

Algal-CAMs: isoforms of a cell adhesion molecule in embryos of the alga *Volvox* with homology to *Drosophila* fasciclin I

Otmar Huber and Manfred Sumper¹

Lehrstuhl Biochemie I, Universität Regensburg, 93040 Regensburg, Germany

¹Corresponding author

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Proof that plants possess homologs of animal adhesion proteins is lacking. In this paper we describe the generation of monoclonal antibodies that interfere with cell–cell contacts in the 4-cell embryo of the multicellular alga *Volvox carteri*, resulting in a hole between the cells. The number of following cell divisions is reduced and the cell division pattern is altered drastically. Antibodies given at a later stage of embryogenesis specifically inhibit inversion of the embryo, a morphogenetic movement that turns the embryo inside out. Immunofluorescence microscopy localizes the antigen (Algal-CAM) at cell contact sites of the developing embryo. Algal-CAM is a protein with a three-domain structure: an N-terminal extensin-like domain characteristic for plant cell walls and two repeats with homology to fasciclin I, a cell adhesion molecule involved in the neuronal development of *Drosophila*. Alternatively spliced variants of Algal-CAM mRNA were detected that are produced under developmental control. Thus, Algal-CAM is the first plant homolog of animal adhesion proteins.

Key words: cell adhesion molecules/embryogenesis/extensin/fasciclin I/*Volvox*

terminated by inversion, a process which turns the embryo inside out to form the spherical daughter colony (for more details see Starr, 1969, 1970). After inversion, *V. carteri* grows in size by deposition of fibrous extracellular matrix material that is secreted by the somatic cells (Kirk *et al.*, 1986; Ertl *et al.*, 1989, 1992). During development, all embryonic cells establish close contacts with their neighbors. Up to now it has been assumed that these contacts are built via a cytoplasmic bridge system that links sister cells in each cleavage furrow by incomplete cytokinesis (Green and Kirk, 1981; Green *et al.*, 1981). No experimental evidence was available for the existence of embryonic cell adhesion molecules, although a model that is able to describe the control of pattern formation was based on properties of cell adhesion molecules (Sumper, 1979). Furthermore, proof that plant cells possess homologs of animal cell adhesion proteins is missing (Chasan, 1994).

To examine the existence, or otherwise, of embryonic cell adhesion molecules, mAbs directed against membrane fractions from *Volvox* embryos were generated. The resulting mAbs were screened for their capability to interfere *in vivo* with cell–cell contact formation during embryogenesis. Several mAbs were isolated that were able to dissociate cell–cell contacts of the 4-cell embryo. In this paper, we describe the influence of these mAbs on development and the primary structure of the corresponding antigen called Algal-CAM. Furthermore, the existence of alternatively spliced variants of Algal-CAM mRNA at defined stages of embryonic development is reported.

Introduction

Specific cell–cell contacts play a key role in the development and morphogenesis of multicellular systems and organisms (Ekblom *et al.*, 1986; Takeichi, 1988, 1990; Edelman and Crossin, 1991; Geiger and Ayalon, 1992). The green algae of the genus *Volvox* represent the simplest multicellular system composed of only two cell types: somatic and reproductive cells (gonidia). In spite of this simplicity, embryogenesis of *Volvox* includes a number of events, like pattern formation and morphogenetic movements, that are key processes in the development of higher organisms. Therefore, these algae are ideal models for a biochemical approach to the study of the functional role of cell–cell contacts in developmental processes.

During asexual development of *Volvox carteri*, a mature gonidium is subdivided by ~11 cleavage divisions to build a hollow, spherical monolayer of 2000–4000 somatic cells and exactly 16 reproductive cells. These reproductive cells are generated at strictly defined positions during the fifth division by an asymmetric cleavage of half of the embryonic cells in the 32-cell embryo. Embryogenesis is

Results

Monoclonal antibodies were raised against an acetone powder of a crude membrane fraction prepared from *Volvox* embryos. To check for the ability to disrupt cell–cell contacts in developing embryos, the resulting mAbs were screened using an *in vivo* assay described as follows. Hybridoma supernatants were added to the uncleaved gonidia that subsequently were allowed to develop up to the 8-cell stage. Hybridoma clones causing any aberrant embryonic development were identified by microscopic examination. The most spectacular effect observed with a few mAbs was the dissociation of the blastomers of the 4-cell embryo. If these particular mAbs were added to a population of synchronously growing gonidia at least 2 h prior to the onset of embryogenesis, nearly all of the embryos were inhibited in cell contact formation. Affected embryos are not able to form the characteristic cross-shaped cell contacts typical of a 4-cell embryo. Instead, cells move away from each other and align at the inner surface of the embryonic vesicle thereby creating a visible hole in the center (Figure 1A). Therefore

