

A Short Consensus Repeat-Containing Complement Regulatory Protein of Lamprey That Participates in Cleavage of Lamprey Complement 3¹

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The prototype of the short consensus repeat (SCR)-containing C regulatory protein is of interest in view of its evolutionary significance with regard to the origin of the C regulatory system. Lamprey is an agnathan fish that belongs to the lowest class of vertebrates. Because it does not possess lymphocytes, it lacks Ig and consequently the classical C pathway. We identified an SCR-containing C regulatory protein from the lamprey. The primary structure predicted from the cDNA sequence showed that this is a secretory protein consisting of eight SCRs. This framework is similar to the α -chain of C4b-binding protein (C4bp). SCR2 and -3 of human C4bp are essential for C4b inactivation, and this region is fairly well conserved in the lamprey protein. However, the other SCRs of this protein are similar to those of other human C regulatory proteins. The lamprey protein binds to the previously reported lamprey C3b/C3bi deposited on yeast and cleaves lamprey C3b-like C3 together with a putative serum protease. The scheme resembles the C regulatory system of mammals, where factor I and its cofactor inactivate C3b. Unlike human cofactors, the lamprey protein requires divalent cations for C3b-like C3 cleavage. Its artificial membrane-anchored form protects host cells from lamprey C attack via the lectin pathway. Thus, the target of this protein appears to be C3b and/or its family. We named this protein Lacrep, the lamprey C regulatory protein. Lacrep is a member of SCR-containing C regulators, the first of its kind identified in the lowest vertebrates. *The Journal of Immunology*, 2004, 173: 1118–1128.

Third component of C is a major molecule of C activation (1). Two distinct C3 convertases (C4b2a and C3bBb) are formed by similar enzymatic processes in different pathways. These convertases have a specific protease activity toward C3 and lead to the activation of C3 fragment, named C3b (1). The activated C3b, in turn, covalently binds through its acyl residue in the thioester group to specific residues of the foreign molecules. This amplification reaction leads to C-mediated cytotoxicity and opsonization. Host cells, in contrast, are protected from targeting by autologous C (2). Therefore, there are mechanisms that protect the host cells from autologous C attack (2). It is generally accepted that C3-step C reg-

ulatory proteins as well as C9-step regulation by CD59, etc., play important roles in the regulation of the C activation (2). The supporting results have been obtained mainly in humans and rodents.

In humans, two soluble proteins, factor H and C4b-binding protein (C4bp),⁷ and four membrane proteins, CR1 (CD35), CR2 (CD21), decay-accelerating factor (DAF; CD55), and membrane cofactor protein (Mcp; CD46), have been identified as C regulatory proteins (1–4). The genes that encode C regulatory proteins cluster on the long arm of chromosome 1 at 1q32 (3–5). This region is called the regulator of C activation (RCA) gene cluster (4–6). The RCA locus is conserved in mice, although the locus splits into two segments (7). The RCA family proteins are composed of multiple copies of tandem structural units termed short consensus repeats (SCRs). Each SCR consists of 60–70 aa, including four highly conserved cysteines (3, 4). The cysteines are disulfide-bonded, holding the SCRs in a rigid triple-loop structure, resulting in similar three-dimensional structures.

From an evolutionary point of view, a nonmammalian, membrane-anchored, C regulatory protein (8) was first identified in the chicken: chicken C regulatory membrane protein bears structural and functional similarity to mammalian Mcp and DAF (9). A nonmammalian soluble-form of C regulator named sand bass protein 1 (SBP1) has been cloned from a bony fish, *Parablax nebulifer* (sand bass) (10), but no such regulator has been identified to date in invertebrates. Sand bass SBP1 is known to be a structural homologue of human factor H (10, 11). SBP1 consists of 17 tandem SCRs

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⁷ Abbreviations used in this paper: C4bp, C4b-binding protein; CHO, Chinese hamster ovarian tumor; CYT, cytoplasmic tail; DAF, decay-accelerating factor (CD55); GVB²⁺, gelatin veronal buffer; huMcp, human Mcp; laC3, lamprey C3; Lacrep, lamprey C regulatory protein; MASP, mannose-binding lectin-associated serine protease; Mcp, membrane cofactor protein (CD46); OG, Oregon Green 488 iodoacetamide; ORF, open reading frame; PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2; RCA, regulator of C activation; SBP1, sand bass protein 1; SCR, short consensus repeat; SH, Src homology; TM, transmembrane.

and binds to both rainbow trout C3b and human C4b (11), serving as cofactor for factor I (11). In its structure and function, SBP1 resembles human factor H, which possesses the ability to inactivate C4b as well as C3b (12). Therefore, mechanisms of C3 inactivation in the C system appear to be conserved across mammals and fish. However, whether the C3 inactivation system is conserved in a jawless fish, *Lampetra japonica* (lamprey), remains unclear (13, 14). Lamprey possesses a C3-like molecule that opsonizes rabbit erythrocytes (13, 14), but the C regulatory proteins, the RCA of higher vertebrates, have not been identified in the lamprey.

In this study we report the identification of a protein in lamprey that consists of eight SCRs and protease cofactor activity for the cleavage of lamprey C3 (C3b-like C3). Structural and functional analyses of this lamprey SCR protein were performed.

Materials and Methods

Ab, cells, proteins, and plasma

Fresh lamprey and human plasma and serum were obtained from each species by standard methods (13, 15). All samples were immediately stored at -80°C until use. Anti-lamprey C3 polyclonal Ab (laC3 Ab) was raised in rabbits by injecting lamprey serum-treated zymosan (13). Chinese hamster ovarian tumor (CHO) cells were obtained from American Type Culture Collection (Manassas, VA). RK13 cells (derived from the rabbit kidney) were obtained from RIKEN Cell Bank (Wako Pure Chemicals, Saitama, Japan). CHO cell clones expressing human Mcp (CD46; CHO/huMcp) were established in a previous study (15). CHO cells were maintained in Ham's F-12/10% FCS, and RK13 cells were maintained in DMEM/10% FCS. These cells were transfected with expression vectors. Lamprey proteins were harvested from the culture supernatant and cell lysate as described previously (9). For RNA and protein blot analysis, total RNA and proteins were obtained from various lamprey tissues and stored at -80°C until use.

Lamprey C3 was purified as described previously (13, 16) and was converted to a C3b-like molecule by incubation with 100 μM methylamine (Wako Pure Chemicals) for 1 h at 37°C in PBS, pH 7.5. To label the Src homology (SH) residue of ^{973}Cys (16), which becomes exposed by the rupture of the thioester bond of laC3 (C3b-like C3) (Y. Kimura and T. Seya, unpublished observation), Oregon Green 488 iodoacetamide (OG; Molecular Probes, Eugene, OR), as previously reported for labeling of human C3b (17), was used. More than 70% of the C3b-like C3 was labeled according to the manufacturer's formula.

Screening a lamprey cDNA library

A lamprey liver cDNA library in pBluescript II SK⁺ at the *EcoRI* site was made from the lamprey hepatopancreas as reported previously (16, 18). A partial lamprey SCR protein (Lacrep; accession no. AB061219) cDNA fragment was obtained by nested PCR using degenerated primers, the vector T3 and SK primers (first and second primers, respectively, in Table I) as previously described (9). A degenerated primer was designed from the junctional sequence of SCR3 and SCR4 of human Mcp (CD46) as described previously for molecular cloning of mouse Mcp (19). The sets of primers used in this study are shown in Table I. The first PCR was performed as follows: denaturation at 94°C for 2 min, followed by denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and polymerization at 72°C for 90 s (typically 20 cycles) with the degenerate and T3 vector primer. In the second PCR, 1 μl of the first PCR product was added to a PCR mixture containing the internal vector primers (Table I), and nested PCR was performed with the degenerate and SK vector primer under the same conditions described above, but for 35 cycles. The PCR products were cloned into the vector pCR-2.1 (Invitrogen Life Technologies, San Diego, CA) and sequenced using ABI 310 DNA autosequencer.

