The Extracellular Matrix of *Volvox carteri*: Molecular Structure of the Cellular Compartment

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Abstract. The extracellular matrix (ECM) of *Volvox* contains insoluble fibrous layers that surround individual cells at a distance to form contiguous cellular compartments. Using immunological techniques, we identified a sulfated surface glycoprotein (SSG 185) as the monomeric precursor of this substructure within the ECM. The primary structure of the SSG 185 polypeptide chain has been derived from cDNA and genomic DNA. A central domain of the protein, 80 amino acid residues long, consists almost exclusively of hydroxyproline residues. The chemical structure of the highly sulfated polysaccharide covalently attached to SSG 185 has been determined by permethylation analysis. As revealed by EM, SSG 185 is a rod-shaped molecule with a 21-nm-long polysaccharide strand protruding from its central region. The chemical nature of the cross-links between SSG 185 monomers is discussed.

THE green algae of the order *Volvocales* range in complexity from unicellular *Chlamydomonas* through colonial genera to multicellular organisms, with differentiated cells in the genus *Volvox*. The individual cells are surrounded by an extracellular matrix (ECM)[1] constructed of hydroxyproline-rich glycoproteins (Miller et al., 1974). The most conserved morphological feature of the Volvocalean ECM is an outer layer. In *Chlamydomonas*, this region is composed of a number of glycoproteins in a distinctive crystalline lattice that can be disassembled by chaotropic agents and recrystallized in vitro (Roberts, 1974; Hills et al., 1975; Catt et al., 1978; Roberts et al., 1985; Goodenough et al., 1986; Goodenough and Heuser, 1988).

The inner portion of the *Chlamydomonas* cell wall is a fibrous network insoluble in chaotropic agents (Roberts et al., 1975; Goodenough and Heuser, 1985). It is within the evolutionary derivatives of this substructure that diversification has occurred to convert a simple cell wall into an ECM of the multicellular *Volvocales*. In these genera the ECM internal to the boundary zone is organized in highly regular patterns into fibrous layers that underlie the crystalline layer, ensheath individual cells, and surround cells at a distance to form contiguous cellular compartments (for review, see Kirk et al., 1986). These cellular compartments exhibit a honeycomb-like organization.

The organization of the ECM of higher *Volvocales* has been analyzed in detail at the light- and electron-microscopic level. Recently, all the known details of the ECM architecture in representative species of *Volvox* were reviewed and a system of nomenclature was proposed (Kirk et al., 1986). According to these proposals, the honeycomb-like cellular compartments are designated cellular zone 3 (CZ 3), schematically shown in Fig. 2 *D*. In this article, we analyze the molecular structure of the glycoprotein that is the biosynthetic precursor of the CZ 3 structure of the ECM of *Volvox carteri*. In a previous article, we have shown that a highly sulfated glycoprotein (SSG 185) serves as the monomeric precursor of an insoluble polymer within the ECM of *Volvox carteri* (Wenzl et al., 1984). This polymer remains insoluble even after boiling with SDS. Thus, low-speed centrifugation of a crude *Volvox* lysate in the presence of 3% SDS allows selective purification of polymeric SSG 185 material. Protease treatment quantitatively converts this insoluble material into a soluble glycopeptide with an apparent molecular mass of 145 kD. Thus, boiling a *Volvox* lysate with SDS followed by proteolytic digestion rapidly yields a defined fragment of the SSG 185 molecule.

In this paper we identify SSG 185 as the precursor of the CZ 3 structure within the ECM, report its primary structure as derived from cDNA and genomic libraries, and describe at a molecular level the saccharide structures attached to this glycoprotein. We also discuss a model for the assembly of the CZ 3 polymer from SSG 185 monomers.

Materials and Methods

Culture Conditions

Volvox carteri f. nagariensis, strain HK 10 (female) was from the culture collection of algae at the University of Texas at Austin (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 lx) cycle (Starr and Jaenicke, 1974).

1. *Abbreviations used in this paper*: CZ 3, cellular zone 3; ECM, extracellular matrix; SSG 185, sulfated surface glycoprotein with apparent molecular mass 185 kD.

Preparation of 145-kD Glycopeptide Monomers and Oligomers

300 liters of a synchronous growing *V. carteri* culture (~15 spheroids/ml, containing late embryos) were harvested by filtration through a nylon screen. The algae were lysed by ultrasonic treatment and stored frozen at −20°C for at least 3 h. After centrifugation at 50,000 *g* for 3 h, the pellet was suspended in extraction buffer (0.2 M NaCl, 3% SDS) using a homogenizer, stirred at room temperature for 20 min and centrifuged at 35,000 *g* for 45 min. The pellet was extracted once under the same conditions, twice with 0.2 M NaCl and once with water. Subsequently, the pellet was delipidated with a mixture of methanol and dichlormethane (1:1) until the material appeared nearly colorless, dried by a gentle stream of nitrogen, and suspended in 50 mM Tris/HCl (pH 8).

For the preparation of monomeric 145-kD glycopeptide, the suspension was heated for 5 min in a boiling-water bath in the presence of 0.5% SDS (wt/vol) and digested with 0.1 mg/ml subtilisin for 30 min at 37°C. After addition of NaCl (final concentration 0.3 M) the mixture was clarified by centrifugation (35,000 *g*, 10 min) and the supernatant applied to a QAE-Sephadex A25 column (bed volume 50 ml; Pharmacia Fine Chemicals, Uppsala, Sweden). The column was washed with 2 vol 0.3 M NaCl, 20 mM Tris-HCl (pH 8) and 10 vol 1 M NaCl, 20 mM Tris-HCl (pH 8). Soluble 145-kD glycopeptide was eluted with the same buffer containing 4 M NaCl. Preparation of 145-kD glycopeptides containing dimers and higher oligomers was performed similarly, except that the suspension was heated in the presence of 0.1% SDS before subtilisin digestion. This procedure yielded ~8 mg glycopeptide.

Proteolytic Digestion and Separation of Peptides

1.2 mg 145-kD glycopeptides (a mixture of mono- and oligomers) were lyophilized and deglycosylated with anhydrous hydrogen fluoride for 90 min at 0°C (Mort and Lamport, 1977; Wieland et al., 1983). After evaporation of hydrogen fluoride, the sample was dissolved in 0.5 ml 0.1 M *N*-ethylmorpholinoacetate buffer (pH 7.5) and dialysed against the same buffer overnight at 4°C. The protein (~0.25 mg) was subsequently digested with 5 μg of trypsin for 8 h at 37°C. After 4 h, an additional 5 μg of trypsin was added. Peptides were separated by reverse-phase HPLC with a Lichrosorb RP 18, 10-μm column (E. Merck, Darmstadt, FRG). Peptides were eluted by a 90-min linear gradient from 5 to 35% acetonitrile in 0.1% trifluoracetic acid with a flow rate of 1 ml/min. If necessary, peptides were rechromatographed using a Lichrosorb RP 18, 5-μm column by a gradient of 1% acetonitrile in 10 min, starting 3% below the calculated elution point. Peptides were sequenced using an automated gas-phase peptide sequencer (Applied Biosystems, Inc., Foster City, CA) as described by Lottspeich (1985).

