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A C-type lectin from the Tunicate, *Styela plicata*, that modulates cellular activity

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Abstract

Previous studies have identified proteins from tunicates (invertebrate members of the Phylum Chordata) that have physicochemical and functional properties similar to those of the inflammatory cytokine, interleukin 1 (IL-1). Here we characterize one of those proteins from the tunicate, *Styela plicata*, that can stimulate tunicate and mammalian cell proliferation, activate phagocytosis, increase interleukin 2 (IL-2) secretion by mammalian peripheral blood mononuclear cells and enhance IL-2 receptor (IL-2R) expression by mammalian EL-4.IL-2 cells. Partial amino acid sequence data showed that the *S. plicata* protein resembles three C-type lectins (TC14, TC14-1 and TC14-2) from a closely related tunicate species, *Polyandrocarpa misakiensis*. Its similarity to carbohydrate recognition domains (CRDs) from *P. misakiensis* lectins suggests that the *S. plicata* protein modulates the activities of mammalian immunocompetent cells by interacting with carbohydrate moieties of glycosylated cell surface receptors.

Keywords: Tunicata (Ascidiacea); Phagocytosis; Thymocyte proliferation; C-Type lectins; Mitogen; Cytokine; Interleukin; *Styela plicata*

1. Introduction

A number of molecules that can modulate cellular immune functions have been identified in invertebrates (Beck and Habicht, 1991; Beck et al., 1989a). These include: plasmocyte depletion factor from the waxmoth, *Galleria mellonella* (Chain and Anderson, 1982, 1983); haemokinins from the moths, *Samia cythia*, *Anthergea polyhemus* and *Hyalophora cercopia* (Cherbas, 1973); encapsulation-promoting factors from the insects, *Heliothis virescens* (Chain and Anderson, 1983); *Heliothis zea* (Schmit, 1977); phagocytosis stimulating factor from *G. mellonella*; sea star factor from *Asteria rubens* (Prendergast and Liu, 1976); opioid homologues and other neuropeptides from mollusks (Stefano et al., 1989a,b, 1993) and inflammatory elicitors and cytokine-like molecules from tunicates (invertebrate members of the phylum Chordata), mollusks and echinoderms (Beck

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and Habicht, 1986, 1996; Beck et al., 1989a,b; Granath et al., 1994; Hughes et al., 1990, 1991; Ottaviani et al., 1993).

Blood cells from a variety of invertebrates can also respond to vertebrate cytokines, including interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 6 (IL-6) and tumor necrosis factor (TNF), and to other stimulatory factors such as opioid neuropeptides (Connors et al., 1995; Magazine et al., 1996; Ottaviani et al., 1995; Raftos et al., 1991a,b; Stefano et al., 1989a,b). This suggests that cytokine receptors and by corollary endogenous cytokines, are expressed by invertebrates.

It has also been demonstrated that some invertebrate proteins can affect mammalian cells in ways that mimic IL-1 (Beck and Habicht, 1986; Beck et al., 1989a,b; Prendergast and Suzuki, 1970; Raftos, 1996). For instance, low molecular weight (15–20 kDa) humoral molecules from tunicates can enhance the proliferation of mouse thymocytes and L929 fibroblasts (Raftos, 1996; Beck et al., 1989b). The mitogenic tunicate proteins stimulate IL-2 secretion and IL-2 receptor (IL-2R) expression by mammalian lymphocytes, as well as increasing the vascular permeability of rabbit skin (Raftos, 1996; Beck et al., 1989b). In tunicates, these low molecular weight proteins can stimulate the chemotactic activity of hemocytes (blood cells), enhance cell proliferation and opsonize target cells for phagocytosis (Beck et al., 1993; Kelly et al., 1992, 1993; Raftos et al., 1991a, 1998).

The tunicate proteins also share a number of physicochemical characteristics with IL-1. They are of comparable molecular weight (approx. 17 kDa) and have isoelectric points that are similar to those of IL-1α and IL-1β. Moreover, polyclonal antibodies to mammalian IL-1 cross-react with a variety of invertebrate proteins and can inhibit the biological activities of the tunicate regulatory molecules (Beck et al., 1986, 1989a,b; Beck and Habicht, 1986; Hughes et al., 1990, 1991).

Despite the functional, physicochemical and serological similarities between tunicate regulatory proteins and IL-1, recent evidence suggests that the tunicate molecules do not gain their effects on mammalian cells via known IL-1 receptors. The binding of antibodies to IL-1 receptors can be blocked by IL-1α and IL-1β, but not by the tunicate regulatory proteins (Raftos, 1996). Moreover, the tunicate proteins have not been sequenced so that their evolutionary relationships to IL-1 remain speculative.

Here, we present a partial amino acid sequence analyses of a 14-kDa protein from the tunicate, *Styela plicata,* that enhances cell proliferation, phagocytosis, IL-2 secretion and IL-2R expression. These data suggest that the protein is a C-type lectin and is not related to IL-1.

2. Experimental procedures

2.1. Tunicates and hemolymph harvesting

S. plicata were collected from Sydney Harbor, New South Wales, Australia. Prior to experimentation, tunicates were held for up to 5 days in refrigerated glass aquaria (60 l, 13°C).

When required for protein purification, hemolymph was collected from incisions in the buccal siphon into chilled polystyrene centrifuge tubes without dilution. Hemocytes were then removed by centrifugation ($50 \times g$, 4°C, 7 min) and protease inhibitor (phenylmethylsulfonyl fluoride, PMSF, 5 mM) was added to the resulting serum. The serum was filtered (glass fiber and 0.22 μm) and stored frozen (−80°C) for up to 1 week.

To collect hemocytes for opsonization assays, hemolymph was harvested into a 3-fold excess of ice-cold filtered seawater (FSW, 0.45 μm filter) and centrifuged ($200 \times g$, 5 min, 4°C). The supernatants were then removed and the hemocytes re-suspended in FSW.

2.2. Fractionation of serum proteins

S. plicata serum was concentrated 50 fold by ultrafiltration through a YM-10 membrane (Amicon, Davers, MA) and filter sterilized (0.45 μm). Five hundred microliters of the concentrated serum were loaded onto a Highload 16/60 gel filtration column (Pharmacia, NSW, Australia) fitted to a Waters 625 HPLC control unit (Waters Scientific, NSW, Australia). The column was calibrated with molecular weight standards (aprotinin: 6.5 kDa; cytochrome *c*: 12.4 kDa; carbonic anhydrase: 29 kDa; bovine serum albumin: 66 kDa) and equilibrated with phosphate buffered saline (PBS, 150 mM NaCl, 10 mM phosphates, pH 7.0). Proteins were eluted with PBS at a flow rate of

0.5 ml/min. Elution was monitored at 214 and 280 nm and 0.5 or 1 ml fractions were collected, filter-sterilized (0.45 μm) and stored at −80°C. The protein content of fractions was determined by the Bradford assay using bovine serum albumin (BSA) to generate standard curves (Bio-Rad, Hercules, CA).

