonas* in their morphology) that are arranged in a monolayer at the surface of a hollow sphere. Sixteen much larger asexual reproductive cells ('gonidia') lie just below the somatic cells. The cells are held together by a complex, glycoprotein-rich extracellular matrix (ECM). Eleven or twelve rapid and synchronous cleavage divisions of a gonidium generate all the cells of the adult organism. The resulting embryonic cells are already arranged in a hollow sphere, but their orientation with respect to the surface is the reverse of that found in the adult: the flagellar ends of the somatic cells are directed towards the centre of the sphere and gonidia protrude from the surface. During inversion, the embryo turns completely inside out through a cross-shaped slit (the 'phialopore') and thereby establishes the adult configuration (Pickett-Heaps, 1970; Kelland, 1977; Viamontes and Kirk, 1977; Viamontes et al., 1979; Green et al., 1981). Inversion is initiated when cells bordering the phialopore undergo a transition in shape and begin to resemble the classic 'bottle cells' of the gastrulating amphibian embryo (Holterfreter, 1943, 1944). Next, the sequence of cell shape changes progresses in a wave-like fashion towards the pole opposite the phialopore. After inversion, the somatic cells begin to secrete ECM material causing each cell to move apart from its neighbours. The organism now grows in size but not cell number. The organization of the *Volvox* ECM has been analysed at the light- and electron-microscopic levels. The details of ECM architecture have been summarized and a system of nomenclature proposed by Kirk et al. (1986). Each organism is surrounded by a boundary zone which includes a crystalline lattice that is very similar to that of *Chlamydomonas reinhardii* (Goodenough and Heuser, 1988; Adair and Appel, 1989; for review, see Roberts et al., 1985). The ECM internal to the boundary zone has been designated cellular zone. The most distinct structural element of this zone is a highly regular pattern of fibrous layers that surround cells at a distance to form contiguous cellular compartments. A sulphated, hydroxyproline-rich glycoprotein (SSG 185) has recently been characterized as the monomeric precursor of this ECM substructure (Ertl et al., 1989).

The mechanism by which *Volvox* cells manage the precise construction of all these defined structures at considerable distance from their own boundaries is unknown. In this paper, we characterize the inversion-specific glycoprotein (ISG) that is synthesized for only a few minutes during embryonic inversion and we present evidence for a functional role of ISG in the organization of ECM architecture.

**Results**

ISG was previously discovered by pulse labelling experiments with radioactive sulphate and characterized as a sulphated glycoprotein (apparent molecular mass 200 kDa) that is synthesized exclusively in inverting embryos for a period of < 10 min (Schlifenbacher et al., 1986). Although ISG is synthesized only during this extremely short period in the 48 h life cycle of *Volvox*, the mature glycoprotein remains stable for at least 24 h, as revealed by a pulse—chase experiment with radioactive sulphate (Figure 1A).

A previously described purification procedure (Schlifenbacher et al., 1986) apparently yields homogeneous material as judged by SDS—polyacrylamide (5%) gel electrophoresis. However, higher percentage polyacrylamide gels revealed that this material was still contaminated by a glycoprotein, so the purification procedure was modified as follows.

**Purification of ISG**

A unique feature of sexual development in the male strain which leads to spheroids containing sperm cells is the occurrence of an additional morphogenetic process: in the first inversion process the embryo turns inside out as during asexual development. Twenty hours later each reproductive cell (now called an androgonidium) undergoes a series of cleavages to form a packet of sperm cells. This bowl-shaped sperm cell mass again performs a morphogenetic process.
(second inversion) to form a sperm bundle which is convex on its anterior side (Starr, 1969). ISG synthesis is again initiated during this second inversion. The period of ISG synthesis during spermatogenesis was analysed by pulse labelling of single *Volvox* spheroids (to circumvent the problem of asynchronous development in the population). As shown in Figure 1B, ISG production during spermatogenesis is again restricted to the short period of inversion.