Mercaptolysis

Mercaptolysis was performed as described by Wenzl et al. (1984). The 90-kD glycopeptide and 28-kD saccharide fragment were separated by ion-exchange chromatography on QAE Sephadex A 25 (Pharmacia Fine Chemicals). In the presence of 1 M NaCl, the 90-kD fragment appeared in the flowthrough, whereas the highly sulfated 28-kD fragment was bound and subsequently eluted with 4 M NaCl.

Preparation of Peptide-specific Antibodies

A synthetic peptide (SGPNVNPIGPA), synthesized by a peptide synthesizer (model SP640; Labortec, Bubendorf, Switzerland) by using F-moc (9-fluorenylmethyloxycarbonyl) amino acid derivatives method was coupled to BSA using either 1-ethyl-3-(3'-diethylaminopropylcarbodiimid) (Steiner, 1974) or glutaraldehyde (Reichlin, 1980) as coupling reagents. A mixture of both BSA-peptide conjugates was used for immunization of a New Zealand rabbit as described (Gerl and Sumper, 1988). Antisera were characterized by Western blotting (Towbin et al., 1979). A crude lysate of *V. carteri* spheroids, deglycosylated and nondeglycosylated oligomers of the 145-kD glycopeptide, a lysate of *Escherichia coli* Y 1090 and BSA were separated by SDS-PAGE and transferred to a nitrocellulose-membrane using a semidry blotting apparatus (LKB Instruments, Bromma, Sweden) according to the manufacturer's instructions. Nitrocellulose sheets were stained as described under immunological screening of the cDNA library. The antiserum exhibited specificity for BSA and the deglycosylated 145-kD glycopeptide.

cDNA Library Construction

RNA and A^+-RNA was isolated as described (Kirk and Kirk, 1985; Mages et al., 1988*a*). First-strand synthesis was performed using M-MuLV-reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) following the manufacturer's instructions. Second-strand synthesis was performed according to the method of Gubler and Hoffman (1983). After treatment with T4 polymerase and addition of Eco RI linkers, the cDNA was digested with Eco RI and size fractionated by chromatography on a Sepharose CL-4B column (Pharmacia Fine Chemicals). Fractions containing the largest cDNAs were precipitated, dissolved in a volume of 10 μl, and ligated with 1 μg of λgt11 arms (Bethesda Research Laboratories). Recombinant DNA was packaged in vitro (Maniatis et al., 1982) and phages propagated in *E. coli* Y 1088. This yielded a cDNA library of 230.000 phages.

Construction of Volvox carteri HK 10 Genomic Library

High-molecular weight Volvox DNA, prepared as described by Mages et al. (1988*b*), was partially digested with Sau3A. Fragments with a size of ~18 kb were selected by preparative gel electrophoresis, purified, cloned into the Bam HI site of λEMBL3 (Frischauf et al., 1983) and packaged in vitro (Maniatis et al., 1982). Propagation of phages in *E. coli* NM 539 (Frischauf et al., 1983) yielded a library of 1×10^6 recombinants.

Cloning of the SSG 185 cDNA

Plating of 220,000 recombinant λgt11 phages (10,000 per 85-mm plate), induction of β-galactosidase with isopropyl thiogalactoside and transfer of expressed fusion protein to nitrocellulose was performed as described by Huynh et al. (1985). Immunological detection of clones containing the SSG 185 cDNA was done as follows. Filters were blocked with 5% horse serum in TBS (0.9% NaCl, 10 mM Tris/HCl [pH 8]) for 90 min at 37°C and washed twice with TNK (0.2 M NaCl, 0.2 M KCl, 50 mM Tris/HCl [pH 8]) for 5 min and briefly with TBS. Subsequently, filters were incubated with dilute antiserum for 1 h at room temperature and washed twice with TNK containing 5% horse serum for 20 min and finally with TBS.

Bound antibody was detected by incubation of the membrane with dilute (1:300 in 2.5% horse serum in TBS) FITC-labeled anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature. Finally, filters were washed as before and dried and inspected under monochromatic light of 490 nm produced by an interference filter (Schott, Mainz, FRG) through a 530-nm cutt-off filter (Schott).

Recombinant λgt11 DNA was prepared according to Frischauf et al. (1983), subcloned into pUC8 (Vieira and Messing, 1982) and sequenced by the chain termination method (Sanger et al., 1977) using T7-DNA polymerase (Pharmacia Fine Chemicals) following the manufacturer's instructions. The insert of the clone λ94, which covered the 5' region of the SSG 185 mRNA was used as a probe to screen a genomic library resulting in clone λSG1. A subfragment (pSG12) of clone λSG1 was sequenced and the obtained information was used to design a 21-mer oligonucleotide, which was subsequently labeled with γ-^{32}P-ATP (Maniatis et al., 1982). Screening of the cDNA library yielded clone λ941 that represented the 3' region of the SSG 185 mRNA.

Electron Microscopy

145-kD glycopeptides and subfragments were rotary shadowed with platinum and visualized as described by Mörgelin et al. (1988).

Monoclonal Antibody Production

100 μg of the 145-kD glycopeptide were injected intraperitoneally into a mouse together with 100 μl of Freund's complete adjuvant. 6 wk later, an intraperitoneal boost of 100 μg antigen with Freund's incomplete adjuvant was given followed by fusion with NSO/U myeloma cells (a generous gift of C. Milstein) on the fourth day. Hybridomas were cloned by selecting, under the microscope, droplets containing single cells. Solid-phase radioimmunoassays and IgG preparation were done by standard methods (Bozarro et al., 1981; Ochiai et al., 1982). Two out of five positive clones proved to be specific for the 145-kD glycopeptide and one of these was selected for further investigation (mAb 302/1).

In Vivo Effect of the Monoclonal Antibody

About 100 isolated *Volvox carteri* embryos just before inversion (Wenzl and Sumper, 1986) in 0.5 ml *Volvox carteri* medium were incubated with 100 μg MCA 302/1. Further development was observed under the microscope compared with a control sample (without antibody, but in the presence of 100 μg mouse IgG) for one life cycle.

Fluorescence Microscopy

Volvox carteri colonies were disrupted by being forced through a 0.5-mm hypodermic needle. The further procedure was done as described previously (Wenzl and Sumper, 1986).

General Methods for Carbohydrate Analysis

Qualitative detection of carbohydrates was performed with a spot test on silica plates (E. Merck) that were sprayed with orcinol (1.5 mg/ml) in 20% sulfuric acid (Vaskovsky et al., 1970). Quantitative determination of neutral sugars was done by the phenol/sulfuric acid method (Dubois et al., 1956) using an arabinose/mannose mixture (10:7.2) as standard. Sugar compositions were determined by gas chromatography of alditol acetates using a model 3700 gas chromatograph (Varian Instruments, Palo Alto, CA) equipped with a Durabond 1701 capillary column (30 m ICT, J & W Scientific, Folsom, CA). The initial temperature (200°C) was raised with 2°C/min.

