J. Biochem. 132, 719–728 (2002)

Mapping of the Sites Responsible for Factor I–Cofactor Activity for Cleavage of C3b and C4b on Human C4b-Binding Protein (C4bp) by Deletion Mutagenesis[1]

Aya Fukui,[*,†,‡,2,3] Tomoko Yuasa-Nakagawa,[*,2,4] Yusuke Murakami,[*,2,5] Kenji Funami,[*,†] Natue Kishi,[*] Tadashi Matsuda,[*] Teizo Fujita,[§] Tsukasa Seya,[†,‡,6] and Shigeharu Nagasawa[*]

[*]*Department of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060;* [†]*Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari-ku, Osaka 537-8511;* [‡]*Department of Molecular Immunology, Nara Institute of Science and Technology, Ikoma, Nara 631-0101; and* [§]*Department of Biochemistry, Fukushima Medical University, Fukushima 960-1295*

Received June 18, 2002; accepted August 15, 2002

Human C4b-binding protein (C4bp) facilitates the factor I–mediated proteolytic cleavage of the active forms of complement effectors C3b and C4b into their inactive forms. C4bp comprises a disulfide-linked heptamer of α-chains with complement (C) regulatory activity and a β-chain. Each α-chain contains 8 short consensus repeat (SCR) domains. Using SCR-deletion mutants of recombinant multimeric C4bp, we identified the domains responsible for the C3b/C4b-binding and C3b/C4b-inactivating cofactor activity. The C4bp mutant with deletion of SCR2 lost the C4b-binding ability, as judged on C3b/C4b-Sepharose binding assaying and ELISA. In contrast, the essential domains for C3b-binding extended more to the C-terminus, exceeding SCR4. Using fluid phase cofactor assaying and deletion mutants of C4bp, SCR2 and 3 were found to be indispensable for C4b cleavage by factor I, and SCR1 contributed to full expression of the factor I–mediated C4b cleaving activity. On the other hand, SCR1, 2, 3, 4, and 5 participated in the factor I–cofactor activity for C3b cleavage, and SCR2, 3, and 4 were absolutely required for C3b inactivation. Thus, different sets of SCRs participate in C3b and C4b inactivation, and the domain repertoire supporting C3b cofactor activity is broader than that supporting C4b inactivation by C4bp and factor I. Furthermore, the domains participating in C3b/C4b binding are not always identical to those responsible for cofactor activity. The necessity of the wide range of SCRs in C3b inactivation compared to C4b inactivation by C4bp and factor I may reflect the physiological properties of C4bp, which is mainly directed to C4b rather than C3b.

Key words: complement regulatory proteins, C3b/C4b INA cofactor (MCF), deletion mutants of C4bp, monoclonal antibodies, short consensus repeat (Sushi domain).

The human complement (C) system comprises soluble effectors and their regulators residing in body fluids and cell membranes. A major effector, C3, plays an important role in the elimination of foreign material including microorganisms. C3 is activated through the C proteolytic cascade and covalently binds foreign materials to tag them for recognition by the host immune system (*1*). Interactions between tagged cells and host C3-receptor–bearing cells lead to activation of Toll-like receptors, NK potentiation, enhancement of phagocytosis, liberation of anaphylatoxins and chemoattractants, and immune clearance (*1, 2*). Deposited C3 fragment C3b also activates the subsequent C cascade resulting in target cell lysis *via* the formation of a membrane attack complex (*1*). These C-mediated cytotoxic effects are severely restricted on foreign cells, thereby circumventing autologous C attack of host cells. Soluble and membrane C regulatory proteins are mainly involved in this host–foreign discrimination (*2, 3*).

Human C4b-binding protein (C4bp) was first identified by Stroud and his colleagues in 1975 as a C4b inactivating

[1]This work was supported in part by CREST, JST, and Grants in-Aid from the Ministry of Education, Science, Sports and Culture (for Scientific Research on Priority Areas), and the Ministry of Health and Welfare of Japan.

[2]The first three authors equally contributed to this work.

Present addresses: [3]Department of Bacterial Toxinology, Research Institute of Microbial Diseases, Osaka University, Suita, Osaka 565; [4]RIKEN Yokohama Institute, Suehiro-cho 1-7-22, Tsurumi, Yokohama, Kanagawa 230-0045; [5]Wellcome Trust Immunology Unit, Department of Medicine, University of Cambridge School of Clinical Medicine, MRC Centre, Hills Road, Cambridge CB2 2SP, UK.

[6]To whom correspondence should be addressed at: Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari-ku, Osaka 537-8511. Tel/Fax: +81-6-6973-3920, E-mail: seya-tu@mc.pref.osaka.jp

Abbreviations: C, complement; C4bp, C4b-binding protein; CR, complement receptor; DACM, *N*-(dimethylamino-4-methylcoumarinyl)-maleimide; DAF, decay-accelerating factor (CD55); flu-C3b/C4b, OG- or DACM-labeled C3b/C4b; MCP, membrane cofactor protein (CD46); OG, Oregon Green 488 iodoacetamide; RCA, regulator of complement activation; SCR, short consensus repeat.

cofactor for factor I (a serine protease responsible for C3b/C4b inactivation) (*4*), and in 1977 as a C3b inactivating cofactor, it being named macromolecular C3b/C4b INA cofactor (MCF) (*5, 6*). Later, the concept was proposed that C4bp mainly participates in prohibiting C3 consumption in the blood plasma by inhibiting C4b-mediated C activation (classical pathway) in the fluid phase (*7, 8*), whereas another soluble cofactor factor, H, participates in protection of C3 consumption through inhibition of the C3b function (alternative pathway) (*9, 10*). In fact, C4bp inactivates C4b through acceleration of the decay of C4b2a convertase and by acting as a cofactor of factor I for the cleavage of C4b (*7, 8, 11, 12*).

The genes of the C3-step regulators, CR1 (CD35), DAF (CD55), MCP (CD46), factor H, and C4bp, were found to be clustered in chromosome 1q32 in humans (*1, 13*). All the C3-step regulatory proteins contain variable numbers of tandemly arranged domains, namely short consensus repeats (SCRs) (*1*). These domains consist of ~60 amino acids, and are framed by two disulfide bonds formed by four cysteines within each SCR module (*1*). Unlike other SCR proteins, which are monomers, the major form of human C4bp in the plasma consists of seven identical α-chains (8 SCRs each) and one β-chain (3 SCRs), and all of these chains are linked together by disulfide bridges (*1, 14*). Electron microscopy of C4bp demonstrated a spider-like conformation, with the seven α-chains extending tentacles (*15*).

Using deletion mutants of C4bp, Hardig *et al.* and Blom *et al.* successfully mapped the C4bp domains functionally essential for C4b-binding and -cleavage by factor I as a cofactor (*16, 17*). Their results in part supported those obtained on functional analysis of proteolytic fragments of C4bp (*18*): the SCRs responsible for C4b-binding and cofactor activity were identified on the N-terminal SCRs of C4bp. These earlier studies, however, focused on the C4b-inactivating function of C4bp. The domains responsible for C3b-inactivation have not been elucidated yet.

Here, we compared the structural requirement of C4bp for supporting the factor I–cofactor activity for cleavage of C4b *vs.* C3b using SCR-deletion mutants, and found that for C3b cleavage by factor I a wider range of SCRs of C4bp is required for full expression of the cofactor activity than for C4b cleavage. The structural difference of C4bp supporting C3b and C4b inactivation will be discussed in comparison with the cases of other C3-step SCR-containing C regulatory proteins.

MATERIALS AND METHODS

Proteins and Reagents—CHO cells were purchased from ATCC. Cells were cultured in Ham's F12 (Sigma) containing 10% FCS, 0.15% $NaHCO_3$, 100 U/ml penicillin G, and 100 μg/ml streptomycin (Wako) at 37°C under an atmosphere of 5% CO_2. Human C4bp cDNA was a kind gift from Dr. Y. Niho (Kyushu University, Fukuoka) (*19*). *N*-(Dimethylamino-4-methylcoumarinyl)maleimide (DACM) was purchased from Wako Pure Chemicals, Osaka. Oregon Green 488 iodoacetamide (OG) was from Molecular Probes, Eugene, OR. The methods used for labeling these SH reagents for C3b/C4b were reported in previous papers (*20, 21*). The mammalian expression vector pME18s was a gift from H. Ariga (*22*). pBluescript was purchased from TOYOBO. Monoclonal antibodies (mAbs) against human C4bp, TK1, TK2, and TK3 were prepared as described previously (*23*), and MFbp4 was kindly denoted by Dr. K Suzuki (Mie University) (*24*).

