Identification and characterization of a putative agglutination/immobilization antigen on the surface of *Cryptocaryon irritans*

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SUMMARY

The ciliated protozoan *Cryptocaryon irritans*, a parasite of seawater fishes, was found to express an antigen that elicits antibodies in rabbits and tiger puffer (*Takifugu ruburipes*). Serum from rabbits and fish immunized with theronts had agglutination/immobilization activity against theronts *in vitro*; fish serum antibody levels (measured by enzyme-linked immunosorbent assays: ELISA) correlated with this activity. Anti-theront antibody levels in fish were significantly higher in the immunized group as compared with control fish at 2 weeks after booster immunization (injection of bovine serum albumin; Student's *t*-test, $P<0{\cdot}01$). Biochemical analyses indicated that a Triton X-114-soluble 32 kDa theront integral membrane protein may be the agglutination/immobilization antigen. Indirect immunofluorescence staining of theronts suggested that this 32 kDa antigen was expressed on the surface of cilia. The full-length 32 kDa antigen cDNA contained 1147 basepairs, encoding a 328-amino acid protein including hydrophobic N- and C-termini. As with *Tetrahymena* and *Paramecium* spp., TAA and TAG appear to be used as glutamine codons in the 32 kDa antigen gene.

Key words: *Cryptocaryon irritans*, white spot disease, vaccination, immobilization/agglutination antigen.

INTRODUCTION

The ciliated protozoan *Cryptocaryon irritans* is a parasite that causes 'white spot disease' in seawater fish, resulting in weakening or death (Colorni and Burgess, 1997). In recent years, *C. irritans* infections of valuable commercial fish species have become a serious problem in Japan. The species that have been affected include Japanese flounder (*Paralichthys olivaceus*), greater amberjack (*Seliola dumerili*), yellowtail (*Seriola quinqueradiata*), red sea bream (*Pugrus major*) and tiger puffer (*Takifugu rubripes*) (Ogawa and Yokoyama, 1998). The parasite life-cycle is similar to *Icthyophthirius multifiliis*, an aetiologic agent of 'white spot disease' of freshwater fish, and includes both a fish-associated trophont stage and a highly motile, free-swimming theront stage (Yoshinaga and Dickerson, 1994). Trophonts reside within the epithelium of the skin and gills where they feed on host tissues and grow large enough to be visible to the naked eye.

Some ciliates, such as *Tetrahymena thermophila*, *Paramecium aurelia* and *I. multifiliis*, have immobilization antigens (i-antigens), which are surface proteins that, when injected into rabbits, elicit the production of antibodies with immobilizing activities *in vitro* (Jones, 1965; Bruns, 1971). Of the protozoan ciliates that infect fish, *I. multifiliis* expresses surface i-antigens, against which host fishes produce immunoglobulin M (IgM) that immobilizes the ciliate *in vitro* (Dickerson *et al.* 1989; Iglesias *et al.* 2002). By several immunological analyses, the *I. multifiliis* i-antigen was shown to localize to the surface of cilia (Dickerson *et al.* 1989). Vaccination of naïve channel catfish (*Ictalurus punctatus*) with the *I. multifiliis* i-antigen elicited immunity in fish, protecting fish from infection by *I. multifiliis* and improving the survival rate against a lethal challenge of the parasite (Wang and Dickerson, 2002), although the role of the i-antigen is still unknown. Also, in the case of *C. irritans*, mummichog (*Fundulus heteroclitus*) immunized with theronts became resistant to subsequent parasite infection (Yoshinaga and Nakazoe, 1997). If the i-antigen is expressed on the surface of *C. irritans*, this antigen is expected to be useful as a component of a vaccine to prevent infections of this parasite in fish.

In the present study, we report that serum from fish or rabbits immunized with theronts causes agglutination of this parasite *in vitro*. The serum antibody level correlated with immobilization activity.

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We identified and purified a putative immobilization antigen extracted by phase separation in theront and trophont Triton X-114 solutions. The putative immobilization antigen, an abundant surface protein of theronts and trophonts, was detected by antisera from rabbits or fish. And the pre-absorption of rabbit antiserum with high concentrations of purified surface antigen of *C. irritans* diminished the agglutination/immobilization activity of the rabbit antiserum. Analysis of the gene sequence indicated that the gene product has a cysteine repeat motif similar to *Paramecium* spp. i-antigen. Also, the putative i-antigen contains a potential N-terminal signal peptide and a potential C-terminal glycosylphosphatidylinositol (GPI) anchor site.

MATERIALS AND METHODS

Fish maintenance

Tiger puffer (~150 g each) hatched in our laboratory were maintained in a 20 000 litre polycarbonate tank. The tank was supplied with seawater at a flow rate of 1·2 l/min; the seawater was sand-filtered and UV irradiated. Tiger puffer was fed a commercial 3 mm pellet diet (Nippon Suisan Kaisha, Ltd) twice a day at a feeding rate of 3% body weight daily. Laboratory seawater conditions throughout the year were as follows: salinity, 34 ppt; pH 8·1; chemical oxygen demand, 1·0 mg/l.

Before each experiment, the gills and skin surface of 10 fish were sampled randomly and examined under a microscope to confirm that the fish were not infected by the parasite.

Parasite maintenance

The *C. irritans* population was maintained using the red sea bream, *Pagrus major*, as a host in a 100 litre polycarbonate tank containing seawater kept at 25±1 °C (Hirazawa *et al.* 2001). As the *C. irritans* cysts adhered to the bottom of the aquarium, we collected *C. irritans* cysts adhering to glass slides that had been placed at the bottom of the aquarium for 15 h. Theronts were obtained from the cysts that were cultured in a 300 ml plastic beaker containing seawater at 25 °C for 5–8 days (Yoshinaga and Dickerson, 1994). Theronts that released from collected cysts within 12 h were used for the experiments. Infected tiger puffer gills were disssected under microscopic observation, and trophonts were collected by Pasteur pipette.

Seawater containing theronts was passed through a cell strainer (2·7 cm in diameter, 5 μm mesh opening) to trap the parasites, which were transferred to a 1·5 ml microcentrifuge tube and centrifuged at 2000 ***g*** for 5 min at 4 °C to remove the remaining seawater. The pelleted theronts were stored at −80 °C.

Fish immunization

To monitor immunoreactivities over time, tiger puffer were immunized with a 1 : 1 (v/v) emulsion of Freund's Complete Adjuvant (FCA) and sonicated theronts in PBS (tiger puffer: $n=11$), or BSA as a negative control (tiger puffer: $n=9$). The dose of injected antigen was 0·5 mg/kg fish body weight. Each tiger puffer was immunized by an intraperitoneal injection of the corresponding emulsion. A booster injection, the same as primary inoculation, was given 2 weeks later. All fish in the immunization experiments were bled at weeks 2, 6 and 10 after the booster injection to obtain small serum samples. Blood was drawn from the caudal vein, and sera were stored at −80 °C.

