The Complement Cofactor Protein (SBP1) from the Barred Sand Bass (Paralabrax nebulifer) Mediates Overlapping Regulatory Activities of Both Human C4b Binding Protein and Factor H*

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We have previously shown that serum of the teleost fish barred sand bass (Paralabrax nebulifer) cleaves the α′-chain of human C4b and C3b. The proteins that participate in these reactions were purified, and a specific protease and a single cofactor protein were identified. Functional characterization of the recombinantly expressed sand bass cofactor protein (SBP1) and truncated forms containing short consensus repeats (SCRs) 1–2, 1–3, 1–4, 1–5, and 12–17 revealed that SBP1 and SCRs 1–4 mediate the functional activities of the human plasma regulatory protein C4bp and factor H. They form a complex with C4b, inhibit the formation, and accelerate the decay of the classical pathway C3 convertase and display cofactor activity for the cleavage of C4b. In contrast, the interaction of SBP1 and SCRs 1–4 with human C3b in all these activities was limited. This difference is due to species-specific incompatibilities between the cofactor protein and human C3b. SBP1 and SCRs 1–5 displayed full binding and cofactor activity for methylamine-treated C3 from trout, a species closely related to the sand bass. The presence of only one cofactor in the fish plasma that combines the functional activities of C4bp and factor H demonstrates that the sand bass cofactor protein is the ancestral precursor to the two complement regulatory proteins in human plasma.

In vertebrates, cleavage of the third component of complement (C3)1 and subsequent assembly of the membrane attack complex (C5–C9) occur as functions of at least two distinct enzymes generated during the activation of the classical (1) and alternative (2, 3) pathways. The classical pathway C3 convertase is formed by a reaction involving the reversible binding of C2 to C4b in the presence of Mg2+ to form the C4b2a complex and by the cleavage of the bound C2 by C1s to generate C4b2a (4). These reactions are remarkably similar to those occurring during the formation of the alternative pathway C3 convertase (2, 3). C3b binds to factor B, which is then cleaved by factor D to generate C3bBb. The formation and function of these two enzymes are regulated by three distinct mechanisms as follows: a temperature-dependent intrinsic decay of the enzymes; an extrinsic decay mediated by the effect of the serum proteins C4-binding protein (C4bp) and factor H, and the proteolytic inactivation of the α′-chain of C4b and C3b by factor I.

Binding of C4bp to C4b directly accelerates the decay of the C4b2a complex (5–7) and prevents further interaction of C4b with C2. Similarly, factor H prevents binding of factor B to C3b and dissociates Bb from the C3bBb complex (8). In addition, C4bp and factor H function as cofactors for the plasma protease factor I that mediates the degradation of C4b and C3b (5, 6, 9). During this reaction the α′-chain of C4b is cleaved into three fragments of 48 (α2), 33 (α3), and 27 (α3) kDa (10). The α′-chain of C3b is first cleaved into two polypeptides of approximately 68 and 46 kDa, followed by a second cleavage of the 68-kDa peptide to a 38- and 30-kDa fragment (5, 9).

Mammalian C4bp is composed of seven identical 75-kDa subunits (α-chains), each of which displays the complement regulatory function, and one distinct β-chain of 45 kDa (11, 12). Human factor H is an elongated single chain glycoprotein with a molecular mass of 150 kDa (13). Human factor H (14) and the α-chains of C4bp (15, 16) are organized in tandem structural units of approximately 60 amino acids, termed short consensus repeats (SCR) (17, 18). Factor H consists of 20 SCRs, and each α-chain of C4bp consists of 8 CSRs and a unique non-SCR C terminus. These repetitive structural elements are found in most plasma or membrane complement regulatory proteins (19) and in a number of non-complement proteins with diverse functions, suggesting that their corresponding genes may have evolved early from an ancestral SCR sequence (20).