2.3. Opsonization assay

The ability of *S. plicata* serum and fractionated serum proteins to enhance the phagocytic activity of hemocytes was quantified by the method of Cooper et al. (1996). Two hundred microliters of hemocyte suspensions (3×10^6 hemocytes per ml in FSW) were cultured (15°C) on autoclaved glass microscope coverslips (22 × 22 mm) for 1 h in a humidified chamber. Adherent hemocytes were then washed with 400 μl FSW before being overlaid with 50 μl target cell (yeast) preparations. Non-ingested target cells were removed by extensive washing with FSW after a 30-min incubation (15°C). The percentage of hemocytes that had ingested at least one target cell was then determined microscopically.

Data are presented as phagocytic stimulation indexes (PSI), which were calculated as the percentage of hemocytes that had ingested at least one target cell opsonized with serum or fractionated serum proteins, divided by the mean percentage of hemocytes that had ingested at least one target cell that had been incubated with FSW rather than serum or fractionated serum proteins.

Target cells were prepared by autoclaving yeast (*Saccharomyces cerevisciae*, Baker's yeast type II, Sigma Chemicals, 5×10^7 cell/ml in FSW) in the presence of 0.4% w/v congo red for 15 min (120°C). The congo red stained yeast were then washed extensively with FSW and stored frozen (−20°C, 5×10^6 yeast/ml). One hundred microliter aliquots of yeast were incubated with *S. plicata* serum, fractionated serum proteins or FSW for 1 h (room temperature, shaking). The yeast was then washed twice through FSW (1 ml per wash), resuspended in 100 μl FSW and overlaid onto adherent hemocytes. In some cases, serum or affinity chromatography fractions were pre-incubated with ethylendiamine tetraacetic acid (EDTA) or a range of carbohydrates for 15 min before being added to yeast.

2.4. Mouse thymocyte, L929 fibroblast and tunicate cell proliferation assays

Tunicate pharyngeal explant cultures were established in tunicate tissue culture medium (T-RPMI) by the methods of Raftos et al. (1991a). T-RPMI contained 454 mg/l RPMI 1640 powder (with L-glutamine, without sodium bicarbonate, Sigma Chemicals, St Louis, MO) in FSW, 10^5 units penicillin sulfate/l and 100 mg streptomycin sulfate/l.

Thymocytes (2×10^6 cells/ml in RPMI-1640 containing 10% v/v fetal calf serum) were prepared from 4 to 6-week-old Balb/c female (Raftos, 1996). L929 fibroblasts were kindly supplied by the Bill Walsh Cancer Centre (Royal North Shore Hospital of Sydney, Australia) and cultured by the method of Raftos (1996).

Pharyngeal explants (1 explant/well), thymocytes (4×10^5 cells/well) and fibroblasts (4×10^5 cells/well) were cultured in 96-well flat-bottomed tissue culture plates (Costar) in fetal calf serum-free media containing putative mitogens or combinations of mitogens. Mitogens included: *S. plicata* serum or fractionated serum proteins; mouse macrophage culture supernatants; and concanavalin A (Con A, Sigma Chemicals). Supernatants from mouse macrophages were prepared using resident peritoneal cells harvested from five Balb/c female mice by peritoneal lavage (Raftos, 1996).

[^{3}H]Thymidine (18.5 MBq, 740 Bq/ mmol, ICN Radiochemicals, Costa Mesa, CA) was added to each well for the last 18 h of a 72-h culture period. Thymocytes and trypsinized fibroblasts were harvested onto glass fiber filters with an automated cell harvester prior to liquid scintillation counting. Pharyngeal explants were harvested and their incorporated radioactivity measured by the method of Raftos (Raftos, 1996).

2.5. Expression of IL-2 receptors by EL-4.IL-2 cells

EL-4.IL-2 mouse lymphoma cells were purchased from the American Type Tissue Culture Collection (ATCC No. TIB 181, Rockville, MD) and cultured in Dulbecco's Modified Eagles Medium (DME, Sigma Chemicals) containing 4.5 g/l glucose and 10% v/v heat-inactivated normal horse serum (NHS). To test for IL-2R expression,

sub-confluent EL-4 cells were harvested, washed twice in DME without NHS and transferred to 24-well tissue culture plates (2×10^6 cells in 500 μl DME without NHS per well). Cells were cultured in the presence of 20 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Chemicals) and varying concentrations of either *S. plicata* gel filtration fractions or human recombinant IL-1β (Collaborative Research, Bedford, MA). After 20 h, cells from individual wells were harvested, washed twice ($16\,000 \times g$, 6 s, 4°C) in PBS and blocked for 30 min (4°C with agitation) in PBS supplemented with 0.5% w/v BSA and 0.02% w/v sodium azide (PBS-BSA). The cells were then incubated (30 min, 4°C, with agitation) in 100 μl of rat anti-IL-2 type I receptor antibody (25 μg/ml in PBS-BSA, Genzyme, Cambridge, MA). After two washes through 1 ml PBS-BSA, cells incubated (30 min, 4°C, with agitation) in 100 μl fluorescene isothiocyanate (FITC)-conjugated rabbit anti-rat IgG (diluted 1:50 in PBS-BSA). Finally, the cells were washed thrice through PBS without BSA, fixed in 1.0% w/v paraformaldehyde in PBS and analyzed on a Becton Dickinson FACscan flow cytometer fitted with an argon-ion laser tuned to 488 nm (Mountain View, CA) to determine the mean fluorescence intensities of anti-IL-2R staining.

2.6. Quantification of IL-2 secretion by human mononuclear cells

Fresh, heparinized human peripheral blood was diluted 1:1 with RPMI-1640 tissue culture medium. Peripheral blood mononuclear cells were then isolated by density centrifugation through Mono-Poly resolving medium (Flow Laboratories, McLean, VA). Adherent mononuclear cells were removed by passage over a 6-ml nylon wool column. The remaining non-adherent peripheral blood mononuclear cells (PBMNC) were washed thrice in RPMI-1640 and finally resuspended to a density of 2×10^6 cells/ml. Two hundred microliters of PBMNC were added per well to 96 well tissue culture plates. Various concentrations of *S. plicata* gel filtration fractions or hrIL-1 and Con A (0.3 μg/ml) were then added to the cells. Cells were cultured for 48 h before the supernatants were removed, centrifuged ($16\,000 \times g$, 6 s, 4°C), filtered (0.22 μm syringe tip filters) and tested for the presence of IL-2 using an IL-ISA 2 ELISA kit according to the manufacturer's instructions (Collaborative Research, Bedford, MA). Standard curves were generated over a concentration range of 1.5–100.0 BRMP units IL-2/ml (Raftos, 1996).