Human C4bp, factor I, C3, and C4 were purified from human serum (*5, 11*). C3b and C4b were prepared as described previously (*21, 25*). OG- or DACM-labeled C3b and C4b were prepared in our laboratory (*20, 21*).

DNA Constructs—cDNA encoding the C4bp α-chain was placed in the *Eco*RI/*Sac*I multicloning site of pME18s. Each SCR and sequential SCRs of the C4bp α-chain were deleted oligonucleotide-directed *in vitro* mutagenesis (*26*), using the pME18s-C4bp α-chain as a template. Briefly, primers for negative and positive strands were designed so as to contain part of the sequence of each SCR to be deleted (*22, 26*). PCR amplification was performed with the C4bp-plasmid as a template, and these primers and vector primers. The PCR products were recovered from the agarose gel and used as templates for the second PCR, in which the deletion sequences were completed. The PCR products were recovered and subcloned into expression vectors. The information on deleted amino acid sequences of the mu-tants is presented in Table I. The plasmid was transformed into *Escherichia coli* DH5α, amplified and then purified as described previously (*22*).

Expression and Purification of C4bp SCR-Deleted Mutants—The plasmid containing each construct (10 μg) was transfected into CHO cells (1×10^6) by calcium phosphate methods (*22*). After 24 h, the cells were transferred to the medium containing 0.5 mg/ml G418 for selection. We collected the culture supernatant and checked the expression of C4bp mutant proteins by immunoblotting analysis with anti-C4bp polyclonal Ab, which was produced in our laboratory. CHO clones exhibiting high levels of wild-type and mutant C4bp expression were detected by Northern blot analysis of total RNA with human C4bp cDNA as a probe, and then expanded in tissue-culture plates or bottles. After reaching 80% confluence, the cells were transferred to FCS-free culture medium. C4bp proteins were harvested from the culture supernatants. Purification of each mutant was conducted as follows. Briefly, the supernatants were concentrated with Q-Sepharose (Sigma) and then purified with anti-C4bp polyclonal Ab–conjugated agarose gels (2 mg/ml). The C4bp proteins were eluted with 0.1% NP-40/3M KSCN/150 mM NaCl/50 mM Tris-HCl (pH 7.4), and then dialyzed against 50 mM NaCl/20 mM phosphate buffer (PB) (pH 7.4). The purity and concentration of each C4bp protein were examined by silver staining and ELISA, respectively (see below). In some experiments, we used the condensed supernatants of CHO transfectants obtained with an ultrafiltration membrane.

SDS-PAGE, Silver Staining, and Immunoblotting—SDS-PAGE was performed by the method of Laemmli (*25*), and gels were stained with silver (Daiichi, Tokyo) according to the manufacturer's booklet.

Immunoblotting was performed as described previously (*22*). An ECL kit (Amersham Pharmacia Biotech, Sweden) was used for detection (*22*).

Sandwich ELISA—The levels of C4bp mutants were determined by sandwich ELISA. Briefly, a 96-well plate (Immuno Plate II, Nunc) was coated with 50 μl of 5 μg/ml anti-C4bp polyclonal antibodies in 50 mM sodium carbonate buffer, pH 9.6, for 5 h at 37°C. After washing, the plates

were quenched in BlockAce (Dainippon Pharm., Tokyo) diluted fourfold with PBS for 15 h at 4°C. After three washes, 50 μl of native C4bp (purified from blood plasma) as a standard and C4bp variants were added, and the plates were allowed to stand for 2 h at 37°C. After incubation, the plates were washed, incubated with 50 μl (1 μg/ml) of mAb, TK1 (for C-terminal deletion mutants), or TK2 (for N-terminal deletion mutants) (*23*), washed again, and reacted with 50 μl of 1,000-fold diluted biotinilated anti–mouse IgG Ab (TAGO), and then HRP-labeled avidin-biotin complex (Amersham Pharmacia Biotech) for 1 h at 37°C. Finally, 100 μl of 50 mM citric acid/100 mM PB, pH 5.0, containing 0.15 mg/ml 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) and 0.02% H_2O_2 was added, followed by incubation for 1 h at room temperature. The absorbance at 405 nm (A_{405}) was measured with a plate reader (Shimadzu, Kyoto). After each step, the plate was washed with 200 μl of phosphate-buffered saline (PBS) containing 0.05% Tween 20 four times. Based on the A_{405} of the standard material (native C4bp), the quantities of C4bp mutants in the samples were estimated.

C3b/C4b Binding Assay—Two direct binding assays were used to assess the ability of C4bp to bind C3b or C4b. First, C4b-Sepharose (4B, Amersham-Pharmacia) or C3b-Sepharose was prepared as 2–3 mg of C4b or C3b bound to 1 ml of Sepharose. Each Sepharose (30 μl) was incubated with 2 μg/ml of wild-type C4bp or one of its variants in 200 μl of 50 or 150 mM NaCl/20 mM PB, pH 7.4, for 15 h at 4°C. Supernatants (sups) were recovered as unbound fractions. The Sepharose was washed with the same buffer containing 0.1% Tween 20 three times and the bound C4bp proteins were recovered as bound fractions by the addition of 2% SDS/10 mM Tris-HCl, pH 6.8. The C4bp levels of these fractions were assessed by SDS-PAGE followed by immunoblotting.

In the other assay, the levels of C4bp in the unbound fractions were quantitatively evaluated by ELISA as described above.

Factor I–Cofactor Assay—We determined factor I–cofactor activity by the methods described previously (*20, 21*). The optimal buffer conditions for C3b cleavage were 50 mM NaCl/10 mM PB, pH 6.0, which were also preferable for C4b cleavage (*27*). Hence, these low ionic conditions were employed for C3b and C4b cleavage. Briefly, OG-C3b (200 ng) or OG-C4b (200 ng), human factor I (50 ng), and serially diluted samples of various C4bp mutants were incubated in 50 mM NaCl/20 mM PB, pH 7.4, for 3 h at 37°C. The samples were separated by SDS-PAGE (8.5% acrylamide) under non-reducing and reducing conditions for C4b and C3b, respectively, and fixed with 5% methanol for 10 min. DACM-C3b and -C4b were used in some experiments. For some of the C4b cleavage assays, the buffer was replaced with PB, pH 7.4, containing 150 mM NaCl. The fluorescence intensity was visualized with a fluorescence imaging analyzer (FLA-2000; Fuji Photo Film, Tokyo) and the activity determined with the following formulae: Factor I–cofactor activity for C3b = α1′ fragment/(α1′ fragment + α′ fragment) × 100 (%); Factor I–cofactor activity for C4b = C4d fragment/(C4d fragment + C4b fragment) × 100 (%).

Blocking studies were performed with 0.1 μg of C4bp and 20 μg of mAbs (*23, 28*, also see Table II) in PBS, pH 7.4. The mixtures were allowed to stand for 1 h at 4°C. The cofactor assay was performed in the presence of a mAb as described above. %Cleavage was determined as described above. %Block was evaluated regarding the cofactor activity in the sample without mAb as 100%.

RESULTS

Expression and Characterization of Recombinant C4bp Mutants—To determine the number of SCRs involved in the factor I–cofactor function of C4bp, we constructed deletion mutants (Fig. 1 and Table I). The mutants we designed for this study were all disulfide-linked multimers. We first remodeled the C4bp molecule such that the scaffold of either SCR1–4 (ΔSCR1–4) or SCR5–8 (ΔSCR5–8) was deleted from each tentacle of the C4bp molecule. Other mutants were multimers in which each of the first five SCRs was individually deleted, or the first 2 or 3 SCRs were deleted (Fig. 1). Single SCR6, SCR7, or SCR8 deletion mutants were produced but the protein recovery was poor (data not shown). We did not use these mutants because ΔSCR1–4 failed to bind to C3b- and C4b-conjugated Sepharose, whereas ΔSCR5–8 greatly bound to C3b- and C4b-Sepharose like the wild-type C4bp (see Fig. 3).