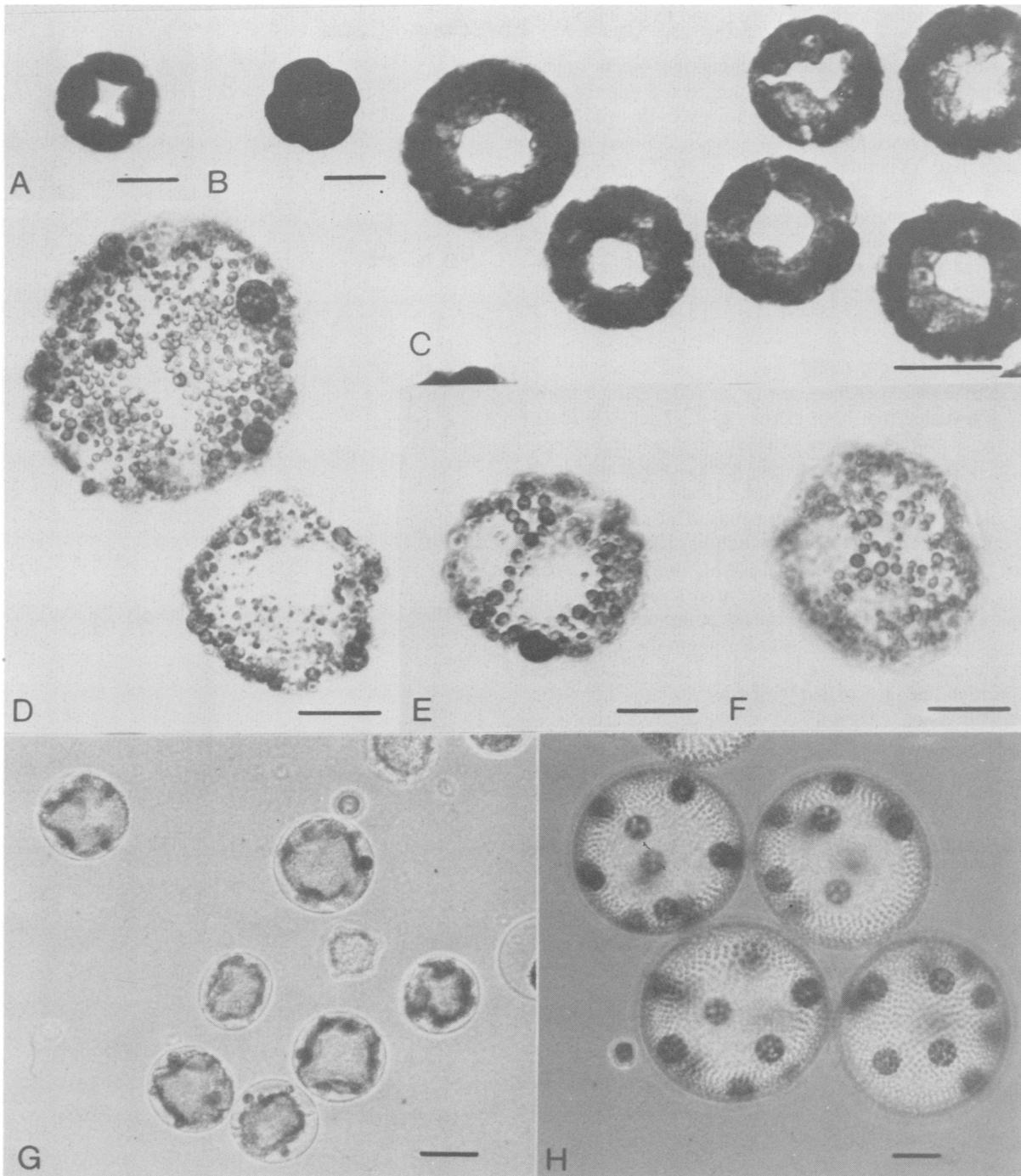


Fig. 1. *In vivo* effect of mAb on embryogenesis and inversion of *Volvox* embryos. 4-Cell embryos in the presence (A) and absence (B) of mAb. (C) *Volvox* embryos as in (A) after three more cleavage divisions. Increasing mAb concentration reduces the number of cell divisions and reproductive cells in further development of the embryos (D: 17.5 µg; E: 35 µg; F: 140 µg of mAb). (G) Inhibition of inversion if mAb is added at late stages of embryogenesis. (H) Control embryos without mAb. The photographs of (D)–(H) were taken 26 h after the addition of mAb. All microscopical photographs are taken from whole embryos. Scale bar: 50 µm.

the antigen was called Algal-CAM. Two of these hybridoma cell lines (25-72-7 and 25-92-4) were selected and used for all further experiments.

Dissociation of blastomers (Figure 1A) requires an antibody concentration of 6 µg/ml of *Volvox* medium. Cell divisions continue in the dissociated 4-cell embryo, but the resulting daughter cells exhibit irregular shapes and the cell borders are hardly visible (Figure 1C). The overall number of cell divisions is reduced, resulting in a daughter colony with a lower number of somatic as well as

reproductive cells (Figure 1D). Increasing the antibody concentration additionally reduces the total number of cell divisions and in parallel generates less reproductive precursor cells (Figure 1E). In extreme cases, embryos develop without the generation of any new reproductive cells (Figure 1F). This type of *in vivo* effect is observed if the antibody is removed at the 4-cell stage of embryogenesis. Thus, antibody binding to the cell adhesion molecules in the 4-cell embryo appears to cause all the dramatic effects on further embryonic development. F(ab) fragments

of the corresponding antibody were found to be unable to inhibit cell contact formation.

If mAbs were added after reaching the 4-cell stage, embryonic development remained unaffected until reaching the stage of inversion. Then, however, this morphogenetic process was inhibited at a stage denoted as the 'Mexican hat' (Viamontes and Kirk, 1977; Viamontes *et al.*, 1979). The affected embryos were unable to complete inversion and consequently the new gonidial precursor cells remained on the outside of the embryo inverted half-way (Figure 1G). This inhibition of inversion is specific in that further development of the reproductive cells proceeds normally.

Purification of Algal-CAM

A Western blot experiment using a crude extract from embryos revealed that both mAbs (25-72-7 and 25-92-4) recognize a protein species with an apparent molecular mass of 150 kDa. However, the stained band appeared rather broad and sometimes could even be resolved in a ladder-like pattern of individual bands. Thus, the antigen may be composed of related but slightly different protein species. Examination of Algal-CAM during embryogenesis by Western blot analysis (Figure 2A) exhibited a steady increase of Algal-CAM during embryonic cleavage divisions. Immediately at or after inversion the amount of detectable antigen decreased rapidly. Thus, Algal-CAM was purified using isolated embryos at late stages of cleavage division. Algal-CAM was extracted from the embryos with detergent. After removal of the extracted embryos and any cell fragments by centrifugation, Algal-CAM was adsorbed onto an affinity column of purified mAbs coupled to CNBr-Sephrose 4B. After extensive washing with extraction buffer, antigen could be eluted with 0.15 M NaCl, 26 mM triethylamine and 0.2% (w/v) deoxycholate, and was finally concentrated in a micro-concentrator. Typically, the yield was 10 µg Algal-CAM from 1000 l *Volvox* culture. Although Algal-CAM was undetectable by protein stains in a crude extract, it became highly enriched after this affinity chromatography and only a few unspecifically bound proteins contaminated the Algal-CAM preparation (Figure 2B, lanes 3 and 4). A nearly quantitative extraction of Algal-CAM was also obtained by washing embryos with 0.1 M NaCO₃ containing 0.02% (w/v) deoxycholate. Thus Algal-CAM appears to be a membrane-associated cell adhesion protein.

Automated Edman degradation of purified Algal-CAM blotted onto polyvinylidene fluoride (PVDF) membrane revealed a blocked N-terminus. To obtain internal peptides derived from Algal-CAM, ~5–10 µg of affinity purified antigen were separated by SDS-PAGE and blotted onto PVDF membrane. The Algal-CAM band was cut out and tryptic digestion was performed directly on the membrane pieces suspended in digestion buffer. The resulting peptide mixture was separated by reversed phase chromatography on a narrow bore HPLC column. Samples of well separated peaks were directly subjected to amino acid sequence analysis on an automated gas-phase sequencer. The amino acid sequences obtained are summarized in Table I and underlined in Figure 4. Blotting the affinity purified antigen onto nitrocellulose membrane and staining with FITC-labeled concanavalin A indicated that Algal-CAM may

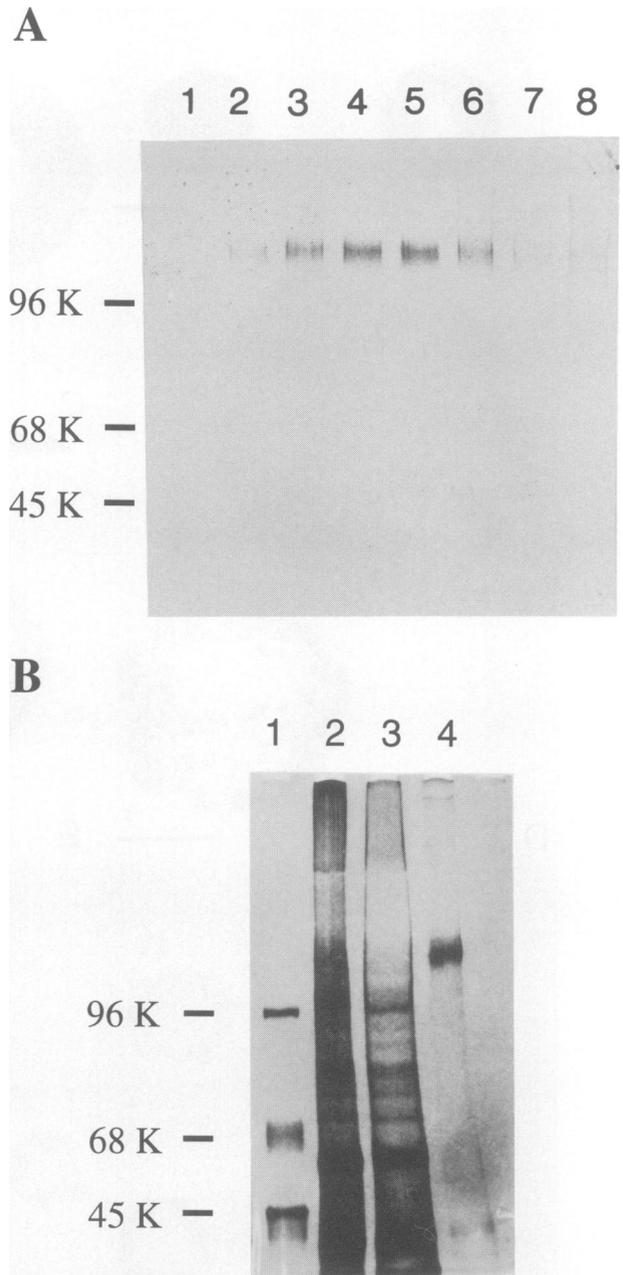


Fig. 2. Expression pattern and purification of the antigen. (A) At different stages of development, embryos (derived from a total of 100 colonies at a given stage) were isolated, SDS extracted and analyzed on a Western blot with mAb 25-92-4 (lane 1, gonidia before initiation of cell division; lane 2, 4/8-cell embryos; lane 3, 32/64-cell embryos; lane 4, embryo after differentiating cleavage; lane 5, post-cleavage embryos; lane 6, embryos at the stage of inversion; lane 7, post-inversion embryos (4 h after inversion); lane 8, post-inversion embryos (8 h after inversion)). (B) Purification of Algal-CAM. Molecular weight standard (lane 1); SDS lysate (lane 2); deoxycholate extract of *Volvox* embryos (lane 3) and concentrated eluate from affinity column (lane 4) as analyzed by SDS-PAGE (8%).