Rabbit immunizations

Sera were obtained from female Japanese White rabbits immunized against theronts. After an initial inoculation of intact theronts ($n=\sim1\,000\,000$; amount of total protein was 400 μg) with FCA, 3 subsequent inoculations of theronts ($n=\sim500\,000$; amount of total protein was 200 μg) were given subcutaneously in multiple dorsal-lumbar sites with Freund's incomplete adjuvant (FIA) at 2-week intervals. Blood was drawn 8 weeks after the booster injection, coagulated at 4 °C, and subsequently centrifuged at 300 ***g*** for 5 min at 4 °C. Sera were collected, inactivated by heating (56 °C for 30 min) and stored at −80 °C.

Agglutination/immobilization assays

The assays were performed essentially according to the method of Clark *et al.* (1988). Filtered and UV-treated seawater (990 μl) containing approximately 100 parasites was added to each well of a 24-well tissue culture plate. Rabbit or fish serum was serially diluted with filtered and UV-treated seawater (10 μl) and added to each well. The plates were incubated for up to 1 h at 25 °C. Agglutination was monitored with a microscope (BX50; Olympus Optical Co.) with a CCD camera (FD-120M; Flovel Co., Ltd). After the number of agglutinated parasites was counted, 50 μl of formaldehyde were added to each well, and the total number of the parasites was counted. The degree of parasite agglutination was calculated as: (number of agglutinated parasites)/(total number of parasites) × 100%. Each fish serum was assayed in triplicate.

Preparation of tiger puffer IgM

Pooled sera from pre-immune and naïve tiger puffer were fractionated by ammonium sulfate precipitation. An equal volume of the saturated ammonium sulfate solution was added to the tiger puffer serum,

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followed by gentle stirring on ice. After incubation on ice for 1 h, the solution was centrifuged at 10 000 ***g*** for 20 min. The pellet was then dialysed against 10 mM PBS. Crude Ig fractions were loaded onto a Sephacryl S-300 HR (Amersham Pharmacia Biotech) gel filtration column equilibrated with 10 mM PBS (pH 7·2); the effluent (flow rate 0·216 ml/min) was monitored by absorbance at 280 nm. Fractions believed to contain IgM heavy chains and IgM light chains, as judged by SDS-PAGE to have molecular masses of approximately 75 kDa and approximately 25 kDa, respectively, were collected. Tiger puffer IgM from the collected fractions was separated by SDS-PAGE, and the 25 kDa and 75 kDa bands were excised and used to immunize rabbits to generate secondary antibodies for ELISA and immunoblotting.

Extraction of membrane proteins

Integral membrane proteins of *C. irritans* were extracted by phase separation in Triton X-114 (Sigma-Aldrich) solution, essentially as described previously (Border, 1981). Frozen parasites (~200 μg; number of theronts = ~500 000) were thawed and resuspended in 100 μl of ice-cold 10 mM Tris-HCl (pH 7·5) in a 1·5-ml microcentrifuge tube. An equal volume of ice-cold extraction buffer (10 mM Tris-HCl, pH 7·5, 300 mM NaCl, 2% [v/v] Triton X-114) was added, and parasites were incubated on ice for 1 h. The cytoskeletal components were removed by centrifugation at 100 000 ***g*** for 1 h at 4 °C. The clear supernatant was overlaid on a 6% (w/v) sucrose cushion containing 10 mM Tris-HCl (pH 7·5), 150 mM NaCl and 0·06% (v/v) Triton X-114, and the tube was incubated at 32 °C for 5 min. The detergent phase was then separated by centrifugation at 300 ***g*** for 3 min at room temperature. The upper aqueous phase was removed from the tube, and Triton X-114 was added to 0·5% (v/v). After incubation on ice, the mixture was again overlaid on the sucrose cushion used previously, and the tube was incubated at 32 °C for 5 min for condensation and then centrifuged again to separate the detergent phase. Integral membrane proteins, which were contained in the detergent phase, were precipitated by adding 9 volumes of ice-cold acetone. After incubation for 1 h on ice, the precipitate was pelleted by centrifugation at 16 000 ***g*** for 20 min at 4 °C. After acetone removal, the pellet was vacuum-dried, resuspended in 100 μl of 10 mM Tris-HCl (pH 7·5) and stored at −80 °C.

ELISA

Frozen theronts were thawed and suspended in PBS, and the protein concentration was adjusted to 50 μg/ml. The theront solution (100 μl) was added to each well of the 96-well ELISA plates (Greiner Labortechnik), and plates were incubated at room temperature for 2 h. After blocking non-specific protein-binding sites with Block Ace (Dainippon Pharmaceutical Co., Ltd) at room temperature for 2 h, tiger puffer serum (1 : 100) diluted in Block Ace, containing 0·1% (v/v) Tween 20 (Sigma-Aldrich), was added, and plates were incubated overnight at 4 °C. Plates were washed 3 times with 0·1% (v/v) Tween 20 in PBS (PBS-T). Rabbit serum anti-tiger puffer IgM diluted 1 : 1000 in PBS-T was added and incubated at room temperature for 2 h. After washing the plates as above, goat anti-rabbit IgG conjugated to alkaline phosphatase (AP; Kirkegaard & Perry Laboratories, Inc.) diluted 1 : 25 000 (40 ng/ml) in PBS-T was added, and plates were incubated 2 h at room temperature. After washing as above, plates were analysed for AP using Alkaline-Phosphatase Substrate (Bio-Rad Laboratories). The absorbance was detected at 405 nm by TECAN Rainbow Thermo (Wako Pure Chemical Industries, Ltd) according to the manufacturer's instructions. The background value at 405 nm (OD_{405}) was defined as the average signal from 3 wells incubated with blocking solution in the absence of primary antibody. Each fish serum was assayed in triplicate.

SDS-PAGE, immunoblotting and N-terminal amino acid sequencing

SDS-PAGE using a discontinuous buffer system was performed on 4–20% gradient gels (Daiichi Pure Chemicals) (Laemmli, 1970) that were stained by Coomassie brilliant blue (CBB). Molecular mass determinations were estimated by comparing the migration of the protein of interest with that of commercial molecular weight size standards (Bio-Rad Laboratories).

Protein bands from SDS-PAGE were electroblotted onto Pall Fluoro Trans W Membranes (Nippon Genetics Co., Ltd) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories) at 2 mA/cm^2 for 1 h. Filters were blocked by incubation for 1 h at room temperature with Block Ace (Dainippon Pharmaceutical Co., Ltd) containing 0·1% (v/v) Tween 20. Filters were then incubated for 1 h with appropriate dilutions of the respective rabbit or fish serum. Bound fish antibodies were detected with a 1 : 1000 dilution of rabbit IgG anti-tiger puffer-IgM for 1 h, followed by a 1 : 25 000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG for 1 h. Bound rabbit antibodies were detected with a 1 : 25 000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG for 1 h. Filters were then developed in nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Roche Diagnostics). All incubations were performed at room temperature.

Integral membrane proteins (~100 μg) were further subjected to reversed-phase chromatography

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on a μRPC C2/C18 SC 2.1/10 column (GE Healthcare Biosciences) with the SMART System (GE Healthcare Biosciences) with a linear gradient of 0–100% acetonitrile containing 0·1% trifluoroacetic acid (TFA) at a flow rate of 0·1 ml/min. Elution proteins were monitored by absorbance at 280 nm. The ~32-kDa protein was then subjected to automated N-terminal amino acid sequencing on a PPSQ-21 protein sequencer (Shimadzu Biotech).