All nine recombinant proteins were expressed in stable CHO cell lines and the expression was confirmed by immu-

TABLE I. **Numbers of amino acid residues deleted in the C4bp mutants used in this study.**

No. of SCR	Relevant sequence*
SCR1	49–110
SCR2	111–172
SCR3	173–235
SCR4	236–296
SCR5	297–361
SCR6	362–424

*Sequence 1–48 represents a signal peptide.

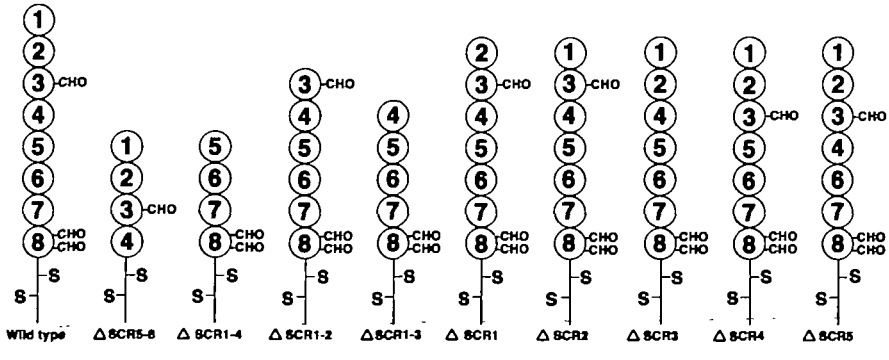

Fig. 1. **Schematic representation of the C4bp mutants used in this study.** Each circle represents one SCR. Top, N-terminal SCR; bottom, C-terminal stretch. The number of the SCRs are shown in the circles. The sugar moiety is indicated as -CHO and the Cys residues involved in the inter-chain disulfide bridges as -S. The names of the C4bp variants are shown at the bottom.

noblotting (Fig. 2A). In most of the experiments, proteins were purified from culture media by affinity chromatography on anti–C4bp polyclonal Ab–coupled Sepharose. The purified proteins were analyzed by SDS-PAGE followed by silver staining (Fig. 2B). As expected, the mutant C4bp proteins formed multimers because the molecular masses of non-reduced single SCR deletion mutants were ~500 kDa under nonreducing conditions and ~70 kDa under reducing conditions. The molecular differences among the deletion mutants were probably due to post-translational sugar modification (Fig. 2A, c and d). Similarly, ΔSCR5–8, ΔSCR1–4, ΔSCR1–2, and ΔSCR1–3 all formed multimers and the expected molecular masses were observed with reducing samples (Fig. 2, A and B). To confirm the uniformity of the multiple chains of a single species of mutant, each variant was transferred to a PDVF membrane and then subjected to N-terminal amino acid sequencing. The C4bp mutants yielded the expected amino acid sequences corresponding to the head of SCRs (data not shown). Since all these variants were found to form polymeric C4bp and judging from the molecular mass of the wild-type C4bp, they formed heptamers (Fig. 2B), and the polymerization process did not appear to be affected by the introduced mutations. The expression levels of all of the recombinant C4bp variants, except for ΔSCR6, ΔSCR7, and ΔSCR8, were similar to that of wild-type C4bp, suggesting their prop-er folding during synthesis.

Reactivity of mAbs with mC4bp—The mutants were probed with a panel of monoclonal antibodies directed against the α-chain of C4bp. The mAbs against C4bp tested were TK1, TK2, and TK3 (*23*), and a mAb MFbp4 (Table II). Judging from the reactivity profiles, TK1 and TK3 re-

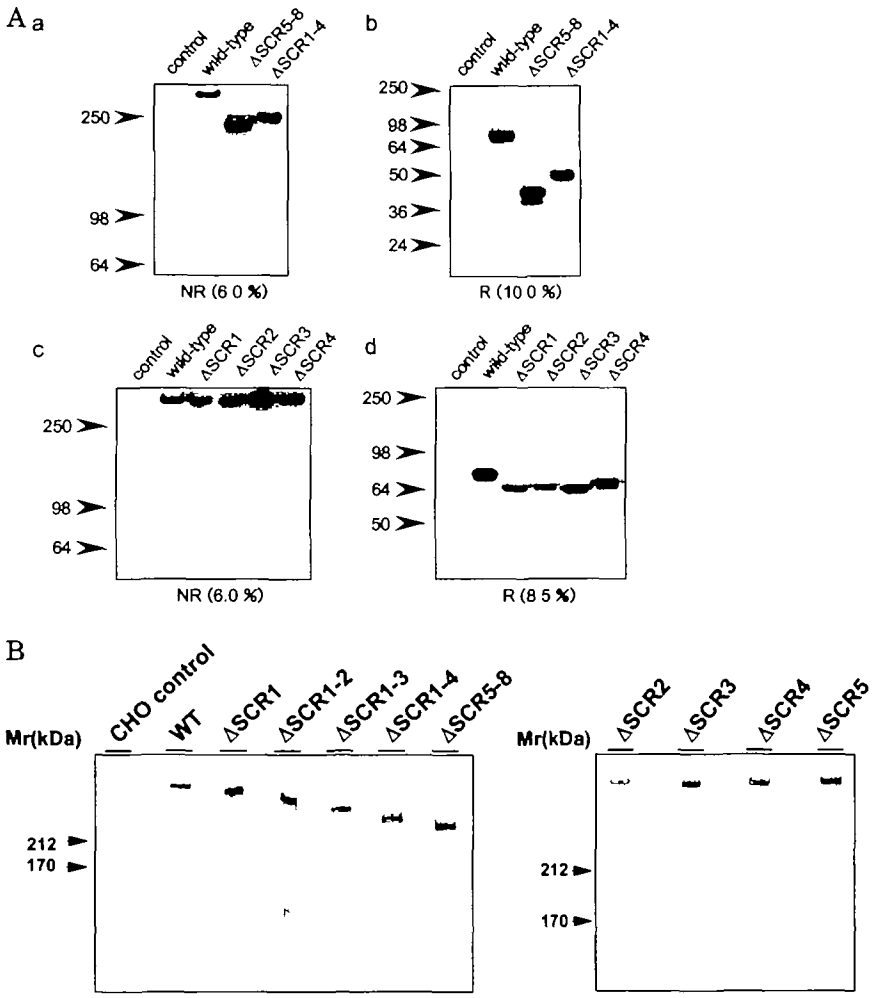

Fig. 2. **A: Immunoblotting analysis of C4bp mutant proteins.** Culture supernatants (sups) of transfectants were subjected to SDS-PAGE (the concentration of acrylamide is given below each panel) and proteins were transblotted onto a PVDF membrane. C4bp mutants were probed with rabbit anti-human C4bp polyclonal Abs. Sup of CHO cells transfected with only a vector was used as a negative control and sup of wild-type C4bp-expressing CHO cells was used as a positive control. The mobility of markers is shown to the left. Panels a and c, non-reduced samples; panels b and d, reduced samples. **B: Silver staining of the purified C4bp mutants.** Purified C4bp and its mutants (150 ng) were subjected to SDS-PAGE (6% acrylamide) under non-reducing conditions and the proteins were stained with a silver staining kit, which demonstrated highly purified materials. The negative control used (CHO control) was the sample obtained from sup of untransfected CHO cells purified by the same procedure as that used for C4bp variant–expressing CHO cells. Based on the molecular masses of variants and compared to that of wild-type C4bp (WT), all mutants formed heptamers. The names of the mutants are shown at the top of the gels and the mobility of markers is shown to the left.