At the first inversion the ratio of somatic cells and embryos is ~2000:16, whereas the ratio of somatic cells and sperm cell packets (at the second inversion) is ~1:1. This explains the higher content of ISG in spheroids containing sperm cell packets which therefore served as the source for ISG purification.

ISG was extracted from the sperm packets by incubating the spheroids in 0.8 M NaCl for 12 h at 4°C. After removal of the spheroids by centrifugation, ISG in the extract was adsorbed to an anion exchange resin (Q-Sepharose). After extensive washing, the highly sulphated ISG could only be eluted at NaCl concentrations as high as 1.35 M. Although ISG represents a minor component of the salt extract, it becomes highly enriched after this step and only a few other sulphated glycoproteins contaminate the ISG preparation (Figure 2A, lane 1). Final purification to homogeneity was achieved by preparative SDS–PAGE (lane 2). ISG is only partially digested by proteinase K; remarkably, a resistant core material remains which exhibits an even higher apparent molecular mass on SDS–PAGE (Figure 2A, lane 3). This behaviour is easily explained on the basis of the ISG primary structure (see below).

Treatment of ISG with anhydrous hydrogen fluoride at 0°C, a procedure that selectively cleaves glycosidic bonds (Mort and Lamport, 1977) drastically reduces the apparent molecular mass to ~60 kDa (Figure 2B). This result indicates a high degree of glycosylation. By quantitative sugar determinations (Dubois et al., 1956), the carbohydrate content was determined to be 70%.

**Protein chemistry studies**

Automated Edman degradation of the purified ISG glycoprotein showed that its N-terminal amino acid sequence was AVSYSVSVYNNIAVTGAPLSGIVSQLLSK. The purified ISG glycoprotein was digested with trypsin and the resulting peptide mixture was separated by reversed phase HPLC. The materials of well separated peaks were directly subjected to amino acid sequence analysis on an automated gas phase sequencer. The amino acid sequence data obtained are summarized in Table I. After chemical deglycosylation of ISG using anhydrous hydrogen fluoride, subsequent digestion with trypsin generated a set of additional peptides. A remarkable feature of these peptides is their extremely high content of hydroxyproline residues (Table I).

**Generation of a 363 bp cDNA probe by PCR**

The amino acid sequence of the N-terminus was used to synthesize a sense oligonucleotide primer, GTNTA T/C AA T/C AA T/C AT T/C/A GC, corresponding to amino acids 8–13. The antisense primer, GG G/T AGGTTNCCGAA G/A TT, was designed from amino acid positions 6–11 of peptide 4 (Table I). The latter primer was used to reverse transcribe mRNA isolated from inverting sperm packets. The resulting cDNA was amplified by the polymerase chain reaction using both the sense and antisense primers. Thirty cycles of amplification produced a DNA fragment of ~360 bp in length that was cloned by blunt-end ligation into pUC18. Sequencing of this fragment revealed an open reading frame matching the amino acid sequences of peptides
Table I. Amino acid sequences of tryptic peptides derived from ISG

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>Val Asn Asp Leu Leu Thr Phe Val Arg</td>
</tr>
<tr>
<td>2</td>
<td>Gly Gin Gly Ser Tyr Trp Ile Thr Glu Gly Leu Thr Ser – Ser Thr Lys</td>
</tr>
<tr>
<td>3</td>
<td>Asn Gly Gly Ser Leu Ile Leu Val Asn Gly Ala Asn Gly Asn Thr Phe Ile Pro Leu</td>
</tr>
<tr>
<td>4</td>
<td>Ile Asp Pro Pro Ser Asn Phe Gly Asn Leu Pro Val Lys</td>
</tr>
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Obtained only from deglycosylated ISG:

