A novel C-type lectin regulating cell growth, cell adhesion and cell differentiation of the multipotent epithelium in budding tunicates

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SUMMARY

We have isolated two Ca^{2+}-dependent, galactose-binding polypeptides from the budding tunicate, *Polyandrocarpa misakiensis*. Based on their partial amino acid sequences, full-length cDNAs were cloned. One of them was identical with a tunicate C-type lectin (TC14-2) reported previously. The other was a novel C-type lectin, referred to as TC14-3. In living animals, they appeared to be coupled. This complex of lectins, when applied in vitro to tunicate multipotent cells of epithelial origin, blocked cell proliferation and induced cell aggregation. The aggregates expressed a homolog of the integrin α-chain and other differentiation markers specific for epithelial cells. Recombinant TC14-3 could reproduce all the activities of native lectins by itself, which was accelerated by recombinant TC14-2. The inhibitory activity of TC14-3 on cell growth was completely abolished by the addition of 50 μM D-galactose. Anti-TC14-3 monoclonal antibody showed that the antigen was expressed constitutively by the multipotent epithelial and mesenchymal cells. These results provide evidence that in *P. misakiensis* a C-type lectin plays a novel, cytostatic role in regulating cell growth, cell adhesion and cell differentiation during asexual reproduction.

Key words: Lectin, α-Integrin, Alkaline phosphatase, Recombinant protein, Cell growth, Multipotency, Budding, Tunicate, *Polyandrocarpa misakiensis*

INTRODUCTION

C-type lectins are calcium-dependent carbohydrate-binding proteins (Drickamer, 1988). They have a common sequence motif of 115-130 amino acid residues, referred to as the carbohydrate recognition domain (CRD). The CRD has four cysteines that are perfectly conserved and involved in two disulfide bonds. It has been found in various kinds of proteins such as hepatic asialoglycoprotein receptor (Drickamer et al., 1984), lymphocyte IgE receptor (Ikuta et al., 1987), mannnose-binding protein (Drickamer et al., 1986), selectin (Lasky et al., 1989) and proteoglycan core protein (Krusius et al., 1987). Their functions include complement activation (Ikeda et al., 1987), endocytosis (Taylor et al., 1990), cell recognition (Weis et al., 1998), defense mechanism (Takahashi et al., 1985), and morphogenesis (Kawamura et al., 1991).

A tunicate C-type lectin of 14 kDa (TC14) has been isolated from the budding animal, *Polyandrocarpa misakiensis* (Suzuki et al., 1990). It consists of a single CRD that binds to D-galactose (Suzuki et al., 1990) and D-fucose (Poget et al., 1999). Mature proteins are secreted into the hemolymph during budding to induce the aggregation of undifferentiated mesenchymal cells, giving rise to the epithelial-forming tissue (Kawamura et al., 1991). A cDNA encoding another C-type lectin has recently been isolated from *P. misakiensis* (Shimada et al., 1995). The deduced amino acid sequence was similar to that of TC14, referred to as TC14-2 with reference to the original one now renamed TC14-1.

In *P. misakiensis*, the atrial (peribranchial) epithelium that underlies the epidermis is the major morphogenetic tissue during blastogenesis (for review see Kawamura and Nakauchi, 1991; Kawamura and Sugino, 1999). The atrial epithelium is multipotent, but not undifferentiated. Cells have pigment granules in the cytoplasm and express several differentiation markers on the cell surface (Fujiwara and Kawamura, 1992; Kawamura and Fujiwara, 1994). They are quiescent mitotically, having a doubling time of more than 170 hours (Kawamura and Nakauchi, 1991). At the onset of blastogenesis, however, a limited number of cells dedifferentiate, initiate rapid cell cycling and transdiffentiate into organ placodes of the next blastogenic generation (Kawamura and Fujiwara, 1994).

Recently, we have described several factors that regulate cell growth and differentiation during budding of *P. misakiensis*. Retinoic acid can induce the secondary bud axis (Hara et al., 1992; Kawamura et al., 1993). Retinoic acid-inducible serine proteases promote cell division and dedifferentiation of the atrial epithelium (Ohashi et al., 1999; K. K., unpublished). If the multipotent cells are continuously exposed to these factors, they may enter the terminal differentiation pathway, resulting in the cessation of asexual reproduction owing to the exhaustion of multipotent cells. We assume that some factor(s)

might counteract these morphogenetic regulators. The hypothetical cytostatic factor is expected to block cell growth and dedifferentiation of the atrial epithelium.

The aim of this study is to find such a factor in *P. misakiensis*. First, native polypeptides were isolated from the living animals. Their effect on multipotent cells was examined in culture. Secondly, the full length cDNA was cloned and the nucleotide sequence was determined, based on partial amino acid sequence data. We prepared recombinant proteins and examined their biological and biochemical characteristics. Lastly, the spatiotemporal expression of proteins was examined, using monoclonal antibodies. The results are discussed in relation to the significance of tunicate C-type lectins in regulating cell growth, cell adhesion and cell differentiation in the process of asexual reproduction.

MATERIALS AND METHODS

Animals

Asexual individuals of *P. misakiensis* were reared in culture boxes settled in Uranouchi Inlet near the Usa Marine Biological Institute, Kochi University.

Protein purification

Proteins were isolated from animals, as described elsewhere (Suzuki et al., 1990). In brief, colonies of *P. misakiensis* were homogenized in 7.5 mM phosphate buffer (pH 7.0) and centrifuged at 15,000 *g* for 20 minutes The supernatant was dialyzed against 20 mM phosphate buffer (pH 8.0) and applied to a column of DEAE-Toyopearl-650M (Tosoh Co.) equilibrated against the same buffer. The column was eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. The eluate was monitored for absorbance at 280 nm. The sample was further fractionated by gel filtration high power liquid chromatography (HPLC), as described elsewhere (Kawamura et al., 1999). In brief, it was dialyzed against 50 mM phosphate buffer (pH 7.2) and applied to a HPLC system (Japan Spectroscopic Co. Ltd.) equipped with a gel filtration column (KW-803, Shodex). The column was eluted with 0.5 M NaCl in the same buffer at a flow rate of 0.5 ml/minutes. β-galactosidase (116 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa) were used as standards of retention time with reference to molecular sizes.

Electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis was done by the method of Laemmli (Laemmli, 1970). Proteins were blotted electrically onto NC membrane (Bio-Rad Lab.) or PVDF membrane (Daiichi Kagaku Co.) at 300 mA for 1.5 hours in the buffer containing 25 mM Tris, 195 mM glycine and 20% methanol.

Protein sequencing

Portions of purified proteins were cleaved at the C terminus of internal methionine residues by cyanogen bromide in 70% formic acid at 37°C for 2 hours. Full length or fragmented polypeptides were electrophoresed and blotted onto PVDF membrane. The bands were cut and applied to a peptide sequencer (476A, ABI) to determine N-terminal amino acid sequences.