TABLE II. **Characterization of mAbs.**

mAb	Reactivity against*								Blocking cofactor activity (%)	
	Control	Wild-type	ΔSCR5–8	ΔSCR1–4	ΔSCR1	ΔSCR2	ΔSCR3	ΔSCR4	C4b	C3b
TK1	–	++	++	–	–	++	++	++	30	45
TK2	–	++	–	++	++	++	++	++	10	40
TK3	–	++	++	–	++	++	–	+	~100	~100
MFb4	–	++	++	–	–	++	++	++	ND	ND

*Assuming that the level of binding of wild-type C4bp to each mAb is 100%. ++ means >90%; +, 90–60%; –, <5%; ND, not determined.

acted with SCR1 and 3, respectively. MFbp4 recognized SCR1. TK2 recognized one of the C-terminal SCRs, 5–8. The results are summarized in Table II. From these results, we decided to use TK1 for detection of the ΔSCR5–8 mutant, and TK2 for detection of the other mutants and the wild-type C4bp. According to the results, ELISA was performed to determine the levels of the C4bp mutants (see Fig. 3B).

C4b- and C3b-Binding Function of C4bp Mutants—Two methods were employed to evaluate C3b- and C4b-binding. First, a direct binding assay was performed using C4b-coupled Sepharose, each supernatant being incubated with the C4b-Sepharose. The supernatants were collected as C4b-unbound material. The Sepharose was washed with 150 mM NaCl/20 mM PB, pH 7.4, containing 0.1% Tween 20, and the bound proteins were recovered with 2% SDS/10 mM Tris-HCl, pH 6.8. Analysis of the C4b-unbound and -bound proteins was performed by SDS-PAGE followed by immunoblotting with anti-C4bp polyclonal Abs (Fig. 3A). The ΔSCR1–4 mutant did not bind to the Sepharose while ΔSCR5–8 bound like the native form. Next, the domains of C4bp responsible for binding to C4b were examined by the Sepharose-binding method. Only ΔSCR2 was found in the unbound fraction and a relatively faint band was observed with ΔSCR3 for the C4b-nonbound sample in some experiments. Thus, SCR2 and, to a lesser extent, SCR3 participate in C4b binding (Fig. 3).

With the Sepharose-binding method with C3b-Sepharose, no bound protein was detected under isotonic conditions (data not shown). We decided to determine the binding efficacy under low conductivity conditions, and the positive results obtained with this direct method and ELISA (see below) indicated that C4bp and ΔSCR5–8 bind C3b-Sepharose.

Second, to quantitatively determine the amounts of C4bp variants in the unbound fractions, we employed the ELISA method (Fig. 3B). Two sets of conditions with different conductivities, 50 mM and 150 mM NaCl in PB/Tween 20, pH

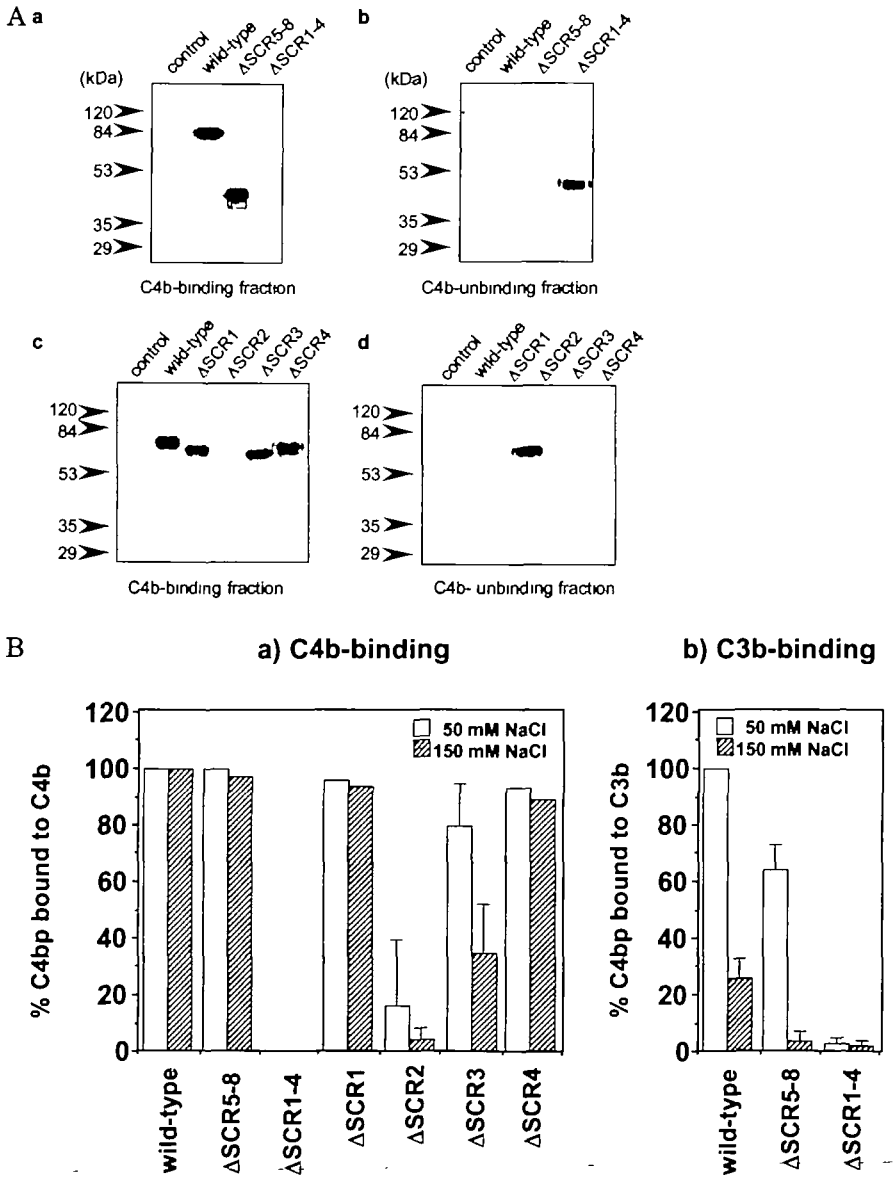

Fig. 3. **A: Binding of C4bp mutants to C4b-Sepharose.** Under physiological buffer conditions, C4bp mutants (0.4 μg) were incubated with C4b-Sepharose (harboring ~90 μg of C4b), namely the C4b-Sepharose binding assay. Sups and bound fractions were obtained and reduced for SDS-PAGE (10% acrylamide) followed by immunoblotting. Panels b and d, C4b-unbinding fractions; panels a and c, C4b-binding fractions. The negative and positive controls were as in Fig. 2A. **B: ELISA for quantitation of C4bp-binding to C4b and C3b.** Panel a: Two different conductivities (50 and 150 mM NaCl) were employed to assess the binding capacities of C4bp mutant proteins as to C4b using the C4b-Sepharose binding assay and sandwich ELISA for determination of C4bp. The binding capacities of wild-type C4bp as to C4b at 50 and 150 mM NaCl conditions were regarded as 100%, respectively, and %binding of each mutant was determined accordingly. The experiments were performed three times and error bars are shown. For some samples no error bars are given since almost 100% of the proteins was bound to the Sepharose. Panel b: A similar assay was performed with C3b-coupled Sepharose under two conductivity conditions (see Panel a). Two experiments were performed, which gave very similar binding profiles.

7.4, were used for evaluation of the C4b-binding activity of C4bp variants. SCR2 was essential for C4b-binding under both low and high ionic strength conditions. SCR3 appears to stabilize the C4b–C4bp interaction under the 50 mM NaCl conditions (Fig. 3B, Panel a).

Under the low ionic strength conditions, C3b-binding was examined, using the purified C4bp material, by ELISA (Fig. 3B, Panel b). About 60% of the ΔSCR5–8 binding was observed under low conductivity conditions, whereas virtually no binding was detected under physiological conditions. Even under the low conductivity conditions, ΔSCR1–4 variant protein binding was barely detected, suggesting that the binding affinity is largely due to SCR1–4.

Unfortunately, we could not examine the subtle binding ability of other deletion mutants using CHO cell supernatants, since the concentrations of the mutants in the CHO cell supernatants were too low for detecting with this method. Purified and concentrated mutants were prepared to check the binding ability but the samples had lost the ability because of a high concentration of coexisting NP-40. Therefore, no definitive data were obtained regarding the SCR range required for C3b binding.