The sequence of the full-length cDNA including the 3'-untranslated region of the lamprey protein, Lacrep, was determined by consecutive

rounds of PCR. The complete Lacrep sequence was confirmed by sequencing 12 RT-PCR products from lamprey liver mRNA.

Tissue RNA blotting analysis

Total RNAs (20 μg) were extracted from various lamprey tissues using TRIzol reagent (Invitrogen Life Technologies) and separated by electrophoresis on 1.0% (w/v) agarose gel. RNAs were transferred onto a Hybond N⁺ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The blot was prehybridized for 30 min at 68°C and hybridized with ^{32}P -labeled, full-length Lacrep open reading frame (ORF) as a probe for 1 h at 68°C in ExpressHybridization buffer (BD Clontech, Palo Alto, CA). The membrane was washed and exposed to x-ray film at -80°C .

Stable transfectants expressing Lacrep-human CD46 fusion protein

The cloned Lacrep cDNA was fused with huMcp transmembrane (TM) and cytoplasmic tail (CYT1) domains as follows. The cloned Lacrep cDNA fragment digested with *EcoRI* and *KpnI* was placed in pCXN-2 mammalian expression vector together with a *KpnI-EcoRI* site-containing PCR product covering human Mcp-TM and CYT1 (Fig. 5A). CHO cells were transfected with this vector using Lipofectamine Plus reagent (Invitrogen Life Technologies). Neomycin-resistant cells were selected by treatment with 0.6 mg/ml G418 (Invitrogen Life Technologies). CHO cells expressing Lacrep-huMcp fusion protein were assessed by immunoblotting and flow cytometric analysis using anti-Lacrep Ab.

Preparation of rabbit anti-Lacrep polyclonal Ab

Rabbit anti-Lacrep polyclonal Ab (anti-Lacrep Ab) was produced by the method established in our laboratory (9). Briefly, RK13 cells (1×10^7) were transiently transfected with a pFlag CMV-Lacrep-His $\times 6$ full-length Lacrep construct using Lipofectamine Plus reagent (Invitrogen Life Technologies). After 48 h, transfected RK13 cells were collected in 10 mM EDTA-PBS, washed three times with PBS, and suspended in 0.5 ml of PBS. The RK13 cell suspensions were then mixed and emulsified with 0.6 ml of CFA (Difco, Detroit, MI) and used for immunization of rabbits. Immunization was performed four times at 7-day intervals, and the rabbits were boosted 3 days before drawing blood. IgG was purified by precipitation with 33% ammonium sulfate, dialyzed against PBS, and stored frozen at -80°C until use.

Protein blot analysis

Various lamprey tissues were solubilized in lysis buffer (1% (v/v) Nonidet P-40, 0.14 M NaCl, 0.01 M EDTA, 0.02 M Tris-HCl (pH 7.4), 1 mg/ml iodoacetamide, and 1 mM PMSF) using a Potter-type homogenizer. After incubation at 4°C for 30 min, each lysate was centrifuged at 15,000 rpm at 4°C for 30 min. The supernatants were collected, and the protein concentration was measured using a protein estimation kit (Bio-Rad, Hercules, CA). Equal amounts of total cellular protein from each lysate were resolved by SDS-PAGE (7.5% gel) and transferred to polyvinylidene difluoride membranes. Lacrep was visualized using an ECL detection system (Amersham Pharmacia Biotech) with rabbit anti-Lacrep Ab and an HRP-linked goat anti-rabbit secondary Ab (BioSource International, Camarillo, CA). Lamprey factor B (20), mannose-binding lectin-associated serine protease (MASP) (21), and C3 (not shown) were detected by a similar blotting method using their specific Abs, reported previously.

Deglycosylation assay of Lacrep

Methods for deglycosylation analysis of the products were described previously (22). Briefly, each transfectant (5×10^6) was solubilized in buffer containing 1% Nonidet P-40, 50 mM Tris-maleate (pH 8.6), 10 mM EDTA, 1 mg/ml iodoacetamide, and 1 mM PMSF for *N*-glycosidase, or 1% Nonidet P-40, 0.02 M Tris-maleate (pH 6.0), 0.01 M EDTA, 1 mg/ml iodoacetamide, and 1 mM PMSF. Solubilized proteins were centrifuged at 15,000 rpm for 30 min at 4°C , and the supernatants were incubated with 100 μU of neuraminidase (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C . Then, the samples were treated with either 250 mU of *N*-glycosidase or 1 mU of *O*-glycosidase (Genzyme, Cambridge, MA) for 16 h at 37°C . The samples were subjected to SDS-PAGE, followed by immunoblotting. Lacrep protein was detected with anti-Lacrep Ab.

Fractionation of plasma proteins responsible for C3 cleavage

Lamprey plasma (250 μl) was applied to Sephadex 200HR 10/30 using an Explorer system (Amersham Pharmacia Biotech). The column was equilibrated with 20 mM phosphate buffer containing 50 mM NaCl and 5 mM EDTA, pH 7.5. In some experiments EDTA was removed by dialysis. Human factor H (150 kDa) and BSA (66 kDa) were used as molecular mass markers.

Table I. Primers used in this study

Vector primers	
First primer	5'-AATTAACCCCTCACTAAAGGG-3'
Second primer	5'-CGCTCTAGAACTAGTGTGGATC-3'
Insert primer	
Degenerate primer	5'-TGTAACCTGGTGAAATGTRAG-3'

Because fraction 5 had proteolytic activity toward C3b-like C3 (see below), we used fraction 5 as a source of a factor I-like C3b-cleaving protease.

Purification of recombinant Lacrep

Lacrep-expressed cloned RK cells were used for the source of recombinant Lacrep. Cells (10^8) were solubilized with 1% Triton X-100/PBS supplemented with protease inhibitors (1 mg/ml iodoacetamide and 1 mM PMSF). Ni^{2+} agarose resin (Qiagen, Valencia, CA) was mixed with the supernatant of the centrifuged lysate ($13,000 \times g$ for 20 min at 4°C) and eluted with imidazole in a batchwise manner. Lacrep was eluted with 250 mM imidazole. The eluate was dialyzed against Tris buffer (9 mS/cm; pH 7.5). The culture medium of the transfected cells also contained Lacrep protein to a lesser extent. Thus, we used the culture supernatant as well as the lysate for the source of Lacrep. We found that both proteins exhibited an indistinguishable effect on C3 cleavage. The protein concentrations were estimated using a protein assay kit (Bio-Rad).

Partial purification of the factor I-like protease

To separate a functionally pure C3b-cleaving protease from cofactors, we applied lamprey plasma to a HiTrap heparin HP column (5 m; Amersham Pharmacia Biotech) equilibrated with the starting buffer (20 mM phosphate buffer (pH 7.0) containing 10 mM EDTA, 10 mM ϵ -aminocaproic acid, and 0.1 M glycine) and eluted with salt gradient (0–500 mM NaCl). C3b-cleaving activity was eluted mainly in unbound fractions. To further isolate the protease, this unbound fraction was applied to a chromatofocusing column (Mono P 5/200 GL; Amersham Pharmacia Biotech). The column was thoroughly washed by 25 mM imidazole-HCl (pH 7.4), then eluted with pH gradient using 1/8 diluted Poly Buffer 74 (Amersham Pharmacia Biotech; pH 4.0), and the C3b-cleaving activity of each fraction (2 ml) was assessed after dialysis against Tris buffer (9 mS/cm; pH 7.5) and 10-fold concentration. Immunoblotting analysis showed that although Lacrep was eluted from pH 4.00–4.85, the C3b-cleaving activity was detected between pH 4.67 and 5.23. Therefore, fraction 4 (pH 5.05–5.23) was used as a functionally isolated protease fraction for further study. To determine the functional activity of Lacrep in serum, fraction 10 was chosen for cofactor assay.