Affinity chromatography

Proteins were dialyzed against 10 mM Tris-HCl (pH 8.0) containing 5 mM EDTA and then 1 mM CaCl$_2$ instead of EDTA. A column of immobilized D-galactose (Pierce Chemical Co.) was equilibrated with the binding buffer (1 mM CaCl$_2$ in 10 mM Tris-HCl, pH 8.0). After loading with the sample, the column was washed with the binding buffer and then eluted with the elusion buffer (5 mM EDTA in 10 mM Tris-HCl, pH 8.0) at a flow rate of 0.1 ml/minute.

PCR and plasmid subcloning

According to partial amino acid sequences, degenerated primers were designed: primer 1, 5′-GA[TC]GA[AG]AC[TCAG]ATGAA[TC]TA-[TC]GC-3′; primer 2, 5′-GA[TC]AA[TC]TA[TC]GA[AG]AT[TCA]-[TC]T[TCAG]ATG-3′. Five kinds of specific primers were also designed: primer 3, 5′-GTGGACTACGACATTCTC-3′; primer 4, 5′-TTAGCATTGCCGCATCCAGT-3′; primer 5, 5′-GATAAC-TATGAAATTCTCATGTCC-3′; primer 6, 5′-GTCGACTATGAA-ATTCTCATGTCC-3′; primer 7, 5′-TCGCGAGCGGCCGCCCT-TTTTTTTTTTTTTTTTT-3′.

For polymerase chain reaction (PCR) (Saiki et al., 1988), rTaq DNA polymerase (Takara Biomed.) was used under the following programmed temperature: 94°C 2 minutes (one cycle); 94°C 30 seconds, 45-55°C 60 seconds, 72°C 90 seconds (30 cycles), 72°C 7 minutes (one cycle).

PCR products were ligated to TA cloning vector (pCR2.1, Invitrogen Co., or pGEM-T, Promega Co.) or subcloned into pBluescript II SK (Stratagene).

Plaque hybridization

Digoxigenin (DIG)-labeled probes were prepared, as described in the instruction manual (Dig DNA labeling kit, Boehringer Mannheim). A λgt11 cDNA library was constructed from poly(A)$^+$ RNA from a colony of *P. misakiensis* (Shimada et al., 1995). Plaques were prepared by infecting bacterial strain (Y1090R$^-$) with lambda phages. A total of 300,000 plaques were hybridized with DIG-labeled probes and visualized by anti-DIG antibody system.

DNA sequencing

For cycle sequencing, Thermo Sequenase Dye Terminator cycle sequencing premix kit (Amersham Pharmacia Biotech.) was used. The products were analyzed using a DNA sequencer (373A, ABI).

Preparation of GST fusion proteins

cDNAs were cut by restriction enzymes *Sal*I and *Not*I from plasmid vectors, subcloned into pGEX vector (Amersham Pharmacia Biotech.) and expressed in the bacterial strain BL21. After induction by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the bacterial cells were harvested and sonicated in 0.1 M Tris-HCl (pH 8.0) containing 6 M urea, 2 mM EDTA and 0.2 mM DTT. After centrifugation, the supernatant was dialyzed against decreasing concentration of urea and finally against phosphate-buffered saline (PBS). GST-fusion proteins were purified by an affinity column of glutathione sepharose 4B. The column was eluted by digestion with 1 U/ml thrombin.

Bioassay

The cell line established from the multipotent epithelium of *P. misakiensis* was cultured in growth medium containing 3% fetal bovine serum in the basal medium that consists of Millipore-filtered seawater and Dulbecco's modified Eagle medium (5:1) (Kawamura and Fujiwara, 1995). For bioassay, cells were suspended in the growth medium at the density of 1×10^5 cells/ml, and 100 μl each was plated on 96-well multiplates. Samples to be examined were dialyzed against PBS and added to the cell suspension. As a control, sterile PBS (10 μl) was added to each well. Cells were photographed every day and suspended in the cell dissociation medium (0.2% trypsin and 2 mM EDTA in PBS). Cells were counted using a hemocytometer.

In situ hybridization

Specimens were fixed in 4% paraformaldehyde for 12 hours at 4°C. They were hybridized with dig-labeled probe as described above, and stained with the anti-dig antibody labeled with alkaline phosphatase as described previously (Hisata et al., 1998).

Monoclonal antibodies and whole-mount immunostaining

BALB/c 3T3 mice were immunized five times with GST fusion protein conjugated with Freund's adjuvant. Spleen cells were fused with NS1/Ag4 myeloma cells and selected by HAT medium. Anti TC14-2 and TC14-3 hybridomas were cloned and grown, respectively, and the culture media were stored in a refrigerator in the presence of 0.05% NaN$_3$. Anti-alkaline phosphatase antibodies have been described elsewhere (Kawamura and Fujiwara, 1994).

When cultured cells were used for immunostaining, they were mounted for 30 minutes on coverslips coated with 0.5 mg/ml poly-L-lysine before staining. Specimens were fixed in Zamboni's fixative at 4°C for 60 minutes, followed by acetone at –20°C for 10 minutes. They were incubated in 1% H$_2$O$_2$ for 10 minutes to inactivate endogenous peroxidase. The following procedures were done on ice. For blocking, the specimens were incubated in 2% skimmed milk (60 minutes) and then in the primary antibody (more than 4 hours). They were washed with 0.1% Tween 20 in PBS, and then reacted for 1 hour with the anti-mouse secondary antibody labeled with peroxidase (Vector Lab.). After washing, they were colored in 50 mM Tris-HCl (pH 7.6) containing 0.2 mg/ml diaminobenzidine and 0.01% H$_2$O$_2$. Some specimens were dehydrated, embedded in JB4 plastic resin (Polyscience Inc.) and sectioned.

RESULTS

Cell growth-inhibitory activity of tunicate galactose-binding proteins

Total homogenates of *P. misakiensis* were fractionated by anion exchange chromatography (Fig. 1A). The substance in the third peak had a strong ability to block cell growth of a clonal cell line established from *Polyandrocarpa* multipotent epithelium (Fig. 2A,B). The peak fraction was further fractionated by gel filtration HPLC (Fig. 1B). Only the fraction in the third, highest peak showed the activity. The retention time of about 20 minutes was comparable to that of carbonic anhydrase with relative molecular mass of 29 kDa. On SDS-PAGE after gel filtration HPLC, the active peak contained two major bands of 18 kDa and 15 kDa and a minor band of about 29 kDa (Fig. 1C). It was purified finally by anion exchange chromatography. It contained only two major peaks (Fig. 1D). They were tentatively named P18 and P15.