Phylogenetic studies of the complement system have proved difficult. Even when the structure of complement proteins of different species is known, the elucidation of their functional properties has been hampered by incompatibilities among complement proteins of heterologous species. The α′-chain of human C4b and C3b is cleaved by the serin of a number of low vertebrates (21), the most primitive of which is the teleost fish barred sand bass (Paralabrax nebulifer). We have purified and characterized the proteins that participate in these reactions (22). Contrary to the findings in higher vertebrates (5, 7, 11, 23), only a single protein serves as cofactor for factor I in the cleavage of C4b and C3b. The cofactor activity correlates with a 110-kDa polypeptide chain of a 360-kDa plasma protein (22). The corresponding cDNA, representing the 110-kDa polypeptide chain, encodes for a sand bass cofactor protein (SBP1) of 1053 amino acids with a calculated molecular mass of 115 kDa. SBP1 has a hydrophobic signal peptide indicative of a secreted function. This work was supported by National Institutes of Health Grant A120067. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: C, complement; C4bp, C4 binding protein; SCR, short consensus repeat; SBP1, sand bass cofactor protein; SB1, sand bass cofactor protein cDNA clone; NHS, normal human serum; VBS, veronal-buffered saline; trout C3b-like, trout C3 converted into a C3b-like molecule by treatment with methylamine; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
translation product, and the remaining part of the molecule is organized in 17 SCRs. A structural comparison of SBP1 (24) to the complement regulatory proteins factor H (14) and C4bp (16, 25) revealed significant homology which exceeded that expected due to structurally conserved amino acids (24). To elucidate whether SBP1 expresses all the functional activities of the two human regulatory proteins, we characterized and localized the functional domains within the protein. Recombinantly expressed SBP1 and truncated forms obtained by the consecutive deletion of SCRs from the C terminus of the molecule were analyzed for binding to human C4b and C3b, inhibition of the formation, and decay accelerating activity for C4b2a and C3bBb and cofactor activity in the cleavage of C4b and C3b. The functional domains of SBP1 were localized to SCRs 1–4.

EXPERIMENTAL PROCEDURES

Isolation of Plasma—P. nebulifer (GIRARD, 1856; class, Ostechthy-thes, order, Perciformes; family, Serranidae; subfamily, Anthiinae; genus, Paralabrax; species, Paralabrax nebulifer; common name, barred sand bass) were captured in the Pacific Ocean. The fish were bled by puncturing the caudal artery, and the blood was collected into vials. The supernatant and incubated for 3 h at 4 °C, pelleted by centrifugation and immediately frozen at –70 °C.

Generation of a Full-length cDNA Clone Representing SBP1—The preparation and screening conditions of the sand bass liver cDNA library have been described (24). The full-length cDNA coding for the sand bass cofactor protein SBP1 was obtained by ligating the restricted overlapping fragments c90 and c120.

PCR Amplification—cDNA fragments were amplified in a polymerase chain reaction (PCR), using Taq-polymerase (Perkin-Elmer) and sequence-specific primers (26). After 30 cycles of amplification (Perkin-Elmer thermocycler: five cycles of 1 min at 95 °C, 1 min at 46 °C, 1 min at 75 °C, 1 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C), the amplified product was cloned into vector pCR II (Invitrogen).

Cloning Vectors Representing SBP1 and Truncated Forms of SBP1—A PCR-generated NotI/Smal-restricted cDNA (SB1) representing SCRs 1–17 of SBP1 was inserted into the NotI/Smal-treated baculovirus expression vector pBSV-8His (27). The presence of a vector-encoded signal peptide upstream from the multiple cloning site and a C-terminal His-tag allows the expression of a secreted His-tagged recombinant protein. Primers were designed that were used in a PCR-generated NotI/Smal-restricted cDNA (SB1) represent- ing SCRs 1–17 of SBP1 was inserted into the NotI/Smal-treated baculovirus expression vector pBSV-8His (27). The presence of a vector-encoded signal peptide upstream from the multiple cloning site and a C-terminal His-tag allows the expression of a secreted His-tagged recombinant protein. A PCR-generated NotI/Smal-restricted cDNA (SB1) representing SCRs 1–17 of SBP1 was inserted into the NotI/Smal-treated baculovirus expression vector pBSV-8His (27). The presence of a vector-encoded signal peptide upstream from the multiple cloning site and a C-terminal His-tag allows the expression of a secreted His-tagged recombinant protein. A PCR-generated NotI/Smal-restricted cDNA (SB1) representing SCRs 1–17 of SBP1 was inserted into the NotI/Smal-treated baculovirus expression vector pBSV-8His (27). The presence of a vector-encoded signal peptide upstream from the multiple cloning site and a C-terminal His-tag allows the expression of a secreted His-tagged recombinant protein. A PCR-generated NotI/Smal-restricted cDNA (SB1) representing SCRs 1–17 of SBP1 was inserted into the NotI/Smal-treated baculovirus expression vector pBSV-8His (27). The presence of a vector-encoded signal peptide upstream from the multiple cloning site and a C-terminal His-tag allows the expression of a secreted His-tagged recombinant protein.