2.7. SDS-PAGE, Western blotting and amino acid sequencing

Fractionated serum proteins were analyzed by SDS-PAGE according to the method of Ausubel et al. (1989). Proteins were reduced in sample buffer containing 10 mg/ml dithiothreitol and separated through 15% gels. Gels were either silver stained by the sodium thiosulfate method of Blum et al. (1987) or subjected to Western blotting for amino acid (aa) sequencing.

Proteins were Western blotted to polyvinyl difluoride (PVDF, Trans-Blot, BioRad) membranes using CAPS transfer buffer by the method of Aebersold et al. (1987). Blotted proteins were stained with Coomassie Blue R-250 (Sigma Chemicals) and the relevant bands were cut from the membranes. PVDF bound proteins, and peptides isolated from tryptic digests of nitrocellulose bound proteins (Aebersold et al., 1987), were sequenced by Macquarie University Centre for Analytical Biochemistry with an HP G100A sequencer using routine 3.1 or 3.1 PVDF chemistries (Hewlett-Packard, NSW, Australia). Short N-terminal aa sequence tags were also obtained from HPLC isolated proteins that were blotted onto polybrene membranes.

Amino acid sequences were compared to protein databases using the Blast and Fast3 algorithms via the Australian National Genomic Information Service or the Expasy molecular biology server (http://www.expasy.ch/). The ClustalW algorithm was used to align tunicate aa sequences with those of other proteins.

2.8. Reversed phase HPLC

Gel filtration fractions were acidified with 0.25% v/v trifluoroacetic acid (TFA). Two hundred microliter aliquots were then loaded onto a C-18 microbore reversed phase HPLC column (Waters Scientific) equilibrated with 0.1% v/v TFA. Proteins were eluted using a linear 100-min gradient (0–80%) of acetonitrile containing 0.09% v/v TFA. Elution was monitored at 214 nm. The sensitivity of the detector was determined using known concentrations of proteins (carbonic anhy-

drase and ribonuclease A) that had approximately the same molecular mass as the biologically active proteins in serum fractions.

2.9. Chromatofocusing

Gel filtration fractions were dialyzed against 25 mM triethanolamine (pH 8.3) before 500 μl aliquots were loaded onto a Mono P HR 5/5 chromatofocusing column (Pharmacia, Uppsala, Sweden). The proteins were bound to the column in 25 mM triethanolamine (pH 8.3) for 10 min and eluted with a 60-min linear gradient of Polybuffers 76 and 94 (pH 5.0) according to the manufacturer's instructions (Pharmacia). Elution was monitored at 214 nm and protein peaks were collected and dialyzed against PBS. The column was calibrated with proteins of known isoelectric point.

2.10. Statistical analysis

Statistical analyses were performed with the Microsoft Excel software package. Differences between mean values were analyzed using Student's two-tailed *t*-test where $P < 0.05$ were considered significant.

3. Results

3.1. S. plicata serum stimulates thymocyte proliferation and phagocytosis

Fig. 1a shows that non-fractioned *S. plicata* serum enhanced the proliferation of mouse thymocytes. Significantly increased [^{3}H]thymidine incorporation, relative to cells cultured without serum ($P < 0.05$), was evident when thymocytes were with co-incubated with as little as 1:500 *S. plicata* serum and 1 μg/ml Con A (Fig. 1a). The concentration of Con A used was sub-mitogenic in the absence of *S. plicata* serum ($P > 0.05$ vs. thymocytes in RPMI alone). Thymocyte proliferation peaked at a 1:200 dilution of serum and then declined at higher concentrations. No alteration of thymidine uptake was evident when thymocytes were incubated in tunicate serum without Con A. The levels of thymidine uptake that could be achieved with tunicate serum were similar to those elicited by mouse macrophage conditioned media, which was used here as a positive control.

Fig. 1b demonstrates that opsonization of target cells with *S. plicata* serum enhanced the phagocytic activity of tunicate hemocytes. Significantly, increased phagocytosis was evident when target cells were incubated with as little as 20% v/v serum ($P < 0.05$ vs. non-opsonized controls). Phagocytosis reached an asymptote at 60% v/v serum. At this concentration, phagocytic activity was 2.25-fold that evident in non-opsonized controls.

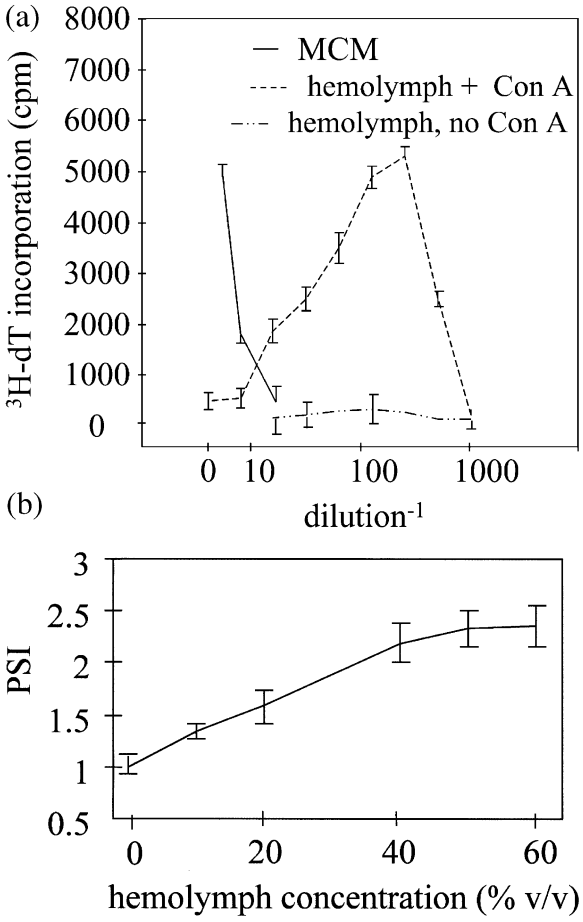

Fig. 1. (a) The effects of various concentrations of *S. plicata* serum (with or without 1 μg/ml Con A) and mouse macrophage conditioned media (MCM) on the uptake of [^{3}H]thymidine by mouse thymocytes. [^{3}H]Thymidine uptake by thymocytes incubated in 1 μg/ml Con A alone was 353 ± 119 cpm. Bars represent standard errors ($n = 4$). (b) The effect of opsonization with varying concentrations of *S. plicata* serum on the ingestion of yeast by *S. plicata* hemocytes (phagocytic stimulation indexes, PSI). Bars represent standard errors ($n = 4$).