The mixture of P18 and P15 not only blocked cell growth but also induced cell aggregation (Fig. 2B). We examined whether these aggregates expressed cell adhesion molecules on the cell surface. They were dissociated by pipetting, suspended in the culture medium, and mounted on cover slips coated with poly-L-lysine. The in vivo multipotent epithelium expressed α-integrin homolog (EMBL/GenBank accession number, AB056090; Fig. 2C), but in vitro cultured cells did not (not shown). P18 and P15 induced this component of cell adhesion molecules on the cell surface (Fig. 2D).

The N-terminal amino acid sequences of the purified proteins were determined. They were VDYDILFSDETMNY-ADA in P18 and DNYEILMSGKAMTYSDAE in P15. In order to obtain the internal sequences, both P18 and P15 were cleaved with cyanogen bromide. Only P18 yielded sequence information: VKAILDFTKDRR. In the SwissProt database, both P18 and P15 showed sequence similarity to TC14-1, the first tunicate C-type lectin (Suzuki et al., 1990).

The mixed solution of P18 and P15 was applied to the affinity column of immobilized galactose. They were bound to the column in the presence of 1 mM CaCl$_2$ and eluted by 5 mM EDTA (Fig. 1E,F), showing that they are indeed calcium-dependent galactose-binding proteins. After affinity chromatography, the proteins (30 µg/ml) had cytostatic activity on cultured tunicate cells, as in Fig. 2B.

Deduced primary structures of P18 and P15

Based on partial amino acid sequences, degenerate primers were designed for PCR. PCR products were inserted into plasmid vectors and used as probes to screen the cDNA library
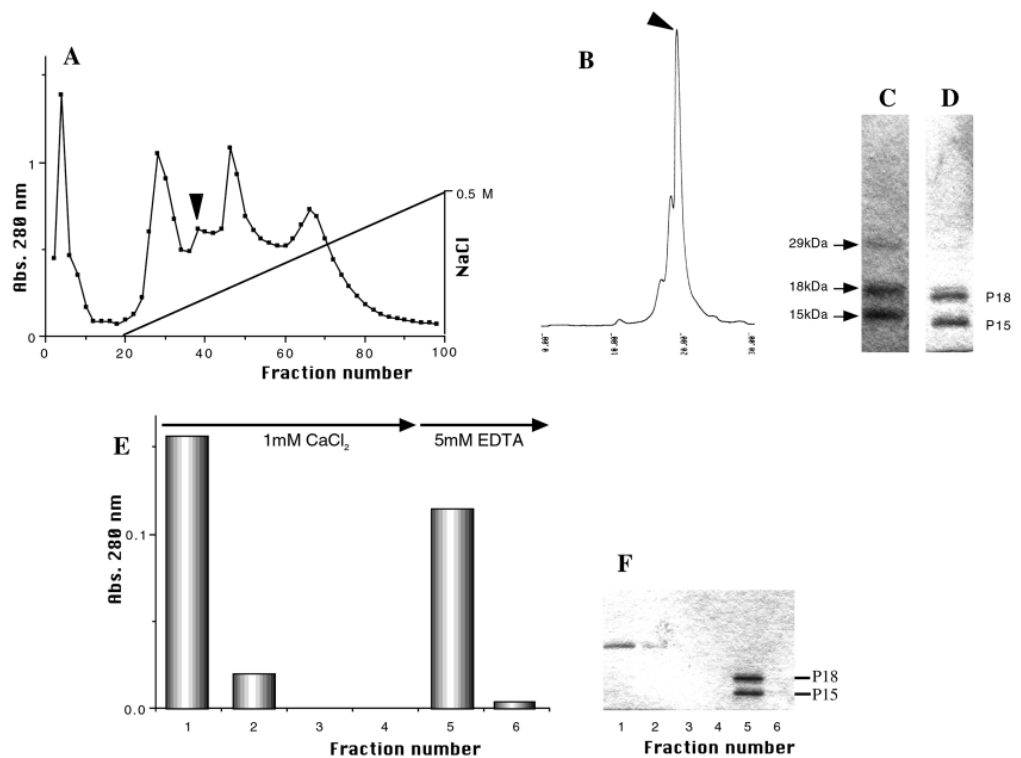

Fig. 1. Purification of novel galactose-binding proteins from *P. misakiensis*. (A) Anion exchange chromatography of crude extracts. The third peak (arrowhead) was further fractionated. (B) Gel filtration HPLC. The highest peak (arrowhead) has a retention time of about 20 minutes. (C) SDS-PAGE of the highest peak after gel filtration HPLC. (D) P18 and P15 after a second anion exchange chromatography step. (E) Affinity chromatography of P18 and P15, on immobilized galactose. After washing with the binding buffer containing 1 mM CaCl$_2$, the column was eluted with 5 mM EDTA. (F) SDS-PAGE of each fraction after affinity chromatography.

Fig. 2. Effect of P18 and P15 on cell growth and aggregation of the atrial epithelium in culture, 4 days after inoculation. (A) Control. Cells were allowed to grow in the serum-supplemented medium. Scale bar, 50 μm. (B) Experiment. The proteins (about 36 μg/ml) were added to the serum-supplemented medium. Note that cells aggregate. Scale bar, 50 μm. (C) In situ hybridization showing in vivo expression of α-integrin homolog (blue) in the atrial epithelium (arrow). Scale bar, 20 μm. (D) In vitro induction of α-integrin homolog by P18 and P15 (brown). Cell aggregates in culture were suspended by pipetting, mounted on cover slips coated with poly-L-lysine, and immunostained (arrow). Scale bar, 10 μm.
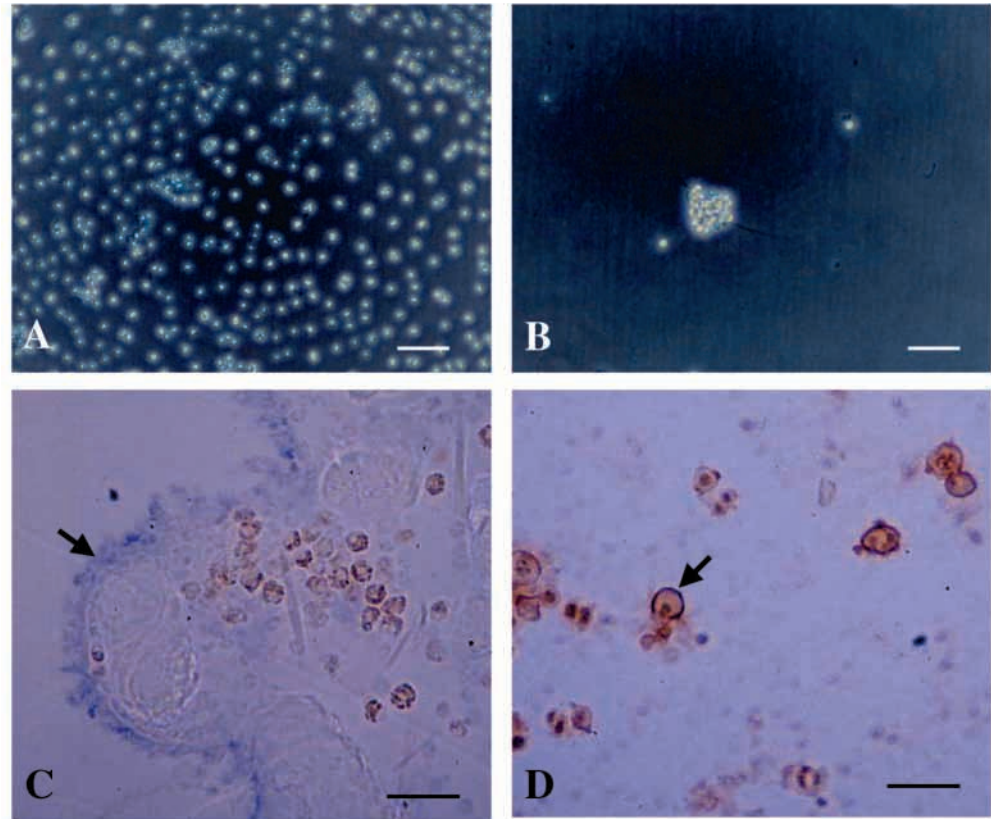
of *Polyandrocarpa* colonies. Cloned cDNAs (accession numbers, AB049564, AB049565) encoding respective P18 and P15 were about 800 bp long and were very similar to each other (Fig. 3A,B). Their open reading frames were of 435 nucleotide long, encoding 145 amino acids that contained the sequences determined by peptide microsequencing (Fig. 3 shaded). The deduced initiation methionine was located at 20 amino acids upstream from the N terminus of the mature protein (Fig. 3A,B double underlines). The initiation codon satisfied Kozak's consensus motif (Kozak, 1986) (Fig. 3A,B dotted letters).