Sequence Analysis of the cDNA Clones—The purified cDNA inserts were sequenced by the dideoxy chain termination method (25), using [α-32P]dATP and Sequenase II (U. S. Biochemical Corp.). Various oligonucleotides were synthesized (gene assembler-I; Amersham Pharmacia Biotech) and used as primers to sequence the cDNA inserts in both orientations.

Insect Cell Culture—Spodoptera frugiperda cells (Sf9) (American Type Culture Collection) were grown at 27 °C in monolayer culture, in Grace’s medium (BioWhittaker) supplemented with 10% fetal calf serum, streptomycin (100 mg/ml), penicillin (100 units/ml), and fungizone (250 ng/ml) or in fetal calf serum-free Express medium (BioWhittaker).

Recombinant Expression and Purification of SBP1 and Truncated Forms of SBP1—The plasmid-DNA used to transfect the insect cells was purified using Nucleobond AX cartridges (Macherey-Nagel). Sf9 cells (6 × 10^6 cells) in a 25-mm cell culture dish were cotransfected with the appropriate plasmid (2 µg) and baculovirus DNA (0.5 µg of BaculoGold DNA, Pharmingen) according to a calcium phosphate precipitation method modified for insect cells (29). Transfected cells were incubated for 4–6 days, and the recombinant virus was isolated from the culture medium by plaque assay (30). Single plaques were purified after 5 days, and viruses were used to infect Sf9 cells (3 × 10^6 cells) grown in 25 ml of Express medium using a multiplicity of infection of 5. The culture medium was harvested 9 days after infection, and the recombinant virus was purified from the supernatant by Ni^2+-nitrilotriacetic acid-agarose (Qiagen) chromatography (31). The resin was added to the supernatant and incubated for 3 h at 4 °C, pelleted by centrifugation (450 × g, 10 min, 4 °C), washed twice with 10-bed volumes of buffer A (0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 5 mM imidazole), and then once with 6-bed volumes of buffer B (0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 60
cells with 350 μg of C3 for 20 min at 21 °C. The cells were washed and incubated for 3 h at 37 °C to allow complete decay of the remaining convertase.

**Hemolytic Assays**—To measure the activity of cell-bound C4b2a or C3bBb, a 50-μl portion of each cellular intermediate (1 × 10⁶ cells/ml) was incubated with an equal volume of NHS-EDTA diluted 1:10 as source of C5–9 for 1 h at 37 °C. After the addition of 650 μl of GDVB, to each sample, the mixtures were centrifuged for 5 min at 1000 × g, and the supernatants were analyzed photometrically at A₅₇₀ to determine the hemoglobin released.

Quantitation of the Cofactor Activity of SBP1 and Truncated Proteins—The cleavage of human 125I-C4b, human 125I-C3b, and trout 125I-C3b-like was measured as follows: 2 μg of 125I-C4b or 125I-C3b was added to 50 μl of cofactor-depleted sand bass serum diluted 1:4 in VBS containing 10 mM CaCl₂. After addition of 200 nM SBP1 or SCRs 1–2, 1–3, 1–4, 1–5, or 12–17 diluted in 25 mM Tris containing 10 mM CaCl₂, the mixtures were incubated for 30 min at 37 °C for 5 h. As controls, 125I-C4b and 125I-C3b were incubated with NHS, sand bass cofactor-depleted serum, or buffer alone. Samples were subjected to SDS-PAGE analysis. In a separate experiment 1 μg of trout 125I-C3b-like was added to 25 μl of the following reagents: NHS, trout serum, sand bass serum, sand bass cofactor-depleted serum, and sand bass cofactor-depleted serum plus 100 nM SCRs 1–5. The mixtures were incubated for 2 h at 20 °C and reduced and analyzed by SDS-PAGE. Human 125I-C3b was used as control. The cofactor activity of the various plasma samples was quantitated by counting the radioactivity in the polypeptides generated from the α’-chains of human 125I-C4b, 125I-C3b, and trout 125I-C3b-like and normalized using the radioactivity present in the β-chain of the same sample.