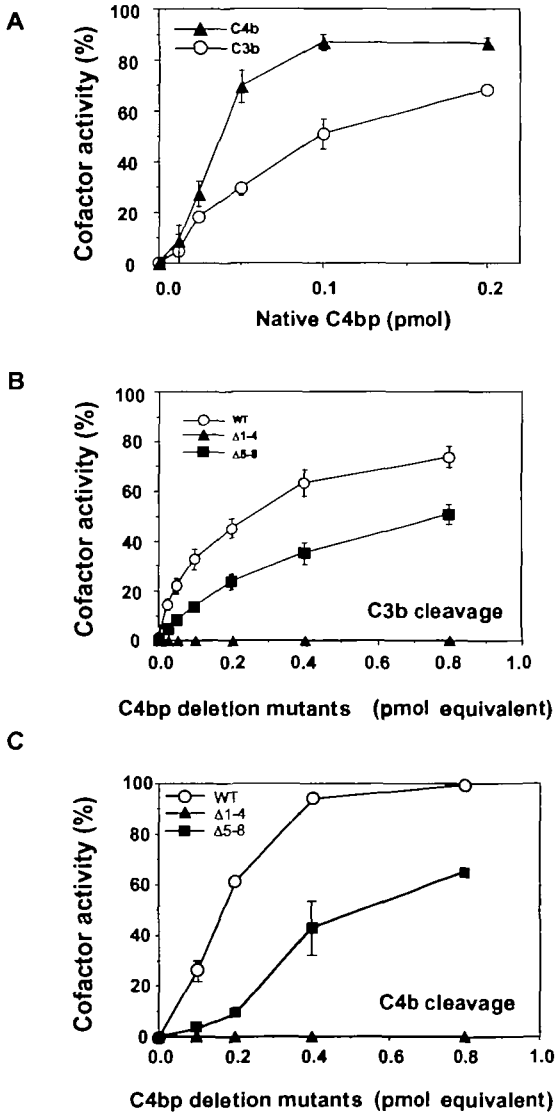

Fig. 4. **Factor I–cofactor activity of C4bp and its mutants.** Wild-type C4bp (WT), and the ΔSCR1–4 and ΔSCR5–8 mutants were incubated with constant amounts of factor I and a fluorescence-labeled substrate, OG-C3b (panel B) or DACM-C4b (panel C). OG-labeled C3b *vs.* C4b cleavage by factor I and native C4bp (purified from human blood plasma) are compared in panel A. The buffer conditions were 20 mM PB, pH 7.4, containing 50 mM NaCl.

Factor I–Cofactor Function of C4bp Mutants—The factor I–cofactor activity for C4b cleavage was examined using the supernatants (sups) of CHO transfectants. The C4b-C4bp interassociation should be more sensitively detected with this method. The sup containing wild-type C4bp efficiently cleaved C4b into C4c and C4d in the fluid phase in a dose-dependent manner without nonspecific degradation (data not shown). Less than 0.20 pmol equivalent of wild-type C4bp sufficiently cleaved ~80% of substrate C4b (Fig. 4C), these results being consistent with those obtained for the standard C4bp material purified from human plasma (native C4bp) (Fig. 4A). No cleavage products were observed if the CHO expressed no C4bp (data not shown), suggesting that the sups of naive CHO exhibited no C4b-cleaving activity. Likewise, the sups of two individual transfectant lines effectively cleaved C4b into C4c and C4d (data not shown). It is notable that ΔSCR1–4 exhibited no cofactor activity while ΔSCR5–8, although having relatively short SCR stretches, are responsible for 50–80% of the full cofactor activity (Fig. 4C). the ΔSCR4 mutant expressed full cofactor activity like the wild-type, while the ΔSCR2 and ΔSCR3 mutants had virtually no cofactor activity (Figs. 5A and 6), suggesting that these two domains are essential for C4b inactivation by C4bp and factor I. The ΔSCR1 mutant expressed moderate (~30%) cofactor activity compared to that of wild-type C4bp (Fig. 5A).

We next determined the domains responsible for C3b cleavage by factor I and C4bp. In this assay, we used purified wild-type C4bp and recombinant variants of it. As shown in Fig. 4B, ΔSCR1–4 possessed no cofactor activity and ΔSCR5–8 showed ~60% of the full cofactor activity of wild-type C4bp. The ΔSCR2, 3, and 4 mutants had completely lost the ability to cleave C3b in the presence of factor I, and ΔSCR1 and ΔSCR5 also exhibited ~50% of the full cofactor activity of wild-type C4bp at 0.2 pmol equivalent (Figs. 5B and 6). These results indicated that C4bp expresses strong cofactor activity for C4b cleavage, which is responsible for SCR2 and 3, and for the cleavage of C3b at least SCR1–5 are required.

This differential profile of C4bp cofactor activity toward C4b *vs.* C3b was confirmed using sequential SCR deletion mutants (Fig. 5C) and a series of mAbs against C4bp (Table II). ΔSCR1–2, ΔSCR1–3, and ΔSCR1–4 mutants had no cofactor activity, while the ΔSCR5–8 as well as ΔSCR5 ones had 60% cofactor activity (Fig. 6). The cofactor activity for C3b cleavage was considerably blocked by mAb TK2, yet this mAb recognizes the C-terminal region of C4bp (Table II). Thus, the cofactor activity for C3b was attributable to SCR1–4 and broad regions of C4bp. Notably, the mAbs that reacted with the C-terminal 160 kDa core domain including 28G-11, which has been characterized as a disruptor of C4bp–protein S association (*24*), severely blocked C3b-cleaving activity, although the reason remains unknown. However, the C4b-cleaving activity was effectively inhibited by TK3 and, to a lesser extent, by TK1, which recognize SCR3 and SCR1, respectively. TK2, which recognizes the C-