Alignment of amino acid sequences showed that, like TC14-1 (accession number, AB049563), both P18 and P15 had four conserved cysteines specific for C-type lectins and had carbohydrate-binding amino acids, E, N, D, and D (Poget et al., 1999) (Fig. 3C). Interestingly, boxed amino acids (102-107), especially those of the third lectin, are quite similar to the sequence, WAPGEP (76-81), which is known to regulate

carbohydrate-binding specificity in E-selectin (Kogan et al., 1995). These results indicate that both P18 and P15 are members of TC14 lectin family. P18 was found to be identical to TC14-2, the partial nucleotide sequence of which had been determined previously (Shimada et al., 1995). In contrast, P15 was found to be a novel protein, referred to as TC14-3.

Characterization of recombinant TC14-2 and TC14-3

Both GST/TC14-2 and GST/TC14-3 fusion proteins were prepared using a bacterial expression system (Fig. 4A,B). They were cut by thrombin after glutathione affinity chromatography. Recombinant TC14-2 (rTC14-2) and rTC14-3, like the native proteins, showed relative electrophoretic mobility of 18kDa and 15 kDa (Fig. 4C,D). They were bound to an immobilized galactose column in a Ca^{2+}-dependent manner (Fig. 4E,F).

Affinity-purified rTC14-2 and rTC14-3 were administered in vitro to tunicate cells. rTC14-3 (3 nmol/ml) blocked cell growth (Table 1) and induced cell aggregation by itself (Fig. 5B). Each cell became larger and expressed alkaline phosphatase antigen specific for the multipotent, atrial epithelium (Fig. 5E) (Kawamura and Fujiwara, 1994). When the atrial epithelium differentiates into the digestive tract, alkaline phosphatase antigens are switched to the intestinal isoform (Kawamura and Fujiwara, 1994). The cells treated with rTC14-3 did not express this intestinal isoform of the antigen (Fig. 5F).

Unlike rTC14-3, rTC14-2 did not have apparent growth-inhibitory activity on cultured cells by itself (Table 1; Fig. 5A,D). By mixing it with rTC14-3, however, the minimal concentration of TC14-3 to exert the cytostatic activity became

Table 1. Cell division-inhibitory activity of recombinant TC14s in *P. misakiensis*

	Cell number (×10⁵ cells)‡		
TC14s added	0 days*	2 days*	4 days*
0 (PBS)	1	7.5±0.8*	60.8±5.8
1 nmol/ml rTC14-2	1	8.2±1.2	84.2±19.1
1 nmol/ml rTC14-3	1	6.8±1.8	58.6±12.1
1 nmol/ml rTC14-2+			
1 nmol/ml rTC14-3	1	1.8±0.8	2.0±0.7
3 nmol/ml rTC14-2	1	4.2±2.9	40.0±18.2
3 nmol/ml rTC14-3	1	0.9±0.4	1.2±0.8