**RESULTS**

Recombinant Expression and Purification of SBP1 and Truncated Forms of SBP1—To localize and characterize further the functional domains in SBP1, SBP1 and truncated forms of SBP1 consisting of SCRs 1–2, 1–3, 1–4, 1–5, and 12–17 were expressed in baculovirus-infected insect cells. The various secreted His-tagged recombinant proteins were purified and analyzed by SDS-PAGE and Western blotting (Fig. 1). The recombinant proteins and peptides contain an interokinase cleavage site and a His tag with a calculated molecular mass of 3.2 kDa. The apparent molecular masses of each of the proteins SBP1 and SCRs 1–2, 1–3, 1–4, 1–5, and 12–17 were 115, 135, 20.5, 28, 35.5 and 43 kDa, respectively. The data are in close agreement with the molecular masses predicted for each protein based on amino acid composition.

Binding Activity of Recombinant SBP1 and the Truncated Mutants to Human C3b, Human C4b, and Trout-C3b-like—The binding of SBP1 to human C3b and C4b and the localization of the binding domains within SBP1 were analyzed by incubating the various proteins with immobilized C4b and C3b. Serial dilutions of 5 μg of human C4b and C4 and human C3b and C3 were bound to nitrocellulose membranes. The filters were blocked and then incubated with either 200 nm recombinant SBP1 or equimolar amounts of each of the five truncated forms of SBP1 diluted in VBS containing 10 mM CaCl₂. Equimolar amounts of human C4b and human factor H were used as positive controls. The membranes were washed and incubated with specific polyclonal antiserum raised against the recombinantly expressed SCRs 1–5 of SBP1; the sand bass cofactor protein was purified from the fish plasma, human factor H, or human C4bp, and the reaction was developed as described. At the same molar concentration, SBP1 and SCRs 1–5, 1–4, and 1–3 displayed the same binding activity for human C4b as shown with C4bp, whereas SCRs 1–2 and 12–17 failed to bind (Table I). SBP1 and SCRs 1–5 and 1–4 bound only 15% of the amount of C3b bound by factor H. The strong binding to C4b and the weak binding to C3b is in good agreement with previous data obtained with the cofactor protein purified from sand bass plasma (23). SCRs 1–2, 1–3, and 12–17 did not bind to human C3b. Neither SBP1 nor any of the mutants tested reacted with the nonactivated complement proteins C4 and C3 (data not shown). Because the noted weak binding of SBP1 to human C3b may be due to the use of a heterologous substrate, the same experiments were performed with trout C3b-like. SBP1 and SCRs 1–4 and 1–5 displayed 100% binding activity (Table I).

None of the proteins bound to nonactivated trout C3 (data not shown).

**Inhibition of the Formation of the Classic and Alternative Pathway C3 Convertase by SBP1 and the Truncated Forms of SBP1**—The previous experiments demonstrated the direct binding of SBP1 and SCRs 1–5, 1–4, and 1–3 to C4b and to lesser extent to C3b but did not address the functional outcome of these interactions. To elucidate whether these proteins participate in the regulation of the formation of the C3 convertases (C4b2a and C3bBb), the following experiments were performed. EC3b and EAC14b were generated and resuspended to a concentration of 1 × 10⁸ cells/ml in Ni-GVB (EC3b) or DGVB²⁺ (EAC14b). Portions of EC3b and EAC14b were incubated with an equal volume of buffer containing either SBP1 or SCRs 1–2, 1–3, 1–4, 1–5, or 12–17 in equimolar concentrations. C4b and factor H were used as controls with EAC14b and EC3b, respectively. The hemolytic activity of each sample was measured by the ability to generate C4b2a in the presence of a limited C2 input or C3bBb after the addition of factors B and D, and the reactions were developed as described above by adding NHS-EDTA.

The generation of C4b2a was markedly reduced in the sam-

| Binding to human C4b* | Binding to human C3b* |
|----------------------|----------------------|
| Protein binding to human C4b was 100% and to human factor H was 0% |
| Protein binding to human C4b was 0% and to human factor H was 100% |
| Protein binding to human C4b was 0% and to human factor H was 0% |

* a: Protein binding to human C4bp was 100% and to human factor H was 0%.
* b: Protein binding to human C4bp was 0% and to human factor H was 100%.
* c: Protein binding to human C4bp was 0% and to human factor H was 0%.