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td>5</td>
<td>Ser Hyp Hyp Hyp Hyp Arg Val Hyp Hyp Hyp Hyp Hyp Val Ala Ser Hyp Hyp Hyp Hyp Hyp Hyp Hyp Hyp Arg</td>
</tr>
<tr>
<td>6</td>
<td>Ala Ser Hyp Hyp Hyp Hyp Ala Ser Ser Hyp Hyp Hyp Hyp Arg Hyp Hyp Hyp Ser Hyp Hyp Ser Hyp Hyp Hyp Hyp Hyp Hyp Hyp Ser Arg</td>
</tr>
<tr>
<td>7</td>
<td>Ser Ser Hyp Ser Hyp Hyp Hyp Val Val Ser Ser Hyp Hyp Hyp Hyp Hyp Hyp Arg</td>
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1–4 (Table I). Therefore this DNA fragment was used as a highly specific probe for the cloning of the ISG gene.

Cloning of the ISG gene

From previous experience with the cloning of a hydroxyproline-rich glycoprotein from *Volvox* (Ertl et al., 1989) it appeared unlikely that a full length cDNA covering the extreme cytidine-rich region encoding the polyproline stretch would be detected. Indeed, repeated screening of an Agt11 cDNA library using the PCR probe mentioned above was unsuccessful. To circumvent these problems, the PCR probe was used to screen a genomic library of *V. carteri* constructed in the replacement vector λEMBL 3. Out of 50 000 phages screened, three positive clones were identified. Digestion of the 16 kb insert of one of these clones with BamHI resulted in a 2.7 kb fragment that hybridized to the 363 bp probe. This fragment was subcloned into pUC18 and sequenced. As shown schematically in Figure 3, the 2.7 kb fragment covered the complete coding region of the ISG gene. The amino acid sequence is encoded in three exons. Exon 1 encodes a typical signal sequence in addition to amino acid positions 1–4 of the mature polypeptide chain. Exon 2 encodes amino acids 5–207 and exon 3 encodes an extremely long stretch of prolines and hydroxy amino acids. All of the prolines in this stretch analysed by peptide sequencing were found to be hydroxyprolines (Table I). The deduced cDNA and amino acid sequence is given in Figure 4. The correct assignment of exon–intron boundaries was confirmed by sequencing the corresponding cDNA fragments. These cDNAs were generated by reverse transcription of mRNA using synthetic antisense oligonucleotides and subsequent amplification by PCR with ISG-specific sense and antisense oligonucleotides. Figure 3 schematically summarizes the strategy applied in order to collect the complete cDNA sequence information.

Primary structure of ISG

The N- and C-terminal halves of the polypeptide chain have completely different amino acid compositions. An N-terminal stretch of 207 amino acids is composed of all naturally occurring amino acids. In sharp contrast, the C-terminal half (248 amino acids) of the polypeptide consists almost exclusively of hydroxyprolines, serines and threonines with arginine residues interspersed within the clusters of hydroxy amino acids. Motifs of Ser–(Hyp)3–7 are a predominant feature. These repeats are diagnostic of extensins, a family of rod-like, hydroxyproline-rich glycoproteins found in the cell walls of higher plants (reviewed by Cassab and Varner, 1988).

ISG is highly glycosylated as indicated by the drastic reduction of the apparent molecular mass after chemical deglycosylation with anhydrous hydrogen fluoride. Arabinose, galactose and xylose in a ratio of 2:1:0.2 were shown to be the main sugar constituents of ISG (Wenzl and Sumper, 1982). The deduced amino acid sequence exhibits a single N-glycosylation site at Asn74 that is indeed glycosylated as indicated by the amino acid sequencing of the corresponding peptide (Table I). Since the hydroxyproline-rich C-terminal domain of ISG is sensitive to digestion with trypsin or proteinase K only after treatment with anhydrous hydrogen fluoride (Table I), glycosylation of all or most of the hydroxy amino acids is highly likely.