3.2. Fractionated S. plicata serum proteins stimulate phagocytosis and thymocyte proliferation

Analysis of serum fractions obtained by gel

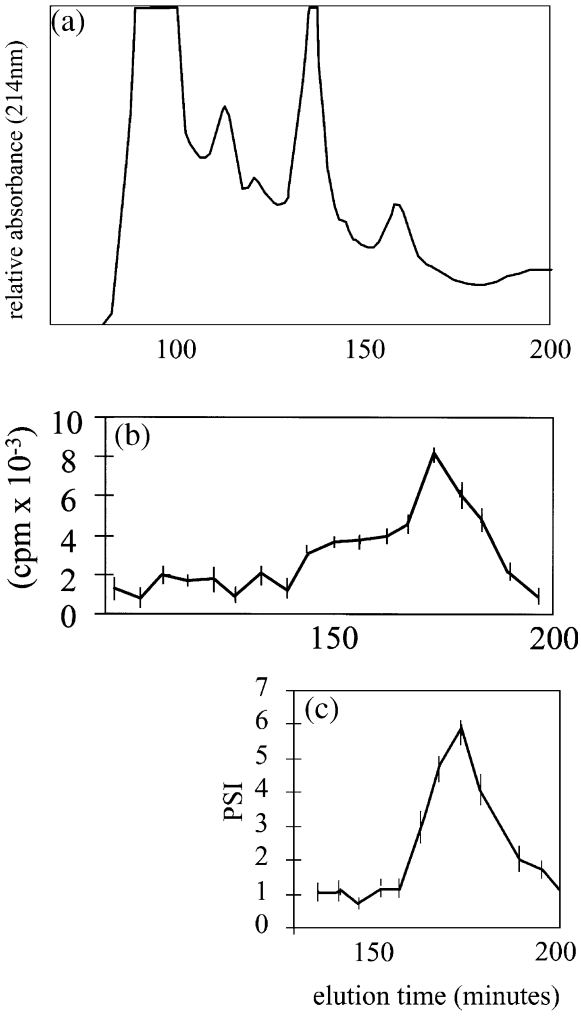

Fig. 2. Fractionation of serum by gel filtration. (a) Elution profile (relative absorbance at 214 nm) for *S. plicata* serum fractioned by gel filtration. (b) Effect of gel filtration fractions on [^{3}H]thymidine ([^{3}H]dT) uptake by mouse thymocytes co-incubated with sub-mitogenic concentrations of Con A (1 mg/ml). Thymocytes incubated with Con A alone incorporated 1129 ± 135 cpm [^{3}H]dT. Bars represent standard errors ($n = 4$). (c) Effect of gel filtration fractions on the opsonization of yeast for phagocytosis by *S. plicata* hemocytes (phagocytic stimulation indexes, PSI). Bars represent standard errors ($n = 4$).

filtration revealed single overlapping peaks for opsonic activity and thymocyte proliferation (Fig. 2). Analysis of seven separate gel filtration experiments revealed that both maximum phagocytic activity (PSI = 5.4 ± 1.8, $P < 0.05$ vs. controls without fractionated protein) and [^{3}H]thymidine incorporation (7940 ± 380 cpm, $P < 0.05$ vs. controls without fractionated protein) was elicited by fractions collected 174 ± 2 min after loading. Interpolation of a standard curve generated with molecular mass standards indicated that fractions collected at 174 min contained proteins of approximately 14 kDa.

3.3. SDS-PAGE, reversed phase HPLC and chromatofocusing of fractionated serum proteins

Reducing SDS-PAGE revealed that fractions from seven different gel filtration experiments that elicited maximum phagocytic and mitogenic activity contained a single protein of approximately 14 kDa (Fig. 3a). Fractions from four of these gel filtration experiments, which were also shown to enhance both phagocytosis and thymocyte proliferation (Fig. 3b), were pooled and subjected to reversed phase HPLC and chromato-

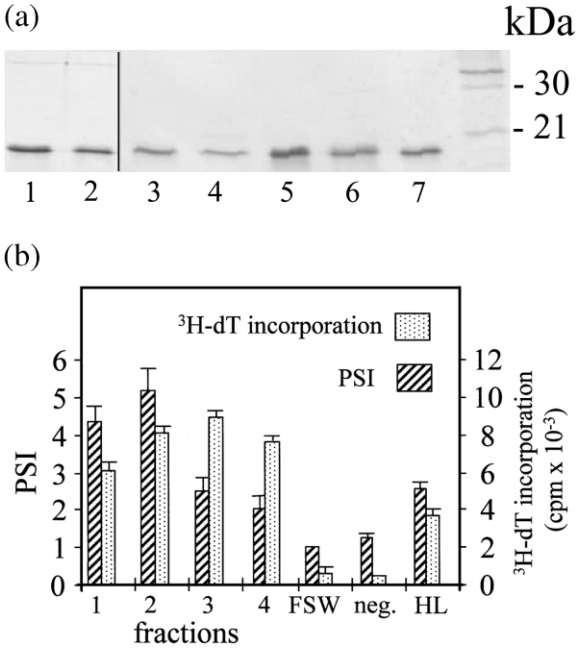

Fig. 3. (a) Silver stained reducing SDS-PAGE of *S. plicata* serum fractions that yielded peak opsonic and mitogenic activity (arbitrarily designated 1–7) from seven gel filtration experiments. Molecular mass markers are shown on the right (kDa). (b) The effect of four of the fractions shown in 3a on the opsonization of yeast for phagocytosis by *S. plicata* hemocytes (phagocytic stimulation indexes, PSI) and on the uptake of [^{3}H]thymidine ([^{3}H]dT) by mouse thymocytes co-incubated with a sub-mitogenic concentration of Con A. [^{3}H]dT uptake by thymocytes incubated in Con A alone was 996 ± 127 cpm. Also shown are the effects of FSW (SW), a gel filtration fraction that did not contain the protein shown in A. (Neg.) and non-fractionated *S. plicata* serum (HL, 50% v/v for opsonization, 1:64 dilution for [^{3}H]thymidine uptake). Bars represent standard errors ($n = 4$).