*Days after inoculation
‡mean±s.d./ml

A
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                5'-TATAACTCCATCAAGCTCTCGACAATATCCATCACC
     ATGGTTTTTCACCGCGATCCTAGTTGCCGTTATCGCTTGCGGTATTCCCACCTCCTACGCT
  1   M V F T A I L V A V I A C G I P T S Y A   20
     GTGGACTACGACATTCTCTTCTCTGATGAAACCATGAATTACGCTGATGCGAGGAGCGTAT
 21   Y D Y D I L F S D E T M N Y A D A   G A Y   40
     TGCCAAAGCAGAGGAATGGTACTAGTCTCAAGTGCGATGCGCGACAGCACAATGGTCAAA
 41   C Q S R G M V L V S S A M R D S T M V K   60
     GCAATTCTCGATTTCACCAAAGACAGACGCTATAACTATTGGGTTGGCGCTGATAACCTA
 61   A I L D F T K D R R Y N Y W V G A D N L   80
     GAAAAAGGAGCGTATAACTTCCTCTGGAACGATGGTGTCTCTCTGCCAACTGACAGCGAC
 81   E K G A Y N F L W N D G V S L P T D S D   100
     TTGTGGTCACCTGATGAACCAAGCAATCCCCAGTCTTCGGAACTGTGCGTCCAAATCTGG
101   L W S P D E P S N P Q S S E L C V Q I W   120
     AACAGGTACAACCTTTTCGATGACACTGGATGCGGCAATGCTAAAAGAGTGATCTGCGAA
121   N R Y N L F D D T G C G N A K R V I C E   140
     AAAGAGTTGGACGACTAATCAGCTATACAATGATCAATTTCTTTTTTGTGTTAGTACGGA
141   K E L D D *
     TGAATTGGATGCATTTGTGATGTACAAGTCTTATAATACTGTTCTATTTCTCGTCTTTAA
     AAAAAAACGGCGGCTAATTTACTTTGATGCTTGTGAATTCAAATTGTAGTGTGTGTTCAAT
     CAGAAATTCAATAAAGTGTTATTATGGG-3'
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B
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                5'-TTCTTCCAAGCTCCCGTCAATATCCATCACC
     ATGGTTTTTATTGCGATTCTAGTTGCCGTTATCGCTTGTGGTATTCCCGCCTCCTATGCT
  1   M V F I A I L V A V I A C G I P A S Y A   20
     GATAACTATGAAATTCTCATGTCCGGTAAGGCGATGACTTACTCTGACGCAGAAACATAC
 21   D N Y E I L M S G K A M T Y S D A E   T Y   40
     TGCGAAAGTATCGGGATGACTGTGGCTGCAAAAGCAATTCGCGACAGTACTACCGTCAAA
 41   C E S I G M T V A A K A I R D S T T V K   60
     GCTATCGTCGATTTCGCCGCAGTTACAGGTAAAAGTTACTGGGTTGGCGCAGACAACAAG
 61   A I V D F A A V T G K S Y W V G A D N K   80
     GAATTGGGAGGATATAACTTCCTCTGGAATGACGGCGTGTCTTTGCCCAGTGACAGCGAT
 81   E L G G Y N F L W N D G V S L P S D S D   100
     TTGTGGGCACCTGGTGAGCCCAGCAATCCCGGCAAGAAAAACTCTATGTGTACAACTTTGG
101   L W A P G E P S N P A G K N L C V Q L W   120
     AAATCGTTGACCAAGTGATCACTGAAGAACGCTTAGTTCATATCTTGATAAGGGATAATT
121   N K Y G L L D D T G C Q K T K N P I C E   140
     CATTGCAATTGTATTTAGGCGTTTAAGAAAGATATTTATGTTTGATTAGGGCATCGGTAGC
141   K S L T K *
     AAATTTGCTTGTCAGTGCTCTCTTTATTCGAAAAATGTTAGCGTGCATTAAAATCTGAAA
     TTCCAATAAATACATTTCATGGGGAAGCTG-3'
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C
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                       25              *           50                      75
TC14-1  MVFTAILVAVIACGIPTSYAMDYEILFSDETMNYADAGTYCQSRGMALVSSAMRDSTMVKAILAFTEVKGHDYWV
TC14-2  -------------------V--D------------A-------V----------------D--KDRRYN---
TC14-3  ---I------------A---DN----M-GKA-T-S--E----E-I--TVAAK-I----T----VD-AA-T-KS---