Permethylation of the 28-kD Saccharide

Complete methylation of the 28-kD saccharide was performed by the method of Haworth (1915). Dry 28-kD saccharide was dissolved in 500 μl 11 M NaOH containing 0.5 mg $NaBH_4$ under a N_2 atmosphere. The solution was maintained at 0°C and stirred vigorously while three times 30 μl dimethylsulfate were added at 2-h intervals. Finally, 100 μl dimethylsulfate was added; after being stirred overnight, the solution was neutralized and dialysed.

Methylation of Partially Desulfated 28-kD Saccharide

This procedure was done according to Hakomori (1964) with the modification described by Waeghe et al. (1983). Sodium dimethylsulfinyl carbanion was prepared as described by Harris et al. (1984). After methylation, the 28-kD derivative was precipitated with 5 ml chloroform and 3 ml ether, washed with 2 ml of ether, and dried with N_2. The precipitate was dissolved in water, applied to a Sep-Pak C 18 cartridge, washed with 10 ml water, and eluted with 18% acetonitrile.

Desulfation

The desulfation of the methylated 28-kD derivative was accomplished by the method of Nagasawa et al. (1977). To convert the sulfated polymer (with $^{35}SO_4$-labeled material as marker) into the free acid, the sample was applied to a 2-ml bed of Dowex AG-50W-X8 (H^+). The effluent was immediately neutralized with pyridine and concentrated to 50 μl. After the addition of 450 μl DMSO, the sample was heated at 100°C, cooled, and diluted fivefold with water. To purify the desulfated polymer, the solution was applied to a Sep-Pak C 18 cartridge. After washing with 10 ml water, followed by 3 ml 15% acetonitrile, the polymer was eluted with 100% acetonitrile.

The degree of desulfation was determined with polyethyleneimine-cellulose plates (eluent: 0.3 M ammonium carbonate; Schleicher & Schüll, Dassel, FRG) by measuring the liberated [^{35}S]sulfate label with a radioscanner (Isomess). Sulfate liberated by hydrolysis (2 M trifluoracetic acid, 100°C, 2 h) was determined by the colorimetric assay described by Terho and Hartiala (1971). Partial desulfation of methylated 28-kD saccharide was performed with the corresponding pyridinium salt in pure DMSO for 5 h at 50°C. The subsequent methylation step was immediately performed without prior isolation of the polymer.

Perethylation

Methyl iodide was replaced by ethyl iodide in an otherwise identical incubation as described for permethylation. The ethylation mixture was diluted 1:1 with water, applied to a Sep-Pak C 18 cartridge and washed with 15 ml water, 6 ml 18% acetonitrile water, 4 ml 25% acetonitrile water, and 100% acetonitrile.

Hydrolysis of Saccharides

The sample was dissolved in 4 M trifluoracetic acid, sealed under N_2, and held at 100°C for 2 h. Sugar derivatives were reduced and acetylated according to standard procedures (Laine et al., 1972; Spiro, 1972).

Preparation of the Mannose Core

Methylated 28-kD was hydrolysed in 50 mM HCl for 90 min at 100°C and then neutralized and dialysed against water.

Gas Chromatography/Mass Spectrometry (GLC/MS)

All GLC-MS analyses were performed with an Hewlett packard model 5995 equipped with a Durabond 1701 fused silica capillary column (30 m ICT, J & W Scientific). Carrier gas was 1 ml/ min high-purity helium. The temperature gradient was 2°/min, starting at 140°C.

The assignment of methylated alditolacetates was accomplished according to reference spectra (Jansson et al., 1976). The identity of partially methylated and ethylated alditolacetates were established from the fragmentation rules described for methylated alditolacetates (Lindberg, 1972).

Results

Indirect Immunofluorescence Microscopy

Mouse mAbs were raised against the purified 145-kD glycopeptide derived from glycoprotein SSG 185. Out of five positive clones, two specifically recognized the 145-kD fragment and did not cross-react with other glycoproteins of the extracellular matrix, as documented in a Western blot experiment (Fig. 1). No component of a total cell lysate is stained by the mAb (lane *3*). Only after proteolytic treatment of this lysate (converting polymeric SSG 185 into a 145-kD glycopeptide fragment) a single immunopositive band becomes detectable (lane *4*).

The localization of this antigen within the ECM was studied by indirect immunofluorescence microscopy. For that purpose, intact *Volvox* spheroids were sliced into hemispheres and smaller fragments, simply by applying mild mechanical stress as exerted by forcing them through a drawn pipette. After fixation with formaldehyde, the fragments were stained with mAb 302/1 and fluorescein-conjugated sheep anti-mouse IgG (Fig. 2 *C*). The antibody selectively stains the cellular compartments CZ 3 within the ECM. From electron microscopic studies, this substructure was described as a coherent network of filaments forming chambers enclosing each individual cell of the organism.

In Vivo Effects of Monoclonal Antibody

mAb 302/1 was tested in vivo for its ability to interfere with the development of the ECM. At the end of embryogenesis, the somatic cells of the embryo begin to secrete sheath material, causing each cell to move apart from its neighbors. The organism now grows in size but not in cell number (Starr, 1969, 1970). This process of enlargement of the young spheroid is strongly suppressed in the presence of mAb 302/1

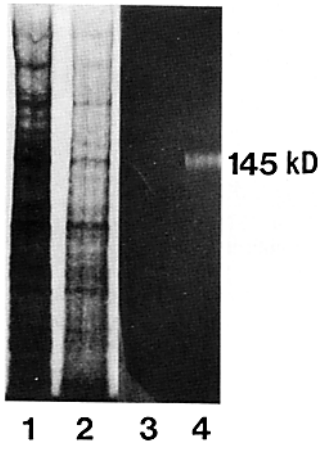

Figure 1. Western blot analysis of mouse mAb 302/1. A total cell lysate (corresponding to the material from 10 *Volvox* spheroids) was applied to a 6% SDS-polyacrylamide gel before (lane *1*) and after (lane *2*) 30-min digestion with 0.5 mg/ml subtilisin. Lanes *3* and *4* show the corresponding immunostaining patterns.