Facilitation of C3-cleaving activity by Lacrep

Purified laC3 (200 μg ; accession no. AY359861) was treated with 100 mM methylamine and then labeled with OG (an SH reagent that specifically labels the SH residue originated from the thioester bond in the α -chain of C3) as described previously (17). The OG-labeled C3b-like C3, but not intact C3, was cleaved by lamprey serum (data not shown). Lamprey serum was used as a control (see Fig. 4). C3b-like C3 (0.2 μg) was incubated at 25°C or for 1 h with lamprey serum (0.5 μl) in Tris-HCl (9 mS/cm; pH 7.4). Alternatively, OG-labeled C3b-like C3 (0.2 μg) was incubated at 25°C for 5 h with 2–5 μl of the fractionated samples to be assayed for protease and cofactor activities (23). In some experiments metal ions were added (1 mM Ca^{2+} and 7 mM Mg^{2+}) along with recombinant Lacrep (0.3 μg) or anti-Lacrep Ab (6 μg) to the incubation mixtures. All samples were reduced by 2-ME and then electrophoresed on SDS-PAGE under reducing or nonreducing conditions. The gels were fixed with 5% methanol, and fluorescence intensity was visualized with a fluorescence image analyzer (FLA-2000; Photo Film, Tokyo, Japan).

Calcein release cytotoxicity assay

The methods for the cytotoxicity assay using a fluorescent tracer were described previously (24, 25). Transfected CHO cells (2×10^4 cells/well) were seeded in 96-well plates. At 90% confluence they were loaded with a fluorescent dye (calcein-AM; Molecular Probes) by incubation with 10 μM calcein-AM in PBS for 30 min at 37°C . After washing the cells four times with PBS to remove the unincorporated fluorescent dye, they were incubated with 50 μl of 400 $\mu\text{g}/\text{ml}$ rabbit anti-CHO cell Ab (precipitated with 33% ammonium sulfate) (15, 26) or tannic acid, followed by yeast mannan (27). For preparation of mannan-coated CHO cells, normal cells were incubated with tannic acid (0.03 mg/ml) at 37°C for 15 min, washed three times, and incubated with yeast mannan (1 mg/ml) at 37°C for 30 min. The cells were washed and suspended in 50 μl of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing glucose-containing gelatin veronal buffer (GVB $^{++}$) (27). In contrast, GVB $^{++}$ without glucose was used as a buffer for anti-CHO Ab-coated cells. The Ab-sensitized cells and mannan-coated cells, which are known to be susceptible to the human alternative and lectin pathways (2, 28), respectively, were incubated with 50 μl of various concentrations (typically 10%) of human or lamprey serum diluted in the suspension buffer for 60 min at 37 or 24°C with gentle shaking (16). The plates were centrifuged at 1500 rpm for 5 min, and the fluorescence intensities of

100- μl aliquots of the supernatants were measured using a fluorescent plate reader with excitation at 488 nm and emission at 514 nm (25). The percent cytotoxicity was calculated as follows: (sample – control)/(MAX – control) \times 100. Untreated CHO cells were used to measure spontaneous calcein release (control), and the cells treated with 1% Triton X-100 were used to measure maximum release (MAX). The experiments were performed three times in triplicate.

C3 deposition assay

The deposition of C3 on yeast cells was determined using a flow cytometer. Fresh lamprey serum was used in all assays. Lamprey C3-depleted serum was prepared by treating the serum with excess anti-laC3 Ab. Rabbit normal IgG-treated serum was used as a control. Samples were treated with protein A-Sepharose to remove Ig and Ag-Ab complexes. Yeast cells (2×10^6) were incubated with YPD medium at 30°C for 16 h. After three washes, yeast cells were incubated with 0–50% lamprey serum in GVB $^{++}$ for 30 min at 25°C to allow C activation. Yeast cells were washed three times with PBS containing 0.1% BSA and 0.05% sodium azide. They were then treated with rabbit anti-laC3 Ab (diluted 1/100) or 25 $\mu\text{g}/\text{ml}$ anti-Lacrep Ab, followed by staining with FITC-conjugated secondary Ab. The stained yeast cells were analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ). Mean fluorescence intensity was evaluated using CellQuest software.

Results

cDNA cloning of lamprey SCR protein

After many PCR trials using a variety of primer sets, we finally obtained a 950-bp cDNA fragment using degenerate primer designed from the conserved sequence of Mcp (29) and vector forward primers as described in *Materials and Methods*. This cDNA fragment was cloned and sequenced. The sequence was found to contain SCR-like domains typical of RCA proteins. By consecutive rounds of PCR (9), we determined nucleotide sequences of the ORF and 3'-untranslated region of the lamprey SCR protein cDNA. This message was confirmed to be present in lamprey hepatopancreas by sequencing 12 independent RT-PCR amplicons.

We named this novel protein lamprey C regulatory protein, Lacrep. The Lacrep cDNA consisted of 2271 bp, which included a complete polyadenylation signal and a poly(A) tail. It encoded a predicted protein of 684 aa (Fig. 1). The amino acid composition of Lacrep suggested it to be a nonmembrane-anchored protein consisting of a 28-aa signal peptide, eight SCR domains, and one SCR-like domain. Lacrep has six putative *N*-glycosylation sites within SCR2, -3, and -4. Homology search analysis revealed that this molecule differs from being a simple homologue of Mcp as well as other human SCR proteins (Table II). At the amino acid level, SCR2/SCR3 (combinational homology of two SCRs) of Lacrep showed the highest homology of 34.3% to SCR2/SCR3 of the human C4bp α -chain. This SCR set was previously identified as an active center for C regulation in human C4bp (2, 30). SCR5 of Lacrep exhibited 39.3% homology to SCR2 of human factor H. SCR6/SCR7 of Lacrep has 34.4% homology to SCR3/SCR4 of human DAF (Fig. 1). Due to the conservation of the SCR2/SCR3 domain of human C4bp regions that regulate the activation of C in Lacrep, we expected that Lacrep had C regulation activity, similar to the SCR proteins of other vertebrates. We therefore hypothesized that Lacrep is a corresponding ancestral molecule of the vertebrate C regulatory proteins.

Lacrep mRNA and protein expression

RNA blotting followed by hybridization with the full-length ORF of Lacrep as a probe (2055 bp), which detects Lacrep mRNA, revealed a single 2.5-kb band only in the hepatopancreas among the various tissues examined (Fig. 2A). However, long exposure of Northern blot revealed trace messages of Lacrep in various organs (data not shown).

To determine the tissue distribution and relative levels of Lacrep protein, we performed immunoblotting analysis (Fig. 2B) using

rabbit anti-Lacrep Ab prepared as described previously (9). Lacrep was detected at 93 and 82 kDa as a doublet in the serum. Only a trace signal of either of these moieties was detected in other tissues, suggesting that the predominance of the two species of Lacrep was tissue dependent. Recombinant Lacrep protein had a molecular mass of 82 kDa, indicating that the cDNA we cloned encoded the lower molecular mass moiety in serum (Fig. 3, *A* and *B*), suggesting that alternative splicing or post-translational modifications, such as glycosylation of Lacrep, may occur. Our findings suggest that Lacrep was synthesized mainly in the hepatopancreas and then was secreted into the systemic circulation.