                           100       *           125      *         *       145
TC14-1  GADNLQDGAYNFLWNDGVSLPTDSDLWSPNEPSNPQSWQLCVQIWSKYNLLDDVGCGGARRVICEKELDD
TC14-2  -----EK--------------------D-------SE------NR---F--T----N-K---------
TC14-3  ----KEL-G------------------S-----A-G-----AGKN----L-N--G----T--QKTKNP----S-TK
```
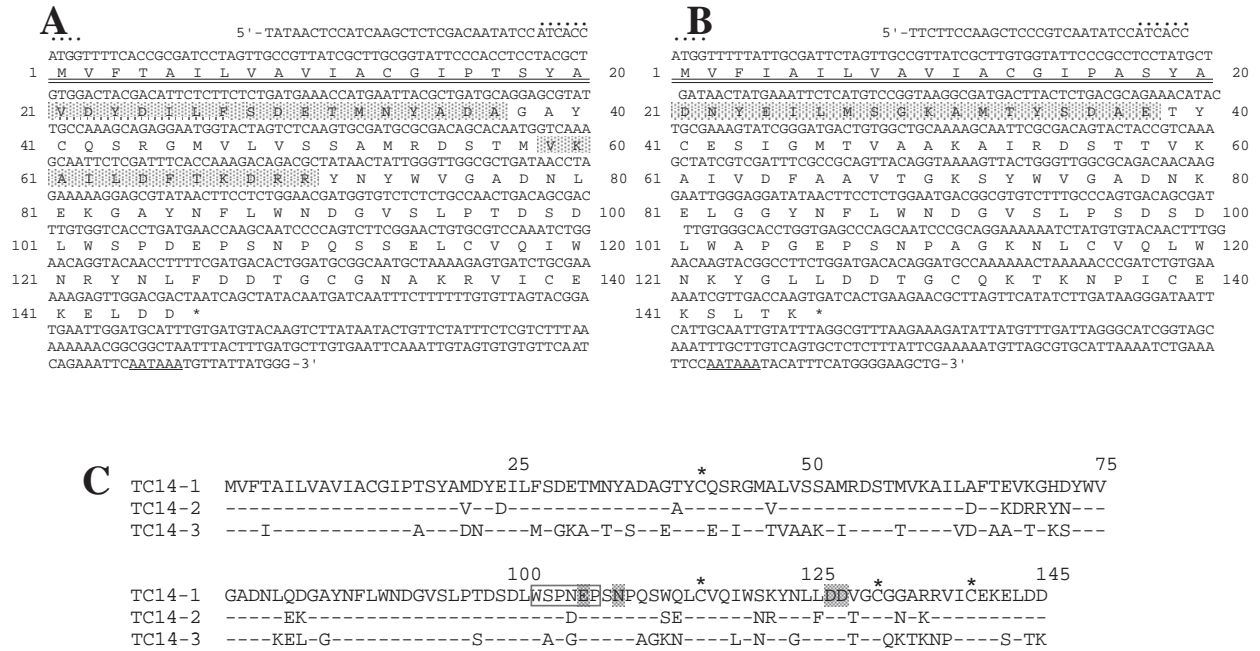
Fig. 3. Nucleotide and deduced amino acid sequences of TC14s. (A) TC14-2. Shaded letters indicate N-terminal and internal amino acid sequences of P18, determined by peptide microsequencing. (B) TC14-3. Shaded letters indicate N-terminal amino acid sequence of P15. Letters marked by dots are Kozak's consensus motif, those with a double underline are the signal peptide and those with a single underline are the polyadenylation signal. (C) Multiple alignment of TC14-1, TC14-2 and TC14-3. Four half cysteines (asterisks) are conserved perfectly. Carbohydrate-binding amino acids, E,N, and DD, are also conserved. A box indicates sequence similarity to E-selectin. These sequence data of TC14-1, TC14-2 and TC14-3 are available from EMBL/GenBank under accession numbers AB049563, AB049564 and AB049565, respectively.

lowered to 1 nmol/ml. The biological activities of TC14-3 were completely abolished by the addition of 50 µM galactose (Fig. 5C,G).

Expression of TC14-3

Crude protein extracts were prepared from young functional animals, adult animals of budding stages, growing buds, developing 1-day-old buds, 2-day-old buds and 3-day-old buds. After SDS-PAGE, they were blotted onto NC membrane and stained with anti-TC14-3 monoclonal antibody. The antibody recognized two bands (Fig. 6A). From the Rf value, the upper band was identified as TC14-3. TC14-3 was expressed constitutively during the asexual life span of this animal. The lower band might be another kind of TC lectin that cross-reacted serologically with anti-TC14-3 antibody (see Discussion). Anti-TC14-2 monoclonal antibody showed that the antigen was expressed constantly in both buds and adult animals (not shown).

In adult animals, TC14-3 was expressed exclusively in mesenchymal cells of the body wall (Fig. 6B) and of the pharynx (Fig. 6C). It was expressed weakly in the pharyngeal epithelium (Fig. 6C). Growing buds expressed TC14-3 in both mesenchymal cells and the atrial epithelium (Fig. 6D,E). The strong signal was maintained during bud development,

but it became exceptionally weak proximally, where dedifferentiation and cell division takes place in the
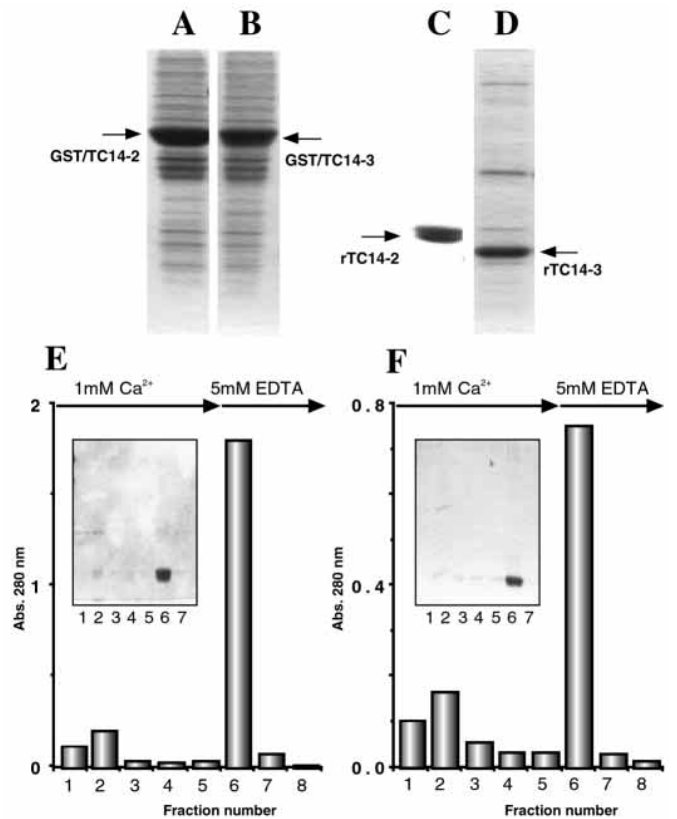

Fig. 4. Preparation of recombinant TC14-2 (rTC14-2) and rTC14-3. (A,B) Bacterial lysates after IPTG induction of GST fusion proteins. (C,D) Affinity purification of rTC14-2 and rTC14-3 after thrombin digestion. (E,F) Affinity chromatography of rTC14-2 (E) and rTC14-3 (F), using immobilized galactose. Elution profiles show that both rTC14-2 and rTC14-3 have calcium-dependent, galactose-binding activity.

Fig. 5. Effects of recombinant proteins on cell growth and differentiation of the atrial epithelium in culture, 5 days after inoculation in the serum-supplemented medium. (A-C) Phase contrast microscopy. Scale bar, 50 µm. (D-G) Immunocytochemistry. Cells were suspended in the medium by pipetting and mounted on poly-L-lysine-coated cover slips. Cells, except those in F, were stained with the monoclonal antibody recognizing specifically alkaline phosphatase of the atrial epithelium. In F, the gastric epithelium-specific antibody was used. (A,D) rTC14-2 (3 nmol/ml). (B,E,F) rTC14-3 (3 nmol/ml). (C,G) rTC14-3 (3 nmol/ml) + 50 µM galactose. Scale bar, 25 µm.
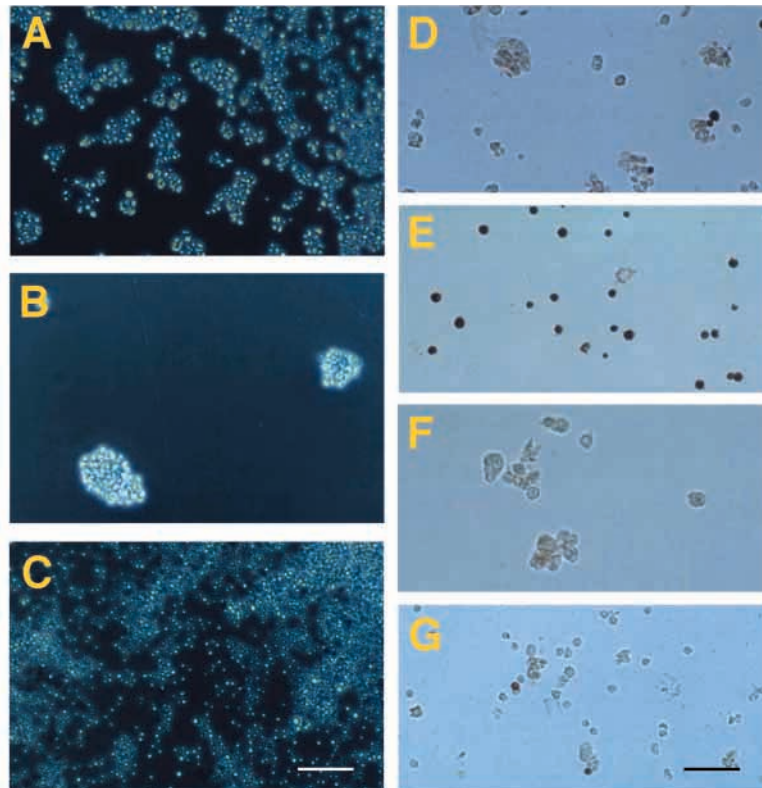
multipotent atrial epithelium (Fig. 6F,G). This finding suggests that in the proximal area cells can escape from cytostatic influence of TC14-3.

DISCUSSION

A C-type lectin family in *P. misakiensis*

Two related TC14 lectins have been described previously in the budding tunicate, *P. misakiensis*. One is TC14-1, for which the complete amino acid sequence of mature protein and the partial nucleotide sequence of a cDNA were determined (Suzuki et al., 1990; Shimada et al., 1995). The other is TC14-2, known only from a partial nucleotide sequence (Shimada et al., 1995). In the present study, we found that an 18 kDa protein (P18) was identical to TC14-2 and we succeeded in determining the complete nucleotide sequence of the corresponding cDNA and demonstrating calcium-dependent galactose-binding activity. We also identified a third polypeptide of 15 kDa (P15) related to TC14. It also bound to galactose in a calcium-dependent manner. The open reading frame of the cDNA, like TC14-1 and TC14-2, encoded 145

Fig. 6. Expression of TC14-3 during the asexual life span of *P. misakiensis*. (A) Blots of crude extracts and immunostaining with anti-TC14-3 monoclonal antibody. Lane 1, young functional animals; lane 2, adult animals of budding stage; lane 3, growing buds; lane 4, 1-day developing buds; lane 5, 2-day developing buds; lane 6, 3-day developing buds.