Electron microscopy

Purified ISG and protease derived fragments thereof were studied by electron microscopy applying the rotary shadowing technique. Intact ISG is composed of a globular domain and a rod-like structure. The mean length of the rod was determined to be 57 ± 3 nm. Both monomers and oligomeric aggregates are detectable; the latter appear as star-like particles with a variable number of the 57 nm arms (Figure 5). The proportions of different oligomers were as follows: 37% of particles had two arms, 18% had three arms, 12% four arms, 10% five arms, 4% six arms, 4% seven arms and 14% more than seven arms. Protease treatment converts ISG into a rod-like molecule of average length 56 ± 4 nm. Most probably, the secondary structure of the hydroxyproline-rich part of the polypeptide chain is the polyproline II helix conformation. This is the most
Fig. 4. Nucleotide sequence of cDNA and predicted amino acid sequence of mature ISG. Amino acid sequences derived from isolated peptides are indicated by broken lines. Ser-(Hyp)_{4-7} repeats within the C-terminal half of ISG are boxed. The nucleotide sequence reported here has been submitted to the EMBL data library and is available under the accession number X65165 V.carteri mRNA ISG.

extended helix formed by polypeptides with three residues per turn and a pitch of 0.94 nm. A rod, 57 nm in length, corresponds to a polypeptide chain of 180 residues, a value that is in good agreement with the length of the hydroxyproline-rich domain.

**Transcriptional control of ISG synthesis**

PCR was used to determine whether the extreme developmental control of ISG synthesis operates at the level of transcription. RNA was extracted from a single embryo collected at a defined stage of inversion. Reverse
transcription and subsequent PCR amplification of ISG cDNA yielded the results shown in Figure 6. mRNA for ISG is virtually absent at the very beginning of the inversion process. However, a strong signal is obtained from inverting embryos. Towards the end of inversion, the level of ISG mRNA decreases significantly. Thus, the developmental control of ISG production operates at the level of transcription.

In vivo effects of a synthetic ISG-derived peptide
A distinct feature at the C-terminal end of ISG is the occurrence of five negatively charged amino acid residues between positions 433 and 448 which are followed by five positively charged amino acid residues between positions 455 and 464. A synthetic decapptide (KKKATGRRLL) matching the latter amino acid sequence was added to developing embryos and analysed for possible interference with ECM biogenesis. Concentrations as low as $5 \times 10^{-6}$ M disturb ECM biogenesis and cause the development of irregularly shaped spheroids. At a concentration of $2 \times 10^{-5}$ M the spheroid begins to disintegrate, producing single cells or cell clumps never observed in normal development (Figure 7). However, the viability of the cells is not affected since further development of the reproductive cells proceeds normally. Interestingly, this peptide no longer disturbs the organization of the ECM if it is added a few hours after embryonic inversion. Commercially available peptides containing positively charged amino acids (GK, LRRASLG and KKGE) used as a control did not affect ECM biogenesis at all, even at concentrations of $10^{-4}$ M.

Indirect immunofluorescence microscopy
The localization of ISG within the ECM was studied by indirect immunofluorescence microscopy using the IgG fraction of a rabbit polyclonal antibody raised against the globular domain of ISG produced in Escherichia coli by recombinant DNA technology. For that purpose, intact Volvox spheroids were broken up into hemispheres and

![Fig. 5. Visualization of ISG by electron microscopy after rotary shadowing. Selected star-like particles with a variable number of 57 ± 3 nm long arms are shown. In a randomly selected field, the number of arms on 50 particles were evaluated (see text). The bar corresponds to 100 nm.](image)

![Fig. 6. Transcriptional control of ISG synthesis. RNA was extracted from a single embryo at the desired stage of inversion (embryos were selected under a stereo microscope). After reverse transcription with an ISG-specific antisense oligonucleotide, a 304 bp cDNA fragment of ISG was amplified by PCR. The sense and antisense oligonucleotides were selected from exons 1 and 2 respectively to prevent amplification of genomic DNA. RNA extraction was done at each of the inversion stages schematically illustrated above the corresponding lanes of the agarose gel (1.5%). For details, see Materials and methods.](image)
fraction) and fluorescein-conjugated anti-rabbit IgG. The antibody selectively stains a narrow layer within the boundary zone of the ECM as well as the flagellar tunnels of each somatic cell (Figure 8C and D). The stained layer and the flagellar tunnels are contiguous. This feature is diagnostic of boundary zone 2 of the ECM (according to the nomenclature introduced by Kirk et al., 1986). In inverted sperm cell packets, the flagellar tunnels again are strongly stained as are the cell–cell contacts between individual sperm cells (Figure 8E).