Glycosylation status of Lacrep

Based on its primary sequence, Lacrep was predicted to have six *N*-linked glycosylation sites. The molecular masses of the recombinant Lacrep protein, as estimated by immunoblotting, were larger than expected (Fig. 3*A*). To determine whether Lacrep has *N*- or *O*-linked sugars, we performed deglycosylation analysis of recombinant and serum Lacrep using *N*-glycosidase (Fig. 3*B*) and *O*-glycosidases (Fig. 3*C*). Both recombinant and serum Lacrep exhibited lower molecular masses by *N*-glycosidase treatment upon immunoblotting (Fig. 3*B*). Lacrep bands were of the size predicted from its amino acid sequence. The mobility of serum Lacrep doublet band was also reduced by 4–6 kDa. In contrast, in a similar analysis the mobility of Lacrep remained unchanged upon *O*-glycosidase treatment (Fig. 3*C*). Thus, it is likely that at least two types of Lacrep are expressed via alternative splicing by post-translational modification other than glycosylation. These results suggested that Lacrep is a glycosylated protein existing in at least two forms *in vivo*.

Complement regulatory activity of Lacrep

We first analyzed the lamprey plasma protease system for the cleavage of lamprey C3b-like C3. OG-labeled C3 was used as a substrate. Lamprey serum was used as a source of the protease and a cofactor (Fig. 4*A*). It is notable that divalent cation was required for the cleavage of laC3b-like C3. Two labeled bands of 60 and 40 kDa were generated from the parent 84-kDa α -chain by the function of serum factors (Fig. 4*A*). The 40-kDa band seems to correspond to the product generated by MASP (T. Fujita, et al., unpublished observations). In fact, of the lamprey plasma fractions eluted from a Sephadex 200HR column, fractions 6 and 7 contained MASP that helped to generate the 40-kDa band (Fig. 4, *C* vs *B*).

As fractions 4 and 5 of the gel filtration column detected Lacrep (Fig. 4*C*), the 60-kDa product was a possible cleavage product by a factor I-like serum protease and Lacrep (Fig. 4*B*). Intact C3 was not cleaved by fraction 5 (data not shown). As shown in Fig. 4*D*, the proteolytic potential of the OG-labeled, C3b-like C3 was augmented by the addition of recombinant Lacrep and was blocked by the addition of anti-Lacrep Ab. Lacrep per se had no proteolytic activity toward OG-labeled, C3b-like C3 (data not shown). Hence, Lacrep together with the lamprey plasma fraction containing factor I-like protease cleaved lamprey C3b-like C3 into an iC3b-like product. Both Lacrep and the serum factor were essential for proteolytic cleavage of the 84-kDa α -chain of lamprey C3b-like C3 to the 60-kDa fragment.

To confirm the cleavage profile of C3b-like C3 by the factor I-like plasma protease and Lacrep, functionally isolated materials were prepared (Fig. 5*A*). Factor B was eluted in the initial heparin column and was not contained in the crude factor I fraction; thus, it played no role in this cleavage process. Anti-Lacrep Ab recognized both plasma and recombinant Lacrep. Recombinant Lacrep was prepared from the transfected cells. When recombinant Lacrep was incubated with crude factor I-like protease and labeled C3b-like laC3, C3b-like C3 was cleaved into C3bi-like product with the expected

size of the α -chain fragment (Fig. 5*B*). Metal ion was required for the C3b-like C3 cleavage. This C3b cleavage was blocked by the addition of anti-Lacrep Ab. Lacrep isolated from lamprey serum (fraction 10) also exerted similar cofactor activity toward C3b-like C3 (Fig. 5*C*). These results suggested that the laC3b cleavage system requires a cofactor that was comparable to the mammalian C regulatory system, and that both recombinant and serum Lacrep possessed similar degrees of cofactor activity. Lacrep should be a C regulatory protein associated with protease cofactor activity.

Protection assay of membrane-anchored Lacrep

It is currently accepted that the lamprey has no lymphocytes that produce effectors for C activation (e.g., Ig). Human and lamprey sera were used as Ig and C sources. To determine whether Lacrep has the ability to protect host cells from attack by C, we generated a membrane-anchored protein possessing the eight SCRs and one SCR-like domain of Lacrep, followed by the TM and CYT1 domains of human Mcp (Fig. 6*A*) and established a CHO cell line stably and constitutively expressing Lacrep-Mcp fusion protein on its cell surface (Fig. 6, *B* and *C*). To measure the C protection activity of CHO/Lacrep-Mcp fusion protein, a CHO clone expressing human Mcp to a similar level (CHO/huMcp) was used as a control. These CHO clones were coated with rabbit IgG or mannan. Cytotoxicity assay was performed with calcein-labeled sensitized CHO as a control, CHO/Lacrep-Mcp or CHO/huMcp cells, and diluted lamprey serum as the C source (Fig. 6*D*). The assay was performed at 24°C when using lamprey serum. The results demonstrated that lamprey serum (10%) damaged mannan-coated CHO cells and the expression of Lacrep-Mcp and partially blocked lamprey serum-mediated CHO cytotoxicity (Fig. 6*D*). IgG sensitization barely conferred susceptibility to lamprey serum on CHO cells. IgG-sensitized CHO showed human C-mediated lysis and under the same conditions CHO/huMcp blocked human C-mediated attack by ~30%, whereas CHO/Lacrep-Mcp cells barely blocked human C-mediated lysis (data not shown). These results indicate that Lacrep possesses the ability to block the lamprey C pathway by acting as a cofactor, which results in protection of host cell from lamprey C-mediated cytolysis. In this case, Lacrep may block the putative lamprey lectin pathway (28). However, which of the C pathways of lamprey is most efficiently blocked by Lacrep has not yet been identified.

C3 deposition assay

Although C3, factor B, and MASP have been identified as lamprey C-related proteins (13, 20, 21), whether and/or how they function in the lamprey C system remain unknown. As MASP and factor B messages are found in the lamprey, we expected that both alternative and lectin pathways work in the lamprey body, because the lamprey C cascade could be activated by the yeast cell wall component of zymosan. To further clarify the role of Lacrep, we performed a C3 deposition assay via alternative and lectin pathways using yeast as a C activator and lamprey serum as a C source (Fig. 7). When yeast cells were incubated with lamprey serum, laC3 was deposited on the yeast surface. Lacrep was also bound to the yeast surface in a manner dependent on the dose of serum. The C3 deposition activity was completely abolished when lamprey serum was depleted of C3 by anti-laC3 Ab. C3 deposition disappeared when the divalent cations were chelated (Fig. 7). These results together with the ability of Lacrep to inactivate C3b and protect host cells from C suggested that Lacrep could act to regulate C activation, which depends on inactivation of C3 or C3-like molecules.