Purification of the 32 kDa C. irritans *surface antigen* (*CISA-32*)

Integral membrane proteins of *C. irritans* were analysed by the SMART System on a Mini Q PC 3.2/3 ion-exchange column (GE Healthcare Biosciences) with a liner gradient of 0–0·5 M NaCl in 50 mM Tris-HCl buffer (pH 9·5) containing 0·5% NP 40 and 0·5% Triton X-100 at a flow rate of 0·2 ml/min. Elution proteins were monitored by absorbance at 280 nm. Fractions containing CISA-32 were concentrated by ultrafiltration using ultrafiltration spin column (Vivascience), and were subject to acetone precipitation to remove residual detergent. The precipitate was resuspended in PBS and stored at −80 °C until use for mouse immunizations.

Mouse immunization with purified CISA-32

Sera were obtained from female mice immunized against purified CISA-32. After an initial inoculation of 40 μg of the CISA-32 with FCA, 3 subsequent inoculations of 20 μg of the CISA-32 were given subcutaneously in multiple dorsal-lumbar sites with FIA at 2-week intervals. Blood was drawn 8 weeks after the booster injection, coagulated at 4 °C, and was subsequently centrifuged at 300 ***g*** for 5 min at 4 °C. Sera were collected, inactivated by heating (56 °C for 30 min) and stored at −80 °C until use for indirect immunofluorescence staining.

Indirect immunofluorescence staining of theronts

Theronts were fixed by incubation for 30 min in 0·5% (v/v) formaldehyde in seawater and were collected by filtration with a 5 μm mesh filter. Formalin-fixed theronts were washed several times with distilled water before use. Approximately 100 formalin-fixed theronts were spread onto 2·5 × 7·5-cm glass slides and air dried at room temperature. After non-specific protein-binding sites were blocked with Block Ace, mouse antiserum or pre-immune serum (negative control) diluted 1 : 200 in PBS-T was added to each square, and slides were incubated in a humidified box for 2 h at room temperature. Slides were washed 3 times with PBS-T, and Alexa-conjugated goat anti-mouse IgG (1 : 400; Molecular Probes) was added to each square. After a 2 h incubation at room temperature, slides were washed and mounted under cover-slips using the Prolong Antifade Kit (Molecular Probes). Theronts were examined with a confocal laser scanning microscope system (FLUOVIEW FV300; Olympus Optical Co.).

cDNA cloning of the CISA-32 and elongation factor-1 α (*EF-1* α)

Total RNA was extracted from theronts using the RNeasy Mini kit (Qiagen). First-strand cDNA was synthesized using a 5′/3′ rapid amplification of cDNA ends (RACE) kit (Roche Diagnostics) for 3′-RACE. The degenerate primer 5′-GCNTGGG-CNCCNAARACNGC-3′ was synthesized based on the N-terminal amino acid sequence. The first cDNA was amplified by polymerase chain reaction (PCR) in a 50 μl reaction volume containing 25 μl of HotStarTaq Master Mix (Qiagen), 10 pM degenerate primer and anchor primer, the specific 3′-end of synthesized cDNA for 3′-RACE, and 1 μl of cDNA template. PCR amplification was performed as follows: an initial denaturation at 94 °C for 15 min, then 30 cycles of 94 °C for 0·5 min, 55 °C for 1 min and 72 °C for 2·5 min, followed by final elongation at 72 °C for 10 min. Amplified products were subcloned into plasmid pT7blue (Novagen) and used to transform JM109 competent cells (Takara Bio, Inc.). For 5′-RACE, first-strand cDNA was synthesized using the 5′-Full RACE Core Set (Takara Bio, Inc.) with a 5′-phosphorylated primer (5′-GATAACAC-AAAAGTAACTT-3′). The first-strand cDNA was amplified by PCR in a 50 μl reaction containing 25 μl HotStarTaq Master Mix, 10 pM primer sets (5′-TTGTTTGGCAACATGTGGTTG-3′ and 5′-GAAAATTGGTACTACTATTG-3′) and 1 μl of cDNA template. PCR amplification was performed as follows: an initial denaturation at 94 °C for 15 min, then 30 cycles of 94 °C for 0·5 min, 58 °C for 1 min and 72 °C for 2 min, followed by final elongation at 72 °C for 10 min. The nucleotide sequences obtained by 3′- and 5′-RACE over the coding regions of CISA-32 were confirmed by PCR in a 50 μl reaction containing 10 × PCR buffer (Stratagene), 200 μM dNTPs, 0·5 U of *Pfu* DNA polymerase, 1 μl of cDNA and 10 pM specific primer sets: 5′-GAT-TTCATCTTTAGCTG TAATGACATCAGC-3′ and 5′-ATCAAAATATGATCTTAATTAGC-3′. PCR amplification was performed as follows: an initial denaturation at 94 °C for 15 min, then 30 cycles of 94 °C for 0·5 min, 58 °C for 1 min and 72 °C for 2 min, followed by final elongation at 72 °C for 10 min. Amplified products were subcloned into plasmid pHSG299 (Takara Bio Inc.) and used to transform JM109 competent cells (Takara Bio Inc.). At least 5 clones were sequenced on a 3100 DNA sequencer (Applied Biosystems).

The partial nucleotide sequence of the EF-1 α gene from *C. irritans* was amplified by PCR in a 50 μl reaction volume containing 25 μl of HotStarTaq

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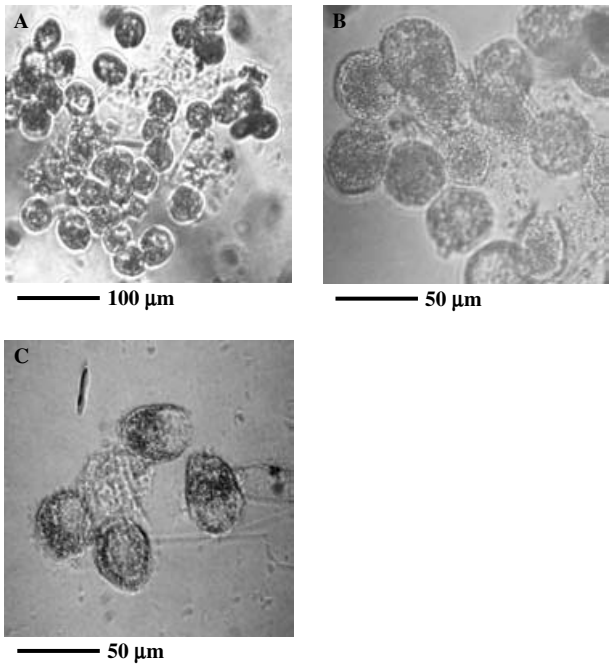

Fig. 1. Agglutination/immobilization of theronts by rabbit (A and B) or tiger puffer (C) antisera obtained by immunization with theronts. Theronts were incubated for 1 h at room temperature in a 1 : 100 dilution of each immune serum.