contain carbohydrates (not shown). This was further substantiated by the amino acid sequence of peptides 7 and 8 that both include a possible *N*-glycosylation motif (peptide 7, amino acids 21–23; peptide 8, amino acids 9–11; amino acid 21 in peptide 7 and amino acid 9 in peptide 8 gave no signal in automated Edman degradation).

Table I. Amino acid sequences of tryptic peptides derived from Algal-CAM

Peptide	Amino acid sequence
1	XXDALGVAR
2	SYESLLR
3	ALSYHVLPTTR
4	XXVAYGWVVD...
5	VSPSSPPATSPEIFILGVSXXAK
6	AGLLPYLPVIK
7	XNSFPTISLALSTANEVATXXDSSQEV...
8	XAQLLKPA XV TSPY TIFVPTDEAFV...
9	XXAGVKPTVASAAVPGSPV...

X, amino acid not identified.

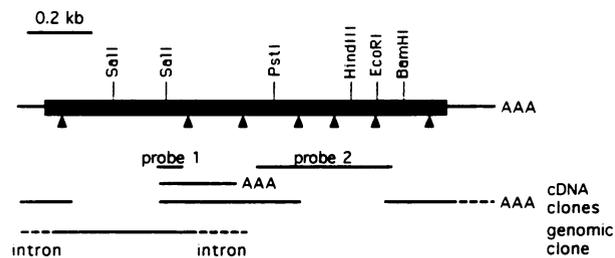


Fig. 3. Strategy applied to collect the complete nucleotide sequence of Algal-CAM cDNA. Amino acid sequence information of tryptic peptides was used to design sense and antisense primers for amplification of cDNA probes. Completion of cDNA was achieved by RT-PCR and a genomic clone. 5' cDNA sequences were established by RACE-PCR. Besides the cDNA clones, the nucleotide sequence of a genomic clone encoding the complete Algal-CAM gene was determined (O. Huber, unpublished results). The positions of introns are indicated by arrows. The EMBL data library accession number is X80416 VCALGALCM.

Cloning of the Algal-CAM gene

The amino acid sequence of peptide 7 (Table I) was used to synthesize an antisense oligonucleotide primer to reverse transcribe mRNA isolated from a mixture of 4-cell to 128-cell embryos. A sense primer derived from the same peptide allowed the amplification by PCR of a cDNA fragment of 80 bp in length (probe 1) that was cloned into the *Sma*I site of pUC18 by blunt-end ligation. Sequencing of this insert revealed a nucleotide sequence matching the amino acid sequence of peptide 7.

A second cDNA probe (probe 2) was generated by PCR on a solid-phase cDNA library coupled to Dynabeads Oligo (dT)₂₅ using the amino acid sequence information from peptides 5 and 9 (Table I). The amplified cDNA fragment revealed an open reading frame of 469 bp matching the amino acid sequences of peptides 2, 4, 5, 8 and 9 (Figure 3).

The remaining 3' stretches of the Algal-CAM cDNA were established by the RACE-PCR technique (Frohman *et al.*, 1988) with oligonucleotide (dT)₁₈ and specific nested sense primers derived from the cDNA probe 1 as well as from cDNA probe 2. Amplification of the 5' stretches by RACE-PCR resulted in a ladder of PCR products that did not allow the identification of the full-length cDNA product. Similar problems observed with hydroxyproline-rich glycoproteins from *Volvox* (Ertl *et al.*, 1989, 1992) suggested that Algal-CAM may also include a proline-rich domain. Thus a genomic library of *V. carteri* constructed in the replacement vector λEMBL3 was

	10	20	30	40
MRMAIAAFMN	YLLACAGLLL	FLTPAWKSNV	LAFTYPLLIA	
50	60	70	80	
SPSSFTSPPL	PSTPSPPPPL	LPALASPPPP	FPNEDVDRPP	
90	100	110	120	
LVKDNTPTSP	ASSQPAIPPP	SPPSPSTPTP	PVSYSIIWDF	
130	140	150	160	
LVKNNSPFTI	SLALSTANEV	ATFNDSQEV	TFFLPTETA	
170	180	190	200	
DKLSDALGVA	RSNRAGLLPY	LPVIKRALSY	HVLPTRISLQ	
210	220	230	240	
SVANQSVGGT	EYYNTLTMG	QSSSIGVRVS	PPSSPPATSP	
250	260	270	280	
E IFILGVSST	AKVLQADVAA	GASCINVVDT	VLQYWNSVD	
290	300	310	320	
EVFASISGAS	TMYQALKTAO	LLKPA NV TSP	Y T I F V P T D E A	
330	340	350	360	
E VSAFGASAA	TTILANLRSY	ESLLRHHVAY	GWVVTDTTSE	
370	380	390	400	
EYVRTSYITL	NSNNVTVVVP	SNDKADAGVK	PTVASAAVPG	
410	420	430	440	
SPVFSILNTF	QVGIEPQVIV	QVINGVLNPA	SSRQTAGGAA	

alternative C-terminus:

GQPQCSSLA FSWGEVVPN R

Fig. 4. Amino acid sequence deduced from Algal-CAM cDNA clones. The arrow marks the potential signal peptidase cleavage site. Amino acid sequences confirmed from isolated peptides are underlined. Potential *N*-glycosylation sites are boxed. The arrowhead marks the position of an alternative splicing event responsible for two different C-terminal amino acid sequences. Characteristic extensin motifs [Ser-Pro₍₃₋₇₎] are italicized. Sequence motifs that are most conserved between fasciclin I-related proteins are highlighted.

screened to obtain the lacking upstream sequence data from a genomic clone. Two positive clones were identified out of 55 000 phages screened. Digestion of the 20 kb insert of one of these clones with *Hind*III resulted in an ~5.5 kb fragment that hybridized to probe 1. This fragment was subcloned and sequenced. Indeed, an open reading frame was identified coding for a proline-rich domain. Synthesizing specific oligonucleotides corresponding to the 5' end of this cytidine-rich exon allowed the characterization of the remaining 5' part of the cDNA by the RACE-PCR technique. The strategy applied to collect the complete cDNA sequence is summarized schematically in Figure 3. The deduced amino acid sequence for Algal-CAM is given in Figure 4.

Algal-CAM cDNA encodes the antigen responsible for the *in vivo* effect

To confirm that the cloned gene encodes the antigen, Algal-CAM partial sequences were expressed in *Escherichia coli* and the purified expression product used to generate polyclonal antibodies in rabbit. Polyclonal serum against amino acids 109–217 did not influence embryogenesis, i.e. it neither generated a hole in 4-cell embryos nor inhibited the inversion process. However, this antiserum was able to detect affinity-purified Algal-CAM in Western blot analysis. In a crude *Volvox* embryo lysate, the polyclonal antiserum specifically recognized the Algal-CAM band (not shown). An immunoprecipitation experiment with this polyclonal antibody and subsequent analysis of the immunoprecipitate by Western blotting and detection with the mAb resulted in the recognition of the 150 kDa protein (Figure 5, lane 2). Control experiments with preimmune serum did not produce a signal (Figure 5, lane 3).