Alternatively, Lacrep has an opsonin-like ability to directly bind to foreign substances, such as yeast, via the lectin or an as yet-unknown classical-like pathway requiring metal ions (Fig. 7, *right*

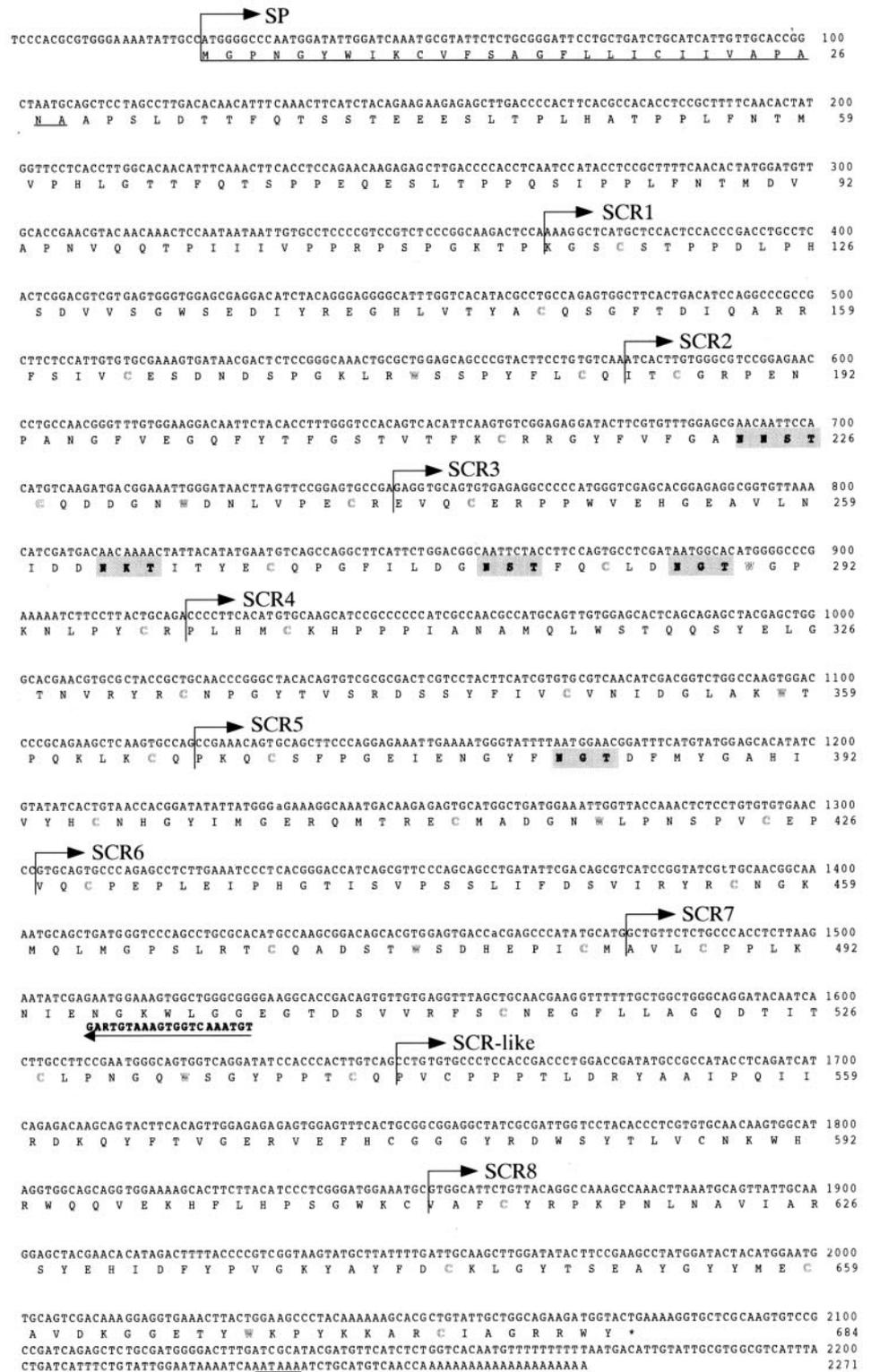
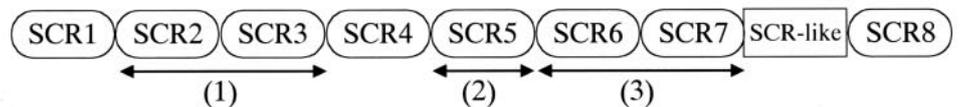


FIGURE 1. Complete Lacrep nucleotide and amino acid sequences. The amino acid sequence is shown under the cDNA nucleotide sequence. The signal peptide (SP) is underlined. The regions encoding SCRs are shown by arrows. Conserved Cys and Trp are shown in bold. The asterisk indicates the stop codon. The long arrow (at nt 1609–1630) indicates the degenerate primer used for PCR. Putative *N*-glycosylation motifs are shaded. The polyadenylation signal is double underlined. The nucleotide sequence has been registered in the EMBL data library/GenBank/DBJ databases (accession no. AB061219). Lower panel, SCR regions 1, 2, and 3 are similar to the SCRs of huC4bp, huCR2, and factor H, respectively (see also Table II).



panels). In fact, even when laC3-depleted serum was used, Lacrep still bound to the yeast surface (Fig. 7). Lacrep may opsonize serum-treated yeast independent of C3 deposition.

Discussion

In this study we identified and cloned an SCR-containing C regulatory protein from the lamprey. Based on the primary protein

Table II. Comparison of the primary structure of each SCR of Lacrep with those of human C regulatory proteins

Lacrep	C Regulators	Homology (%)	Combination of SCR Domains	Homology (%)
SCR1	SCR4 of DAF	37.1	SCR3-4 of DAF SCR2-3 of C4bp α SCR3-4 of MCP	32.6 34.3 30.3
SCR2	SCR2 of C4bp β	36.3		
SCR3	SCR3 of DAF	34.8		
SCR3	SCR4 of MCP	38.0		
	SCR3 of C4bp α	34.4		
SCR4	SCR7 of CR2	32.7	SCR3-4 of DAF	34.4
SCR5	SCR2 of fH	39.3		
SCR6	SCR3 of DAF	35.9		
SCR7	SCR2 of CR2	40.5		
	SCR4 of DAF	37.0		
SCR8				
SCR-like	SCR1 of MCP	38.9		

sequence and analysis of the culture supernatant of cells transfected with relevant cDNA, it was shown to be a secretory protein. It consisted of eight SCRs and thus has a framework similar to the α -chain of C4bp. The functional properties were vested with SCR2 and SCR3, which contained an SCR encoded by a split exon, similar to human C4bp (2, 30). This protein served as a protease (presumably, factor I-like) cofactor for the cleavage of laC3b. Furthermore, its artificial membrane-anchored form was able to protect

host cells from laC, presumably through the lectin pathway. Contrary to the conventional C3b inactivation systems, this SCR protein and serum protease required divalent cations to cleave laC3b-like C3. We named this protein laC regulatory protein (Lacrep), which represents an SCR-containing C regulatory protein in the jawless fish.

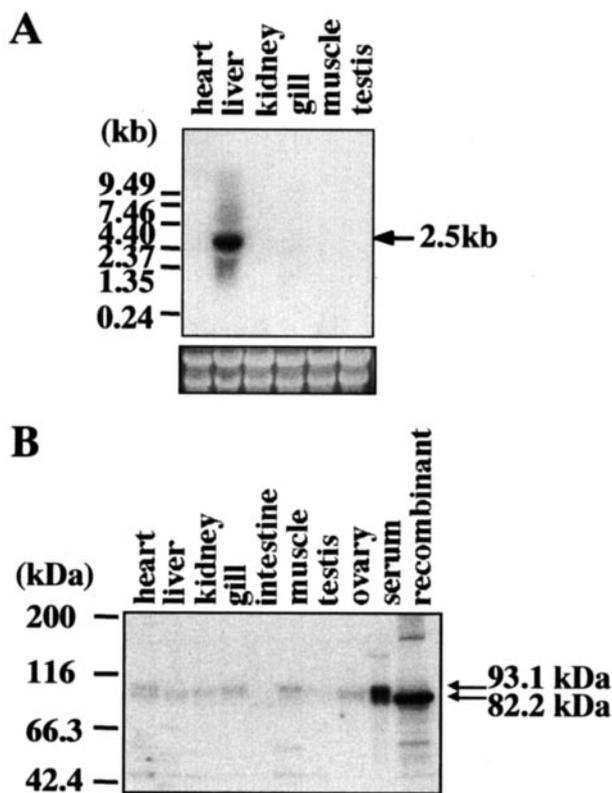


FIGURE 2. Tissue distributions of Lacrep mRNA and protein. *A*, RNA expression of Lacrep in various lamprey tissues. Total RNA (20 μ g) from various tissues was separated by electrophoresis in a 1.0% (w/v) agarose gel and hybridized with a 32 P-labeled Lacrep probe. *Upper panel*, Notice the 2.5-kb band in liver RNA. *Bottom panel*, Total RNA extracted from specified lamprey tissues and stained with ethidium bromide. In lamprey, the liver is synonymous with the hepatopancreas. *B*, Immunoblotting analysis of various lamprey tissues. Solubilized proteins (extracted from 100 μ g of tissue) from various tissues were separated by SDS-PAGE in a 7.5% polyacrylamide gel under nonreducing conditions, transblotted onto a membrane, and detected with anti-Lacrep Ab. Notice two close bands at 93 and 82 kDa in serum.