Master Mix (Qiagen), 10 pM degenerate primers (AGRGAYTTYATHAARAAYATG and GCDATNTGNGCNGTRTGRCARTC) and 1 μl of cDNA template. PCR amplification was performed as follows: an initial denaturation at 94 °C for 15 min, then 30 cycles of 94 °C for 0·5 min, 58 °C for 1 min and 72 °C for 2·5 min, followed by final elongation at 72 °C for 5 min. Amplified products were subcloned into plasmid pT7blue (Novagen) and used to transform JM109 competent cells (Takara Bio Inc.). At least 3 clones for each isoform were sequenced.

Amino acid sequence alignment of EF-1 α

The deduced amino acid sequence of *C. irritans* EF-1 α obtained was compared with other species EF-1 α sequences on GenBank database, *Tetrahymena pyriformis* (BAA01856), *Paramecium tetraurelia* (AAD46607), *Stylonychia lemnae* (CAA41001), *Blepharisma japonicum* (AAD03252), *Danio rerio* (NP_571338), *Mus musculus* (P10126), *Xenopus laevis* (CAA39027), *Gallus gallus* (AAA48757) and *Saccharomyces cerevisiae* (AAA34585). Multiple alignment of sequences was conducted using GENETYX-MAC version 10.1.

RT-PCR

Reverse transcription (RT) was carried out using total RNA (1 μg) extracted from theronts and trophonts, as described above, using the ProSTAR First-Strand RT-PCR kit (Stratagene). Reverse transcription products were amplified by 35 cycles of PCR using specific primers: 5′-GATTTCATCTTTAGCTGTAATGACATCAGC-3′ and 5′-ATCAAAATATGATCTTAATTAGC-3′. PCR amplification was performed as follows: an initial denaturation at 94 °C for 15 min, then 30 cycles of 94 °C for 0·5 min, 58 °C for 1 min and 72 °C for 2 min, followed by final elongation at 72 °C for 10 min.

RESULTS

Agglutination/immobilization activities in rabbit and fish serum

In the presence of rabbit and fish serum immunized with theronts, parasites ceased to swim and began to form large aggregates that settled to the bottom of the well (Fig. 1). A 1 : 100 dilution of immunized serum caused agglutination of live parasites, whereas a 1 : 10 dilution of rabbit pre-immune serum and fish serum immunized with BSA caused little agglutination.

Antibody levels in fish sera

Figure 2A shows the time-course of the immunoreactivity of fish sera generated against theronts, as analysed by ELISA. Two weeks after booster immunization, detectable levels of antibodies against theronts were found in the group immunized with theronts. There was a significant difference between each of the groups and the control group (Student's *t*-test; $P<0{\cdot}001$). The OD_{405} values for the immunized group became manifest within 2–6 weeks. The OD_{405} value and the degree of agglutination of the sera varied among the fish, but there was a detectable correlation between these characteristics within a given individual (Fig. 2B).

Identification of the putative agglutination/immobilization antigen

Membrane compartments of theronts are thought to be associated with the propensity with which they can be immobilized; thus, theront membranes were extracted by differential centrifugation and phase separation in the non-ionic detergent Triton X-114. Figure 3A shows the protein composition of whole theronts, as resolved by non-reducing SDS-PAGE (lane 1), and of theront integral membrane compartments, as resolved by non-reducing (lane 2) or reducing (lane 3) SDS-PAGE. The whole-theront fraction contained a complex mixture of proteins, including a prominent ~32 kDa protein. In the integral-membrane protein fraction, however, the ~32 kDa protein, both in the non-reduced and the reduced form (named CISA (*Cryptocaryon irritans* surface antigen))-32 was predominant. The apparent molecular mass of protein band of CISA-32 on SDS-PAGE under reducing conditions was

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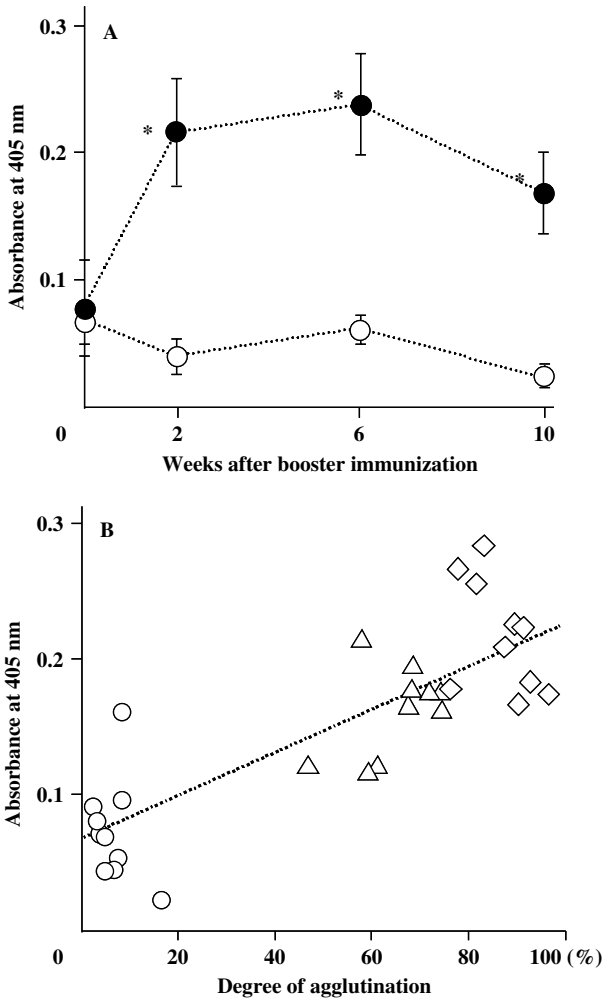

Fig. 2. Serum from tiger puffer inoculated with theronts causes agglutination. (A) Time-course of the humoral response of tiger puffer immunized with theronts (closed circles) or BSA (open circles; negative control) plus the adjuvant FCA. Each point represents the mean ± S.E. of absorbance at 405 nm for each individual serum sample as determined by ELISA. $*P<0{\cdot}001$, with respect to the control fish (Student's t-test). (B) Correlation between ELISA reactivity and agglutination/immobilization activity of antisera from tiger puffer. The graph shows the relationship between the degree of agglutination in a 1 : 100 dilution and ELISA reactivity of sera from fish before immunization (open circles), 2 weeks (open triangles) or 6 weeks (open rectangles) after booster immunization. The regression line (dashed) is shown ($y=0{\cdot}0016x+0{\cdot}0675$, $R^2=0{\cdot}6796$).