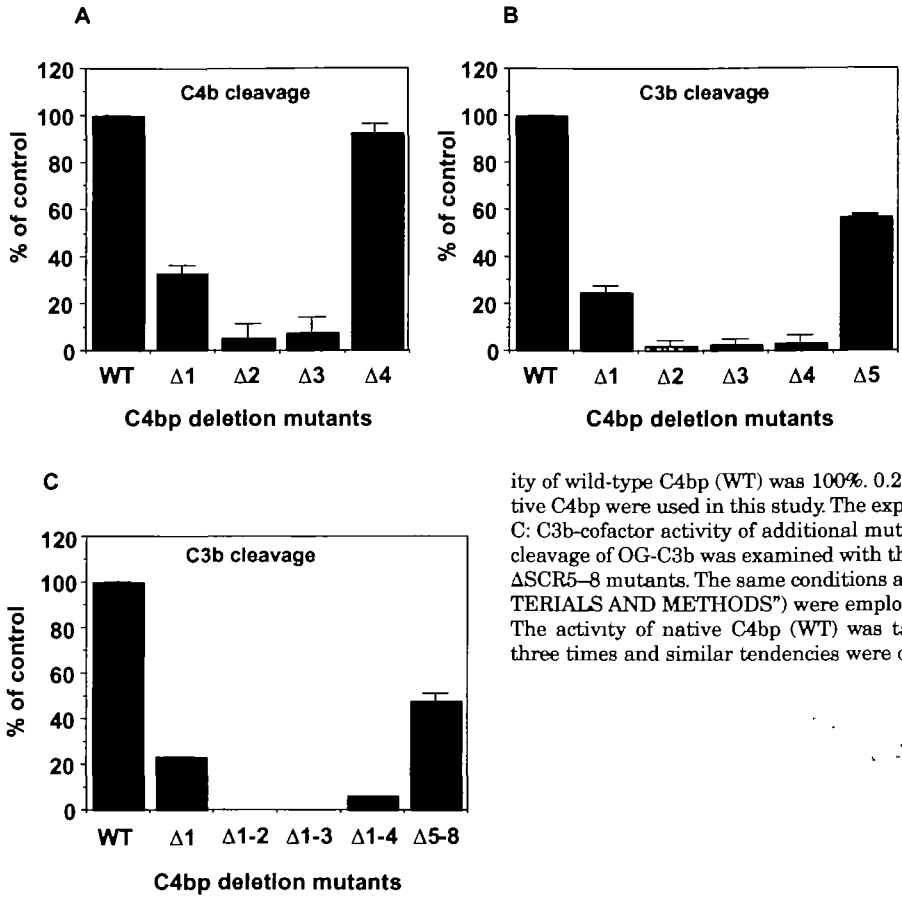

Fig. 5. **Factor I–mediated C4b- and C3b-inactivating ability of purified C4bp mutant proteins.** Panel A: The OG-labeled C4b-cofactor capacities of C4bp mutants were measured assuming that the factor I–cofactor activity of wild-type C4bp (WT) was 100%. ΔSCR4 cleaved C4b together with factor I nearly 100%. 0.2 pmol equivalents of mutants compared to native C4bp (purified from human blood plasma) were used in this study. The buffer conditions were as in Fig. 4. Samples were analyzed by SDS-PAGE followed by analysis with an image analyzer. %Cleavage was evaluated as under "MATERIALS AND METHODS." Panel B: The OG-labeled C3b-cofactor capacities of C4bp mutants were evaluated assuming that the factor I–cofactor activity of wild-type C4bp (WT) was 100%. 0.2 pmol equivalents of mutants compared to native C4bp were used in this study. The experimental conditions were as in panel A. Panel C: C3b-cofactor activity of additional mutants of C4bp. The factor I–cofactor activity for cleavage of OG-C3b was examined with the ΔSCR1, ΔSCR1–2, ΔSCR1–3, ΔSCR1–4, and ΔSCR5–8 mutants. The same conditions as those in panels A and B (also see under "MATERIALS AND METHODS") were employed to confirm the results in the above panels. The activity of native C4bp (WT) was taken as 100%. These assays were performed three times and similar tendencies were observed.

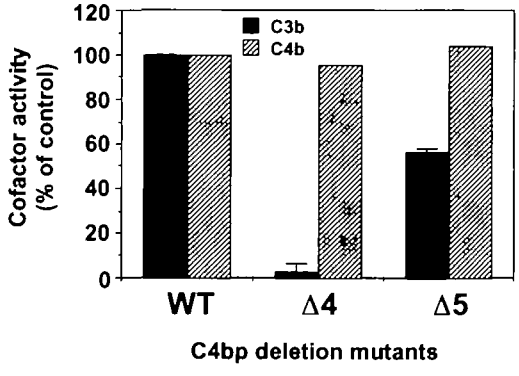

Fig. 6. **Comparison of C3b-cofactor *vs.* C4b-cofactor activities of D4 and D5 mutants under low conductivity conditions.** Purified C4bp mutants ΔSCR4 and ΔSCR5 were incubated with OG-labeled C3b or C4b and factor I under low conductivity conditions (50 mM NaCl/PB, pH 7.4). After incubation, samples were analyzed by SDS-PAGE and then with an image analyzer. %Cleavage was evaluated as under "MATERIALS AND METHODS." The degree of cleavage of wild-type C4bp (WT) for both C3b and C4b was assumed to be 100%.

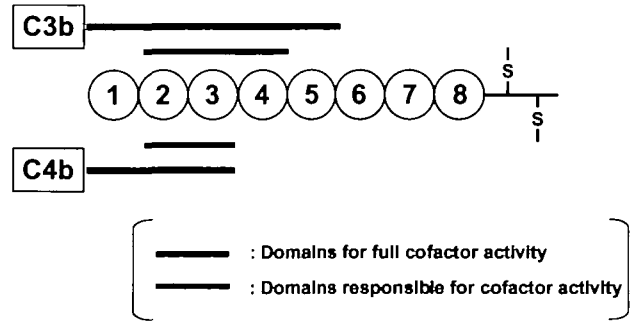

Fig. 7. **Modules of C4bp associated with C3b- and C4b-inactivation as factor I–cofactor.** The SCR domains of C4bp participating in C3b- and C4b-inactivation are summarized. See the text for details.

terminal core, barely inhibited C4b cleavage by factor I and C4bp, similar to other C-terminal-recognizing mAbs, 24H-1 and 28G-11 (*24*). In the mAb-blocking and deletion mutant studies, some of the C-terminal portion other than the SCR1–4 domain was found to be important for C3b inactivation, and N-terminal SCR1–3 prima-rily participate in factor I–mediated C4b cleavage. Under 0.2 pmol equimolar conditions, the ΔSCR5–8 mutant and factor I caused 60% cleavage of C3b, this degree being consistent with that in the case of ΔSCR5 and factor I. Thus, SCR6–8 be responsible for the additional 40% activity. This point was not clarified in this study since the expression levels of recombinant C4bp variants ΔSCR6, 7, and 8 were too low for affinity purification. Our current conclusion is schematically presented in Fig. 7, where the domain combination for factor I–mediated C4b inactivation is different from that for C3b inactivation.

DISCUSSION

The goals of this study was to delineate the complement regulatory sites for C3b and to compare them with those for C4b within the eight SCR modules of the C4bp α-chain. Several different regions of the α-chain have been nominated as accepting sites for C4b (*16, 29–31*). In particular, Blom *et al.* recently looked into the structural essentials of each SCR domain in C4bp for C4b inactivation using C4bp mutants (*17*). This study and theirs will clarify the different structural requirements of C4bp for inactivation of C4b *vs.* C3b. Our conclusions are: 1. SCR2 and 3 are essential and SCR1 is optionally required for C4b inactivation by factor I; 2. SCR2, 3, and 4 are essential, and SCR1 and 5 are optionally required for C3b inactivation by factor I. The ΔSCR1–4 and ΔSCR5–8 mutants were useful for discriminating the structural essentials for C3b and C4b inactivation. For the heptamer, compared to the monomer, of C4bp, C3b binding and inactivation activity become decreased, which may reflect steric hinderance and well match the physiological properties of C4bp.

Regarding C4b binding and cleavage, we confirmed the results of Blom *et al.* (*17*), which are mostly consistent with ours. Their approach as well as ours supported the previous finding that an N-terminal 48 kDa α-chain fragment liberated on chymotrypsin digestion was sufficient for expression of the factor I–cofactor activity for C4b (*18, 31*). These results also reinforced the finding that the N-terminal SCRs are indispensable for the binding of C4b. Minor differences from Blom's results are 1. in the domain sets essential for C4b binding (SCR2 and 3 *vs.* SCR2) (Fig. 3A, panel c) and 2. the relatively lower contribution of SCR1 to factor I–mediated C4b cleavage (Fig. 5A). However, these are peripheral, probably reflecting the different conditions employed.

The main point of this study was to define the differences in the properties of C4bp in C3b cleavage compared to C4b cleavage using the SCR deletion mutants. For expression of factor I–cofactor activity for cleavage of C3b, SCR2, 3, and 4 are essential, and SCR1 and 5 further support complete activity. C3b inactivation requires wider regions of C4bp than C4b inactivation. The SCR sets responsible for binding to C3b, in contrast, could barely be identified under the conditions under which C4b binding sites could be defined as SCR2, suggesting that the binding affinity of the SCR repertoire for C3b is much lower than that for C4b. C3b inactivation may require more C-terminal SCRs subsequent to SCR5. In the C4bp heptamer, the SCR repertoire responsible for binding to C3b barely functions as a C3b catching-up arm under physiological conditions, although SCR2 of C4bp works sufficiently for C4b binding. At least SCR1–4 are needed to sustain C4bp binding to C3b.

This study reinforced the previous report on functional studies of membrane-bound monomeric C4bp (*32*), in which some C-terminal portion was found to be important for the C3b-cleaving cofactor activity of monomeric C4bp. Although we could not find an independent C3b-binding portion, at least in multimeric C4bp molecules, the requirement of the broad stretch of SCRs for C3b inactivation by C4bp was confirmed in this study. Thus, the C3b inactivation activity of C4bp would be partly hampered by the formation of a multimer that causes steric hinderance. There are two possible interpretations of the participation of unexpected broad SCR domains in the C3b-inactivating cofactor activity of C4bp; the α chain of C4bp primarily serves as a C3b as well as a C4b inactivating cofactor with different SCR sets, or C4bp essentially acts as a C4b inactivator and this function optionally extends to cover C3b inactivation. We favor the former interpretation: C4bp acts primarily on C3b and C4b, and together with factor I cleaves these substrates in a similar manner (*32*). That is, multimer C4bp and monomer C4bp effectively mediate factor I–dependent C4b cleavage, and only monomer C4bp exhibits effective cofactor activity towards C3b. Indeed, most other animals produce monomeric C4bp without multimer formation (*33, 34*).