(B) Section of the body wall of adult animal. Mesenchymal cells are stained. Scale bar, 25 µm.
(C) Section of the pharynx of adult animal. Mesenchymal cells are stained heavily, and the pharyngeal epithelium is stained faintly (arrow). Scale bar, 25 µm.
(D) Whole-mount of growing bud. Arrows indicate the atrial epithelium. Scale bar, 250 µm.
(E) Section of growing bud. Both mesenchymal cells and atrial epithelium are stained. Scale bar, 25 µm. (F) Whole mount of 1-day-developing bud. Signal becomes weak at the proximal (boxed) area where morphogenetic events are in progress. Scale bar, 1 mm.
(G) Section of boxed area in F. Scale bar, 25 µm. ae, atrial epithelium; e, epidermis (white dashed lines); m, mesenchymal cell.
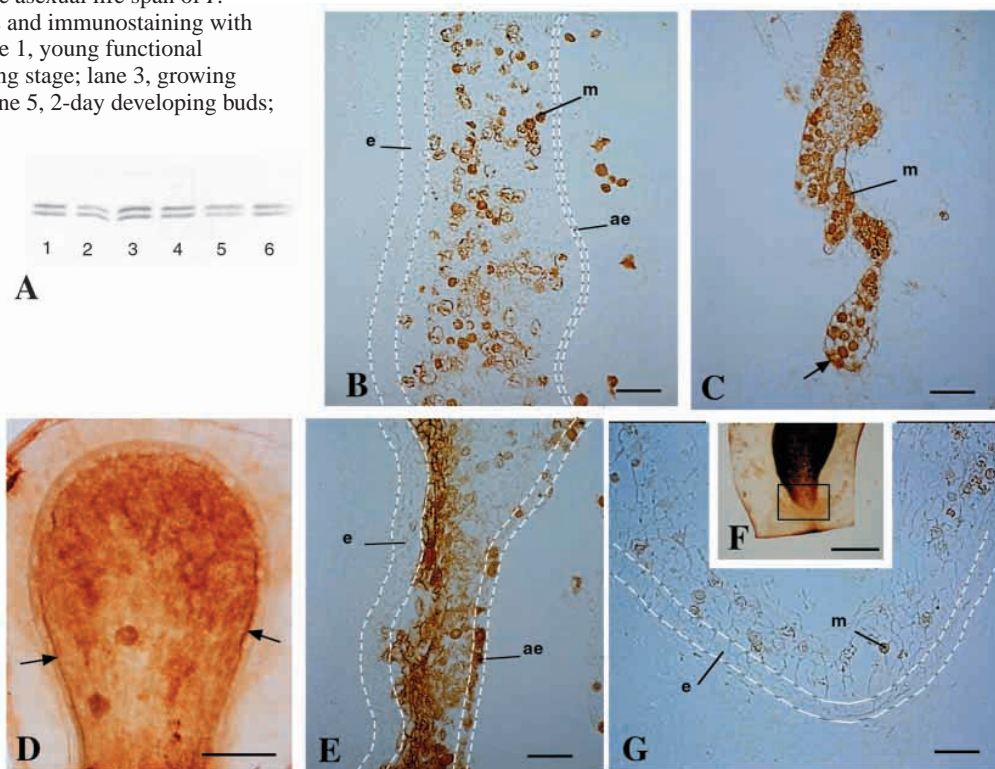
Fig. 7. Possible role of TC14-3 in asexual reproduction of *P. misakiensis*. (A) The atrial epithelium is composed of differentiated pigment cells. (B) During blastogenesis, dedifferentiation takes place. (C) Dedifferentiated cells become progenitors of various tissues and organs. (D) Respective progenitor cells enter the terminal differentiation pathways to become nerves, gastric epithelium, pharyngeal epithelium and others. TRAMP is a retinoic acid-inducible serine protease that promotes cell growth and, possibly, dedifferentiation of the atrial epithelium. TC14-3 is expressed constitutively by mesenchymal cells and by the atrial epithelium during budding stages. It blocks cell growth, dedifferentiation and the resultant terminal differentiation of the atrial epithelium, thus contributing to the maintenance of multipotent cells. The effect of TC14-3 is strong but reversible, as it is cancelled with ease by galactose.
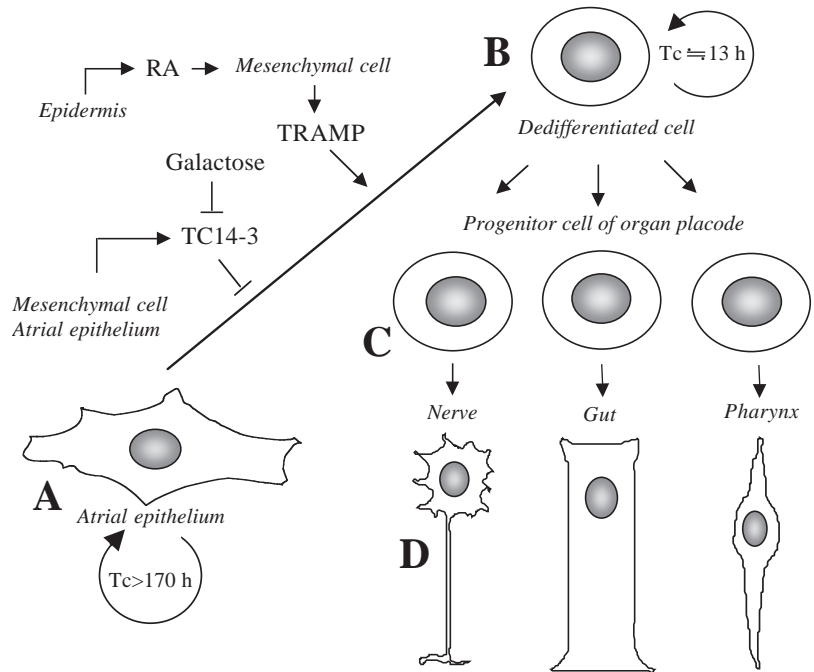
amino acids, including a signal peptide. It had four cysteines conserved in C-type lectins (Drickamer, 1988) and had four carbohydrate binding residues, E, N, D and D (compare with Poget et al., 1999). Therefore, we named this 15 kDa protein TC14-3.

In vivo products of TC14-2 and TC14-3 were always associated with each other after anion exchange chromatography, gel filtration chromatography and affinity chromatography, suggesting that they form a heterodimer in the native condition. Actually, TC14-2 and TC14-3 gave approximately the same retention time as carbonic anhydrase of 29 kDa by gel filtration HPLC. This result is not so surprising, because TC14-1 is known to form a dimer under physiological conditions, where the helix α2 of TC14 is important in forming the dimeric interface (Poget et al., 1999). Recently, a fourth type of lectin (TC14-4) (accession number, AB049566) has been found in *P. misakiensis* (Kawamura and Fujiwara, 2000). It can bind to immobilized TC14-1 affinity column (C. N., unpublished data).

Anti-TC14-3 antibody recognized two bands after western blotting of *Polyandrocarpa* crude extracts (Fig. 6A). The lower band could be TC14-4, from its electrophoretic mobility. Anti-TC14-2 monoclonal antibody cross-reacted with TC14-1 that showed electrophoretic mobility equivalent to 17 kDa (not shown). The results strongly suggest that single asexual *Polyandrocarpa* colonies express four types of TC lectins. We propose that in *P. misakiensis* TC14s constitute a gene family, in which products of redundant genes collaborate with each other in a dimeric form.

A novel, cytostatic activity of C-type lectin

Members of the TC14 family consist only of a single C-type lectin domain or carbohydrate recognition domain (CRD; Drickamer, 1999). The CRD has been found in various kinds of protein. It is involved in a variety of biological functions: endocytosis via a macrophage receptor (Taylor et al., 1992) and

thymic endothelium receptor (Jiang et al., 1995), cell adhesion via a selectin (Lasky et al., 1989), resistance to compression via a proteoglycan core protein (Krusius et al., 1987) and defense mechanisms of invertebrates (Takahashi et al., 1985) and vertebrates (Ikeda et al., 1987). The present study has shown that at least one form of the tunicate CRD-containing TC14 has a novel cytostatic activity.

Although it was uncertain whether native P18 and P15 performed the cytostatic activity in a single form or a combined form, recombinant proteins have shown clearly that TC14-3 (P15) is responsible for the activity. As already mentioned, TC14-3 had sugar-binding amino acid residues, and it was actually bound to galactose. Galactose blocked the cytostatic activity of TC14-3. Site-directed mutagenesis of sugar-binding residues resulted in the loss of the activity (K. K., unpublished data). All these data indicate that the galactose-binding site of TC14 is essential for this activity. An interesting question is why TC14-2 does not exhibit the cytostatic activity by itself, although it has the CRD. Recently, we constructed chimeric proteins consisting of N-terminal TC14-2 and C-terminal TC14-3 and vice versa. The results suggest that the C-terminal region of TC14-3 has an unknown sequence motif necessary for the cytostatic activity (K. K., unpublished data).