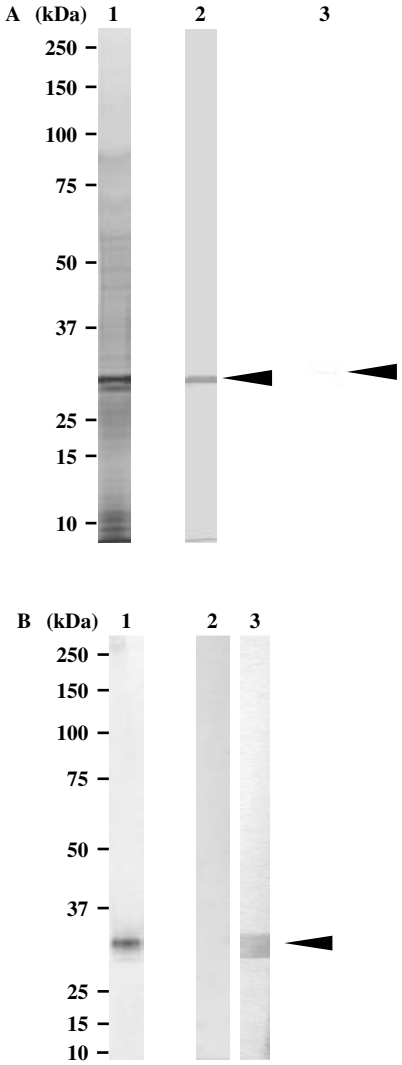

Fig. 3. SDS-PAGE and immunoblot analyses of theront integral membrane proteins. (A) Intact theronts (lane 1) and integral membrane proteins isolated from theronts (were solubilized in Laemmli sample buffer, resolved by non-reducing (lane 2) and reducing (lane 3) SDS-PAGE on 4–20% polyacrylamide gradient gels (10 μg/lane) and visualized using CBB. (B) Immunoblot analysis: 10 ng (for detection with rabbit serum) or 5 μg (for detection with tiger puffer serum) of theront integral membrane proteins were resolved by non-reducing SDS-PAGE and then immunoblotted using the following sera: lane 1, serum from rabbits immunized with theronts; lane 2, serum from tiger puffer injected with BSA as a negative control; lane 3, serum from tiger puffer immunized with theronts. Relative molecular masses of standard markers are indicated on the left. The arrowhead indicates the position of the 32 kDa antigen.

slightly higher than that under non-reducing conditions, suggesting that CISA-32 has a compact folded structure due to (an) intramolecular disulfide bond(s). To characterize CISA-32 as a potential agglutination/immobilization antigen, immunoblot analyses were carried out with antigenic proteins of theronts that were immobilized by rabbit and fish immune sera. Under non-reducing conditions, CISA-32 was strongly detected by immune serum from a rabbit injected with intact theronts (Fig. 3B, lane 1) and by serum from immunized tiger puffer (Fig. 3B, lane 3). On the other hand, under reducing conditions in the presence of 2-mercaptoethanol, CISA-32 was only weakly detected by immunized rabbit serum and was not detected by immunized tiger puffer serum (data not shown).

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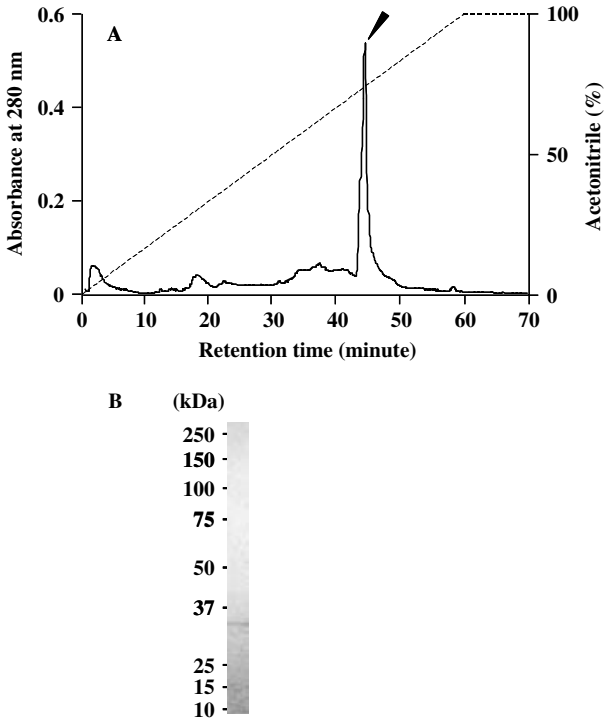

Fig. 4. Purification of CISA-32 from theront integral membrane fraction by reverse phase chromatography. Integral membrane fraction of theronts was applied to μRPC C2/C18 SC 2.1/10 column (GE Healthcare Biosciences) with a liner gradient of acetonitrile containing 0·1% TFA at a flow rate of 0·1 ml/min (A). Dashed line shows the gradient of acetonitrile. The peak fractions indicated by the arrowhead were resolved by reducing SDS-PAGE (B).

The CISA-32 was successfully purified by reverse phase chromatography for the N-terminal amino acid sequence (Fig. 4). The N-terminal amino acid sequence of CISA-32, as determined by automated sequencing (data not shown), was determined to be:

NH_2-Ala-Trp-Ala-Pro-Lys-Thr-Ala-Ala-Ala-Asp-Trp-Lys-Gly-Thr-Phe-Val-Val-Thr-.

Purification of CISA-32 by anion-exchange chromatography

The purification of CISA-32 from theront integral membrane fraction extracted with Triton X-114 was achieved by anion-exchange chromatography (Fig. 5A). The CISA-32 did not bind to the Mini Q column in 50 mM Tris-HCl buffer containing 0·5% NP 40 and 0·5% Triton X-100 (pH 9·5) and was found in the flow through fractions (Fig. 5B), while the small amounts of contaminant proteins bound to the column and were eluted with a gradient buffer from 0 to 0·5 M NaCl.

Indirect immunofluorescence staining of theronts

Antiserum from rabbit immunized with the theronts that promoted agglutination seemed to react with

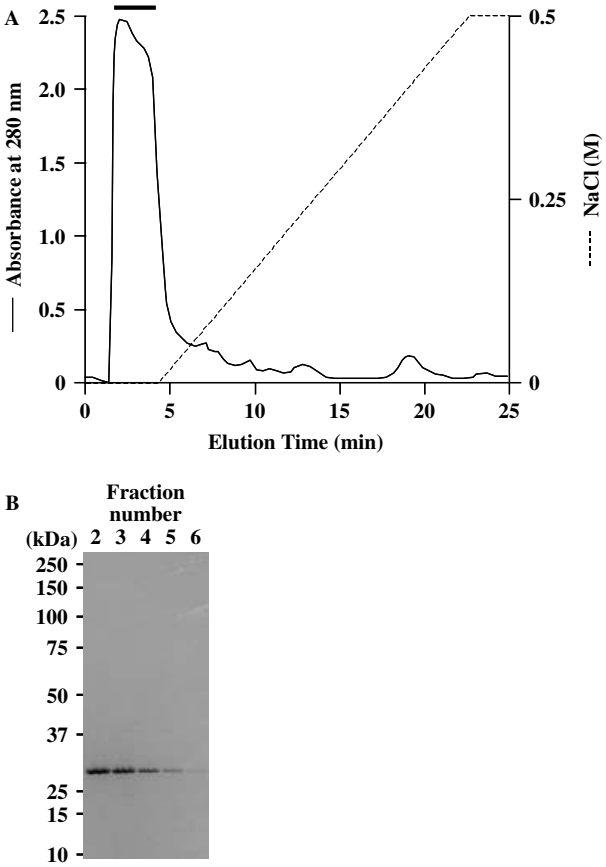

Fig. 5. Purification of CISA-32 from theront integral membrane fraction by anion-exchange chromatography. Integral membrane fraction of theronts was applied to Mini Q ion-exchange column (GE Healthcare Biosciences) with a liner gradient of NaCl in 50 mM Tris-HCl buffer (pH 9·5) containing 0·5% NP 40 and 0·5% Triton X-100 at a flow rate of 0·2 ml/min (A). The flow through fractions containing CISA-32 indicated by bar were resolved by reducing SDS-PAGE (B). Dashed line shows the gradient of NaCl.