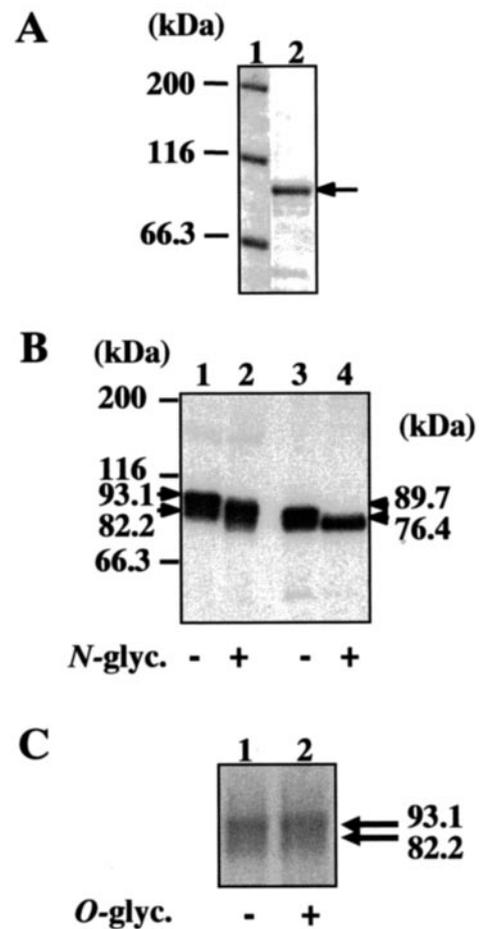


FIGURE 3. Deglycosylation analysis of Lacrep. Lacrep was isolated from cell lysate of RK13 cells transfected with the plasmid DNA containing Lacrep full-length cDNA (*A*). Recombinant Lacrep and lamprey plasma proteins were treated with *N*-glycosidase (*B*) or *O*-glycosidase (*C*; indicated by +). Negative controls were treated under the same conditions in the absence of *N*- or *O*-glycosidase (indicated by -). The samples were subjected to immunoblotting with anti-Lacrep Ab. *Lanes 1* and *2*, Lacrep in lamprey plasma; *lanes 3* and *4*, recombinant Lacrep.

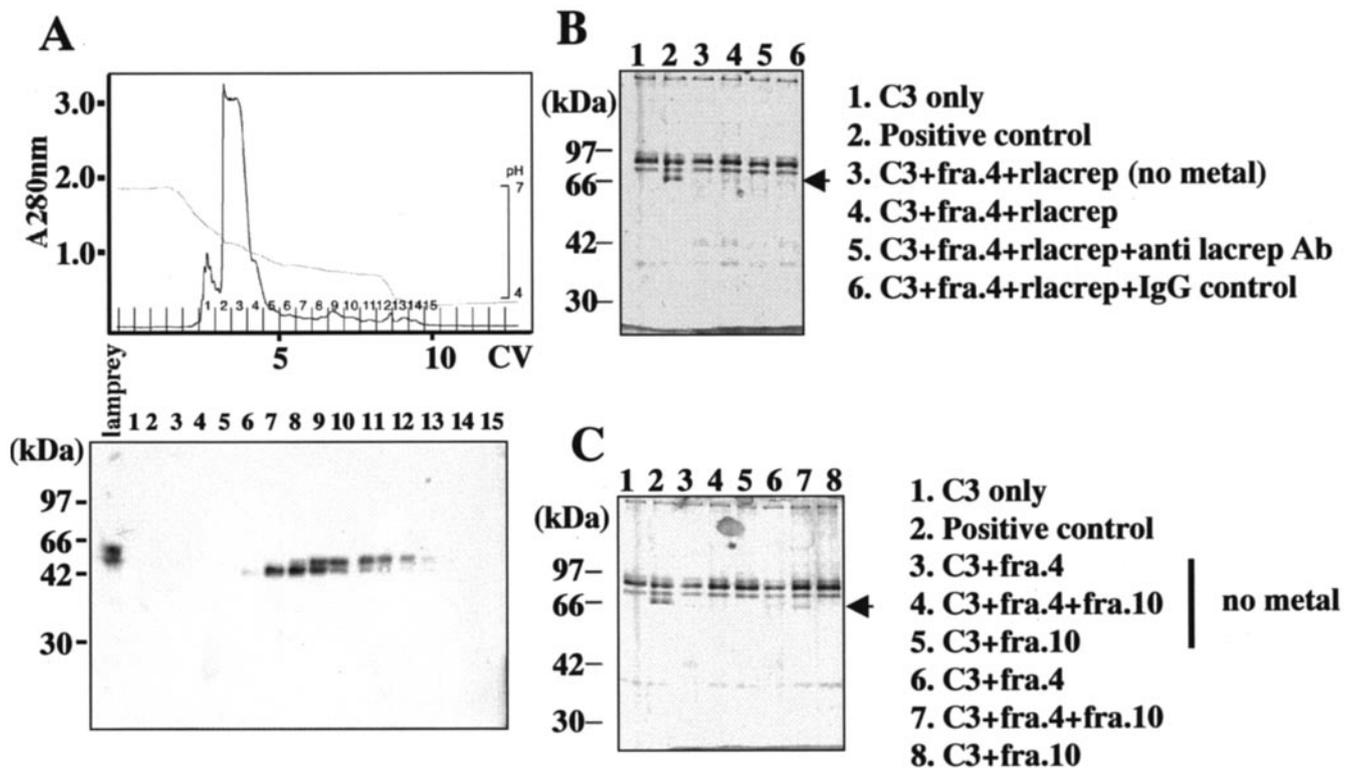


FIGURE 5. Analysis of Lacrep cofactor activity. *A*, Elution profile of a heparin-unbound fraction of lamprey plasma from the chromatofocusing column. The C3b-cleaving protease-containing material was eluted by pH gradient (pH 4–7) as described in *Materials and Methods*. According to the immunoblot analysis of each fraction, fraction 4 was used for crude factor I-like protease, and fraction 10 was used as a source of serum Lacrep. *B*, Cofactor assay for cleavage of IaC3b-like C3. Purified recombinant Lacrep was used as a cofactor and the cleavage of IaC3b-like C3 was confirmed using isolated factor I-like protease (fraction 4). The protease was partially purified as described in *Materials and Methods*. Negative and positive controls are shown in *lanes 1* and *2*. C3b-like C3 and the protease were incubated with purified recombinant Lacrep in the absence (*lane 3*) or the presence (*lane 4*) of metals. The α -chain was cleaved in the presence of metals, and this cleavage was specifically blocked by anti-Lacrep Ab (*lane 5*), whereas control IgG did not affect the cleavage of α -chain (*lane 6*). *C*, Serum Lacrep has cofactor activity. Negative and positive controls are shown in *lanes 1* and *2*. Fraction 4 reflects a factor I-like protease fraction, and fraction 10 is a cofactor fraction. Metal-minus controls are shown in *lanes 3–5*. In metal-plus samples, either fraction 4 (*lane 6*) or fraction 10 alone (*lane 8*) barely expressed C3b-cleaving activity toward C3b-like C3 (designated C3). C3b-like C3 cleavage activity was exerted in combination with fractions 4 and 10. Lacrep-containing fraction cleaved α -chain of C3b-like C3 together with the protease and metals (*lane 7*). The arrows indicate the C3 fragments generated by Lacrep and factor I-like serum protease.