For the other C regulators, CR1, MCP, DAF, and factor H, the involvement of individual SCR domains in C binding and the regulatory function of C4b/C3b have been investigated. In the case of DAF (4 SCRs), which has decay-accelerating activity but not cofactor activity, the regulatory function for the classical pathway C3 convertase, C4b2a, resides in SCR2 and SCR3, whereas regulation of the alternative pathway requires SCR1, SCR2, and SCR3 (*35*). In MCP (4 SCRs), sites for the C4b/C3b interaction have been mapped to SCR2, 3, and 4 (*36, 37*). In factor H (20 SCRs), there are three C3b binding sites localized to SCR1–4 (*38*), SCR12–14, and SCR19–20 (*39*). CR1 (30 SCRs) is organized into four long homologous repeats each consisting of 7 SCR units. Full ligand binding (C3b and C4b) and cofactor activity require the first four SCRs in the first three long homologous repeats (*40*). The preferential selectivity toward C3b or C4b differs among the functional units of these proteins, and the essential structure discriminating C3b and C4b has remained undetermined. The present analysis indicated the importance of the different sets of SCR domains of human C4bp for discrimination of C3b and C4b.

The physiological significance of C4bp in C3b inactivation may be less than that in C4b inactivation in humans since factor H is known to be a main inhibitor of C3b inactivation in the fluid phase (*1, 9, 10*), and soluble C4bp barely acts as an inhibitor of membrane-bound C4b at natural doses, leading to a failure to suppress hemolysis (*20, 41*). Nevertheless, we performed this analysis because comparison of C3b *vs.* C4b inactivation should be important from an evolutional view. All reported C regulatory SCR proteins serve as not only C3b inhibitors but also C4b inhibitors (*42, 43*). Even human factor H can act as a cofactor for factor I in the cleavage of C4b under certain conditions (*43*). Furthermore, a sand bass factor H–like SCR protein also acts as a cofactor for both C3b and C4b (*44*). Membrane cofactors MCP and CR1 also regulate both C3b and C4b together with factor I (*1, 42*). More interestingly, fishes and lampreys possess genes that encode C4bp-like proteins (Inoue, N., Oshiumi, H., Seya, T., *et al.*, manuscript in preparation). On Fugu genome analysis, we found a C4bp-like gene in the RCA region (Oshiumi, H, Seya, T., unpublished observation). Our results infer that differential usage of a SCR repertoire in C4bp results in C3b and C4b inactivation. This and previous results (*17, 42, 43*) suggest that C4bp-mediated inactivation of the C cascade encompasses C3b and C4b, that is, alternative and classical pathways. In humans, the classical pathway is preferentially inhibited by C4bp and factor I; multimer formation may facilitate

this functional tendency.

The concept has been accepted that C3, C4, and C5 originated from a single gene through tetrologous gene multimerization (*45*). The genes of the SCR proteins are also clustered in mammals and birds (*46*) in a limited region of the chromosomes, namely the RCA region, through gene duplication. Thus, it is not surprising that current SCR-containing C regulatory proteins retain the inhibitory function across the C3 family proteins C3 and C4.

It is notable that human C4bp is the only C regulator that comprises multiple identical subunits. In rodents (*47*), chicken (*46*), Fugu fish (*Fugu rubripes*) (*46*), and lamprey (Inoue, N. and Seya, T., unpublished), we and others have identified the genes of C4bp-like SCR proteins consisting 8–9 SCRs, which presumably inhibit the host C together with factor I–like proteases. Most of them are mapped on the RCA region in a chromosome, based on the locations of other marker genes. They have no C-terminal Cys participating in multimerization, and recombinant proteins produced in mammalian cells showed monomers (Oshiumi, H., Tsujita, T., *et al.*, unpublished observation), suggesting that a C4bp-like SCR protein is a prototype of a number of the RCA family proteins, and human C4bp evolved to become particular in its multimeric structural signature. Requirement of the narrow region for C4b inactivation, and the wide range containing SCR4 and 5 for C3b recognition and inactivation may reflect the general properties of an ancient prototype of the SCR-containing C regulatory proteins, which could have adapted to all C3 family proteins through gene shuffling and duplication. The properties of the RCA family proteins herein reported may also be important for the reported bacterial recognition and/or virus receptor functions (*48*).

We wish to thank Drs. K. Toyoshima, M. Matsumoto, M. Tatsuta, and H. Koyama (Osaka Medical Center for Cancer) for supporting this work. We also thank Drs. K. Takahashi, T. Yamashita, H. Nishimura, and K. Iwata (Hokkaido University, Sapporo), and Dr. K. Suzuki (Mie University, Mie) for the critical discussions.

REFERENCES

1. Morgan, B.P. and Harris, C.L. (1989) *Complement Regulatory Proteins*, pp. 41–135, Academic Press, San Diego
2. Fearon, D.T. (1997) Seeking wisdom in innate immunity. *Nature* **388**, 323–324
3. Hourcade, D., Holers, V.M., and Atkinson, J.P. (1989) The regulators of complement activation (RCA) gene cluster. *Adv. Immunol.* **45**, 381–416
4. Shiraishi, S. and Stroud, R.M. (1975) Cleavage products of C4b produced by enzymes in human serum. *Immunochemistry* **12**, 935–939
5. Nagasawa, S. and Stroud, R.M. (1977) Mechanism of action of the C3b inactivator: requirement for a high molecular weight cofactor (C3b-C4bINA cofactor) and production of a new C3b derivative (C3b'). *Immunochemistry* **14**, 749–756
6. Nagasawa, S. and Stroud, R.M. (1980) Purification and characterization of a macromolecular weight cofactor for C3b-inactivator, C4bC3bINA-cofactor, of human plasma. *Molec. Immunol.* **17**, 1365–1372
7. Fujita, T. and Nussenzweig, V. (1979) The role of C4-binding protein and β1H in proteolysis of C4b and C3b. *J. Exp. Med.* **150**, 267–276
8. Gigli, I., Fujita, T., and Nussenzweig, V. (1979) Modulation of the classical pathway C3 convertase by plasma protein C4b binding protein and C3b inactivator. *Proc. Natl. Acad. Sci. USA* **76**, 6596–6600
9. Whaley, K. and Ruddy, S. (1976) Modulation of the alternative complement pathways by beta 1 H globulin. *J. Exp. Med.* **144**, 1147–1163
10. Schreiber, R.D., Pangburn, M.K., Lesavre, P.H., and Muller-Eberhard, H.J. (1978) Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. *Proc. Natl. Acad. Sci. USA* **75**, 3948–3952
11. Nagasawa, S., Ichihara, C., and Stroud, R.M. (1980) Cleavage of C4b by C3b inactivator: production of a nicked form of C4b, C4b', as an intermediate cleavage product of C4b by C3b inactivator. *J. Immunol.* **125**, 578–582
12. Fujita, T. (2002) Evolution of the lectin-complement pathway and its role in innate immunity. *Nature Rev. Immunol.* **2**, 346–353
13. Bora, N.S., Lublin, D.M., Kumar, B.V., Hockett, R.D., Holers, V.M., and Atkinson, J.P. (1989) Structural gene for human membrane cofactor protein (MCP) of complement maps to within 100 kb of the 3' end of the C3b/C4b receptor gene. *J. Exp. Med.* **169**, 597–602
14. Hillarp, A. and Dahlback, B. (1988) Novel subunit in C4b-binding protein required for protein S binding. *J. Biol. Chem.* **263**, 12759–12764
15. Dahlback, B., Smith, C.A., and Muller-Eberhard, H.J. (1983) Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement C4b. *Proc. Natl. Acad. Sci. USA* **80**, 3461–3465
16. Hardig, Y., Hillap, A., and Dahlback, B. (1997) The amino-terminal module of the C4b-binding protein alpha-chain is crucial for C4b binding and factor I-cofactor function. *Biochem. J.* **323**, 469–475
17. Blom, A.M., Kask, L., and Dahlback, B. (2001) Structural requirements for the complement regulatory activities of C4BP. *J. Biol. Chem.* **276**, 27136–27144
18. Nagasawa, S., Mizuguchi, K., Ichihara, C., and Koyama, J. (1982) Limited chymotryptic cleavage of human C4-binding protein: isolation of a carbohydrate-containing core domain and an active fragment. *J. Biochem.* **92**, 1329–1332
19. Matsuguchi, T., Okamura, S., Aso, T., Sata, T., and Niho, Y. (1989) Molecular cloning of the cDNA coding for proline-rich protein (PRP): identity of PRP as C4b-binding protein. *Biochem. Biophys. Res. Commun.* **165**, 138–144
20. Seya, T. and Nagasawa, S. (1982) A fluorometric method for determination of C3b inactivator. *Clin. Chim. Acta* **119**, 189–196
21. Murakami, Y., Fukui, A., Seya, T., Ueda, S., and Nagasawa, S. (1999) Effect of mutations at the residues R25, D27, P69, and N70 of B95a-MCP on receptor activities for the measles viruses Nagahata wild-type strain and CAM vaccine strain. *Int. J. Mol. Med.* **3**, 25–32
22. Iwata, K., Seya, T., Ariga, H., and Nagasawa, S. (1994) Expression of a hybrid complement regulatory protein, membrane cofactor protein (MCP)-decay-accelerating factor (DAF) on Chinese hamster ovary cells; comparison of its regulatory effect with those of DAF and MCP. *J. Immunol.* **152**, 3436–3444
23. Fujita, T., Kamato, T., and Tamura, N. (1985) Characterization of functional properties of C4-binding protein by monodonal antibodies. *J. Immunol.* **134**, 3320–3324
24. Suzuki, K. and Nishioka, J. (1988) Binding site for vitamin K-dependent protein S on complement C4-binding protein. *J. Biol. Chem.* **263**, 17034–17039
25. Ichihara, C., Nagasawa, S., and Koyama, J. (1981) Characterization of an amine-reactive, heat-labile acyl residue in the fourth component of human complement, C4. *J. Biochem.* **90**, 423–432
26. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488–492
27. Seya, T. and Nagasawa, S. (1985) Limited proteolysis of complement protein C3b by regulatory enzyme C3b inactivator: isolation and characterization of a biologically active fragment, C3d,g. *J. Biochem.* **97**, 373–382