ciliary proteins of theronts. Therefore, CISA-32 is thought to be expressed on the surface of cilia. To determine whether cilia contain CISA-32, indirect immune staining was performed using mouse antiserum immunized with purified CISA-32. Ciliary surface proteins were strongly recognized by mouse antiserum (Fig. 6A). On the other hand, treatment with pre-immunized mouse serum showed little fluorescent staining (Fig. 6C).

cDNA cloning of CISA-32

CISA-32 cDNA fragments were generated from theront RNA by 3′-RACE using degenerate primers. Subsequently, the 5′ untranslated region of CISA-32 was identified by 5′-RACE using specific primers. The CISA-32 cDNA contained a 984 bp open reading frame encoding 328 amino acids, including a putative 19-residue signal peptide (Fig. 7A) (GenBank; BAF37969). In the gene coding in CISA-32, TAA (nts: 388–390, 463–465, 478–480,

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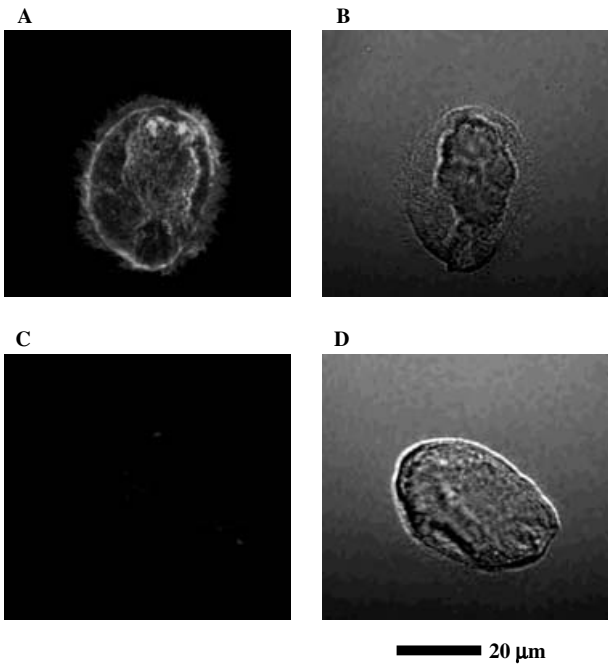

Fig. 6. Indirect immunofluorescent staining of theronts using mouse antiserum immunized with purified CISA-32. Fixed theronts were incubated in mouse antiserum (A and C) or pre-immunized mouse serum (B and D). Bound antibodies were detected with Alexa-conjugated goat anti-mouse IgG. Theronts were visualized by fluorescence microscopy (left column; A and C) or phase contrast (right column; B and D).

496–498, 517–519, 538–540 and 655–657) and TAG (nts: 19–21) codons appeared to be used as glutamine codons, as has been observed in *Tetrahymena* and *Paramecium* species (Caron and Mayer, 1985; Kuchino *et al*. 1985; Preer *et al*. 1985; Hanyu *et al*. 1986). Furthermore, the threonine codons ACT and ACA were used preferentially in the CISA-32 gene, as was seen in *Paramecium* species (Martindale, 1989). A Kyte-Doolittle hydropathy plot of the derived amino acid sequence is shown in Fig. 7B. This plot showed that the C-terminal portion of CISA-32 contained a hydrophobic segment characteristically found in proteins containing a C-terminal GPI anchor (Gerber *et al*. 1992). The potential omega (ω) site in CISA-32 for propeptide cleavage and GPI attachment – as predicted by the big-PI Predictor program – is 305th-serine (Eisenhaber *et al*. 1999). The SMART program (Schultz *et al*. 1998) identified a domain in CISA-32 that is similar to the repeated surface antigen (PSA) motif delineated by cysteine residues in *Paramecium* spp. (Fig. 8).

cDNA cloning of EF-1 α

To determine whether non-canoninal genetic code is common to other *C. irritans* genes, cDNA cloning of EF-1 α gene was carried out. Three types of cDNA clones (GenBank; AB275855, AB275856 and AB275857 for isoform-1, -2 and -3, respectively) were obtained, and these clones show high homology with other EF-1 α sequences. The isoform-3 encoded TAA codons at position 6, 30, 163, 186 and 224, both position at 6 and 30 are highly conserved for glutamine (Fig. 9). On the other hand, isoform-1 and -2 encoded CAA codons at position 6 and 30.

Immunoblot and RT-PCR analyses of trophonts

We investigated whether trophonts express the CISA-32 gene product on their surface. The ~32 kDa protein was predominant in the Triton X-114-soluble fraction of trophonts resolved by SDS-PAGE (Fig. 10A, lane 1), and was detected by immunoblotting with serum from rabbits immunized with theronts (lane 2). The PCR products amplified from theront and trophont cDNA provided bands of the predicted size (984 bp) (Fig. 10B), and thus were subcloned, sequenced, and confirmed to be the CISA-32 cDNA sequences.

DISCUSSION

In the present study, sera from tiger puffer or rabbits that had been immunized with theronts caused agglutination of this parasite *in vitro*. This agglutination activity demonstrates that theronts express agglutination/immobilization antigens on their surface, as do the ciliates *T. thermophila*, *P. aurelia*, *I. multifiliis*, *P. dicentrarchi* and *N. gillerae* (Jones, 1965; Bruns, 1971; Dickerson *et al*. 1989; Iglesias *et al*. 2002; Hatanaka *et al*. 2005). In agglutination/immobilization tests using rabbit or fish serum, agglutinated theront cilia were observed by microscopy; thus, theronts are thought to express agglutination/immobilization antigens on the cilium surface – as do the ciliate species mentioned above. Agglutination/immobilization activity of serum from rabbits and fish immunized with theronts and the OD_{405} value of serum from each individual, as determined by ELISA, were correlated. These results suggest that specific antibodies against theront surface protein(s) were produced in rabbit and fish and contribute to the agglutination/immobilization activity against theronts.