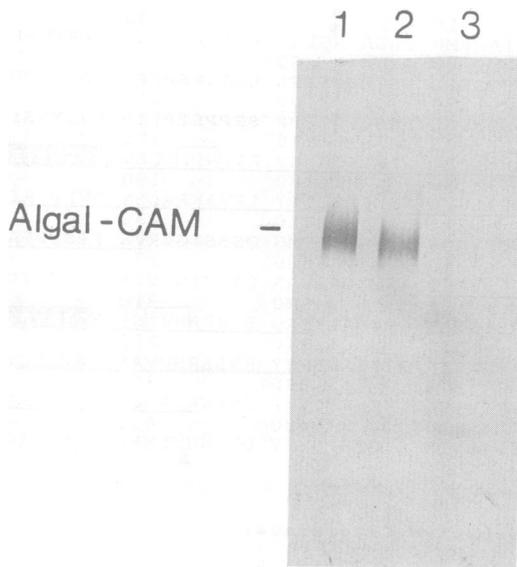


Fig. 5. Algal-CAM encodes the antigen responsible for the *in vivo* effect. Algal-CAM was immunoprecipitated from a NDC extract with a polyclonal antiserum against a heterologously expressed domain of Algal-CAM (amino acids 109–217). Subsequently, the immunoprecipitate was subjected to Western blot analysis and stained with the original mAb. SDS extract of embryos as a control (lane 1); immunoprecipitate with polyclonal antiserum against recombinant polypeptide (lane 2); immunoprecipitate obtained with the preimmune serum (lane 3).

Indirect immunofluorescence microscopy

The localization of Algal-CAM in *Volvox* embryos was studied by indirect immunofluorescence microscopy. Isolated embryos bound to poly-L-lysine-treated slides were fixed with paraformaldehyde and stained with anti-Algal-CAM mAb (purified by protein A column chromatography) and fluorescein-conjugated anti-mouse IgG. Indeed, the antibodies stained cell–cell contacts in the embryo as expected for the localization of a cell adhesion molecule (Figure 6A and C). Confocal laser scanning immunofluorescence microscopy verified the localization of Algal-CAM at the sites of cell–cell contacts (Figure 6B).

The amino acid sequence predicts a three-domain structure

All amino acid sequences of peptides collected from the tryptic digest could be found in the deduced Algal-CAM amino acid sequence (Figure 4) confirming the correct assignment of the open reading frame. Algal-CAM cDNA encodes a 440 amino acid polypeptide chain including an N-terminal amino acid sequence representing a putative signal peptide with a characteristic signal peptidase cleavage site Ala–Cys–Ala (Perlman and Halvorson, 1983; von Heijne, 1983, 1986). The calculated molecular mass of the polypeptide chain is 46.3 kDa. This value is much lower than the apparent molecular mass of the protein derived from SDS–PAGE (see also Discussion). A high degree of glycosylation of the polypeptide chain may be responsible for this difference. The amino acid sequence predicts six potential *N*-glycosylation sites, and at least two of them turned out to be glycosylated as shown by the peptide sequencing data (Table I, peptides 7 and 8). A large number of potential *O*-glycosylation sites are

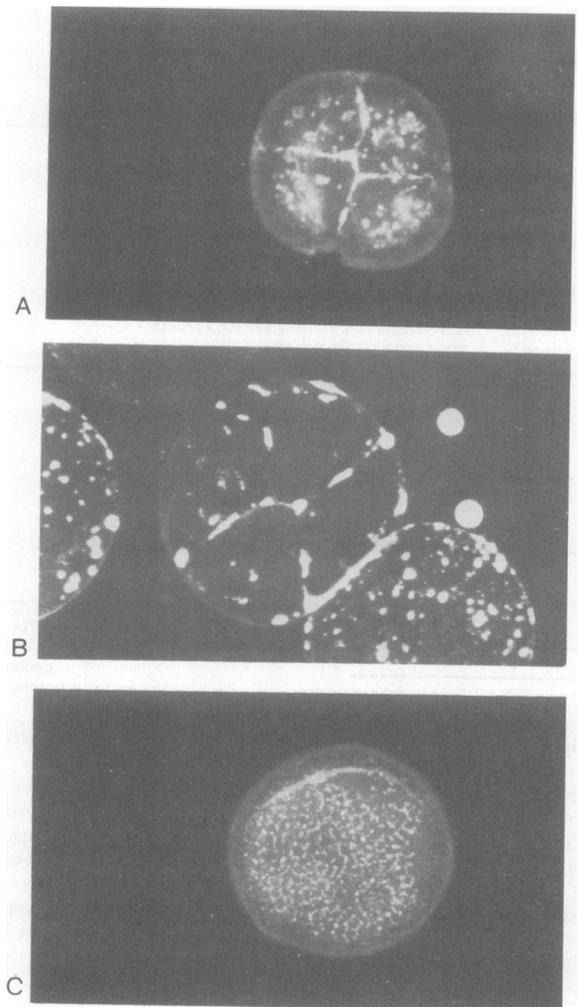


Fig. 6. Localization of Algal-CAM by indirect immunofluorescence microscopy. mAbs stain the cell–cell contacts of a 4-cell *Volvox* embryo (A: immunofluorescence micrograph; B: confocal laser scanning immunofluorescence micrograph). Immunofluorescence micrograph of a late (post-cleavage) embryo (C). Scale bar: 25 μ m.

located within the N-terminal domain (amino acids 17–116) of Algal-CAM. This domain exhibits repeats of the motif Ser–Pro_(3–7) characteristic of extensins, a family of rod-shaped, hydroxyproline-rich glycoproteins found in cell walls of higher plants (reviewed by Cassab and Varner, 1988) and more recently in algae (Adair *et al.*, 1987; Ertl *et al.*, 1989, 1992). Thus, most prolines of this domain are assumed to be modified to hydroxyproline. Since not a single tryptic peptide could be found originating from this extensin-like domain, glycosylation of all or most of these hydroxy amino acids is very likely. Extensins are known to be covalently crosslinked. Therefore, it is also possible that mature Algal-CAM exists as a covalently crosslinked dimer or trimer. The C-terminal stretch of the protein exhibits no particular prevalence in the amino acid composition.

A BLASTP search (Altschul *et al.*, 1990) of the Swiss-Prot Protein Sequence Database with the C-terminal amino acid sequence revealed that this part of the protein includes regions with significant homology to fasciclin I (Zinn *et al.*, 1988). Fasciclin I was discovered as a peripheral membrane protein involved in the development of the neuronal network in *Drosophila*. *Drosophila* fasciclin I

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AlCAM 1  NSF-PTIS-LA[STANEVATFNDS[SQEVTEFLPTEAF]DK-20-V[IKR]AD[SYHVL]P[TRIS]- 25 -
AlCAM 2  ISGASTM[MQ--A]L[KTAQ]L[KPAN]VTSPYTFVPT[DEAFV[S]- 5-AA[TTI]L[ANLRSY]ESLL- 12 -
Fasc 1    DSELSQFYS--L[ESN]QI[ANSTL]SLR[SCTIFVPTNEAF]QR-10-H[ITTE]AYTQ[RLP]NTV- 2 -
Fasc 3    NGALLR[KFEY]EVIMDNGGAVL[DDI]NSL[TEVTI]L[CA]S[NEAF]NS-20-I[IKR]D[RI]NVD[IKR]QKNA- 5 -