**Discussion**

The polypeptide chain of ISG consists of two completely different domains. The C-terminal half of the polypeptide chain is composed of a many repeating units of (Ser–Hyp$_{2–8}$) and has a rod-like structure. These characteristics are diagnostic of a member of the extensin family. Extensins have been described at protein and gene levels from dicotyledonous plants (Cassab and Varner, 1988); they are structural proteins of the higher plant cell walls. Upon secretion to the cell wall, extensins are rapidly insolubilized, presumably through the formation of covalent cross-links. They are thought to contribute to the structural integrity of the cell wall throughout plant development. Furthermore, synthesis of extensins is induced by fungal infection and wounding (Shawalter et al., 1985). Although the ECMs of algae in the order Volvocales are made entirely from hydroxyproline-rich glycoproteins, most of the glycoproteins characterized so far were reported to be different from typical higher plant extensins (Adair and Appel, 1989; Ertl et al., 1989). In contrast, a zygote-specific cell wall glycoprotein (Woesnser and Goodenough, 1989) and ISG are the first hydroxyproline-rich glycoproteins from algae shown to be closely related to the extensins from higher plants.

The extensin domain of ISG is N-terminally extended by >200 amino acid residues. This extension exhibits no unusual amino acid preferences and is visualized by electron microscopy as a globular domain attached to a rod-like element. This additional protein domain supports the idea of a functional role of ISG. The extreme developmental control of ISG synthesis further supports this idea. ISG is synthesized in embryos for only a few minutes towards the end of inversion and is also synthesized in inverting sperm packets. During inversion, all cells of the embryo are joined by numerous cytoplasmic bridges (Green et al., 1981). Immediately after inversion these bridges disappear in *V. carteri* and the production of ECM material is initiated. It is probably an important function of one of the early ECM glycoproteins to hold the embryonic cells together, thereby replacing the function of the cytoplasmic bridges. ISG is probably the first ECM component produced in the embryo and, therefore, ISG is a candidate for being involved in this function. The *in vivo* effects caused by an ISG-derived peptide further support this idea. Synthesis of the glycoprotein SSG 185, precursor of the insoluble ECM sub-zone CZ3 (Figure 8A) is initiated as late as 4–6 h after the end of inversion (S. Wenzl and M. Sumper, unpublished).

Unfortunately, ISG antibodies do not penetrate intact *Volvox* spheroids, preventing the observation of effects caused under *in vivo* conditions.

The immunofluorescence data locate ISG within the boundary zone of ECM, most probably within sub-zone BZ 2 (Figure 8A) according to Kirk et al. (1986). This sub-zone represents the tripartite layer, corresponding to layers W2–W6 of *Chlamydomonas* in the terminology of Roberts (1974) and includes the chaotrope-soluble crystalline layer. The tripartite layer is highly conserved within the order Volvocales (Catt et al., 1978) and has been shown to possess the ability of self-assembly as well as nucleated assembly (Adair et al., 1987). The observations described in this paper suggest that ISG is likely to be involved in the early processes of ECM biogenesis.

A unique feature of ISG synthesis is its extreme developmental control. Even the exquisitely sensitive method of PCR amplification does not detect any ISG mRNA molecules until the initiation of inversion. Thus, the control of ISG production is regulated at the transcriptional level. During the period between the first and second inversion events (sexual male development) only very low levels of ISG mRNA are detectable by PCR amplification, indicating the instability of this message. Inversion takes place at the end of embryogenesis or spermatogenesis when all embryonic cells are differentiated. Since the ISG gene is activated in assexual embryos as well as in sperm packets, this signal cannot depend on a particularly differentiated cell type. A common feature of cells engaged in the process of inversion is a change of cell shape. It is tempting to speculate that the reorganization of the cytoskeleton delivers the signal for ISG gene activation.