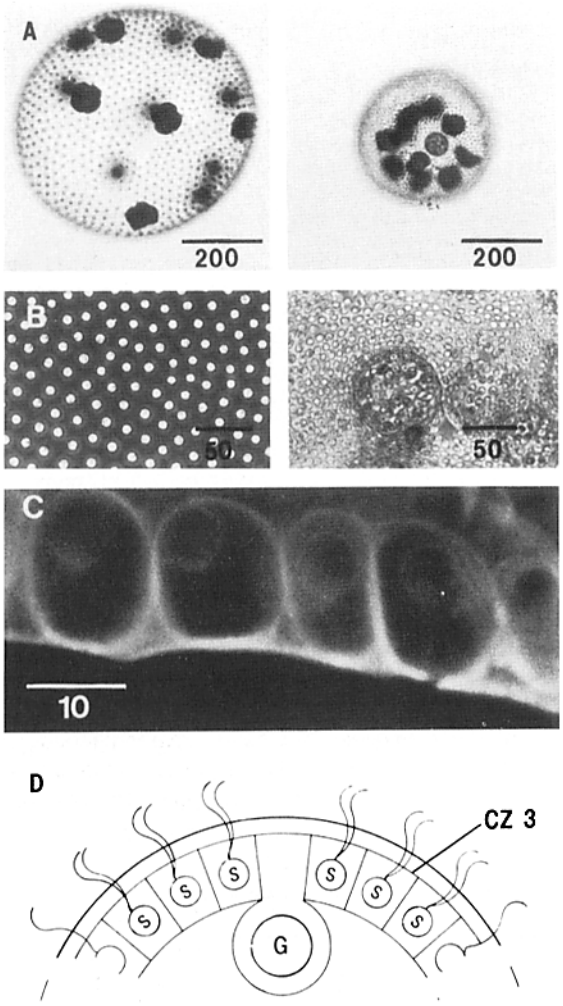

Figure 2. In vivo effect of mAb 302/1 and localization of its antigen, the extracellular glycoprotein SSG 185. (*A*) Growth of *Volvox* spheroids in the absence (*left*) and presence of mAb 302/1 (200 μg IgG/ml). *B* is similar to *A*, but somatic cell sheets seen at higher magnification. (*C*) Localization of SSG 185 glycoprotein by indirect immunofluorescence microscopy. mAb 302/1 selectively stains the cellular compartments (CZ 3 according to Kirk et al., 1986) within the ECM, which are schematically shown in *D*. *S*, somatic cell, *G*, gonidium. Sizes are indicated in microns.

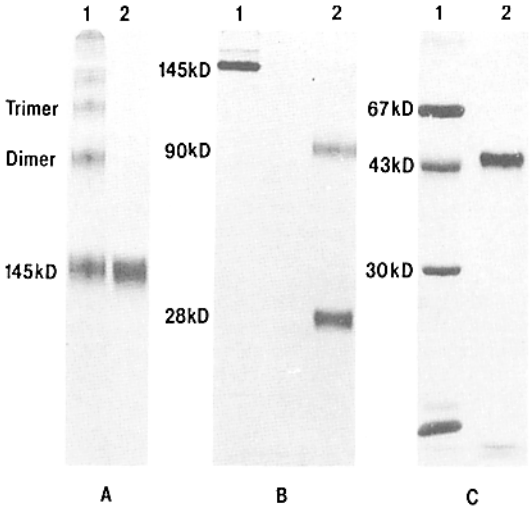

Figure 3. Sequential degradation of polymeric SSG 185. (*A*) *1*: Polymeric SSG 185 after digestion with 100 μg/ml subtilisin in the presence of 0.1% SDS. *2*: Similar to *1*, but in the presence of 0.5% SDS. Analysis was performed on a 6% SDS-polyacrylamide gel. (*B*) The proteolytic degradation product of SSG 185, the 145-kD glycopeptide, before and after mild mercaptolysis. Analysis was performed on 12% SDS polyacrylamide gel. (*C*) *1*: Molecular weight markers. *2*: Oligomeric degradation product of SSG 185 (as shown in *Al*) after complete deglycosylation by HF (10% SDS polyacrylamide gel). *A* and *B* were stained with Stains All; *C* is a silver stain.

(Fig. 2, *A* and *B*). The inhibition is specific for the growth of ECM, because the somatic and the reproductive cells remain viable and the latter cells enter embryogenesis again.

Protein Chemical Characterization of SSG 185

Purified polymeric SSG 185 becomes degradable by proteases only after incubation at 95°C in the presence of SDS. Depending on the experimental conditions during the subsequent digestion, the polymer is either completely degraded to the 145-kD glycopeptide or to a mixture of soluble oligomers of SSG 185. Complete degradation by subtilisin is observed in the presence of 0.5% SDS, whereas in the presence of 0.1% SDS, degradation results in the formation of oligomeric products (Fig. 3 *A*). These highly soluble oligomers were further purified by anion-exchange chromatography. Due to the high degree of sulfation, the oligomers remain bound to the ion-exchange resin up to NaCl concentrations of 3.5 M. Thus, all contaminating proteins are easily removed by this chromatographic step. Subsequently, the oligomers were deglycosylated by treatment with anhydrous HF. The product of deglycosylation is a single polypeptide chain with an apparent molecular mass of 50 kD (Fig. 3 *C*). This deglycosylated polypeptide was readily digested by trypsin and the resulting peptide mixture was separated by reverse phase C 18 HPLC. Well-separated peaks were directly submitted to amino acid sequence analysis on an automated gas-phase sequencer. All amino acid sequence data obtained are summarized in Table I. All of these peptides exhibit amino acid sequences that appeared unfavorable for designing an oligonucleotide probe that would specifically detect the SSG 185 gene. Therefore, the amino acid sequence information was used to raise a peptide-specific antibody. For that purpose, peptide 6 (Table I) was selected and chemically synthesized in its unmodified form, i.e., SGPNVNPI-

Table I. Amino Acid Sequences of Tryptic Peptides Derived from SSG 185

Peptide	Amino acid sequence
1 *a*	Phe Ala Asp Leu Cys Pro Gly Arg
1 *b*	Gly Cys Thr Val Ala Val Phe
2	Ser Ser Phe Ser Trp Asp Ser Thr Arg
3	Ser Pro Ser Ser Try Pro Trp Arg
4	Lys Hyp Hyp Ser Hyp Ser Hyp Hyp Val...
5	Thr Leu Ser Asn Thr Ala Glu Ile Arg
6	Ser Gly Pro Asn Val Asn Hyp Ile Gly Hyp Ala Hyp Asn Asn Ser Hyp Leu Hyp Hyp Ser Hyp Gln Hyp Thr

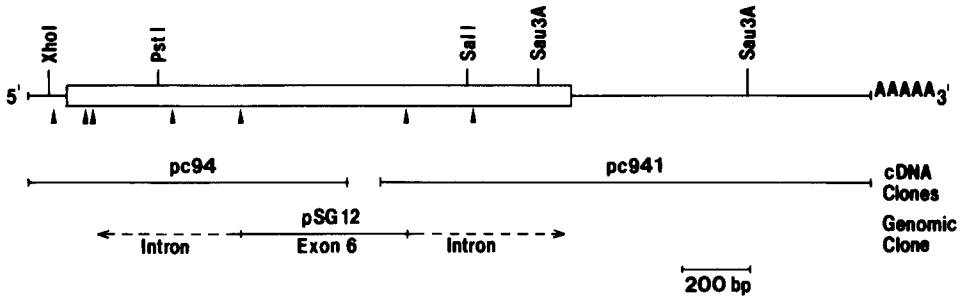

Figure 4. Comparison of SSG 185 cDNA and genomic clones used to establish the complete cDNA sequence. Besides the cDNA clones, the nucleotide sequence of a genomic clone encoding the complete SSG 185 gene was determined (Ertl, H., unpublished results). The cDNA sequence turned out to be interrupted by seven introns. Arrows indicate the positions of these introns. A short stretch of the exon 6 nucleotide sequence was required to fill the gap between cDNA clones pc94 and pc941.