AlCAM 1  S[IG]M[RV]S[E]P[S]-PPAT[S]E---[F]I[L]G[V]S[S]T[A]K[M]L[Q]A[D]V[A]A[G]A[S]CIN[M]V[D]T[V]L[Q]Y[W]N[S]M[D]E[V]F[A]S
AlCAM 2  TTSE[Y]V[RT]S[M]-I[T]L[NS]N[V]-T[V]V[M]P[S]-NDKADAG[V]K[P]T[V]A[S]A[AP]G[S]P[M]F[S]I[L]N[T]F[Q]V[G]I[E]P[Q]M[I]V[Q]
Fasc 1    S[SD]MAGN[E]P[PLY]-I[T]K[NS]N[G]-D[IF]M[N]NARIIP[SL]S[M]E[T]NSD[G]K[R]OIM[H]I[D]E[V]L[E]P[L]T[V]KAGH[S]D[T]P
Fasc 3    VPT[V]N[N]N[I]F[L]Y[F]-NVRGE[G]S[D]T[V]I[T]V[E]G[G]V[N]A[T]M[I]Q[A]D[V]A[Q]TNGYV[H]I[D]H[V]L[G]V[P]M[T]T[V]L[G]K[L]E[S]

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Fig. 7. Comparison of the two fasciclin homologous domains of Algal-CAM and the *Drosophila* fasciclin I domains 1 and 3. Alignments of the two *Drosophila* fasciclin I domains (Fasc 1 and Fasc 3) are taken from Zinn *et al.* (1988) and the two Algal-CAM domains (AlCAM 1 and AlCAM 2) are adopted to this alignment. Numbers give amino acids that are not shown because they are not homologous between the domains. To maximize homology, gaps are introduced and shown as dashes. Residues of Algal-CAM domains identical to residues at the same position in one of the fasciclin I domains are boxed.

polypeptide contains four homologous repeats, each consisting of ~150 amino acids in length. Sequence alignments added to that given by Zinn *et al.* (1988) indicate that the Algal-CAM amino acid sequence also includes two fasciclin I homologous domains (Figure 7). Comparison of these repeats reveals interdomain identities similar to those reported for *Drosophila* fasciclin I domains (Zinn *et al.*, 1988). The Algal-CAM 1 and Fasc 1 domains share two stretches with a high degree of conservation: one stretch shares nine out of 10 amino acids [TIFVPT-(D/N)EAF] and the other seven out of eight amino acids [YIT(L/K)NSN]. The TIFVPT(D/N)EAF motif can also be detected in the Fasc 2 and 4 domains (see Discussion). A third conserved amino acid stretch with seven amino acids identical out of nine [A(K/T)V(IL)QADAVA] is found in the Algal-CAM 1 and Fasc 3 domains. Algal-CAM 1 is most homologous to Fasc 3 (21.2% identity) and Algal-CAM 2 to Fasc 1 (20.3% identity).

Hydropathy analysis (Kyte and Doolittle, 1982) of Algal-CAM does not reveal a membrane-spanning domain. These data are consistent with the extraction behavior of Algal-CAM described above. However, analysis of the alternatively spliced mRNA variants (see below) indicates the existence of an Algal-CAM variant with a predominantly hydrophobic sequence at its C-terminus, similar to the amino acid sequences that signal the addition of glycosylphosphatidylinositol (GPI) anchors (Englund, 1993). Therefore, this type of post-translational modification might be present in this particular Algal-CAM variant.

Alternative splicing and differential processing of Algal-CAM transcripts

As mentioned above, the RACE-PCR technique was used to establish the 3' end of the mRNA. Unexpectedly, this approach resulted in the amplification of different PCR products. Cloning and sequencing of all of these products proved the existence of transcripts with different 3' ends. To ensure that these transcripts are derived from one and the same gene, we sequenced the complete genomic DNA of Algal-CAM. Indeed, all detected variants turned out to be transcripts of the same Algal-CAM gene, created by differential RNA processing and alternative splicing. Figure 8 schematically represents the detected variants of Algal-CAM mRNA. Variant 1 is encoded by the first three exons of the Algal-CAM gene and ends at amino acid position 217. The stop codon present in this variant at the 3' end of exon III is removed by alternative splicing in all the other variants. Variant 2 is encoded by

exons I–VIII. In variant 3, exon VIII is removed and replaced by exon IX. Exon IX encodes a new C-terminus of the polypeptide that might represent a signal for attachment of a GPI anchor, as mentioned above.

As indicated in Figure 8, five different polyadenylation sites could be detected. Variant 1 uses polyadenylation site 1. Variant 2 mRNA uses either polyadenylation signals 2A or 2B. Again two different polyadenylation signals (3A and 3B) were found to be in use for variant 3 mRNA.

Developmentally controlled production of mRNA variants

RT-PCR was used to address the question of whether the observed mRNA variants are produced under developmental control. As the growth of *Volvox* spheroids can be highly synchronized, it is possible to prepare stage-specific cDNA libraries. RNA was extracted from synchronously growing *Volvox* embryos (with >80 % of the population in the same cleavage stage) collected at a defined stage of embryogenesis, hybridized to oligo (dT)₂₅ coupled to magnetic beads and reverse transcribed. Corresponding cDNA libraries were prepared from 4-, 16-, 64- and 128/256-cell embryos, as well as embryos at the end of cleavage divisions. Variant-specific oligonucleotide primers were used to detect the presence of a particular variant at a given developmental stage. The results obtained for the mRNA variants 1, 2 and 3 are given in Figure 9. mRNA encoding variant 1 was detected at a fairly constant level throughout all stages of embryogenesis (Figure 9A). In contrast, the level of mRNA encoding variant 2 continuously increased until the final stages of embryogenesis (Figure 9B). The production of mRNA encoding variant 3 was found to be under striking developmental control: this particular message was found to be absent at all early cleavage stages up to the 16/32-cell stage. At or immediately after the differentiating cell cleavage (32- to 64-cell stage), production of that splicing variant was initiated. This variant remained detectable at all late stages of embryogenesis (Figure 9C).

Discussion

Embryogenesis of the multicellular algae *Volvox* includes a fascinating process of pattern formation. In the 32-cell embryo only half of the embryonic cells at defined positions undergo an asymmetric cleavage to produce new reproductive initials. A model to explain this cell counting mechanism is based on known properties of cell adhesion

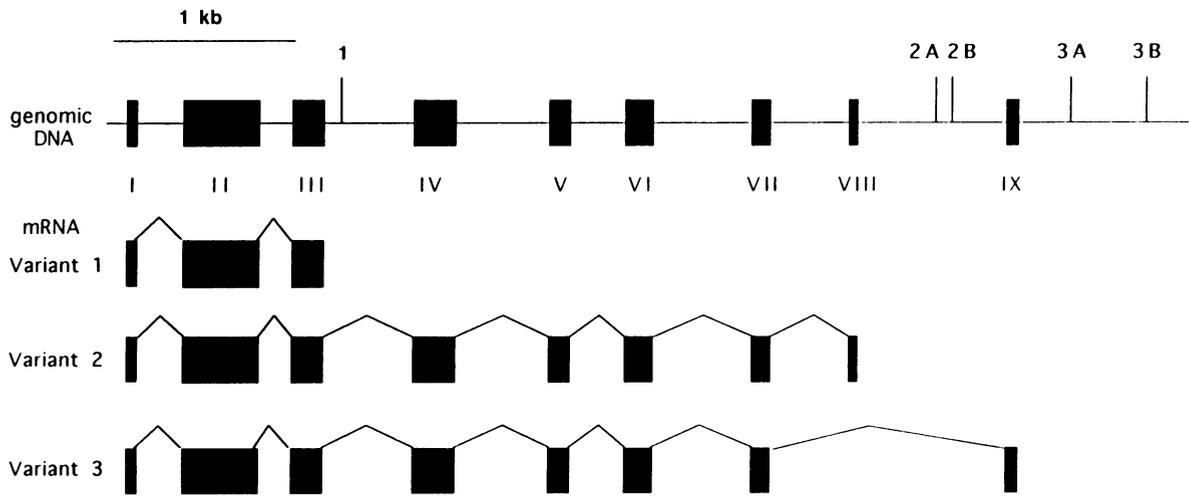


Fig. 8. Analysis of alternative splicing variants of Algal-CAM mRNAs. A map of 5 kb genomic DNA coding for the Algal-CAM gene consisting of nine exons (I–IX) and the composition of alternative splicing variants of Algal-CAM mRNAs. Three different mRNA variants and their downstream untranslated stretches are shown. Variant 1 is transcribed from the first three exons. Variant 2 is built from exons I–VIII, with the different lengths of the 3' untranslated regions created by using different polyadenylation signals (2A and 2B). In variant 3 the C-terminus of the corresponding polypeptide is encoded by exon IX instead of exon VIII. Again, two different polyadenylation signals (3A and 3B) are in use.