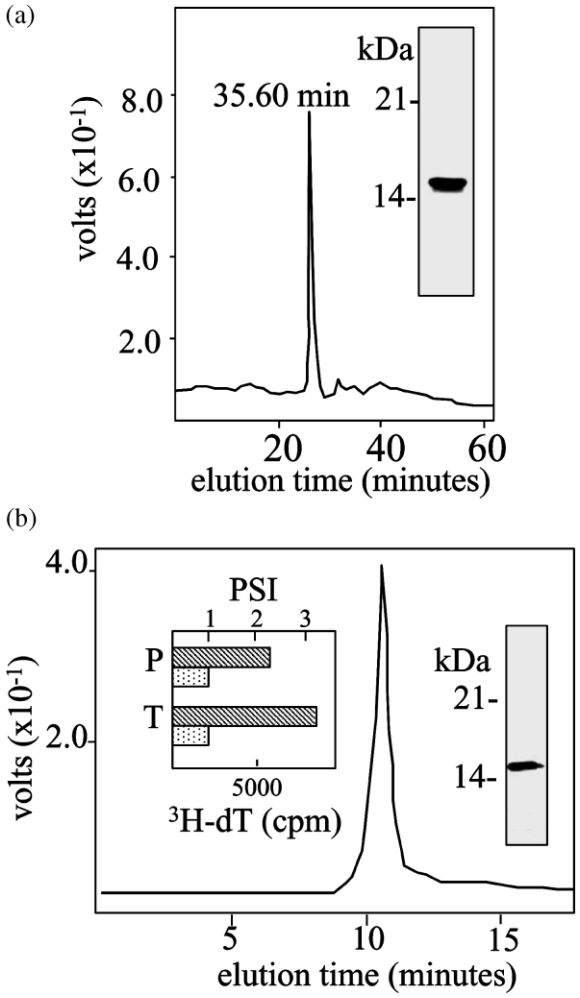

Fig. 4. (a) Reversed phase HPLC of pooled gel filtration fractions that elicited peak opsonic and mitogenic activity. Inset: reducing SDS-PAGE of the peak eluted at 35.6 min with approximate position of molecular mass markers shown on the left (kDa). (b) Chromatofocusing of pooled gel filtration fractions that elicited peak opsonic and mitogenic activity. Insets: reducing SDS-PAGE of the peak eluted at 10.5 min (approximate position of molecular mass markers, kDa, are shown on the left); and opsonic activity (P) and [^{3}H]thymidine (T) uptake by mouse thymocytes (co-incubated with 1 μg/ml Con A) stimulated by the fraction eluted at 10.5 min■ or by FSW■.

focusing. Fractions from the remaining three gel filtration experiments were not included in further analyses because SDS-PAGE showed evidence of degradation (Fig. 3a).

Reversed phase HPLC eluted a single protein peak, which was shown by SDS-PAGE to contain the 14-kDa protein (Fig. 4a). Standardization with known concentrations of carbonic anhydraze or ribonuclease A indicated that the reversed phase HPLC column could detect proteins at concentrations ≥ 50 pM.

Chromatofocusing also eluted a single protein peak that contained the 14-kDa protein (Fig. 4b). Interpolation of a standard curve generated using proteins of known pI indicated that the 14-kDa protein had a pI of approximately 5.5. Chromatofocusing fractions that contained the 14-kDa protein elicited significantly enhanced phagocytosis by tunicate hemocytes and [^{3}H]thymidine incorporation by mouse thymocytes ($P < 0.05$ vs. controls without fractionated protein) (Fig. 4b inset).

3.4. Additional biological activities of fractionated serum proteins

Pooled gel filtration fractions containing the 14-kDa *S. plicata* protein were also found to enhance L929 fibroblast and tunicate pharyngeal explant proliferation, elicit IL-2 secretion by PBMNC and enhance IL-2R expression by EL-4.IL-2 cells. Fig. 5a shows that 100 or 250 ng/ml of the pooled fractions significantly increased [^{3}H]thymidine incorporation by fibroblasts (2 and 2.5 fold increases, respectively) when compared to cultures established without the 14-kDa *S. plicata* protein ($P < 0.05$). The levels of enhanced fibroblast proliferation induced by gel filtration fractions were comparable to those elicited by mouse macrophage condition media.

Substantial IL-2 secretion was evident when PBMNC were incubated in 0.5 or 1.0 μg/ml of the 14-kDa *S. plicata* protein in pooled gel filtration fractions (Fig. 5b). No IL-2 could be detected in supernatants from PBMNC that had not been stimulated with gel filtration fractions. The level of IL-2 secretion that could be elicited by gel filtration fractions was equivalent to that induced by incubation with 10 pg/ml human recombinant IL-1β.

Fig. 5c shows that IL-2R expression was increased by approximately two fold when EL4 cells were incubated with PMA and either 0.5 or 1.0 μg/ml of the pooled gel filtration fractions ($P <$ 0.05 vs. controls incubated in PMA only). A similar level of IL-2R expression was induced by incubating EL4 cells in 1.0 ng/ml human recombinant IL-1β.

The incorporation of [^{3}H]thymidine by *S. plicata* pharyngeal explants was enhanced, in a dose-dependent manner, by concentrations of the 14-kDa protein ranging from 100 to 500 ng/ml