GPA. The resulting rabbit antisera were analyzed by Western immunoblotting and shown to bind to the polypeptide derived from the SSG 185 oligomers (not shown).

cDNA Cloning

The peptide antibody was used to screen a *Volvox* cDNA library constructed in a λgt11 vector. Screening of 220,000 clones resulted in the identification of a single immunopositive clone (clone 94). The *Volvox* insert DNA of this clone was transferred into a pUC8 vector and its nucleotide sequence determined. Since the insert DNA encoded the amino acid sequence of peptide 6 (Table I), the cloned cDNA was identified definitively as derived from the SSG 185 gene. However, the cloned cDNA encoded only 265 amino acids and lacked the information for the COOH-terminal portion of the polypeptide. The open reading frame of this cDNA fragment was found to be terminated in a region encoding a large cluster of proline residues. Because we were unable to identify a full length cDNA, this cytidine-rich sequence

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   1    G GAA TCC CTT TTT CAC GAA AAG GCG TGG AGT GGT CCG TTA TTC CAT TTC TGT CAC GAC TCG AGA CTT GGC TAC TGG TGC TCA CCG GGA CCA ATT

  95  TTA TAC GCA AAG ATG TCT AAG CTG CTG CTC GTG GCG CTT TTT GGC GCC ATA GCA GTG GTT GCT ACG AGT GCT GAG GTA CTC AAC CTC AAC GGA AGG
   1                  Met Ser Lys Leu Leu Leu Val Ala Leu Phe Gly Ala  Ile Ala Val Val Ala Thr Ser Ala Glu Val Leu Asn Leu Asn Gly Arg

 191  TCT CTT TTG AAC AAC GAC GAC CCG AAC GCC TTC CCT TAC TGC AAA TGT  ACT TAT CGT CAG CGC CGG AGC CCA TAC CGC CTG AAG TAT GTT GGC GCC
  29  Ser Leu Leu Asn Asn Asp Asp Pro Asn Ala Phe Pro Tyr Cys Lys Cys  Thr Tyr Arg Gln Arg Arg Ser Pro Tyr Arg Leu Lys Tyr Val Gly Ala

 287  GAG AAC AAC TAC AAG GGC AAT GAC TGG CTG TGC TAC AGC ATT GTC CTT  GAC ACC ACG GGC ACT GTT TGC CAG ACA GTT CCG TTG ACG GAG CCT TGC
  61  Glu Asn Asn Tyr Lys Gly Asn Asp Trp Leu Cys Tyr Ser Ile Val Leu  Asp Thr Thr Gly Thr Val Cys Gln Thr Val Pro Leu Thr Glu Pro Cys

 383  TGC AGC GCT GAC CTG TAC AAG ATT GAG TTT GAC GTC AAA CCC TCC TGC  AAG GGC ACT GTT ACA CGT GCT ATG GTG TTC AAG GGC ATT GAC AGG ACT
  93  Cys Ser Ala Asp Leu Tyr Lys Ile Glu Phe Asp Val Lys Pro Ser Cys  Lys Gly Thr Val Thr Arg Ala Met Val Phe Lys Gly Ile Asp Arg Thr

 479  GTT GGC GGC GTA CGT GTG TTG GAG TCC ATC AGC ACC GTC GGC ATT GAT  GAC GTC ACC GGT GTT CCG GGC GCG GCC ATC CTT CGC ATC GTG AAG GAC
 125  Val Gly Gly Val Arg Val Leu Glu Ser Ile Ser Thr Val Gly Ile Asp  Asp Val Thr Gly Val Pro Gly Ala Ala Ile Leu Arg Ile Val Lys Asp

 575  CTT GCC CTC CCG TAC AGC GTC GTG GCT TCC TTC TTG CCT AAT GGC TTG  CCG GTC TGC ATC AAC AGG GTG CCC GGC TCG TGC ACG TTC CCG GAA CTG
 157  Leu Ala Leu Pro Tyr Ser Val Val Ala Ser Phe Leu Pro Asn Gly Leu  Pro Val Cys Ile Asn Arg Val Pro Gly Ser Cys Thr Phe Pro Glu Leu

 671  TTC ATG GAC GTT AAC GGC ACT GCC TCG TAC TCG GTC TTC AAC TCT GAT  AAG GAT TGC TGC CCA ACC GGC CTT TCC GGC CCC AAT GTC AAT CCA ATT
 189  Phe Met Asp Val Asn Gly Thr Ala Ser Tyr Ser Val Phe Asn Ser Asp  Lys Asp Cys Cys Pro Thr Gly Leu Ser Gly Pro Asn Val Asn Pro Ile

 767  GGC CCG GCA CCC AAC AAC AGC CCG TTG CCA CCA AGC CCG CAG CCA ACA  GCA AGC TCG CGG CCA CCA AGC CCG CCG CCC AGC CCG CGG CCG CCA AGC
 221  Gly Pro Ala Pro Asn Asn Ser Pro Leu Pro Pro Ser Pro Gln Pro Thr  Ala Ser Ser Arg Pro Pro Ser Pro Pro Pro Ser Pro Arg Pro Pro Ser

 863  CCG CCG CCT CCC TCG CCC AGC CCG CCG CCC CCA CCG CCG CCG CCC CCT  CCA CCC CCA CCG CCG CCC CCC CCA AGC CCC CCA CCG CCG CCA CCG CCT
 253  Pro Pro Pro Pro Ser Pro Ser Pro Pro Pro Pro Pro Pro Pro Pro Pro  Pro Pro Pro Pro Pro Pro Pro Pro Pro Ser Pro Pro Pro Pro Pro Pro

 959  CCC CCA CCT CCT CCT CCA CCA CCA CCG CCA CCC TCT CCC TCC CCG CCA  CGC AAG CCA CCG TCC CCA TCC CCA CCT GTT CCC CCA CCG CCA TCA CCA
 285  Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Ser Pro Ser Pro Pro  Arg Lys Pro Pro Ser Pro Ser Pro Pro Val Pro Pro Pro Ser Pro

1055  CCG AGC GTC TTG CCC GCT GCT ACC GGC TTC CCC TTC TGC GAG TGC GTG  TCC CGG TCC CCT TCC AGC TAC CCA TGG CGC GTG ACC GTC GCC AAC GTT
 317  Pro Ser Val Leu Pro Ala Ala Thr Gly Phe Pro Phe Cys Glu Cys Val  Ser Arg Ser Pro Ser Ser Tyr Pro Trp Arg Val Thr Val Ala Asn Val

1151  TCC GCG GTC ACC ATC TCT GGC GGC GCT GGC GAG CGC GTG TGC CTG AAG  ATT TCG GTC GAC AAC GCG GCC GCT GCC ACC TGC AAC AAC GGC CTG GGC
 349  Ser Ala Val Thr Ile Ser Gly Gly Ala Gly Glu Arg Val Cys Leu Lys  Ile Ser Val Asp Asn Ala Ala Ala Ala Thr Cys Asn Asn Gly Leu Gly