molecules (Sumper, 1979). However, up to now proof that plant cells possess homologs of animal adhesion proteins has been lacking. Furthermore, it was concluded from electron microscopic investigations that embryonic cells of the green algae *V. carteri* are exclusively linked by cytoplasmic bridges (Green and Kirk, 1981; Kirk and Harper, 1986) and do not possess cell–cell contacts mediated by cell adhesion molecules. Considering the known importance of cell adhesion molecules in developmental processes like differentiation and morphogenetic movements, we reinvestigated the nature of cell–cell contacts of *Volvox* embryos using a biochemical approach. A mAb raised against a crude membrane fraction from *Volvox* embryos was found to be able to disrupt cell–cell contacts of the 4-cell embryo. Confocal laser scanning immunofluorescence microscopy localizes the antigen in cell–cell contact sites, as expected for a potential cell adhesion molecule. Therefore, we denoted this antigen Algal-CAM.

Analysis of the cDNA of this Algal-CAM revealed an open reading frame encoding a polypeptide with a molecular mass of 46.3 kDa. A typical signal peptide indicates that Algal-CAM is transported to the cell surface. The mature protein exhibits a three-domain structure, with an N-terminally located extensin-like domain and two domains with homology to fasciclin I, a cell adhesion molecule found originally in the nervous system of *Drosophila*. Extensins were first described as proteins that contribute to the structural integrity of the higher plant cell walls. Repeating units of Ser–Pro_(3–7) responsible for a rod-like structure are a diagnostic motif for extensins. Most of these prolines are hydroxylated and *O*-glycosylated (reviewed in Cassab and Varner, 1988). Extensins are secreted to the cell wall and rapidly insolubilized by the formation of covalent crosslinks between tyrosine residues (Fry, 1982). Furthermore, extensin synthesis is also induced by fungal infection and wounding (Showalter *et al.*, 1985). Detection of organ-specific extensin-like mRNAs in tobacco flower development suggests that extensin-like proteins may also have specific functions

during developmental processes (Chen *et al.*, 1992; Goldman *et al.*, 1992). A zygote-specific cell wall glycoprotein in *Chlamydomonas* (Woessner and Goodenough, 1989) and an inversion-specific glycoprotein in *V. carteri* that is expressed for only a few minutes (Ertl *et al.*, 1992) were detected recently as the first algal extensin-like proteins. Denaturation of the Algal-CAM protein in 6 M guanidine hydrochloride and acylation of the polypeptide with succinic anhydride to prevent reassociation of subunits revealed no change in the apparent molecular mass on Western blots (not shown), indicating that covalent crosslinks and/or glycosylation may be responsible for the much higher apparent molecular mass determined by SDS–PAGE. In addition, from experience with another extensin-like protein from *V. carteri* (Ertl *et al.*, 1992), we know that extensin domains strongly increase the apparent molecular mass exhibited on SDS–polyacrylamide gels.

Computer analysis of Algal-CAM revealed homology of the C-terminal portion to *Drosophila* fasciclin I. The fasciclin I protein in *Drosophila* is a homophilic cell adhesion molecule with a molecular mass of 70 kDa (Elkins *et al.*, 1990a). Fasciclin I is expressed on the surface of a subset of fasciculating axons and seems to be involved in growth cone extension and/or guidance (Zinn *et al.*, 1988). Analysis of the amino acid sequence showed that fasciclin I is composed of four homologous repeats of ~150 amino acids each (Zinn *et al.*, 1988). Algal-CAM contains two repeats with homology to fasciclin I domains. The fasciclin I-related domains are 158 and 133 amino acids in length. In *Drosophila*, the four aligned repeats share 7–15% identity in pairwise comparison. The aligned Algal-CAM repeats exhibit ~15% identity in pairwise comparison and 7–21% identity in comparison with the four *Drosophila* fasciclin I repeats.

In *Drosophila* embryos, fasciclin I is found in soluble as well as insoluble membrane-bound forms. Attachment to the cell surface is achieved by GPI linkage (Hortsch and Goodman, 1990). GPI-anchored proteins are synthesized as precursors with a C-terminal extension that is cleaved off for attachment of the lipid modification

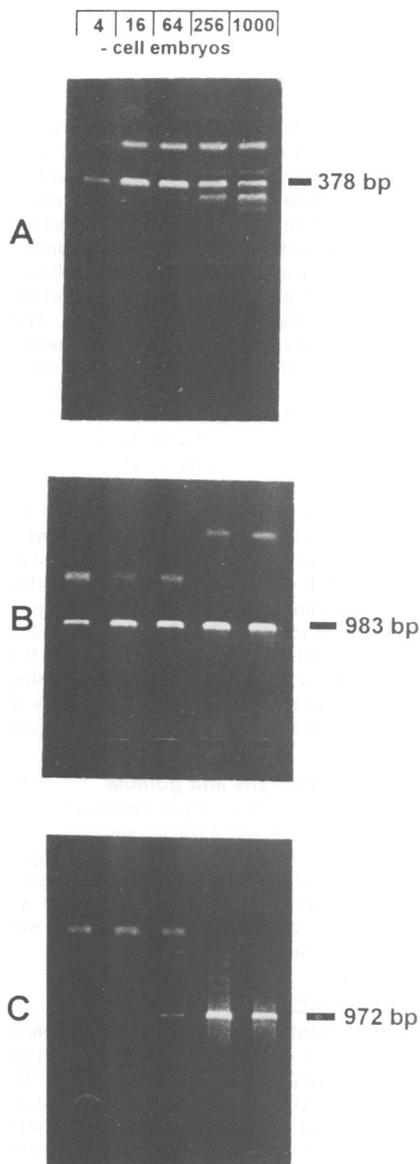


Fig. 9. Expression of Algal-CAM mRNA variants is developmentally regulated. At defined stages during *Volvox* embryogenesis (shown on top) the presence of different mRNA variants was analyzed by RT-PCR using variant-specific oligonucleotide primer combinations. (A) 378 bp fragment derived from variant 1 mRNA; (B) 983 bp fragment derived from variant 2; (C) 972 bp fragment derived from variant 3.

(reviewed by Englund, 1993). During *Drosophila* development, the ratio between membrane-bound and soluble fasciclin I protein varies considerably and therefore it was assumed that the organism uses developmentally regulated cleavage of its GPI linkage as a way of regulating cell adhesion (Hortsch and Goodman, 1990). Biochemically, Algal-CAM behaves like a membrane-associated protein because ~80% of the protein is released from isolated embryos after treatment with 0.1 M Na₂CO₃. This treatment only removes peripheral membrane proteins, indicating that Algal-CAM must be attached to the cell surface via protein-protein or protein-carbohydrate interactions. During phase separation experiments with Triton X-114, a detectable amount of Algal-CAM remained in the detergent phase (not shown). Therefore it is also possible that membrane-anchored variants of Algal-CAM exist.

Two variants of Algal-CAM created by alternative splicing events differ in their C-termini. One of these variants (using exon IX) contains a potential GPI-anchor addition signal (Englund, 1993). Each of the two different C-termini is encoded by a separate exon, and RT-PCR analysis revealed that these two variants are expressed under developmental control. The potential GPI-anchored variant of Algal-CAM is absent in early embryos and becomes detectable at or immediately after the differentiating cleavage (i.e. the transition from the 32- to the 64-cell embryo), suggesting an important function of Algal-CAM variants during *Volvox* embryogenesis. A GPI-linked variant and further isoforms are also described for N-CAM, a member of the immunoglobulin superfamily mediating homo- and heterophilic cell-cell interactions. N-CAM is specified by a single gene and regulated expression during development at different times and places by alternative splicing events is reported (reviewed in Edelman, 1988).