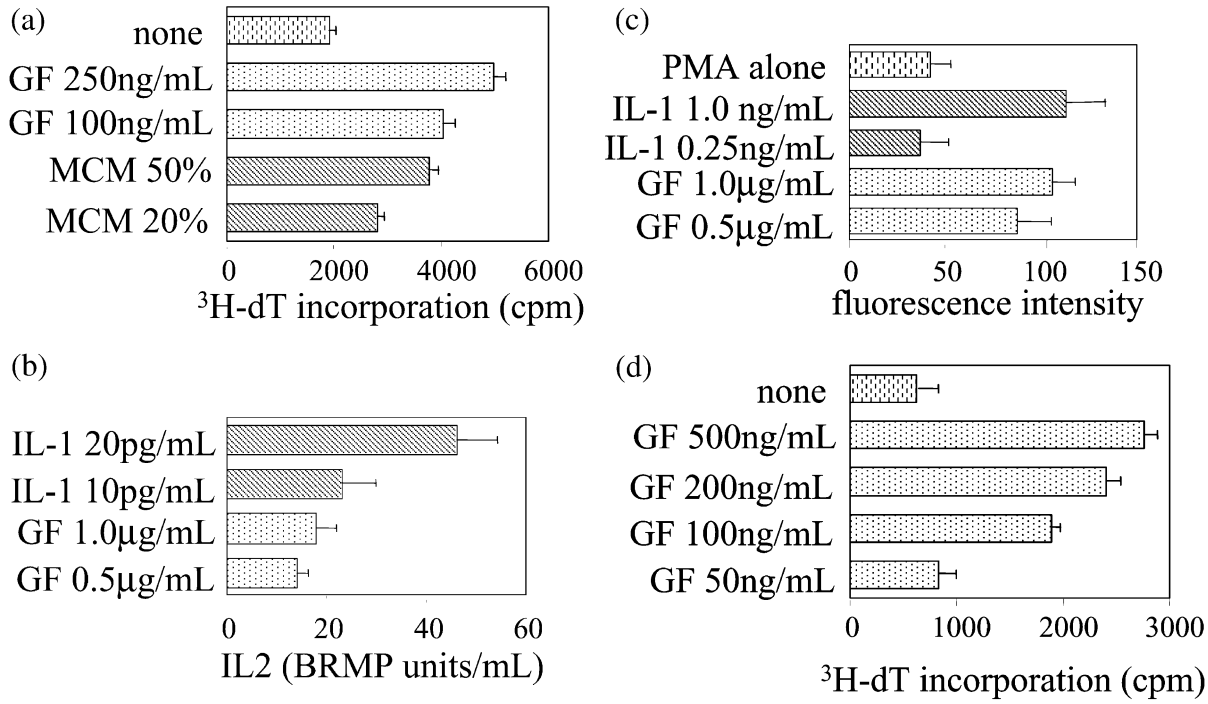

Fig. 5. Effects of pooled gel filtrations fractions containing the 14 kDa *S. plicata* serum protein (GF), mouse macrophage condition media (MCM), or human recombinant IL-1 (IL-1) on: (a) [^{3}H]thymidine ([^{3}H]dT) incorporation by L929 fibroblasts ($n \geq 8$, bars = S.E.M.); (b) IL-2 secretion by EL-4.IL-2 lymphoma cells ($n \geq 4$, bars = S.E.M.); (c) mean fluorescence intensities of EL-4.IL-2 cells co-incubated with 20 ng/ml PMA and stained with rat anti-IL-2R and anti-rat Ig-FITC ($n \geq 4$, bars = S.E.M) or; (d) [^{3}H]thymidine ([^{3}H]dT) incorporation by *S. plicata* pharyngeal explants ($n \geq 9$, bars = S.E.M.).

($P < 0.05$ vs. explants incubated in T-RPMI only). Lower concentrations of the 14-kDa protein (50 ng/ml) did not significantly alter [^{3}H]thymidine uptake by explants, relative to untreated controls ($P > 0.05$).

3.5. EDTA and monosaccharides inhibit the opsonic activity of the 14-kDa S. plicata protein

Fig. 6 shows that EDTA, D-galactose and *N*-acetyl-D-galactosamine (galNAc) significantly inhibited the phagocytic activity elicited by gel filtration fractions containing the 14-kDa protein. The phagocytic activity evident when EDTA, D-galactose and galNAc were co-incubated with pooled fractions and target cells did not differ significantly from negative controls in which target cells were incubated in FSW ($P > 0.05$). None of the other monosaccharides tested (fucose, ribose, D-glucose and *N*-acetyl-D-glucosamine) inhibited the phagocytic activity stimulated by gel filtration fractions ($P > 0.05$ vs. target cells incubated in pooled gel filtration fractions alone).

3.6. Sequence analyses

N-terminal aa sequencing of PVDF blotted gel filtration fractions identified the first 23 aa of the 14-kDa *S. plicata* protein. A sequence tag identical to the first six aa of this sequence was obtained from HPLC isolated protein. In addition, a 33-aa sequence was generated from a tryptic digest of PVDF bound protein. The sequence for the tryptic peptide overlapped exactly with the last seven amino acids of the N-terminal sequence. The 49-aa sequence obtained by overlapping the N-terminal and tryptic peptide sequences is shown in Fig. 7a.

Fast3 and Blast similarity searches of the Swiss-Prot and GenPep databases showed that the *S. plicata* sequence was closely related to three C-type lectins from the tunicate, *Polyandrocarpa misakiensis*. A ClustalW alignment of the *S. plicata* 14-kDa protein with these three lectins from *P. misakiensis* is shown in Fig. 7b. The closest match to the *S. plicata* sequence was with the

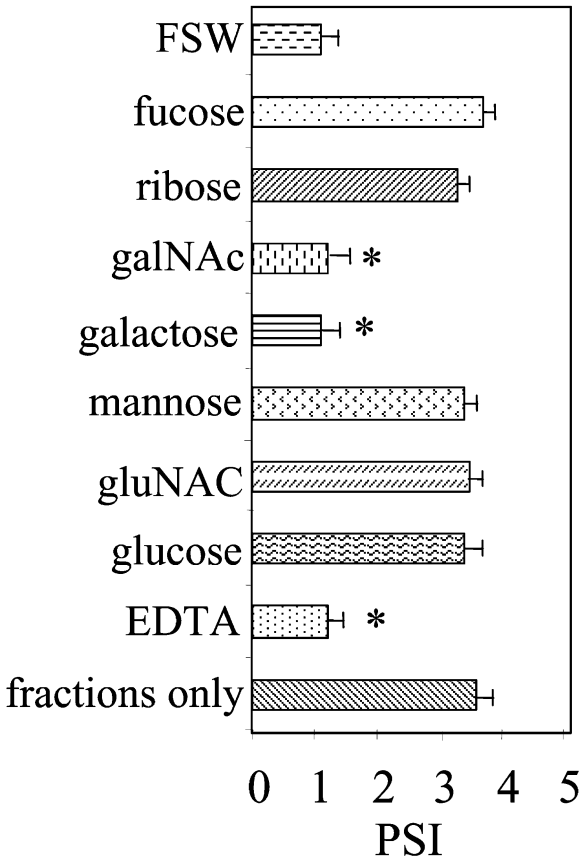

Fig. 6. Phagocytic stimulation indexes for yeast incubated with gel filtrations fractions containing the 14-kDa *S. plicata* serum protein in the presence of various carbohydrates (all 20 mg/ml) or 20 mM EDTA ($n \geq 4$, bars = S.E.M.). Asterisks denote significant differences from yeast incubated in gel filtrations fractions containing the 14-kDa *S. plicata* serum protein without carbohydrates or EDTA ($P < 0.05$).

N-terminal carbohydrate recognition domain of a defensive lectin, TC14, from *P. misakiensis* (accession no. P16108) (Suzuki et al., 1990). TC14 and the *S. plicata* protein shared 44.9% identity in a 49-aa overlap, whilst a further 22.4% of residues represented conservative substitutions that did not alter the charge or polarity of the residue. Similar levels of homology were evident between the *S. plicata* protein and the other two *P. misakiensis* lectins that are closely related to TC14, TC14-1 (42.8% identity with *S. plicata* protein, 49 aa overlap) and TC14-2 (46.2% identity, 39 aa overlap) (Kawamura et al., 1991).

Far lower levels of aa identity were evident between the *S. plicata* 14-kDa protein and other C-type lectins of comparable molecular weight and carbohydrate specificity. For instance, the *S. plicata* protein shared 18.4% identity with a 15-kDa galactose-specific agglutinin (BRA-3) from the acorn barnacle, *Megabalanus rosa* (Takamatsu et al., 1993), 14.2% identity with the 30-kDa α sub-unit of a galactose-specific defensive lectin from the flesh fly, *Sarcophaga peregrina* (Takahashi et al., 1985) and 10.6% identity with a 14-kDa lectin isolated from the cartilage of the reef shark, *Carcharhinus springeri* (Neame et al., 1992) and an 18-kDa galactose-specific defensive lectin (protein A-16) from the African malaria mosquito, *Anopheles gambiae*. Similarly low levels of homology were also evident when the *P. misakiensis* lectins were compared to these other species. For instance, the TC14 from *P. misakiensis* and BRA-3 from *M. rosa* shared only 16.3% aa identity. Despite these low levels of aa identity between the tunicate proteins and lectins from other species, ClustalW analysis of all the lectins described above revealed a ubiquitously conserved cysteine (C^{23} in the *S. plicata* sequence).