1247  GGG TGC TGC TCG GAT GGA CTG GAG AAG GTC GAG CTC TTT GCT AAC GGC  AAG TGC AAG GGC TCC ATT TTG CCG TTC ACT CTG TCC AAC ACC GCG GAG
 381  Gly Cys Cys Ser Asp Gly Leu Glu Lys Val Glu Leu Phe Ala Asn Gly  Lys Cys Lys Gly Ser Ile Leu Pro Phe Thr Leu Ser Asn Thr Ala Glu

1343  ATT AGG TCG TCA TTC TCC TGG GAC TCC ACT CGC CCA GTC CTC AAA TTC  ACC CGC CTG GGC CTC ACC TAT GCT CAA GGT GTC GCC GGC GGC AGC CTC
 413  Ile Arg Ser Ser Phe Ser Trp Asp Ser Thr Arg Pro Val Leu Lys Phe  Thr Arg Leu Gly Leu Thr Tyr Ala Gln Gly Val Ala Gly Gly Ser Leu

1439  TGC TTC AAC ATC AAG GGT GCC GGC TGT ACC AAG TTT GCA GAT CTC TGT  CCT GGC CGC GGC TGC ACC GTC GCG GTA TTC AAC AAC CCC GAC AAC ACA
 445  Cys Phe Asn Ile Lys Gly Ala Gly Cys Thr Lys Phe Ala Asp Leu Cys  Pro Gly Arg Gly Cys Thr Val Ala Val Phe Asn Asn Pro Asp Asn Thr

1535  TGT TGC CCG CGG GTC GGC ACC ATC GCG TAA AGC GTT TCG CAT GAC GAT  GCA AAA AAA AGG GTG GTG CAG CGC AAC ACC CCC CCG TGG GTG TGT GGG
 477  Cys Cys Pro Arg Val Gly Thr Ile Ala ***

1631  TGT GTG TGC CCG CGC AAG AAG ATT GTG CGT GAG TGG ATG TGC GCG GAT  GTG GGG GGG AAA ACC GCC AGT CCC TTT CTT ATA TGC TGC TAC ACA TGA
1727  CTG AGG TTG CTG CCG GGG AGG CGG AGG AGG CTT GTG GGT GTG GCG TCG  GCG TCG ATG CCA AGT CAC CGC TGT GCC ACT GCT GCG GCG GGC TTG GAT
1823  AGC AGC AGC AGC GGC GCA GCA GCA GCA GCA TCC GCA GCA TCA GCA GCA  TAG GGA CGC ATG CAT GCC TTT AGC CGG AAT GGG AGG GCT GCG TGC TGC
1919  TTT TGC AAC CGC AGG GGT GAA AGT AGG TAG TCC TGC ATA AAT GAT TAA  GCA CTG GTC AAA TGT CGC TGA GAT GAA CAT AAC ACG ATT ATA TAT GAT
2015  ATG ATG CGC GAC ATG ATT GTT GGA TGC GGC CAG TAC TTT TTT CCG CTG  CGC AGG TGT CAG TGT TGC ACT TGG GTT TGA TCC AGT TAC GGG CCT CTC
2111  GGC GTC TAC CAA ACG CAA ATT GTC GTT AAT TGA TAT TCT CTT GCT TTT  TGC GCA TCT CGT CAA GTG AGG GCA GCG TCC CCC TCC TGT GCT GCT GCA
2207  CTT GAC GTG GCT GTT GGA ATG TTC CGT GCA TGG CCG CTG GCC CGA ACC  ATT TGT AGT TGA GTG ACT ACT GCG CTA ACA TTC AGT TAT TTC GTA ATA
2303  ATC ACC ATC ATA ATT ATC ATC ATC GCT ACC TGC CTA TGG CCG GGA AGT  TGA GCA GGT TTT AAC GTT TTA CGT TCA ATC CCG ATG GCC CAC GTC ACG
2399  TGT AGC AGG CCT TTG CGT TCC CGC TCT CTT GTA ACT TAT TTC TCA TAA  AAA AAA
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Figure 5. The predicted amino acid sequence of sulfated glycoprotein SSG 185. The dark bar underlines a central domain that consists nearly exclusively of hydroxyproline residues. Amino acid sequences derived from isolated peptides are indicated by broken lines.

probably terminates the oligo(dT)-primed reverse transcription. Transcripts beyond this cytidine-rich area (as our clone 94) may result from an unspecific priming of reverse transcription. To circumvent these problems, clone 94 was used to screen a genomic library of *Volvox* carteri constructed in the replacement vector EMBL 3. Out of 100,000 phages screened, 7 positive clones were isolated. One of these was subcloned (pSG12) and sequenced. It was possible to cover the cytidine-rich region and to run into sequences encoding another known peptide (peptide 4 of Table I), thereby confirming the correct sequencing of the cytidine-rich region. The new sequence information derived from the genomic clone was finally used to rescreen the original λgt11 library with an oligonucleotide. This resulted in the identification of oligo(dT)-primed cDNA clones (e.g. λ941) encoding the COOH-terminal portion of the SSG 185 polypeptide. Fig. 4 schematically summarizes the strategy applied to collect the complete cDNA sequence information. The nucleotide sequence is given in Fig. 5 together with the deduced amino acid sequence of the SSG 185 glycoprotein. The predicted amino acid sequence contains 485 amino acids with a putative NH_2-terminal signal sequence. The calculated molecular mass of the polypeptide chain is 50,434 D. The most remarkable feature is a cluster of 40 proline residues interrupted only by single serine residues at positions 257, 259, and 277. Amino acid analysis from isolated peptides (Table I) and the complete 145-kD polypeptide (Wenzl et al., 1984) indicate that most if not all of these proline residues are hydroxyproline in the mature glycoprotein.

The Structure of the Sulfated Saccharide

On mild mercaptolysis, the 145-kD glycopeptide is further cleaved into a large fragment (90 kD) and a smaller fragment (28 kD), as shown in Fig. 3 *B*. The large fragment contains all of the amino acids and the neutral sugars galactose and arabinose, but no sulfate. The 28-kD fragment contains no amino acids, but mannose, arabinose and all of the sulfate residues found in the SSG 185 glycoprotein. The molar ratio of arabinose and mannose was determined by gas chromatography of the corresponding alditol acetates and found to be 10:7.2 (±4%). The molar ratio of covalently bound sulfate per sugar residue was determined to be 1.3, indicating the existence of sugarbissulfates in the 28-kD polysaccharide. Methylation analysis of the 28-kD polysaccharide, using the Hakomori procedure (Hakomori, 1964), was impossible because of the insolubility of the sulfated macromolecule in DMSO. In contrast, the complete methylation was achieved using the classical Haworth procedure (Haworth, 1915). Because the methylated 28-kD saccharide is soluble in DMSO, the product obtained by the Haworth procedure was again subjected to methylation with the Hakomori procedure. No difference in the pattern of partially methylated sugars was found after the second round of methylation, indicating that the Haworth procedure yields a completely methylated product. The product of methylation was hydrolyzed, reduced, acetylated, and investigated by GLC-MS. The collected data are summarized in Table II. 1,2,3,4-Tetra-*O*-acetyl-5-*O*-methyl-arabinitol and peracetylated arabinitol were found to be the main derivatives of arabinose, indicating substitutions at three and four positions, respectively. Only a single main derivative of mannose is found, namely 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-mannitol. Thus, the polysaccharide contains mannose residues with four substitutions.