28. Nukatsuka, M. and Nagasawa, S. (1987) Characterization of the interaction between human protein S and C4b-binding protein (C4bp). *J. Biochem.* **102**, 599–605
29. Hessing, M., Kanters, D., Takaeya, H., van't Veer, C., Hackeng, T.M., Iwanaga, S., and Bouma, B.N. (1993) The region Ser333-Arg356 of the alpha-chain of human C4b-binding protein is involved in the binding of complement C4b. *FEBS Lett.* **317**, 228–232
30. Chung, L.P. and Reid, K.B.M. (1985) Structural and functional studies on C4b-binding protein, a regulatory component of the human complement system. *Biosci. Rep.* **5**, 855–865
31. Nagasawa, S., Unno, H., Ichihara, C., Koyama, J., and Koide, T. (1983) Human C4b-binding protein, C4bp. Chymotryptic cleavage and location of the 48 kDa active fragment within C4bp. *FEBS Lett.* **164**, 135–138
32. Mikata, S., Miyagawa, S., Fukui, A., Murakami, Y., Shirakura, R., Matsuda, H., Hatanaka, M., Matsumoto, M., Seya, T., Suzuki, K., and Nagasawa, S. (1998) A monomeric human C4b-binding protein (C4bp) more efficiently inactivates C3b than natural C4bp: participation of C-terminal domains in factor I-cofactor activity. *Mol. Immunol.* **35**, 537–544
33. Rodriguez de Cordoba, S., Perez-Blas, M., Ramos-Ruiz, R., Sanchez-Corral, P., Pardo-Manuel de Villena, F., and Rey-Campos, J. (1994) The gene coding for the beta-chain of C4b-binding protein (C4BPB) has become a pseudogene in the mouse. *Genomics* **21**, 501–509
34. Hillarp, A., Wiklund, H., Thern, A., and Dahlback, B. (1997) Molecular cloning of rat C4b binding protein alpha- and beta-chains: structural and functional relationships among human, bovine, rabbit, mouse, and rat proteins. *J. Immunol.* **158**, 1315–1323
35. Coyne, K.E., Hall, S.E., Thompson, S., Arce, M.A., Kinoshita, T., Fujita, T., Anstee, D.J., Rosse, W., and Lublin, D.M. (1992) Mapping of epitopes, glycosylation sites, and complement regulatory domains in human decay accelerating factor. *J. Immunol.* **149**, 2906–2913
36. Adams, E.M., Brown, M.C., Nunge, M., Krych, M., and Atkinson, J.P. (1991) Contribution of the repeating domains of membrane cofactor protein (CD46) of the complement system to ligand binding and cofactor activity. *J. Immunol.* **147**, 3005–3011
37. Iwata, K., Seya, T., Yanagi, Y., Pesando, J.M., Johnson, P.M., Okabe, M., Ueda, S., Ariga, H., and Nagasawa, S. (1995) Diversity of sites for measles virus binding and for inactivation of complement C3b and C4b on membrane cofactor protein CD46. *J. Biol. Chem.* **270**, 15148–15152
38. Giannakis, E., Male, D.A., Ormsby, R.J., Mold, C., Jokiranta, T.S., Ranganathan, S., and Gordon, D.L. (2001) Multiple ligand binding sites on domain seven of human complement factor H. *Int. Immunopharmacol.* **1**, 433–443
39. Sharma, A.K. and Pangburn, M.K. (1996) Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. *Proc. Natl. Acad. Sci. USA* **93**, 10996–11001
40. Kalli, K.R., Hsu, P.H., Bartow, T.J., Ahearn, J.M., Matsumoto, A.K., Klickstein, L.B., and Fearon, D.T. (1991) Mapping of the C3b-binding site of CR1 and construction of a $(CR1)_2$-$F(ab')_2$ chimeric complement inhibitor. *J. Exp. Med.* **174**, 1451–1460
41. Fujita, T. and Tamura, N. (1983) Interaction of C4-binding protein with cell-bound C4b. A quantitative analysis of binding and the role of C4-binding protein in proteolysis of cell-bound C4b. *J. Exp. Med.* **157**, 1239–1251
42. Seya, T., Holers, V.M., and Atkinson, J.P. (1985) Purification and functional analysis of the polymorphic variants of the C3b/C4b receptor (CR1), and comparison to H, C4b-binding protein (C4bp), and decay-accelerating factor (DAF). *J. Immunol.* **135**, 2661–2667
43. Seya, T., Nakamura, K., Masaki, T., Ichihara-Itoh, C., Matsumoto, M., and Nagasawa, S. (1995) Human factor H and C4b-binding protein serve as factor I-cofactors both encompassing inactivation of C3b and C4b. *Mol. Immunol.* **32**, 355–360
44. Kemper, C., Zipfel, P.F., and Gigli, I. (1998) 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. *J. Biol. Chem.* **273**, 19398–19404
45. Ohno, S. (1993) Patterns in genome evolution. *Curr. Opin. Genet. Dev.* **3**, 911–914
46. Oshiumi, H., Shida, K., Kato, J., Ohba, S., Tamaki, Y., Hattori, T., Yamada, N., Inoue, N., Matsumoto, M., Mizuno, S., and Seya, T. (2002) Regulator of complement activation (RCA) locus of chicken. *Jpn. Compl. Symp.* **40**, 11–12
47. Holers, V.M., Kinoshita, T., and Molina, H. (1992) The evolution of mouse and human complement C3-binding proteins: divergence of form but conservation of function. *Immunol. Today* **13**, 231–236
48. Seya, T. (1995) Human regulator of complement activation (RCA) gene family proteins and their relationship to microbial infection. *Microbiol. Immunol.* **39**, 295–305