Alternative splicing of micro-exons has been detected in *Drosophila*, creating multiple forms of fasciclin I (McAllister *et al.*, 1992). Besides the three mRNA variants of Algal-CAM described in this paper, additional splicing variants are likely to exist according to preliminary observations. In particular, two more mRNA variants were found using a transcriptional start site located within intron III thereby encoding a new signal peptide. Further experiments are required to confirm the existence of the corresponding polypeptide variants that would lack the extensin-like domain.

Up to now little is known about intracellular signalling pathways in *V. carteri*. Fasciclin I seems to interact with a signal transduction pathway involving the Abelson tyrosine kinase (Elkins *et al.*, 1990b). With the help of the recently developed transformation (Schiedlmeier *et al.*, 1994) and reporter gene system (Hallmann and Sumper, 1994) for *V. carteri*, a detailed genetic analysis of the functional role of Algal-CAM during *Volvox* embryogenesis is now possible. Compared with neuronal networks, *Volvox* embryogenesis offers a much simpler developmental process that should allow the elucidation of the biochemical function of this type of cell adhesion molecule.

Recently it was reported that transforming growth factor (TGF)- β induces transcription of a fasciclin I homologous protein (β IG-H3) in human lung adenocarcinoma cells (Skonier *et al.*, 1992). Osteoblast-specific factor 2 (OSF-2) cDNA coding for another fasciclin I-related protein was cloned from mouse and human and speculated to act as a homophilic adhesion molecule in bone formation (Takeshita *et al.*, 1993). The functions of these proteins remain to be elucidated. Considering the extremely wide range of distribution among multicellular organisms that is now established for fasciclin-like proteins, an important function for these proteins in developmental processes must be anticipated. One highly conserved amino acid motif is common to all fasciclin-related polypeptides published to date. In comparison (Table II), the following consensus sequence can be deduced: TX(F/L)(A/V)P-(T/S)(N/D)EA(F/W). Therefore, we propose that fasciclins are a new family of cell adhesion molecules comparable with cell adhesion molecules of the immunoglobulin, cadherin, integrin and selectin family. Algal-CAM is likely

Table II. Comparison of the conserved repeat sequences in fasciclin-like proteins

Protein	Sequence
<i>Drosophila</i>	
D1	TIFVPTNEAF
D2	TFLVPVDEGF
D3	TILAPSNEAW
D4	TYFVPRDKGW
Grasshopper	
D1	TVFAPTNLAF
D2	TFFIPVDEGF
D3	TLFAPSNAAW
D4	TYFVPRDLAW
βIG-H3	
D1	TIFAPSNEAW
D2	TLLAPTNEAF
D3	TLLAPLNSVF
D4	TVFAPTNEFR
mOSF-2	
D1	TYFAPSNEAW
D2	TLFAPTNEAF
D3	TLLAPVNNAF
D4	TLFAPTNDAF
hOSF-2	
D1	TYFAPSNEAF
D2	TLFAPTNEAF
D3	TLLAPVNNAF
D4	TLFVPTNDAF
Algal CAM	
D1	TFFLPTETAF
D2	TIFVPTDEAF

Consensus motif: (T)(-)(F/L)(A/V)(P)(T/S)(N/D)(E)(A)(F/W).

to represent an archetype of the fasciclin I family of cell adhesion molecules. Furthermore, it represents the first plant homolog to animal cell adhesion molecules, but it contains in addition an extensin-like domain, a structural element unique to plants.

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 (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. A fernbach flask containing 800 ml *Volvox* medium was inoculated with five spheroids of the female strain HK10 (at the stage of embryogenesis). After 7 days, this flask was used as inoculum for a 20 l glass flask. Growth was continued for a further 3 days. Spheroids were harvested at the stage of embryogenesis. Continuous growth conditions could be maintained in 20 l glass fermenters (Bio-engineering, type L1523). Fermenters with ~500 000 spheroids were harvested by removing 90% of the culture medium, refilled with sterile *Volvox* medium and harvested again after 2 days. Usually, this procedure could be repeated four times.

Preparation of immunogen

Isolated embryos were disintegrated by ultrasonic treatment and the lysate was centrifuged at 135 000 g for 2 h. The green pellet was dissolved in 10 mM potassium phosphate buffer pH 6.8 and used for the preparation of an acetone powder (Morton, 1955). The acetone powder was resuspended in 150 µl PBS, 0.1% (w/v) SDS, mixed with the same volume of the appropriate adjuvant and used for a single immunization.

Monoclonal antibody production

The acetone powder preparation was injected intraperitoneally into a mouse together with Freund's complete adjuvant. After 6 weeks, an intraperitoneal boost with Freund's incomplete adjuvant was given. Fusion was performed with X63-Ag8.653 myeloma cells 4 days later. Hybridoma supernatants that interfere with cell-cell contacts during embryogenesis of *V. carteri* were detected in an *in vivo* assay. About 100 isolated gonidia in 0.5 ml *Volvox* medium were mixed 2 h before the onset of embryogenesis with concentrated hybridoma culture supernatants prepared as follows: 0.7 ml aliquots of hybridoma culture supernatants were precipitated by the addition of 0.7 ml saturated ammonium sulfate, followed by centrifugation and resuspension of the pellet in 50 µl 0.5 g/l glycylglycine, 0.7% NaCl, pH 8.0. After dialysis against the same buffer, this solution was added quantitatively to the gonidia. After the start of embryogenesis all samples were checked for disruption of cell-cell contacts. Positive hybridomas were cloned as described (Wenzl and Sumper, 1986). Antibody classes were determined with a mouse hybridoma subtyping kit (Boehringer Mannheim). Only IgM-producing clones could be detected in the first fusion. A second fusion resulted in five IgG producing clones. Two of them (25-72-7 and 25-92-4) were used for further experiments.

Large-scale production of mAbs was performed in a roller incubator (Vismara). Antibodies were precipitated from hybridoma culture supernatant with ammonium sulfate (50% saturation), dialyzed against 0.14 M phosphate buffer, pH 8.0 (Ochiai *et al.*, 1982), and purified by protein A-Sepharose column chromatography (Pharmacia, Uppsala). Antibody was eluted with 26 mM triethylamine, 0.15 M NaCl. Eluted antibodies were dialyzed against 0.14 M phosphate buffer pH 8.0 and stored at 4°C after the addition of 0.2% (w/v) azide. For coupling to CNBr-Sepharose 4B (Pharmacia, Uppsala), eluted antibodies were dialyzed against coupling buffer following the manufacturer's instructions.

Isolation of *Volvox* embryos and gonidia

Spheroids were concentrated on a 100 µm mesh nylon cloth and washed with water. After dissociation with a dounce homogenizer (loose fitting), embryos were washed through a 100 µm mesh nylon cloth and concentrated by low speed centrifugation (30 s, 1500 r.p.m., 20°C) using a swing-out rotor. The pellet of embryos was washed with water and recentrifuged to remove single somatic cells. Gonidia were isolated as described in Wenzl and Sumper (1986).

Purification of Algal-CAM

Embryos were suspended in 0.1 M NaCl, 0.1 M phosphate buffer, pH 8.0, and 0.2% (w/v) deoxycholate. After 2 h stirring at 4°C, the suspension was centrifuged for 2 h at 135 000 g and 4°C. The supernatant (extract) was loaded (overnight, circulating, 12 ml/h) on an antibody affinity column (coupled to BrCN-activated Sepharose). The affinity column was washed with at least 5 vol of extraction buffer and eluted with 0.15 M NaCl, 26 mM triethylamine, 0.2% (w/v) deoxycholate. Fractions containing antigen were concentrated on a Centricon 30 microconcentrator (Amicon) and analyzed on an 8% SDS-polyacrylamide gel. Typically the yield was 5–10 µg antigen from 1000 l *Volvox* culture.