Table II. Partially O-Methylated Alditol Acetates Derived from Permethylation Analysis of 28-kD Saccharide

Alditol	Postion of *O*-acetyl groups	Position of *O*-methyl groups	
			mol %
Ara	1,2,4	3,5	11.2
Ara	1,2,3,4	5	20.7
Ara	1,2,3,4,5		24.1
Man	1,3,4,5	2,6	7.3
Man	1,3,5,6	2,4	2.4
Man	1,3,4,5,6	2	31.5

Additional structural information was obtained by partial acid hydrolysis studies of the methylated 28-kD polysaccharide. Hydrolysis with 50 mM HCl at 100°C for 90 min leads to the loss of nearly all of the arabinose and sulfate residues, whereas the mannose residues remain stable in a polymer. This polymannan core structure was characterized by methylation analysis. The main product obtained was 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-mannitol (74.2 mol%). This result proves that a chain of 1,3-linked mannose residues represents the core structure of the 28-kD polysaccharide. The ratio of 1,3-linked residues to reducing end residues was found to be 30. Proton nuclear magnetic resonance studies indicated the presence of β 1–3 linkages in the polymannan core, since the chemical shift of the H1 signal was determined to be $\delta = 4.73$ ppm (relative to tetramethylsilan). The same conclusion was drawn from optical rotation experiments ($[\alpha]_D^{20} = -51°$ 0.08 H_2O).

The 28-kD polysaccharide contains more than one sulfate residue per sugar molecule. Information regarding the positions of sulfate attachment should be obtained if the methylated polysaccharide is desulfated and the product is realkylated using ethyl iodide. Usually, desulfation can easily be accomplished due to the extreme acid lability of sulfate esters. However, in the case of the 28-kD polysaccharide, selective desulfation was made impossible because of the presence of highly labile arabinose residues. Therefore, the experimental approach was slightly modified as follows. In a first step, the most labile sulfate and arabinose substituents were removed from the pyridinium salt of the 28-kD polysaccharide by mild solvolysis (in DMSO; 5 h at 50°C). This procedure removes ~30% of the sulfate and 20% of the arabinose residues bound to the polymer. This product was subsequently methylated, using the Hakomori procedure, and purified. In the second step, solvolysis was repeated for 1 h at 100°C. These conditions selectively remove all of the remaining sulfate residues without substantial cleavage of glycosidic bonds (<5% of arabinose is lost). The desulfated polymer was then realkylated using ethyl iodide, hydrolyzed, reduced, acetylated, and analyzed by GLC-MS.

The methylated polymer resulting from the partial solvolysis in step 1 still contains highly substituted arabinose and mannose residues. Again, the main products of the methylation analysis were found to be 1,2,3,4-tetra-*O*-acetyl-5-*O*-methyl-arabinitol (25% of total arabinose derivatives), peracetylated arabinitol (25% of total arabinose derivatives) and

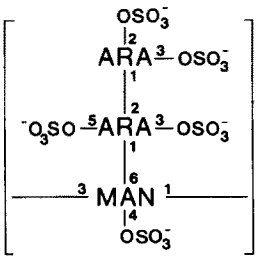

Figure 6. Proposed chemical structure of the sulfated 28 kD saccharide covalently linked to SSG 185.

1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-mannitol (53% of total mannose derivatives). After complete desulfation and ethylation of the polymer the following new derivatives of these molecules were produced: the 5-*O*-methyl-arabinitol is mainly converted to 1,4-di-*O*-acetyl-2,3-di-*O*-ethyl-5-*O*-methyl-arabinitol, the peracetylated arabinitol to 1,2,4,-tri-*O*-acetyl-3,5-di-*O*-ethyl-arabinitol and the 2-*O*-methyl-mannitol to 1,3,5,6-tetra-*O*-acetyl-4-*O*-ethyl-2-*O*-methyl-mannitol. These data indicate that three different types of sulfated sugar residues are main constituents of the 28-kD polysaccharide: furanosidic arabinose sulfated at positions 2 and 3, furanosidic arabinose sulfated at positions 3 and 5, and pyranosidic mannose sulfated at position 4. A minor fraction of the total arabinoses (~20%) was found to be sulfated only at position 2 and these residues were derived from non-reducing ends (permethylation data not shown).

In ^{13}C nuclear magnetic resonance experiments with the intact 28-kD molecule, the chemical shift of the C1 atoms of arabinoses was found to be δ = 106.7 ppm (H_2O, relative to TMS) indicating the α-furanosidic structure.

The structural element shown in Fig. 6 is in agreement with all the results of the degradation and methylation studies and therefore is proposed as the main structural feature of the 28-kD glycoconjugate of SSG 185.

Electron Microscopy

The 145-kD monomer and fragments thereof were studied by EM applying the rotary shadowing technique (Fig. 7). The 145-kD monomer is a T-shaped molecule with a thick rod-like structure with a length of 28.7 ± 3.4 nm. Attached to the central region of this rod (10 ± 3 nm from the nearest end) is a thinner structural element with an extension of 21.4 ± 2.2 nm (Fig. 7 *A*). By comparing the pictures of defined fragments obtained by mercaptolysis of the 145-kD monomer, the thick rod is identified as the 90-kD fragment containing the glycosylated hydroxyproline-rich peptide (Fig. 7 *B*). The thinner element represents the sulfated 28-kD polysaccharide (Fig. 7 *C*). Dimers isolated from partial proteolytic digests of the polymeric SSG 185 glycoprotein are shown in Fig. 7 *D*. The average length is 38.5 ± 5.5 nm, indicating an overlapping staggered arrangement of the monomeric units within the dimer.

Discussion

The extracellular glycoprotein SSG 185 is the monomeric precursor of a fibrous structural element that gives rise to the honeycomb-like cellular compartments within the ECM of *Volvox carteri*. The polypeptide chain of SSG 185 contains a central domain, 80 amino acid residues long, that is composed nearly exclusively of hydroxyproline residues. Preliminary experiments indicate that most of these hydroxyproline residues are glycosylated with 1,2-linked di- and tri-arabinosides. Proteolysis of SSG 185 results in a large, completely resistant 145-kD fragment with a high content of hydroxyproline. Most probably, the secondary structure of such a domain is the polyproline II helix conformation. This is the most extended helix formed by polypeptides with three residues per turn and of a pitch 0.94 nm. Our electron micrographs of the 145-kD glycopeptide show an extended rod structure, 28.7 nm long. This corresponds to a polypeptide chain of ~90 residues, a value that is in good agreement with the length of the central hydroxyproline-rich domain of SSG 185. Both the NH_2-terminal (~230 amino acid residues) and the COOH-terminal extensions (165 amino acid residues) of the central hydroxyproline cluster exhibit no unusual amino acid preferences except for cysteine. In the COOH-terminal domain 13 out of 165 amino acid residues are cysteines. SSG 185 differs from the extensins characterized from plant cell walls in being not highly basic and having most of its hydroxyprolines in an extended cluster instead of repeats of the typical Ser-$(Hyp)_4$ peptide sequence (Cassab and Varner, 1988).