Neither Fast3 searches nor ClustalW alignments revealed any significant similarity between the *S. plicata* sequence and either the IL-1α or the IL-1β/IL-1 receptor antagonist (IL-1ra) sub-families (data not shown). The consensus sequence of the IL-1α sub-family (ProDom family no. PD004587) is characterized by seven ubiquitously conserved amino acids within the first 49 residues (S^{1}, Y^{12}, N^{26}, D^{27}, L^{29}, N^{30}, Q^{31}, L^{42}), whilst the IL-1β/IL-1ra sub-family (ProDom family no. PD002536) has three absolutely conserved residues (L^{37}, A^{39}, L^{42}) and five amino acids that are conserved in all but one of available sequences (D^{23}, K^{27}, V^{30}, L/M^{31}, P^{34}). ClustalW aligned none of the conserved residues with the tunicate sequence.

4. Discussion

This study has characterized a low molecular weight humoral protein from the tunicate, *S. plicata,* that exerts a number of modulatory effects on both tunicate and mammalian cells. Whole *S. plicata* serum could opsonize target cells for phagocytosis by *S. plicata* hemocytes and increased mouse thymocyte proliferation. Gel filtration of *S. plicata* serum yielded single, precisely overlapping peaks for both enhanced phagocytosis and thymocyte mitogenesis, and SDS-PAGE showed that the fractions yielding peak biological

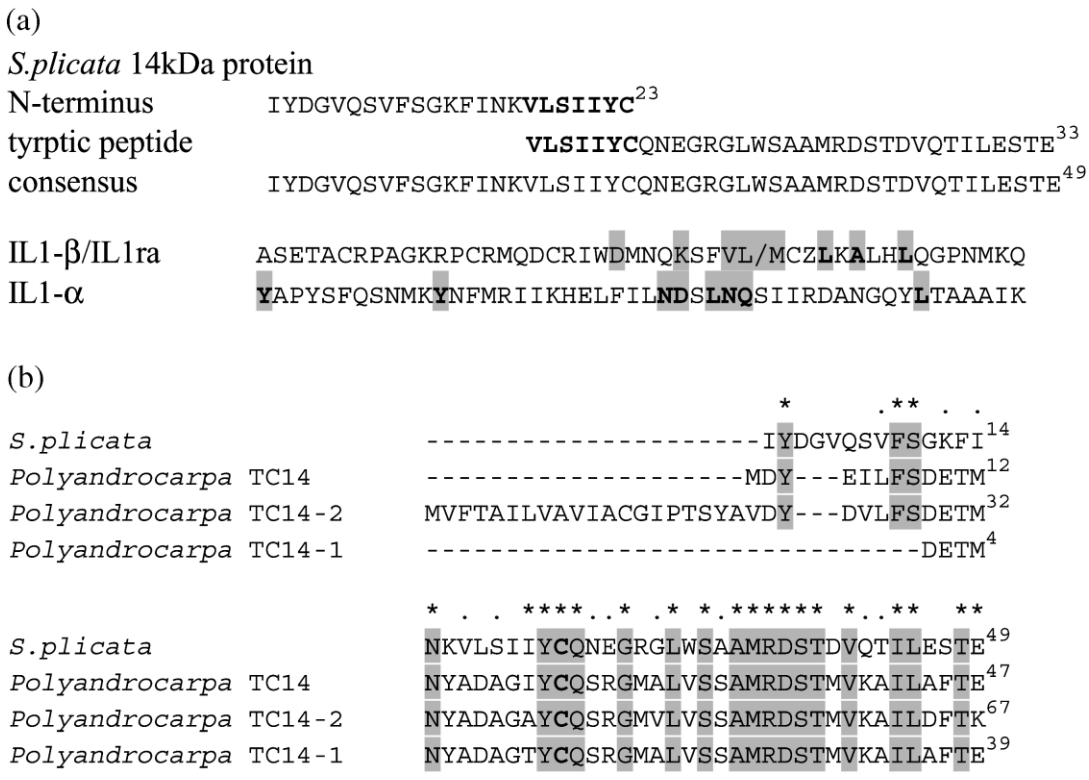

Fig. 7. (a) Amino acid sequences of the N-terminus of the *S. plicata* 14-kDa protein, a tryptic peptide from the *S. plicata* 14-kDa protein, the 49 aa N-terminal consensus sequence for the *S. plicata* 14-kDa protein generated by overlapping the N-terminal and tryptic sequences and consensus sequences for the IL-1β/IL-1ra (ProDom family no. PD002536) and IL-1α (ProDom family no. PD004587) sub-families from the Pfam database. Boxed aa are conserved in all but one of the available IL-1β/IL-1ra or IL-1α sub-family sequences. Amino acids in bold are conserved in the N-terminal and tryptic sequences for the *S. plicata* 14-kDa protein, or in all available sequences for the IL-1 sub-families. (b) ClustalW alignment of the *S. plicata* 14-kDa serum protein with three C-type lectins from the tunicate, *Polyandrocarpa misakiensis: Polyandrocarpa* TC14, 14 kDa antibacterial lectin; *Polyandrocarpa* TC14-1 and TC14-2, 14 kDa lectin developmentally expressed lectins. Asterisks denote aa that are conserved between the *S. plicata* sequence and *Polyandrocarpa* TC14, dots denote conservative substitutions between these two sequences that do not alter the charge or polarity of the residue. Boxed residues represent aa that are conserved in all of the sequences and the residue in bold type represents a cysteine that is conserved in all C-type lectins.

activity contained a single protein with a molecular weight of approximately 14 kDa. Gel filtration fractions that contained only the 14-kDa protein could also enhance tunicate pharyngeal cell and L929 fibroblast proliferation, as well as stimulating IL-2 secretion by PBMNC and IL-2R expression on mammalian EL-4 lymphoma cells.

The purity of the 14-kDa protein in gel filtration fractions was confirmed by reversed phase HPLC, which could detect proteins at concentrations of as little as 50 pM. Chromatofocusing also indicated that gel filtration fractions with peak biological activity contained only the 14-kDa protein. Moreover, functional assays of chromatofocusing fractions containing only this protein confirmed its capacity to enhance phagocytosis and thymocyte proliferation.