**TABLE I**

Inhibition of the Formation of the Classic and Alternative Pathway C3 Convertase by SBP1 and the Truncated Forms of SBP1—The previous experiments demonstrated the direct binding of SBP1 and SCRs 1–5, 1–4, and 1–3 to C4b and to lesser extent to C3b but did not address the functional outcome of these interactions. To elucidate whether these proteins participate in the regulation of the formation of the C3 convertases (C4b2a and C3bBb), the following experiments were performed. EC3b and EAC14b were generated and resuspended to a concentration of 1 × 10⁸ cells/ml in Ni-GVB (EC3b) or DGVB²⁺ (EAC14b). Portions of EC3b and EAC14b were incubated with an equal volume of buffer containing either SBP1 or SCRs 1–2, 1–3, 1–4, 1–5, or 12–17 in equimolar concentrations. C4b and factor H were used as controls with EAC14b and EC3b, respectively. The hemolytic activity of each sample was measured by the ability to generate C4b2a in the presence of a limited C2 input or C3bBb after the addition of factors B and D, and the reactions were developed as described above by adding NHS-EDTA.

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**FIG. 1. Expression of SBP1 and the truncated forms of SBP1.**

SBP1 and SCRs 1–2, 1–3, 1–4, 1–5, and 12–17 were separated by SDS-PAGE (12%) under nonreducing conditions and were visualized by silver staining (A) and Western blotting (B) with an affinity purified rabbit antibody raised against the purified plasma cofactor protein.
respectively. All reactions were incubated at 30 °C. At zero
factor H were used as controls with EAC14b2a and EC3bBb,
concentrations of SBP1, SCRs 1–5, or SCRs 12–17. C4bp and
samples received equal volumes of buffer containing equimolar
of EC3bBb received an equal volume of buffer, and the other
divided into equal samples. One aliquot of EAC14b2a and one
as controls. C2 (0.25 m
forms of SBP1.
accelerating the decay of EAC14b cells was incubated with SBP1,
SCRs 1–4, or 1–5 of SBP1 (Fig. 2A). This inhibition was
concentration-dependent and of the same extent as that obtained
with C4bp. SCRs 1–3 partially inhibited the formation of the
classical pathway C3 convertase (25%), and SCRs 1–2 and
12–17 had no effect. The effect of SBP1 and the truncated forms
on the formation of C3bBb is shown in Fig. 2A. Incubation with
SBP1 and SCRs 1–4, 1–5, and 1–3 resulted in inhibition of lysis
but to a lesser degree than that obtained with factor H (SBP1,
SCRs 1–2, and 12–17 had no effect.

Acceleration of the Decay Rate of the Classical and Alternative Pathway C3 Convertases by SBP1 and the Truncated Forms of SBP1—The effects of SBP1 and SCRs 1–2, 1–5, and
12–17 on the decay of EAC14a2b and C3bBb were investigated
kinetically. EAC14a2b and C3bBb were prepared as described.
The cells were washed in ice-cold buffer and resuspended to 1 × 10⁶
cells/ml, and each cellular intermediate was divided into equal samples. One aliquot of EAC14a2b and one of EC3bBb received an equal volume of buffer, and the other samples received equal volumes of buffer containing equimolar concentrations of SBP1, SCRs 1–5, or SCRs 12–17. C4bp and factor H were used as controls with EAC14a2b and EC3bBb, respectively. All reactions were incubated at 30 °C. At zero
time and at various time intervals thereafter, samples were
removed and added to NHS-EDTA to measure the residual C3
convertase activity.

The decay of C4b2a (7 min half-life) was markedly accelerated
by the addition of C4bp (3 min half-life), SBP1, and SCRs
1–5 (4.5 min half-life) (Fig. 3A). The loss of convertase activity
followed first-order kinetics for each of the proteins. SCRs 1–2
and 12–17 did not affect the decay rate of the enzyme. The sand
bass cofactor proteins had a minimal effect on the decay of
C3bBb as compared with the control with factor H (Fig. 3B).

Determination of the Cofactor Activity and Localization of the Domains in SBP1 in the Cleavage of Human C4b, Human C3b,
and Trout C3b-like—The structural changes in C4b and C3b
mediated by SBP1 and the truncated proteins were analyzed as
described under “Experimental Procedures.” Human C4b was
effectively cleaved by sand bass serum (Fig. 4); the α’-chain
produced by sand bass serum (Fig. 6A). The α’-chain was degraded into three peptides of α8 (α2), α27 (α3), and α19
(α4) kDa. The molecular masses of these products agree with
those generated by NHS. Cofactor-depleted sand bass serum
did not degrade C4b (lane 3). The addition of SCRs 1–5 (lanes
4–6) or 1–4 (data not shown) to cofactor-depleted sand bass
serum restored 100% of the C4b cleavage activity, whereas the
addition of SCRs 1–3 resulted in restoration of only 40% of the
activity. SCRs 1–2 and 12–17 had no effect (Fig. 5A). A similar
experiment performed with C3b showed a common cleavage
pattern of the α-chain produced by sand bass serum (Fig. 6B,
lane 3) and reconstituted cofactor-depleted sand bass serum:
α-68, α-46, α-43, and α-30 (lane 5). Although quantitatively