TC14-3 induced in vitro cell aggregation. This was consistent with our previous report that TC14-1 plays a role in aggregation and epithelial transformation of in vivo mesenchymal cells during budding (Kawamura et al., 1991). The present study has shown that TC14-3 can induce a homolog of the integrin α chain that is normally expressed in vivo by the atrial epithelium. The result suggests that the in vitro cell aggregation is induced by the de novo synthesis of cell adhesion molecules on the cell surface. It is, of course, possible that dimeric TC14-3 acts as multivalent ligands at the initial stage of cell aggregation.

C-type lectins resemble endostatin in three-dimensional structure (Hohenester et al., 1998). Endostatin is a C-terminal

proteolytic fragment of collagen and a potent inhibitor of endothelial cell proliferation and angiogenesis (O'Reilly et al., 1997). It may inhibit angiogenesis by binding to the heparan sulfate proteoglycans involved in growth factor signaling, although this hypothesis has been challenged (Chang et al., 1999). Comparison of three dimensional structures of TC14s will give information about how TC14-3 exerts the cytostatic activity.

Possible role of TC4-3 in asexual reproduction

In *P. misakiensis*, the atrial epithelium is a major formative tissue (Kawamura and Sugino, 1999). It is composed of pigment cells that express alkaline phosphatase (Kawamura and Fujiwara, 1994) and an α-integrin homolog on the cell surface (this work). The cell divides very slowly with a doubling time of more than 170 hours (Kawamura and Nakauchi, 1991) (Fig. 7A). Retinoic acid is one of chemical signals that break this static state of the atrial epithelium (Hara et al., 1992; Kawamura et al., 1993) (Fig. 7A,B). It is thought to be synthesized by the epidermis and secreted into the lumen (Kawamura et al., 1993), where mesenchymal cells receive the signal and secrete secondary signals such as serine protease (TRAMP; Ohashi et al., 1999) (Fig. 7A,B). TRAMP has a mitogenic activity and is a probable dedifferentiation factor of the atrial epithelium. Dedifferentiated cells form a variety of organ placodes (Fig. 7C), and enter terminal differentiation pathways to form the dorsal ganglion, pharynx, digestive tract and other organs and tissues (Kawamura and Sugino, 1999) (Fig. 7D). As already mentioned, the gastric epithelium-specific isoform of alkaline phosphatase is one of terminal differentiation markers of the digestive tract (compare Fig. 5) (Kawamura and Fujiwara, 1994).

The body fluid of *P. misakiensis* shows a high level of enzymatic activity of serine protease (Ohashi et al., 1999; K. K., unpublished data). If the atrial epithelium is continuously exposed to this kind of protease, multipotent cells may be exhausted owing to dedifferentiation and subsequent terminal differentiation. We assumed that some humoral factor(s) might suppress these processes (see Introduction). The results of the present study have shown that TC14-3 is able to block cell growth and dedifferentiation of the atrial epithelium (Fig. 7A,B). TC14-3 may also act as a differentiation-inducing factor. Both α-integrin and alkaline phosphatase induced in vitro were specific for the atrial epithelium (Figs 2, 5). These activities of TC14-3 can work continuously, as the lectin is expressed constitutively during asexual life of *Polyandrocarpa*. Interestingly, it was cancelled with ease by D-galactose. It may be important, therefore, to determine whether there is expression and/or secretion of galactose-containing polysaccharides that would counteract in vivo TC14 at the morphogenetic area during budding (Fig. 7A,B).

As mentioned above, the effect of TC14-3 is strong but reversible, which may bring about the developmental flexibility of the atrial epithelium. If a large amount of polysaccharides is added to living animals, TC14-3 would no longer sustain the differentiated state of multipotent cells. If the lectin loses the sensitivity to galactose and becomes an irreversible cytostatic factor, it would never permit dedifferentiation of the atrial epithelium. In both cases the atrial epithelium would be deprived of the developmental flexibility, changing *Polyandrocarpa* into a solitary tunicate similar to *Styela* and *Ciona*.

In conclusion, TC14-3 is a cytostatic factor that keeps the atrial epithelium in a differentiated, but multipotent state, and consequently serves as a negative regulator of terminal differentiation. It may contribute to the maintenance of asexual reproductive potential in *P. misakiensis*.

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