**Materials and methods**

**Culture conditions**

*Volvox carteri* f. *nagariensis*, strains HK 10 (female) and 69-1b (male), were obtained from the culture collection of algae at the University of Texas (Dr. R.C. Starr). Synchronous cultures were grown in *Volvox* medium

![Fig. 7. In vivo effects of a synthetic decapeptide (KKKATGRRLL) matching the C-terminal sequence of ISG. Preinversion embryos were incubated in Volvox medium containing 10^{-4} M (A2) or 2 \times 10^{-5} M (B2) peptide. A1 and B1 show untreated controls. Photographs were taken 24 h (A) or 48 h (B) after the addition of peptide.](image-url)
Fig. 8. Localization by indirect immunofluorescence of ISG in asexual spheroids (C and D) and in sperm cell packets (E). (A) Stylized drawing of a portion of a *Volvox carteri* spheroid illustrating ECM zones and the corresponding nomenclature as proposed by Kirk et al. (1986). BZ, boundary zone; CZ, cellular zone; DZ, deep zone. (B) Western blot analysis of polyclonal antibody (purified IgG fraction) raised against recombinant ISG polypeptide (amino acids 5-172, see Materials and methods). Lane 1, authentic ISG, lane 2, deglycosylated ISG. (C) Left, a somatic cell sheet photographed with Nomarski optics; right, immunofluorescence of the same area (stained with purified anti-ISG IgG and FITC-labelled anti-rabbit IgG). In this orientation, both flagellar tunnels of each somatic cell are mainly visible. (D) As (C), but a side view of somatic cells bordering the edge of the sheet. (E) Left, inverted sperm packets photographed with Nomarski optics; right, immunofluorescence of the same area.

(Provasoli and Pintner, 1959) at 28°C in an 8 h dark/16 h light (10 000 lux) cycle (Starr and Jaenicke, 1974). Large scale growth of strain 69-lb was performed according to a protocol described previously (Tschochner et al., 1987).

**Pulse labelling of ISG**
Pulse labeling with $^{35}$Ssulphate was performed as described by Wenzl and Sumper (1986b). Pulse labelling of a single *Volvox* spheroid was performed as described by Schlipfenbacher et al. (1986) for individual embryos.

**Purification of ISG**
*Volvox* spheroids from six 20 l cultures containing inverted sperm packets were harvested by filtration through a nylon screen. The spheroids were broken up by forcing them through a 0.6 mm hypodermic needle. The cell suspension was adjusted to 0.82 M NaCl and incubated overnight at 4°C. After centrifugation at 26 000 g for 20 min, the supernatant was adjusted to 1% N-lauroylsarcosine, 20 mM Tris - HCl (pH 8.0) and heated to 95°C for 10 min. After cooling to 4°C, Q-Sepharose corresponding to a bed volume of 10 ml was added and the suspension was stirred overnight. The suspension was poured into a column and the Q-Sepharose was subsequently
washed with 100 ml of a solution containing 8 M urea, 0.85 M NaCl and 0.5% N-lauroylsarcosine. ISG could be eluted by raising the NaCl concentration to 1.35 M. After extensive dialysis, the eluate was concentrated by lyophilization. The concentrated material was applied to a 6% SDS-polyacrylamide gel. ISG was eluted from the gel by diffusion. Typically, the yield was 200–300 μg of homogenous ISG.