The biological functions reported here for the 14-kDa *S. plicata* protein correspond closely with those of cytophilic molecules from other tunicates. Two proteins with molecular masses of approximately 17 kDa from the congeneric tunicate, *Styela clava,* can opsonize target cells for phagocytosis and induce mouse thymocyte proliferation (Beck et al., 1989b, 1993). The functional spectrum of the *S. plicata* protein also closely resembles that of vertebrate IL-1. IL-1 is known to enhance thymocyte and fibroblast proliferation and can induce IL-2 secretion and IL-2R expression (Dinarello, 1986; Gery and Lepe-Zuniga, 1984; Schmidt et al., 1982). There is also some evidence that IL-1 increases phagocytosis (Connors et al., 1995). However, our sequence analysis of the 14-kDa protein from *S. plicata* has failed to

reveal any obvious evolutionary relationship with IL-1. N-terminal and internal amino acid sequencing deciphered the first 49 amino acids of the tunicate protein. This may represent up to 50% of the entire protein. ClustalW alignments of this sequence with consensus sequences for the two sub-families of IL-1 (IL-1α and IL-1β/IL-1ra) failed to reveal any significant homology, even though the N-termini of both IL-1 sub-families contain a number of highly conserved amino acids. There seems little possibility that the *S. plicata* sequence is related to IL-1α. The IL-1α sub-family is characterized by numerous absolutely conserved aa within its N-terminus (S^{1}, Y^{12}, N^{26}, D^{27}, L^{29}, N^{30}, Q^{31}, L^{42}) and phylogenetic trees suggest that this sub-family diverged only recently from the IL-1β/IL-1ra lineage.

There are, however, a number possible explanations for the lack of concordance in aa sequence between the tunicate protein and the IL-1β/IL-1ra sub-family. The tunicate protein might represent an IL-1β/IL-1ra domain with an extended N-terminus, so that the limited number of conserved N-terminal residues that are diagnostic of the IL-1β/IL-1ra sub-family (L^{37}, A^{39}, L^{42}) may not have been included in the 49 aa sequence obtained for the *S. plicata* protein. Alternatively, the phylogenetic distance between *S. plicata* and the species used to generate consensus sequences for the IL-1β/IL-1ra sub-family may have been so great that the diagnostic residues are not conserved. This seems unlikely though, because the consensus sequence for the IL-1β/IL-1ra domain includes two sequences from phylogenetically primitive vertebrates (rainbow trout and carp), which are relatively closely related to tunicates. Moreover, the conserved residues appear to be central to the biological functions of IL-1β/IL-1ra.

Whilst further sequencing of the *S. plicata* protein may eventually reveal a relationship with IL-1, the most obvious explanation for the lack of sequence similarity is that the tunicate protein does not share ancestry with IL-1 molecules. In contrast, the aa sequence obtained here for the tunicate protein shows obvious evidence of homology with C-type lectins. The 49 aa sequence for the *S. plicata* protein aligns closely with the carbohydrate recognition domains (CRDs) of three C-type lectins from the tunicate, *P. misakiensis,* which belongs to the same family (Styelidae) as *S. plicata* (Suzuki et al., 1990). Forty five percent of aa was absolutely conserved between the *S. plicata* protein and the CRD of a galactose-specific defensive lectin, TC14, from *P. misakiensis*. Sixty seven percent of residues were either identical or represented conservative aa substitutions. Similar levels of homology were apparent between the *S. plicata* protein and two other lectins from *P. misakiensis* (TC14-1 and TC14-2) that are involved in colonial budding.

Far lower levels of similarity were evident when the *S. plicata* protein was compared to C-type CRDs from other animal groups. However, this limited conservation is not unexpected since the levels of homology of C-type CRDs from different phyla are often extremely low (Drickamer, 1988; Drickamer and Taylor, 1993; Suzuki et al., 1990). Comparisons with other C-type lectins did, however, show that the tunicate protein shares a ubiquitously conserved cysteine (C^{23} in the *S. plicata* sequence). This is one of six cysteines in C-type CRDs that are known to form intrachain disulfide bonds and are critical for carbohydrate binding (Drickamer, 1988). Neither the *S. plicata* sequence nor TC14 have two other N-terminal cysteines that are often found in the long form of the C-type CRD.

Given its obvious relationship with other tunicate C-type lectins, it is relatively easy to re-evaluate the *S. plicata* protein's biological activities. Numerous plant lectins, such as con A, phytohemagglutinin, wheat germ agglutinin and jacalin can stimulate proliferation or other cellular activities in mammals (Drickamer and Taylor, 1993; Kery, 1991; Lis and Sharon, 1986; Raftos, 1994; Vasta, 1991). Similarly, a galactosyl-specific lectin from another tunicate, *Didemnum candidum*, is mitogenic for mouse thymocytes and splenocytes (Vasta et al., 1986). The consensus of opinion is that such effects result from the ability of lectins to bind carbohydrate side chains on glycosylated cell surface receptors (Lis and Sharon, 1986). For instance, the plant lectin, jacalin, can stimulate sub-cellular signaling, intracellular calcium release and cytokine secretion by interacting with carbohydrates on the CD4 antigens of mammalian lymphocytes (Lafont, et al., 1994, 1998). Similar carbohydrate binding activity is evident for the *S. plicata* protein. The target cells used here to test phagocytic activation were washed extensively after incubation the *S. plicata* protein and before they were presented to tuni-

cate phagocytes. Hence, the activation of phagocytosis must have resulted from binding or modification of target cell surfaces by the *S. plicata* protein. Moreover, opsonic activity could be abrogated by EDTA, galactose and galNAc, but not by other monosaccharides, suggesting that the tunicate protein bears a galactose/galNAc-specific, divalent cation-dependent CRD that has the capacity to bind glycosylated cell surface structures.

Even though this interpretation implies that the tunicate protein's ability to modulate the activity of immunocompetent mammalian cells results from chance cross-reactivity, it remains likely that the functions of this molecule in tunicates include defensive reactivity. C-type lectins, particularly those with specificity for galactose, have often been associated with the innate immunological reactions, such as agglutination and opsonization, in both invertebrates and vertebrates (Drickamer and Taylor, 1993; Vasta, 1991).

5. Nomenclature

aa:	Amino acid(s)
CAPS:	3(Cyclohexylamino)-1-propanesulfonic acid
Con A:	Concanavalin A
CRD:	Carbohydrate recognition domain
EDTA:	Ethylenediaminetetraacetic acid
DME:	Dulbecco's modified Eagles medium
FITC:	Fluorescene isothiocyanate
FSW:	Filtered seawater
galNAc:	*N*-Acetyl-D-galactosamine
gluNAc:	*N*-Acetyl-D-glucosamine
[^3H]*dT*:	[^{3}H]Thymidine
HPLC:	High performance liquid chromatography
IL-1:	Interleukin 1
IL-1ra:	Interleukin 1 receptor antagonist
IL-2:	Interleukin 2
IL-2R:	Interleukin 2 receptor
PBS:	Phosphate-buffered saline
PMA:	Phorbol 12-myristate 13-acetate
PMSF:	Phenylmethylsulfonyl fluoride
PSI:	Phagocytic stimulation index
PVDF:	Polyvinylidene difluoride
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TFA:	Trifluoroacetic acid
T-RPMI:	Tunicate tissue culture media

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