Proteolytic digestion and separation of peptides

The eluate from the affinity column containing ~10 µg antigen was loaded on an 8% SDS-polyacrylamide gel. After blotting to PVDF membrane (Millipore) by the semi-dry technique (Towbin *et al.*, 1979), the membrane was soaked in water and stained in 40% (v/v) methanol, 5% (v/v) acetic acid, 0.025% (w/v) Coomassie R250 for 3 min and destained for 5 min in 30% (v/v) methanol, 5% (v/v) acetic acid. The antigen band was fully destained with 90% (v/v) ethanol, cut into small pieces, saturated with 0.2% (w/v) polyvinylpyrrolidone 40 in 50% (v/v) methanol twice for 10 min. Afterwards membrane pieces were washed twice in water and in 0.1 M Tris-HCl, pH 8.0. For digestion, membrane pieces were resuspended in 150 µl 0.1 M Tris-HCl pH 8.0, 10 mM CaCl₂, and 1 µg TPCK-trypsin was added. After digestion overnight at 37°C, the resulting peptides were recovered from the membrane pieces with 100 µl 80% (v/v) formic acid and 2 × 200 µl water. The combined fractions were dried in a vacuum concentrator. The peptides were redissolved in 150 µl 6 M guanidine hydrochloride and subjected to a C18 reversed phase chromatography on a VYDAC 218 TP, 5 µm column. Peptides were eluted by a linear gradient from 5 to 40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min. Peptides were sequenced using an automated gas-phase peptide sequencer (Applied Biosystems Inc., Foster City, CA) as described by Lottspeich (1985).

Generation of cDNA probes by PCR

The amino acid sequence of the peptide -NSFPTISLALSTANEVAT-XXDSSQEVV was used to synthesize the antisense oligonucleotide primer GTNACYTC (corresponding to amino acid sequence EVT). This primer was used to reverse transcribe mRNA isolated from a mixture of 4- to 128-cell embryos. The antisense primer GTNACYTCYGRCT derived from the amino acid sequence SQEVV and the sense primer AAYAGYTTYCCNAC corresponding to amino acid sequence NSFPT of the same peptide were used to amplify the resulting cDNA by PCR. A total of 35 cycles of amplification generated a cDNA fragment of 80 bp in length (probe 1) that was cloned into the *Sma*I site of pUC18 by blunt-end ligation.

RNA and poly(A)⁺ RNA were extracted from *Volvox* embryos 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 dithiothreitol, 20 U RNAGuard (Pharmacia), 400 pmol antisense oligonucleotide (GTNACYTC), 1 mM each dNTP, 1 µg poly(A)⁺ RNA and 200 U of MoMuLV reverse transcriptase. Incubation was at 42°C for 45 min. After the addition of 80 µl of 1× PCR buffer containing 400 pmol sense and 400 pmol antisense oligonucleotide and 2.5 U *Taq* polymerase, thermal cycling (Perkin-Elmer cyclor 480) was initiated (35 cycles: denaturation at 94°C for 45 s, annealing at 45°C for 30 s and extension at 72°C for 5 s).

A second cDNA probe was generated by PCR on a solid-phase cDNA library coupled to Dynabeads Oligo (dT)₂₅. From the amino acid sequence TSPEIFI (peptide 5) the sense oligonucleotide ACKT-CKCKGAGATHHTTYAT was derived; from the sequence AAVPG (peptide 9) the antisense oligonucleotide CCKGGKACKGCKGC was derived. A PCR utilizing these oligonucleotides was performed for 35 cycles. A second round of PCR (30 cycles) was initiated with the nested antisense primer GCKACKGKGGYTT corresponding to the amino acid sequence KPTVA (peptide 9). The amplified DNA fragment was cloned and sequenced. It revealed an open reading frame of 469 bp (probe 2).

Cloning of the Algal-CAM gene

The sequences of the remaining 5' and 3' stretches were established by the RACE-PCR technique (Frohman *et al.*, 1988). The *V. carteri* genomic library in λEMBL3 (Frischauf *et al.*, 1983) described by Ertl *et al.* (1989) was used to clone the Algal-CAM 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).

Preparation of recombinant Algal-CAM polypeptide

A cDNA fragment encoding amino acid positions 109–217 was generated by PCR and subsequently cloned into pET11a (Novagen) 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 washed in 2% (w/v) Triton X-100, 0.1 M Tris-HCl, pH 6.8, 1 mM EDTA (2×), resuspended in 0.1 M Tris-HCl, pH 6.8, 1 mM EDTA, applied to a 40% (w/v) sucrose cushion and centrifuged for 10 min at 12 000 g and 4°C. The resulting pellet was washed repeatedly in 4 M urea and the resulting supernatant containing the expression product was dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. During dialysis the expression product precipitated and was concentrated by centrifugation for 10 min at 12 000 g and 4°C. The pellet was solubilized in SDS sample buffer and loaded on preparative 12% SDS-polyacrylamide gels. Only one major band was detected; this material was eluted, concentrated in a microconcentrator (Centricon 10, Amicon) and used to raise polyclonal antibodies in rabbit.

Detection of variant-specific mRNA by RT-PCR

Synchronously growing *Volvox* spheroids (800 ml culture with ~15 spheroids/ml) containing embryos at the desired stage of development were collected on a nylon screen and disrupted by forcing them through a 0.5 mm hypodermic needle. Somatic cell sheets were removed by filtration through a 100 µm mesh nylon cloth. Embryos were collected and washed repeatedly on a 10 µm mesh nylon cloth. Any remaining somatic cells were completely removed by this procedure. The embryos were concentrated to a volume of 30 µl by low speed centrifugation and immediately lysed by the addition of 300 µl lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EGTA and 2% SDS). After 10 min at 30°C, the extracted embryos were removed by low speed centrifugation (1 min at 3000 r.p.m.) and the supernatant mixed with magnetic beads coupled with oligo (dT)₂₅ suspended in 150 µl buffer (20 mM Tris-HCl, pH 7.5, 1 M LiCl, 1 mM EDTA). Subsequently, the

cDNA library covalently linked to magnetic beads was constructed according to the instructions of the manufacturer of the magnetic beads (Deutsche Dynal, Hamburg, Germany). PCR amplification of variant-specific cDNA was performed in a total volume of 100 µl containing the following reagents: 4 µl suspension of the stage-specific cDNA library covalently linked to magnetic beads, 20 µl 5× PCR buffer, 50 pmol of each sense and antisense primer, 100 µM dNTP and 2.5 U *Taq* polymerase. A total of 35 cycles of PCR amplification (Perkin-Elmer cyclor 9600) were performed (denaturation at 94°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 20 s). The following oligonucleotides allowed selective amplification of a specific variant cDNA: for variant 1, CCCCTCCTGTCAGCTACTCTTCCA (sense) and CCGCGTCCCATCACAAACCCA (antisense); for variant 2, sense oligonucleotide as above and GGCTGTCTGCCGACTGCT (antisense); for variant 3, sense oligonucleotide as above and GAAGGC-TGCATTGAGGTTG (antisense).

Fluorescence microscopy

Fluorescence microscopy with *V. carteri* embryos was as described previously (Wenzl and Sumper, 1986). For confocal laser scanning immunofluorescence microscopy, isolated embryos were bound to poly-L-lysine-treated embryos and fixed in 2% (w/v) paraformaldehyde for 15 min at 20°C. After washing with PBS and blocking with 5% (v/v) horse serum, embryos were labeled with 5 µg/ml mAb in PBS for 60 min. After washing, FITC-conjugated anti-mouse IgG was applied for 1 h. Labeled embryos were washed and viewed using a Zeiss LSM10.

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