Proteolytic digestion and separation of peptides

180 μg ISG were dissolved in 400 μl 50 mM Tris–HCl (pH 8.0) and digested with 3 μg of trypsin at 37°C. After 3 h, an additional 3 μg of trypsin was added and digestion was continued for 6 h. Peptides were fractionated by reversed phase HPLC on a Lichrosorb RP 18, 10 μm column (E. Merck, Darmstadt, FRG) as described by Ertl et al. (1989). Deglycosylation of ISG and subsequent proteolytic digestion were performed as described for the glycoprotein SSG 185 by Ertl et al. (1989).

Generation of a cDNA probe by PCR

RNA and poly(A)+ RNA were extracted from Volvox spheroids as described by Kirk and Kirk (1985). Reverse transcription was performed in a final volume of 20 μl containing 50 mM Tris–HCl (pH 8.3), 40 mM KCl, 6 mM MgCl2, 1 mM diithothreitol, 20 U RNaseguard (Pharmacia), 150 pmol antisense oligonucleotide (GG G/T AGGGTCCGAA G/A TT), 1 mM each dNTP, 250 ng poly(A)+ RNA and 200 U of MuMoLV reverse transcriptase. Incubation was at 42°C for 45 min. After addition of 80 μl of 1 × PCR buffer containing 500 pmol of sense oligonucleotide (GTNTA TC/A A/C T/A/C AT/T/C AG) and 2.5 U of Taq polymerase, thermal cycling (Perkin–Elmer cycler 480) was initiated (30 cycles: denaturation at 94°C for 45 s, annealing at 50°C for 30 s and extension at 72°C for 45 s). The resulting 360 bp DNA fragment was ligated into the Smal site of pUC18 and sequenced.

Cloning of the ISG gene

The V. carteri genomic library in λEMBL3 (Frishauf et al., 1983) described by Ertl et al. (1989) was used to clone the ISG 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). Synthetic oligonucleotides were used to sequence the ISG gene in both directions.

PCR with RNA from a single embryo

Synchronously growing Volvox spheroids containing preinversion embryos were disrupted by forcing them through a 0.5 mm hypodermic needle. Somatic cell sheets were removed by filtration (100 μm mesh nylon cloth). A single embryo was then identified under the stereo microscope, picked up with a drawn plastic pipette and transferred into 10 μl sterile lysis buffer (30 mM Tris–HCl, pH 8, 300 mM NaCl, 5 mM EGTA and 2% SDS) containing 1 μg E.coli 55 RNA as carrier. After 10 min at 28°C, RNA was precipitated with 30 μl ethanol. The washed precipitate (70% ethanol) was dissolved in 10 μl reverse transcription buffer and further processed as described above. The sense and antisense oligonucleotides used were 5'-GATGTCGCTCCAGTCCGTCG (exon 1) and 5'-GGTCCAGGTTTCCGTAATCGACTA (exon 2), respectively. 35 cycles of PCR amplification were performed (94°C, 45 s; 60°C, 30 s; 72°C, 45 s).

Electron microscopy

ISG and subfragments were rotary shadowed with platinum and visualized as described by Mörgelin et al. (1988).

Preparation of recombinant ISG polypeptide

A cDNA fragment encoding amino acid positions 5–172 was generated by PCR and subsequently cloned into pET11 and expressed in E.coli according to the method of Studier et al. (1990). The recombinant peptide was overexpressed and located in inclusion bodies. The isolated inclusion bodies were repeatedly washed with 4 M urea and solubilized in SDS sample buffer. Only a single major band was detected on an SDS–polyacrylamide gel (128 kDa); this material was eluted, concentrated in a microconcentrator (Centricon 10, Amicon) and used to raise polyclonal antibodies in rabbit.

Fluorescence microscopy

V. carteri spheroids were disrupted by forcing them through a 0.4 mm hypodermic needle. The subsequent procedure was as described previously (Wenzl and Sumper, 1986a).

Acknowledgements

We wish to thank Dr J.Engel for taking the electron micrographs, Dr R. Deutzmann for sequencing peptides and Elke Berg for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 43).

References


Received on January 30, 1992; revised on February 27, 1992