A unique feature of SSG 185 is an extremely sulfated polysaccharide attached close to the center of the hydroxyproline cluster in an as yet unknown covalent linkage. This linkage is cleavable by thiols but only under acid catalysis excluding a disulfide linkage. The polysaccharide exhibits a length of 21.4 nm, indicating a degree of polymerization ~40. Detailed chemical analysis has revealed a 1,3-linked mannose backbone with diarabinosides attached via the 6 positions. Sulfate residues are linked to the 4 positions of the mannoses and to the 3 and 5 positions of the arabinoses attached to the backbone, whereas the arabinoses at the nonreducing ends are sulfated at positions 2 and 3. It is interesting to note that the degree of sulfation of this saccharide is under developmental control (Wenzl et al., 1984). SSG 185 isolated at the beginning of the cleavage period of the reproductive cells exhibits a lower degree of sulfation than material isolated from colonies containing late embryos. From our permethylation data, we conclude that mainly the degree of sulfation at position 4 of mannose is under developmental control and increases during the period of embryogenesis. The unusually high density of negative charges should exert a strong influence on the overall physicochemical properties of the ECM. Positively charged proteins within the ECM should bind to this strong cation exchanger. The chaotrope-soluble crystalline layers from both *Chlamydomonas reinhardtii* and *Volvox carteri* were characterized as positively charged glycoproteins (Goodenough et al., 1986; Goodenough and Heuser, 1988). Aggregation in vitro was shown to be enhanced in the presence of chaotrope-insoluble cell wall material acting as nucleation centers (Hills, 1975; Adair et al., 1987). The sulfated polysaccharide extending from polymeric SSG 185 may be involved in this nucleation process.

Pulse-chase experiments demonstrate that monomeric SSG 185 is made insoluble in the ECM by the formation of highly polymeric material, which is completely resistant towards chaotropic agents or boiling in the presence of de-

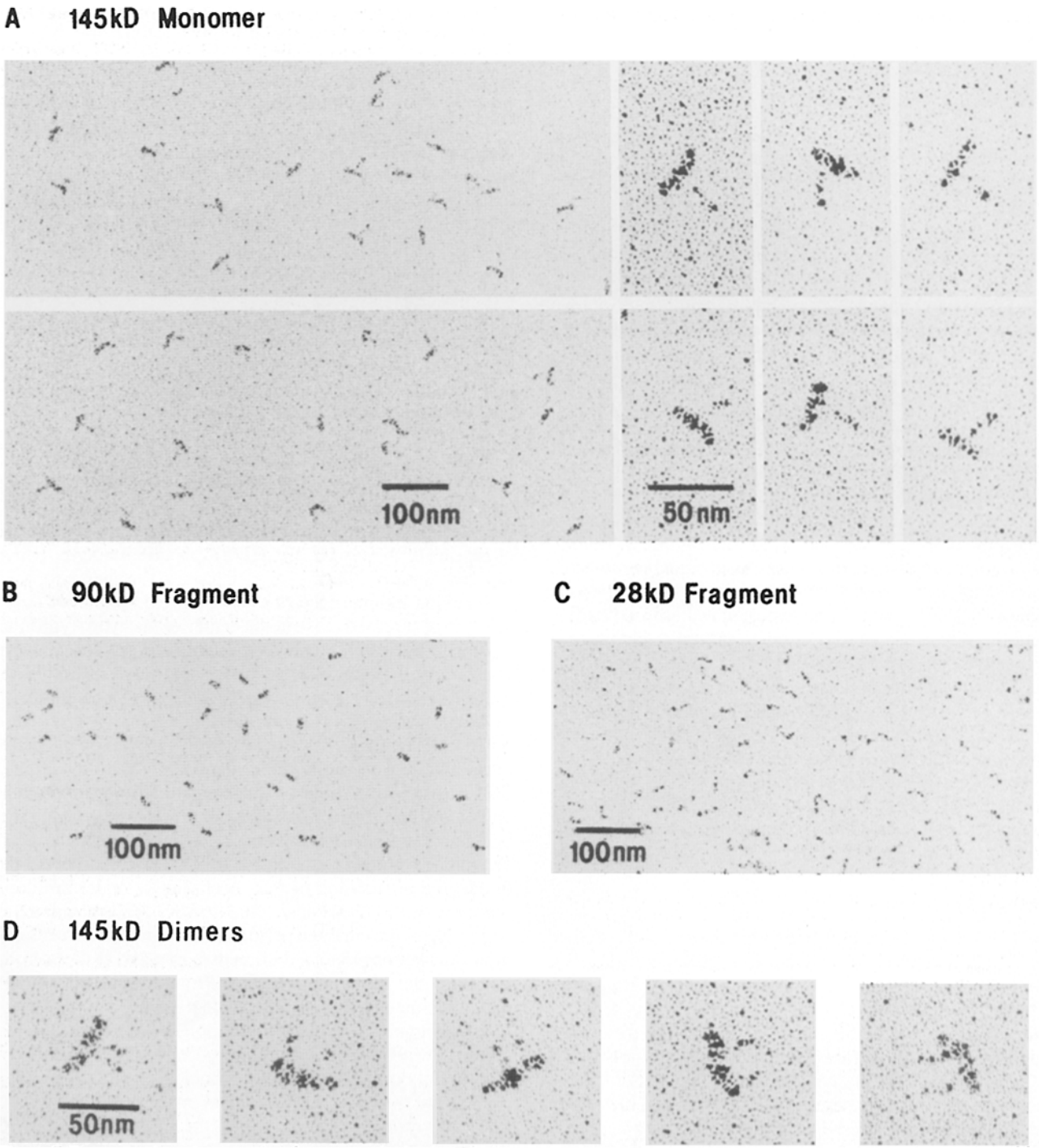

Figure 7. Electron microphotographs after rotary shadowing of (*A*) 145-kD glycopeptide monomers. (*B* and *C*) Degradation products 90-kD glycopeptide and 28-kD sulfated polysaccharide, respectively. (*D*) Dimers of 145 kD glycopeptide, obtained by limited proteolysis of polymeric SSG 185.

tergents. Therefore, the presence of covalent cross-links appears highly likely. Chemical deglycosylation of the polymeric material with anhydrous hydrogen fluoride causes complete depolymerization, resulting in the formation of a single polypeptide chain with apparent molecular mass ~50 kD. This indicates that covalent cross-links are formed between the saccharide chains of the SSG 185 molecule rather than between the polypeptide chains. Recently we have identified a phosphodiester bridge between the C-5 atoms of two arabinose residues as an integral structural element of SSG 185 (Holst et al., 1989). Obviously, this diester is a plausible candidate to function as the covalent cross-link of SSG 185 in the ECM of *Volvox*.

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