According to Kruskal et al. (31), the SCR-containing C regulatory proteins can be categorized into groups 1 and 2. In humans, factor H and its related proteins fall into group 1, and their genes are located on chromosome 1q32, >7 Mbp from the RCA cluster, which is classified into group 2. Group 2 genes are located within the 0.9-Mb region, which contains the RCA cluster C4bp α , C4bp β , Mcp, Mcp-like, DAF, CR1, CR2, and CR1-like (2, 6, 7), although the group 2 proteins identified to date are C4bp α , CR1,

CR2, Mcp, and DAF. These genes are clustered together with a C-unrelated gene, *PFKFB2* (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2). In fish, no group 2 proteins were identified, whereas a group 1 protein, SBP1, has been identified (10, 11). Another protein, the sand bass cofactor-related protein 1, with an additional SCR-containing gene, shares structural similarity with SBP1 (32). The relationship between these two appears to be similar to that between factor H and its related proteins (33). Lacrep,

(*lane 6*) of metal ions were analyzed by SDS-PAGE, followed by fluorometry. OG was specifically incorporated into the α -chain of C3b-like C3. The α -chain and putative α' -chain were stained with OG (*lanes 3–6*). The molecular mass markers and the three chains of C3b-like C3 are indicated. The lamprey serum-derived metal ion-dependent 60-kDa α -chain fragment and 40-kDa band are indicated by arrows. *B*, Cleavage of lamprey OG-labeled C3b-like C3 by lamprey plasma. A typical cleavage profile of OG-labeled C3b-like C3 by lamprey plasma is shown (see *right panels*, Positive Cont.). The labeled substrate was 84-kDa α -chain, and a 74-kDa fragment reflects putative α' -chain (both designated as C3 on the *top*). Lamprey plasma was separated by molecular sieve HPLC (*left panel*). The positions of the eluted marker proteins are indicated. C3 α -chain cleaving activity was tested in terms of the defined fraction numbers. OG-labeled α -chain of C3-like C3 was cleaved into the 40-kDa fragment within 1 h by fractions 6 and 7 (*right upper panel*) and into the 60-kDa fragment after long incubation with divalent metal cations by fraction 5 (*lower panel*). The common 36-kDa band is a contaminant of the C3 preparation. *C*, HPLC elution profiles of LaC-related proteins. Five microliters of each fraction were immunoblotted under nonreducing conditions. Bold italics and arrows show MASP, Lacrep, and factor B of the lamprey in the specified fractions. Arrows indicate the relevant proteins. These results, with those of *A* and *B*, 40-kDa fragment generation occur in parallel with the presence of MASP (fractions 6 and 7), whereas the 60-kDa generating activity partly coincides with Lacrep (fraction 5). *Lanes 4* and *5* in the sheet of factor B contained unidentified ~200-kDa bands recognized by the anti-factor B Ab. *D*, Termination of C3-cleaving activity of fraction 5 by anti-Lacrep Ab. Minor cleavage of OG-labeled α -chain was seen by the addition of fraction 5 (*lane 1* vs *lane 2*). Fraction 5 activity was not affected by IgG (*lane 3*), but augmented by the addition of Lacrep (*lane 4*). Fraction 5-mediated cleavage of α -chain was completely blocked by the addition of anti-Lacrep Ab (*lane 5*). The 60-kDa band generated by fraction 5 aligned with that generated by lamprey serum (*lane 6*). Arrows indicate the bands generated by lamprey serum.

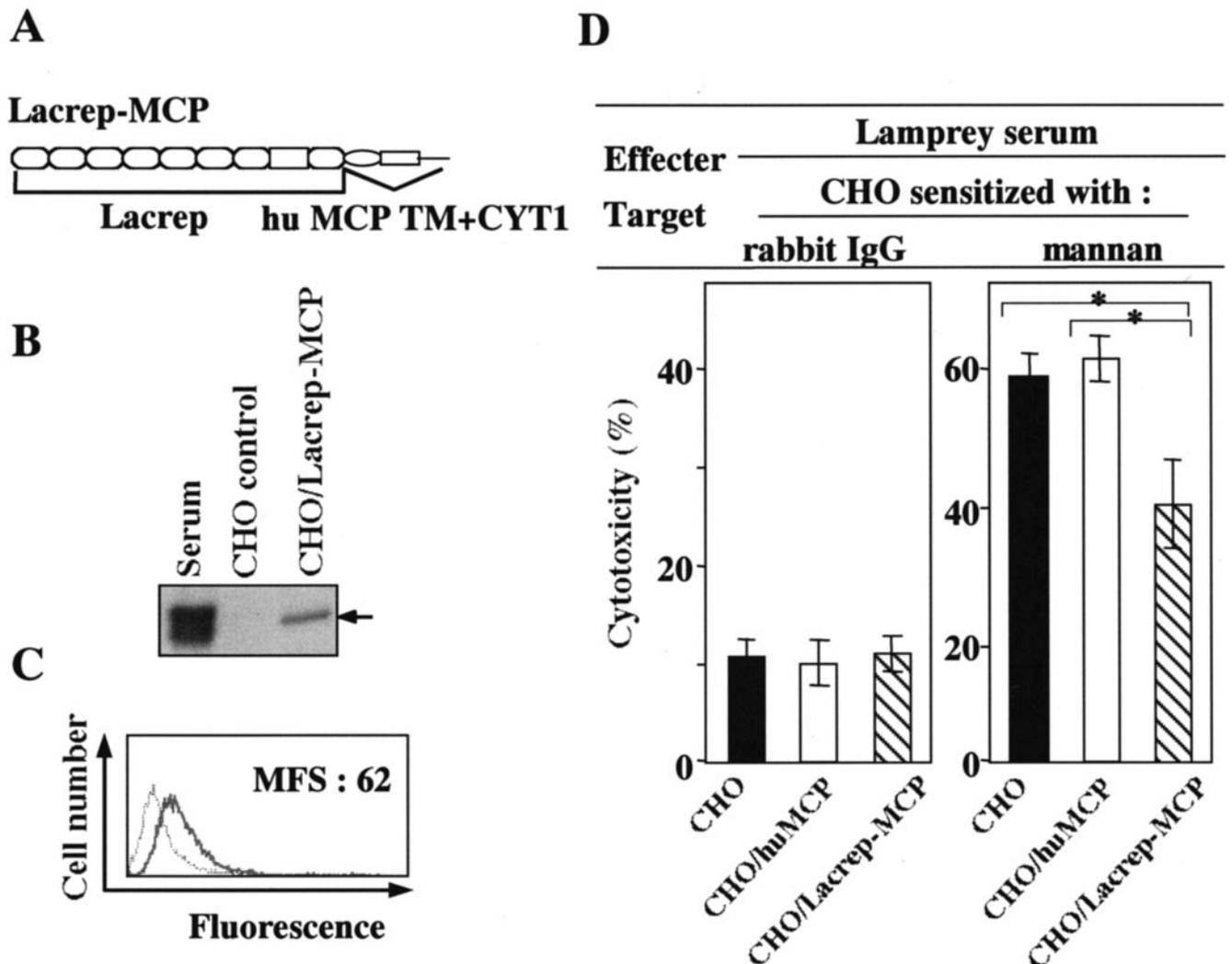


FIGURE 6. Complement inhibition assay for Lacrep. *A*, Scheme of the construct encoding the Lacrep and huMcp fusion protein. The chimeric protein has all SCR domains of Lacrep and the TM and CYT1 domains of huMcp. Immunoblotting (*B*) and flow cytometric analysis (*C*) were performed for testing the establishment of a CHO cell subline stably expressing Lacrep-Mcp fusion protein (CHO/Lacrep-Mcp). *D*, Calcein release cytotoxicity assay. CHO/Lacrep-Mcp (▨), CHO/huMcp (□), and control CHO cells (■) labeled with calcein-AM were sensitized with rabbit anti-CHO Ab (*left panel*) or mannan (*right panel*) and incubated with 10% lamprey serum in GVB⁺⁺ or glucose-GVB⁺⁺. The percent cytotoxicity was calculated as described in *Materials and Methods*. The experiments were performed three times in triplicate. The values represent the mean \pm SD; $p < 0.02$, by two-tailed test.