FIG. 2. Regulation of the formation of the classical and alternative pathway C3 convertases by SBP1 and the truncated forms of SBP1. Aliquots of 50 μl of EAC14b (A) and EC3b (B) were incubated for 30 min at 30 °C with an equal volume of buffer containing increasing amounts (0.75, 1.5, 3.0, and 6.0 μM) of SBP1 (●); SCRs 1–5 (○), 1–4 (×), 1–3 (■), 1–2 (□), and 12–17 (▲); or C4bp (▲) or factor H (●) as controls. C2 (0.5 μg/ml) diluted in 50 μl buffer was added to EAC14b; 0.5 μg of factor B and 10 ng of factor D diluted in 50 μl of buffer were added to EC3b. The mixtures were incubated for 10 min at 30 °C, and the C3 convertase generated was developed with 50 μl of NHS-EDTA diluted 1:10. The samples were incubated for 1 h at 37 °C, and the hemoglobin released was measured and compared with a 100% lysed sample (% lysis).

FIG. 3. Acceleration of the decay rate of the classical and alternative pathway C3 convertases by SBP1 and SCRs 1–5 and 12–17. One-ml aliquots of EAC14b2a (A) and EC3bBb (B) were incubated at 30 °C, with an equal volume of buffer or buffer containing 200
μg C4bp (EAC14b2a), factor H (EC3bBb), SBP1, SCRs 1–5, or SCRs 12–17. 50-μl samples were removed from each of the mixtures at 0, 2, 4, 6, 8, and 10 min and added to 50 μl of NHS-EDTA diluted 1:10. The decay rates of C4b2a (A) and C3bBb (B) in the presence of buffer (●), SBP1 (○), SCRs 1–5 (×), SCRs 12–17 (■), C4bp (□), and factor H (▲) are shown as percent lysis.
different, the molecular masses of these fragments agreed with those of the peptides generated by NHS. No cleavage occurred with cofactor-depleted sand bass serum alone (lane 4) or when SCRs 1–2 or 12–17 was added (Fig. 5B). The cofactor activity of SBP1 for human C4b (Fig. 4) and C3b (data not shown) was concentration-dependent. To investigate whether the lower efficiency in the cleavage of C3b by sand bass serum was due to incompatibility between the substrate and the ligand, a similar experiment was performed with C3 isolated from rainbow trout (S. gairdneri), a closely related fish.

As demonstrated by the disappearance of the α’-like chain, sand bass serum cleaved trout C3b-like (Fig. 6A, lane 3) more effectively than human C3b (Fig. 6B, lane 3). NHS and cofactor-depleted sand bass serum did not degrade trout C3b-like (Fig. 6A, lanes 1 and 4), whereas the addition of 5 μg of SCRs 1–5 to the cofactor-depleted sand bass serum fully restored cofactor activity (Fig. 6A, lane 5). Although the cleavage pattern was similar to that obtained using human C3b, the molecular masses of the peptides generated were different, being approximately 34 and 37 kDa (Fig. 6A, lanes 2, 3 and 5), as opposed to 43 and 46 kDa for the human C3b fragments (Fig. 6B, lanes 2, 3 and 5). On longer exposure of the gel a product of 30 kDa was also detected. Because the α-chain of trout C3b-like still contains the N-terminal portion (C3a), the α-68 fragment is larger, and the molecule has the electrophoretic mobility of the β-chain; thus it cannot be detected as a separate band (Fig. 6A, lanes 2, 3, and 5).