The ~32 kDa protein, CISA-32, seemed to be an agglutination/immobilization antigen and preabsorption of rabbit antiserum with high concentrations of purified CISA-32 diminished the agglutination/immobilization activity of the rabbit antiserum (data not shown). This putative agglutination/immobilization antigen CISA-32 was found to be expressed abundantly on the surface of the cilia of theronts by indirect immunofluorescence staining of theronts. CISA-32 has the characteristics of an agglutination/immobilization antigen and may prevent fish from infection following a lethal challenge with parasites, similar to the i-antigen of *I. multifiliis*

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A

```
AATAATAAAAAAGAGATGTAGAAAATTATATCAATTTTATTGATTTCATCTTTAGCTGTA   60
                  M  Q  K  I  I  S  I  L  L  I  S  S  L  A  V   15
ATGACATCAGCTGCATGGGCTCCAAAAACTGCAGCTGCAGATTGGAAAGGTACATTTGTT  120
 M  T  S  A  A  W  A  P  K  T  A  A  A  D  W  K  G  T  F  V    35
GTTACAAAAAGTAGTTGTTTGGCAACTTGTGGTTGGAAAATTGGTACTACTATTGTTATT  180
 V  T  K  S  S  C  L  A  T  C  G  W  K  I  G  T  T  I  V  I    55
GCTGATAAAACAGGAGATAGCTCAAAAGTAACTTGGATGGGAACAGTACATACAACTGAT  240
 A  D  K  T  G  D  S  S  K  V  T  W  M  G  T  V  H  T  T  D    75
TCTACAAATGTTGATGTTGTAAGCGGAAGTTGTAAATATGTTAGTGTTGTAGCAACTGCA  300
 S  T  N  V  D  V  V  S  G  S  C  K  Y  V  S  V  V  A  T  A    95
GGTACTGCTGGAACACCAGCTGAAGTTTTGAAAAACAGTGATGAATGTGCTTTTGCAACT  360
 G  T  A  G  T  P  A  E  V  L  K  N  S  D  E  C  A  F  A  T   115
GGAACTTGCGGTATTATGGGAAGAAAATAATCAACTCCTGGAACAGTAACTTTCAATAGG  420
 G  T  C  G  I  M  G  R  K  Q  S  T  P  G  T  V  T  F  N  R   135
GATACGACTTTAGATACTAAGCCTTTGCAAATTCTTTATAAATAATTTGCAATGGTATAA  480
 D  T  T  L  D  T  K  P  L  Q  I  L  Y  K  Q  F  A  M  V  Q   155
AAAACATCAACCACCTAAAAAGCCACAGCTACTGACTAAGCAGCAAACTGTGATACTTAA  540
 K  T  S  T  T  Q  K  A  T  A  T  D  Q  A  A  N  C  D  T  Q   175
GCTTCATTCGTAGATACTACAACTGATGCTAAAGCAATTGTAGGATCATTAAAACTATCT  600
 A  S  F  V  D  T  T  T  D  A  K  A  I  V  G  S  L  K  L  S   195
AAAGCTAGTTGTGATAAATGCTCTTGGGATACAACTAAAGATTTAAAAATTACATAAGAT  660
 K  A  S  C  D  K  C  S  W  D  T  T  K  D  L  K  I  T  Q  D   215
GCTTCAAAAAAATATATGGTCACATTGGCAGGAACTATCAAAGAAACTGCAACTGGTGAT  720
 A  S  K  K  Y  M  V  T  L  A  G  T  I  K  E  T  A  T  G  D   235
TGTAATAATAAACTTACAGCTTCTGAAGCATGTTATGTCACAAAAAAAGATGATAAGACT  780
 C  N  N  K  L  T  A  S  E  A  C  Y  V  T  K  K  D  D  K  T   255
TATGTTCTCGTTAGTTGTGCTACTCTTGACACAACTAGTAAAGGTATTCCAATTGCCATT  840
 Y  V  L  V  S  C  A  T  L  D  T  T  S  K  G  I  P  I  A  I   275
ACAACAGCAAATTCAAAAACCACTTTAACCATGACATGGACTGATTCAGCTTCTAAAGCA  900
 T  T  A  N  S  K  T  T  L  T  M  T  W  T  D  S  A  S  K  A   295
TGTAGTGTTGTTGGAGAAGTAACTTCTAGTTCTAATAGTGTAAAACTTATTTCAGGATTC  960
 C  S  V  V  G  E  V  T  S  (S) S  N  S  V  K  L  I  S  G  F   315
AGTGCTATGTTAATCCTCTCTTTAGCTCTTTTATTCAAATGAGCTAATTAAGATCATATT 1020
 S  A  M  L  I  L  S  L  A  L  L  F  K  *                     328
TTGATGAAATTTTATATATAAAGCATAAAGCCTTTAAATATTTGATATTCATGAATTAAG 1080
TACTTTAATAGTTTCATATTTATGCAAAACAATTTGAATTAAATATAAAAAAAAAAAAAA 1140
```

B

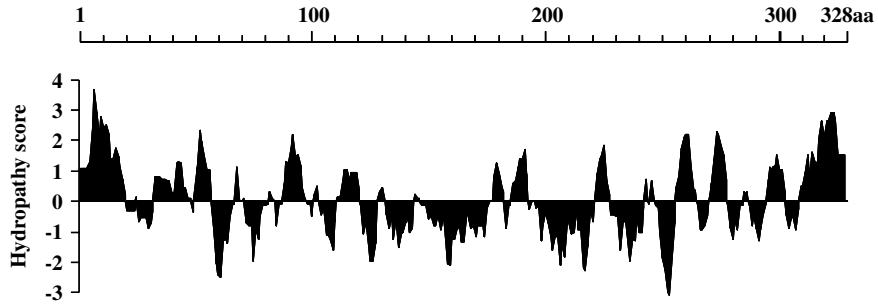

Fig. 7. Sequence analysis of CISA-32. (A) Nucleotide sequence of the CISA-32 cDNA and derived amino acid sequence. The full sequence of the CISA-32 cDNA was determined from total theront RNA using 3′- and 5′-RACE with degenerate primers. Eighteen N-terminal amino acid residues determined from Edman degradation are underlined. The potential N-terminal signal peptide is boxed. TAG and TAA in the cDNA sequence, thought to be used as glutamine codons, are underlined. The position of the putative omega (ω) site for propeptide cleavage and GPI attachment is circled (Ser305). (B) Kyte-hydropathy plot of the CISA-32 amino acid sequence (window size, 7 residues).

(Wang and Dickerson, 2002). The sizes of agglutination/immobilization antigens of *T. thermophila*, *P. aurelia* and *I. multifiliis* vary from approximately 40 kDa to 250 kDa (Doerder *et al*. 1985; Hansma, 1985; Dickerson *et al*. 1989). CISA-32 is somewhat smaller than this range (~32 kDa according to reducing SDS-PAGE), but is a major component of the integral membrane protein fraction of theronts

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```
CISA-32    178 ASCDKCSWDTTKDLKITQNAS-----KKYMVTLAGTIKETATGDCNNK--------LTAS  225
51A PAR    349 SSNQDCIWDTTCKEKTCANAPTTNNTHDLCTSYLSTCTVKTGGGCQNRTCANAPVTLTTN  408
                *   * ****   *   **               *      * * *          **

CISA-32    226 EAC--YVTKKDDKTYVLVSCATLDTTSKGIPIAIATANSKTTLTMTWTDSASKAC       277
51A PAR    409 DACEAYLTGNNCITKSGGGCVT-NTTCAAITLEAACVKNSSGQTCFW-DSASSSC       461
               **  * *     *     * *  **   *    *        *  * **** *
```

Fig. 8. Alignment of the partial amino acid sequence of CISA-32 and *Paramecium tetraurelia* 51A surface antigen (51A PAR). Asterisks indicate identical residues. Cysteine residues delineating the *Paramecium* spp. cysteine repeated motif are boxed.