as shown by its structure and function, would be a group 2 protein. The existence of the Lacrep gene in close proximity to the *PFKFB2* gene (H. Oshiumi and T. Seya, unpublished observations) further supports this classification. Hence, this is the first report on RCA-like C regulator in lower vertebrates. If this represents an ancestral member of the RCA proteins of mammals and birds (2, 4, 8, 34), lower vertebrates, such as fish, amphibians, and reptiles, should also possess a group 2 soluble RCA protein.

Lacrep served as a cofactor for the lamprey protease, and the chimeric membrane form of Lacrep exhibited the ability to protect host cells from laC. This ability was barely expressed on human C (Fig. 6D). It appears that laC regulator displays considerable species specificity. Earlier studies by Kaidoh et al. (35, 36) suggested the presence of factor I-cofactor activity toward human C3b in fish, including osteichthyes and even chondrichthyes. Lacrep may confer molecular basis for the previously reported functional entities of the C regulatory proteins of lower vertebrates. Alternatively, Lacrep may modulate opsonin activity through its ability to bind C3b deposited on foreign material such as yeast. Similar opsonin-modulating features were reported in human soluble C regulators, factor H (37, 38) and C4bp (39, 40).

Lampreys lack Ig because they do not have the acquired immune system including MHC and T/B cell receptors (41, 42). Thus, they must be devoid of the typical classical C pathway (2). In accord with this, rabbit IgG-sensitized CHO cells were resistant to lysis by diluted lamprey serum (Fig. 5D). Although the entire C system of the lamprey has not yet been elucidated, a C3-like effector molecule was inactivated by proteolytic cleavage (13), which may be mediated by a trypsin-like protease (16). Thus, the natural target of Lacrep might be this C3-like molecule. However, the possibility still remains that laC3 is a member of a family with a number of C3-like proteins, and only limited members of this family serve as targets of Lacrep. In fact, this is true for the C3 family of carp (43). Furthermore, the reported laC3 consisted of three chains, resembling human C4 in its general profile (13, 24). As our data suggested that C3b-like C3 can be weakly cleaved by the factor I-like protease and an unidentified cofactor (data not shown), the presence of another cofactor with different specificity is further supported.

Lamprey Lacrep and human C4bp share the active center of SCR2 and SCR3 with $\sim 34\%$ homology. Other SCRs of Lacrep showed low similarity to those of C4bp, whereas SCRs of other

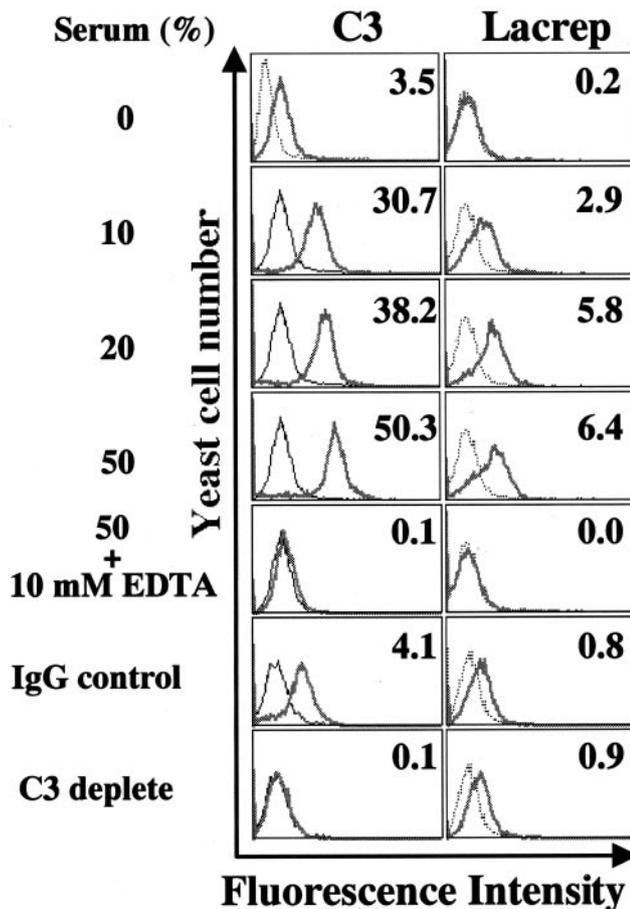


FIGURE 7. C3 deposition assay on yeast cells. Lamprey C3 deposition on yeast cells was determined using flow cytometry. Yeast cells were incubated with 0–50% lamprey serum in GVB⁺⁺ for 30 min at 24°C to allow C activation. C activation was inhibited by chelating 10 mM EDTA or C3-depleted serum. Lamprey C3 (left panel) and Lacrep (right panel) were detected with anti-laC3 Ab and anti-Lacrep Ab, respectively. The dotted line indicates control IgG. Mean fluorescence shifts reflecting the binding degrees of C3 or Lacrep are shown in the insets.

group 2 proteins, particularly DAF and Mcp, have high similarity to the remaining SCRs of Lacrep. Hence, Lacrep is not simply a homologue of human C4bp, but possibly is a chimera of the human SCR C regulatory proteins. This is consistent with the results of the phylogenetic gene analysis judged by the neighbor-joining method (data not shown).

Blom et al. (44) and our group (45) identified the functional cassette of human C4bp. The region of human C4bp participating in C4b inactivation was SCR2 and -3, whereas that for C3b was SCR1 to -5. We currently project that the active center is SCR2 and -3 in both Lacrep and C4bp. SCR deletion analysis will facilitate identification of the active center of the laC regulator.

Elucidation of pathways that trigger activation of laC still remains a critical issue. The lectin pathway may need C4 for efficient amplification of C activation (28). Recent findings suggested that lymphocyte-like cells are produced in lamprey, which express the genes essential for activation of mammalian lymphocytes (41, 42). This means that a prototype of acquired immune system was established in the ancestor of the lamprey. Thus, the possibility that this system contained a factor substituting Ig, resulting in the capability of activating a classical-like C pathway, is reasonably high. Indeed, a C1q-like molecule exists in lamprey serum (46). The finding that the IgG-sensitized CHO cells are susceptible to

C-mediated lysis in a longer incubation might be related to this issue.

In addition to the emergence of the C1q-like molecule, the TLR system appears to be established in lamprey (H. Oshiumi, K. Shida, T. Seya, et al., unpublished observations) as well as in fish (47). These molecules unequivocally predict the process of establishment of the acquired immune system and its related responses in lamprey. We imagine that generation of the TLR and C systems coincided in parallel with emerging lymphocyte-like cells in the jawless fish (41, 42). More extensive studies of the laC system, including the SCR-containing RCA proteins and their relationship to the innate and acquired immune system, have yet to be performed.

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