**DISCUSSION**

The complement system of contemporary mammals, consisting of more than 25 plasma and cell membrane proteins, constitutes a highly successful recognition and effector system against invading microorganisms, parasites, and viruses. The proteins of the complement system appear to be phylogenetically old. Invertebrates possess a cytolytic system that can be activated by cobra venom factor (41), and Cyclostomes, the most primitive existing vertebrate, has a complement system that appears to consist of only the alternative pathway (42). A C3 gene has been isolated from the sea urchin (43), and in different bony fish cDNAs coding for complement proteins for both the alternative and the classic pathways has been isolated, C3 from rainbow trout (S. gairdneri) and sea bream (Sparus aurata) (33, 44), C4 from carp (45), Bf/C2 from medaka

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**Fig. 4.** Cofactor activity of SCRs 1–5 of SBP1 in the cleavage of human 125I-C4b. SDS-PAGE of 125I-C4b incubated with 1:4 dilutions of NHS (lane 1), sand bass (Sb) serum (lane 2), cofactor-depleted sand bass serum (Sb serum depl.) (lane 3), or cofactor-depleted sand bass serum reconstituted with different amounts of SCRs 1–5 of SBP1 (lanes 4–6). 125I-C4b incubated with VBS served as Control. The traditional nomenclature of the generated fragments of the α’-chain is indicated on the left.

**Fig. 5.** Quantitation of cofactor activity of SCRs 1–2, 1–3, 1–4, 1–5, and 12–17 in the cleavage of human 125I-C4b and 125I-C3b. 125I-C4b (A) and 125I-C3b (B) were incubated with 1:4 dilutions of NHS, sand bass serum, cofactor-depleted sand bass serum, or cofactor-depleted sand bass serum reconstituted with SCRs 1–2, 1–3, 1–4, 1–5, or 12–17. The cleavage of 125I-C4b was quantitated by the amount of α2 fragment generated and that of 125I-C3b by the amount of α-68 generated.
Ancestral Precursor to Human Plasma Complement Regulators

Fig. 6. Cofactor activity of SCRs 1–5 of SBP1 in the cleavage of trout (25)C3b-like and human (25)C3b. SDS-PAGE of trout (25)C3b-like (A) and human (25)C3b (B) incubated with NHS (lane 1), trout serum (lane 2), sand bass (Sb) serum (lane 3), cofactor-depleted sand bass serum (Sb serum dep.) (lane 4), or cofactor-depleted sand bass serum reconstituted with 5 μg of SCRs 1–5 of SBP1 (lane 5). Trout (25)C3b-like and human (25)C3b incubated with VBS served as controls.

Activation of complement either via the classical or the alternative pathway allows the assembly of the membrane attack complex that leads to the osmotic lysis of target cells. The evolutionary older alternative pathway is activated directly by microorganisms, whereas the younger classic pathway is triggered by antibody-antigen complexes. In mammals, the formation and function of the C3-cleaving enzymes (C3 convertases) are carefully regulated by the plasma cofactor proteins C4bp and factor H and the plasma enzyme factor I. Similar data in lower vertebrates are limited. In previous studies we have demonstrated that although with considerable quantitative differences human C4b and C3b can be degraded by serum from animal species throughout evolution (21). The most primitive species displaying these activities, so far identified, are the teleost fish barred sand bass (P. nebulifer) and the rainbow trout (S. gairdneri). In contrast to higher vertebrates (5–7, 11), in sand bass only one protein serves as cofactor for factor I in the cleavage of C4b and C3b. The cofactor involved in this reaction has been purified and characterized at both the protein (23) and DNA level (24).

To confirm our hypothesis that the fish cofactor molecule is an ancestor precursor protein to the human plasma complement regulators C4bp and factor H, we characterized its functional properties. The full-length cDNA and truncated forms obtained by the consecutive deletion of SCRs from the C terminus of the molecule were recombinantly expressed (SBP1 and SCRs 1–2, 1–3, 1–4, 1–5, and 12–17) in the baculovirus system and purified to homogeneity (Fig. 1). Expression of SCRs 6–11 in this system was not successful. The C4b binding domain in SBP1 was localized to SCRs 1–3 (Table I). This finding is in agreement with the localization of a single C4b-binding site in the N-terminal portion of the α-chains of human C4bp (49, 50). In humans (51) and mice (52) this site resides in SCRs 1–3. When the effects of SBP1 and truncated mutants on the formation and decay rate of the classical pathway convertase were analyzed with C4bp as standard, it was found that equimolar amounts of SBP1, SCRs 1–4, and 1–5 inhibited the formation of the classical pathway convertase to approximately the same extent as C4bp (Fig. 2A). In addition, SBP1 and SCRs 1–5 displayed decay-accelerating activity toward C4b2a comparable to that of the control (Fig. 3A). SCRs 1–4, 1–3, and 1–2 were not tested. It appears that the binding of C4b to SBP1 requires different SCRs from those involved in the inhibition of the formation of cell-bound C4b2a. This concept is supported by the fact that whereas SCRs 1–3 displayed 100% binding activity to fluid phase C4b (Table I), the formation of the classic pathway convertase was inhibited by only 30% (Fig. 2A), and full inhibition required SCRs 1–4 (Fig. 2A).