```
                          ▼                                ▼
Isoform-1            1 ITGTSQADCGVLIVAAGVGEFEAGISKNGQTREHALLAFTLGVKQLIVGVNKMDSTEPPY  60
Isoform-2            1 ITGTSQADCAVLVISSQAGEFEAGIAKEGQTREHALLAYTLGVKQMVVAINKMDHPSTNY  60
Isoform-3            1 ITGTS*ADCALLMIASPQGEFEAGISKDG*TREHALLSFTLGVKQMIVCVNKMDEKTVNY  60
T. pyriformis      103 ITGTSQADVAILMIASPQGEFEAGISKDGQTREHALLAFTLGVKQMIVCLNKMDEKTVNF 162
P. tetraurelia     102 ITGTSQADVALLMIASPAGEFEAGISKEGQTREHVLLAYTLGVKQMICATNKMDEKTVNY 161
S. lemnae          102 ITGTSQADAAILIIASGQGEFEAGISKEGQTREHALLAFTMGVKQMIVAVNKMDDKSVNW 161
B. japonicum        89 ITGTSQADVAILIIAAGKGEFEAGYSKNGQTREHALLAFTLGVKQMVVGVNKMDDKSVEW 148
D. rerio           102 ITGTSQADCAVLIVAGGVGEFEAGISKNGQTREHALLAFTLGVKQLIVGVNKMDSTEPPY 161
M. musculus        102 ITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPY 161
X. laevis          102 ITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLGVKQLIVGINKMDSTEPPY 161
G. gallus          102 ITGTSQADCAVLIVAAGVGEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPY 161
S. cerevisiae      102 ITGTSQADCAILIIAGGVGEFEAGISKDGQTREHALLAFTLGVRQLIVAVNKMDSV--KW 161
```

Fig. 9. Alignment of derived amino acid sequences of *Cryptocaryon irritans* EF-1 α and other EF-1 α. Arrowheads above sequence indicate TAA codons in *C. irritans* EF-1 α isoform-3.

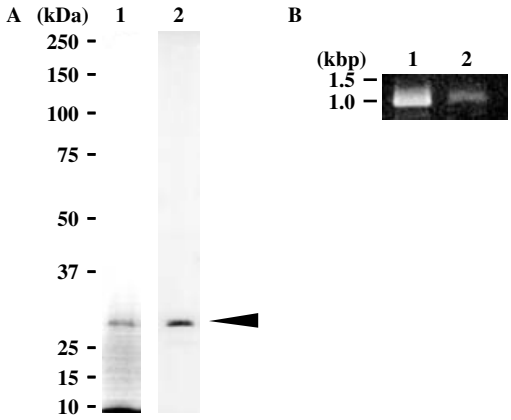

Fig. 10. Immunoblot and RT-PCR analyses of trophonts. (A) SDS-PAGE (lane 1) and immunoblot analysis (lane 2) of integral membrane proteins from trophonts. The resolved proteins were immunoblotted under non-reducing conditions using serum from rabbits immunized against theronts. Relative molecular masses of standard markers are indicated on the left. The arrowhead indicates the position of the 32 kDa antigen. (B) RT-PCR analysis of theronts (lane 1) and trophonts (lane 2). Total RNA from theronts and trophonts was isolated and reverse-transcribed to generate cDNA. Amplification products (984 bp) were electrophoresed in 1·5% agarose gel and stained with ethidium bromide. DNA size standard markers are shown on the left.

and trophonts and was detected by immunoblot analysis using rabbit and fish antisera, each of which had agglutination/immobilization activity. Vaccination of naïve channel catfish with i-antigen of *I. multifiliis* elicits immunity in fish, prevents infection by *I. multifiliis* in fish, and improves the survival rate against a lethal challenge of infectious parasite (Wang and Dickerson, 2002). Thus, CISA-32 from *C. irritans* could be used in a vaccine therapy in marine aquacultured fish to prevent infection by this parasite.

The *C. irritans* CISA-32 gene sequence suggests that TAA and TAG are used as glutamine codons rather than termination codons, which are often found in ciliate protozoa (Prescott, 1994), although TAG codon was unfortunately not found in *C. irritans* EF-1 α sequences. These non-canonical genetic codes seem to be infrequently used in *C. irritans*. Although the CISA-32 gene is being considered for use in production of recombinant protein for further vaccination trials, these differences in the genetic code make it necessary to change TAA codons in the CISA-32 gene to CAA or CAG (universal glutamine codons) using DNA mutation techniques, as was the case for the production of i-antigen of *I. multifiliis* (Lin *et al*. 2002). In addition, intramolecular sulfhydryl bonds of CISA-32 seemed to be responsible for maintaining the conformation for binding of tiger puffer agglutination/immobilization antibodies, because tiger puffer serum antibodies detected CISA-32 only under non-reducing conditions, but did not detect this antigen under

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reducing conditions in immunoblot analyses. Therefore, it may be necessary to make bacterial hosts express CISA-32 as a native formation of intramolecular sulfhydryl bonds, and it may alternatively be necessary to use other expression systems, as mammalian cells, insect cells or yeast expression systems, for correct intramolecular folding of recombinant CISA-32.

The CISA-32 gene product is thought to be a GPI-anchored protein that may have extracellular functions in *C. irritans*. This gene has some homology with other genes: *Paramecium tetraurelia* 51A surface protein (GenBank: 159973), *Paramecium primaurelia* G surface protein (GenBank: 3452505) and *Tetrahymena thermophila* SerH3 immobilization antigen (GenBank: 102396), according to the Blast Search program. The gene also encodes a motif similar to the periodic repeated motif of *Paramecium* spp., according to the SMART program (Schultz *et al*. 1998). Repeated motifs from the above surface antigen sequences are delineated by periodic cysteine residues (Nielsen *et al*. 1991; Doerder, 2000). The *I. multifiliis* i-antigen contains 4 repeated motifs (Clark *et al*. 1992); however, CISA-32 does not have any such repeated motif, even though it contains 12 cysteine residues throughout its sequence (CX_3-CX_{40}-CX_{24}-CX_6-CX_{53}-CX_{26}-CX_2-CX_{33}-CX_9-CX_{14}-CX_{34}-CX_{32}). The surface antigens from *Tetrahymena* and *Paramecium* spp. are GPI-anchored proteins (Azzouz *et al*. 1990; Ko and Thompson, 1992) that are switched to alternative variants in response to environmental conditions such as temperature (Caron and Meyer, 1989; Smith *et al*. 1992). However, their functions in ciliates are not well known. *C. irritans* may also express different antigens on the surface in response to changing environmental conditions, although the same antigen was expressed during both the theront and trophont stage. Therefore, further investigation will be necessary to develop CISA-32 for use as a vaccine to prevent infection of this parasite in aquacultured fish.

In conclusion, this study demonstrates that *C. irritans* expresses a 32 kDa immobilization/agglutination antigen on its surface. This antigen, CISA-32, is recognized by the tiger puffer immune system and may be useful for vaccine development. We are currently working to develop recombinant CISA-32 as a vaccine using several kinds of hosts for lethal challenges. This DNA recombinant technique will make it possible to produce a large amount of vaccine with an easily cultured organism.

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