Sand bass serum degrades the α-chain of human C4b, yielding the same fragments, α2, α3, and α4, as those produced by NHS (Fig. 4). At equimolar concentrations SCRs 1–5 and 1–4 fully reconstituted the activity of cofactor-depleted sand bass serum, whereas SCRs 1–3 provided 30% of cofactor activity as assessed by the generation of the α2 fragment (Fig. 5A). These differences in the requirement of SCRs for binding, inhibition of convertase formation, decay accelerating activity, and cofactor function of SBP1 may be explained by the contribution of tertiary structures of neighboring SCRs. Antibodies that bind to SCR 1 or SCR 2 completely inhibit C4b binding and abolish cofactor activity of C4bp (53). As it is for human C4bp, the cofactor activity of SBP1 in the cleavage of C4b is concentration-dependent (Fig. 4). Although a heterologous substrate was used, these results show that the sand bass cofactor protein mediates all the functional activities ascribed to C4bp, suggesting that this system has not been significantly altered during evolution.

SBP1 binds to human C3b far more weakly than to C4b (Table I). In addition SBP1, SCRs 1–5, and SCRs 1–4 in equimolar concentrations inhibited the formation of the alternative pathway C3 convertase by only 30% compared with the inhibition achieved with human factor H (Fig. 2B). SCRs 1–3 did not bind C3b and had a minimal effect on the inhibition of the formation of C3bBb (Fig. 2B). Similarly, the decay accelerating activity of SBP1 and SCRs 1–5 was limited (Fig. 2B). SCRs 1–4, 1–3, and 1–2 were not tested. Despite the closer sequence homology between factor H and SBP1 (24), at the functional level the interaction with human C3b is weaker. Complete binding of factor H to human C3b requires at least three distinct binding sites (SCRs 1–4, 6–10, and 16–20) as shown by the fact that mutant proteins lacking any one of these sites exhibit a 6–8-fold reduction in affinity for C3b (54). This may be explained by the localization of only a single C3b binding domain in SCRs 1–4. Another possibility may be differences between human and fish C3 resulting from amino acid...
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mutations during the long evolutionary divergence between fish and mammals (55). In experiments with methylamine-treated C3 (C3b-like) from rainbow trout, closely related species, SBP1, SCRs 1–5, and 1–4, demonstrated 100% binding (Table I) compared with the interaction between human factor H and human C3b. These results are similar to those obtained with human factor H, in which domains required for cofactor and decay accelerating activity have been mapped to SCR 1–4 (31, 56, 57). Thus, the weak interaction of SBP1 and human C3b is at least in part due to species-specific differences between human and fish C3.

Sand bass serum and NHS cleaved the α-chain of human C3b in the same way (Fig. 6B), but the efficiency of this reaction was low. In contrast, sand bass serum cleaved 100% of the α′-chain of trout C3b-like (Fig. 6A), in a cleavage pattern similar to that obtained with human C3b but with different molecular masses of the polypeptides generated. The first cleavage generated a 34-kDa polypeptide and a fragment of approximately 75 kDa that also contains the N terminus of the trout C3b-like. Two additional fragments of 37 and 30 kDa appeared that correlated with the reduction of the 75-kDa band. Both fragments were visible only upon longer exposure. The difference in the size of the products may be due to differences in the position of the cleavage sites in the trout C3.

Sand bass cofactor-depleted serum, used as source of factor I, did not cleave human C4b, human C3b, or trout C3b-like unless SBP1 or SCRs 1–4 or 1–5 were added (Fig. 4 and Fig. 6, A and B). Thus the sand bass cofactor protein is the only protein in the fish plasma that serves as a cofactor in the cleavage of C4b and C3b. The sand bass cofactor protein can therefore be considered an ancestral precursor protein for both mammalian complement regulatory proteins, C4bp and factor H. These findings are in good agreement with studies (58) that suggest that factor H is of ancient origin, whereas C4bp is a novel gene that has emerged due to gene duplication after the separation between fish and mammals. The close relationship of the sand bass cofactor protein and human factor H agrees with the idea of the more ancient origin of the alternative pathway of complement, which does not require the presence of antibodies.

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