Identification of Residues within the 727-767 Segment of Human Complement Component C3 Important for Its Interaction with Factor H and with Complement Receptor 1 (CR1, CD35)*

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Mapping approaches employing blocking antibodies and synthetic peptides have implicated the 727–767 segment at the NH₂ terminus of C3b α-chain as contributing to the interactions with factor B, factor H, and CR1. Our previous mutagenesis study on the NH₂-terminal acidic cluster of this segment identified residues Glu-736 and Glu-744 as contributing to the binding of C3b to factor B and CR1 but not factor H. We have now extended the charged residue mutagenic scan to cover the remainder of the segment (728–767) and have assessed the ability of the C3b-like C3(H₂O) form of the mutant molecules to interact with factor H, CR1, and membrane cofactor protein (MCP) using a cofactor-dependent factor I cleavage assay as a surrogate binding assay. We have found that the negatively charged side chains of Glu-744 and Glu-747 are important for the interaction between C3(H₂O) and factor H, a result in general agreement with an earlier synthetic peptide study (Fishelson, Z. (1991) Mol. Immunol. 28, 545–552) which implicated residues within the 744–754 segment in H binding. The interactions of the mutants with soluble CR1 (sCR1) revealed two classes of residues. The first are residues required for sCR1 to be an I cofactor for the first two cleavages of the α-chain. These are all acidic residues and include the Glu-736/Glu-737 pair, Glu-744, and the Glu-754/Asp-755 pairing. The second class affects only the ability of sCR1 to be a cofactor for the third factor I cleavage and include Glu-747 and the Lys-757/Glu-758 pairing. The dominance of acidic residues in the loss-of-function mutants is striking and suggests that H and CR1 contribute basic residues to the interface. Additionally, although there is partial overlap, the contacts required for CR1 binding appear to extend over a wider portion of the 727–767 segment than is the case for factor H. Finally, none of the mutations had any effect on the interaction between soluble MCP and C3(H₂O), indicating that despite its functional homology to H and CR1, MCP differs in its mode of binding to C3b/C3(H₂O).

Stringent regulation of the alternative complement pathway C3 convertase is essential for the prevention of C3 hyperactivation and for the protection of host tissue from the deleterious effects of alternative pathway propagation. In particular, it is important to regulate alternative pathway propagation that can employ as a nidus C3b molecules that have become adventitiously bound to host cell membrane. In primates, this regulation involves primarily the soluble serum proteins factor H and factor I, as well as the membrane-resident proteins decay-accelerating factor (CD55), membrane cofactor protein (MCP, CD46), and complement receptor 1 (CR1, CD35) (reviewed in Ref. 1). With the exception of the serum protease factor I, the other regulatory molecules are members of a superfamily consisting almost entirely of varying numbers of a sequence motif alternatively referred to as a short consensus repeat (SCR) or complement control protein (CCP) module. Although this motif is also found in many non-complement proteins, the genes encoding the complement regulatory molecules are clustered on the long arm of chromosome 1 at what has been termed the RCA locus, for regulators of complement activation. Structural analyses of single and paired CCP modules have directly demonstrated that they each form independently folded compact globular domains consisting of two interacting layers of anti-parallel β-sheet (2–4). It has been speculated that the protruding loops, some of which are of variable length, are good candidates for mediating interaction with ligand.

CCP-containing proteins that bind to C3b exert their regulatory effect via two mechanisms as follows: decay-accelerating activity and factor I-cofactor activity. Specifically, the binding of H, decay-accelerating factor, and CR1 to C3bBb, the alternative pathway C3 convertase, leads to an accelerated rate of unidirectional dissociation of the serine protease Bb from the C3b modulatory subunit of the convertase. I cofactor activity refers to the accessory role of the SCR-containing protein in the I-mediated cleavage of C3b COOH terminus to residues 1281 and 1298 (mature C3 numbering), yielding the major fragmentation product iC3b and a minor fragment termed C3f. Since the iC3b fragment can no longer associate with factor B, this permanently inactivates the C3 molecule with respect to being a nidus for alternative pathway C3 convertase formation. However, this factor I-mediated cleavage can only take place when the C3b is complexed with one of three cofactors, these being the soluble protein factor H and the host membrane-associated

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1 The abbreviations used are: MCP, membrane cofactor protein; CCP, complement control protein; CR, complement receptor; CVF, cobra venom factor; DMEM, Dulbecco’s modified Eagle’s medium; DMEM/ K76, DMEM containing K76-COOH-treated fetal calf serum; EAC, sheep erythrocytes coated with antibody and the indicated complement components or fragments thereof; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; PBS, phosphate-buffered saline; rC3, recombinant C3; RCA, regulators of complement activation; RIA, radioimmunoassay; SCR, short consensus repeat; sCR1, soluble CR1; sMCP, soluble MCP; VBS, veronal-buffered saline; PAGE, polyacrylamide gel electrophoresis; bp, base pair; Hu, human; Tr, trout; Xe, Xenopus.
proteins CR1 and MCP. Although there is some suggestive evidence in the literature that binding of H to C3b causes a conformational change in the latter (5), thereby increasing the affinity for factor I, there is also recent evidence that factor I can simultaneously bind to both H and C3b, thereby stabilizing the intrinsically weak C3b-I interaction sufficiently to permit cleavage (6). Whether factor I similarly binds directly to cofactors CR1 or MCP is as yet unknown. At physiological ionic strength, only CR1 can efficiently act as an I cofactor for the further cleavage of iC3b COOH-terminal to residue 932, yielding fragments C3c and C3dg (7).

Domain deletion and domain exchange studies have succeeded in identifying CCP/SCR regions within the complement regulatory proteins that are required for their C3b-binding and I cofactor activity (8–13). Usually 3–4 SCR domains are required for functional activity. For example, in the case of factor H, SCRs 1–4 are minimally required for the expression of its I cofactor activity (8, 9). Factor H has also been shown to possess C3b-binding sites involving SCRs 6–10 and 16–20, although these sites do not contribute to the I cofactor activity of the molecule (9). The two C3b-binding sites of human CR1 are contributed by SCRs 8–11 and 15–18, respectively, and these entities are each capable of mediating full I cofactor activity (10). A recent study on CR1 has shown that the first three SCR domains in each case account for most of the functional activity (11) and critical residues within these functionally important domains have also been identified (11, 14, 15).

In contrast to the situation in the complement regulatory molecules where the repeating sequence motifs suggested an obvious experimental approach for functional site-mapping studies, the absence of any identifiable sequence motifs within C3 precludes the domain deletion or domain exchange approach. Nevertheless, several independent groups have used a variety of other approaches to putatively identify segments within C3 that mediate association with its membrane-bound receptors and soluble protein ligands. The approaches have included functional site blocking antibodies against C3, together with the identification of the polypeptide segments to which they bind (16, 17) and the combined use of proteolytic fragments and synthetic peptides derived from C3 as functional mimetics of the intact physiologic fragments (18, 19). Additionally, once one has a candidate site, xenogeneic sequence comparisons, together with knowledge about whether a particular non-human species of C3b can or cannot interact with the human ligands, can provide additional evidence for the involvement of a particular C3 segment in a binding interaction (20). Lambris and co-workers (21) have in some cases used this information to construct and assess the ligand binding activities of C3 molecules in which a segment of human C3 has either been deleted or replaced with the homologous segment of the non-human ligand-binding xenogeneic species in order to verify a proposed binding site. Ultimately, one can employ site-directed mutagenesis to test further the validity of proposed binding sites, to identify important residues, and in some cases to establish the chemical nature of the side chains that are required for a particular binding reaction (22, 23).

All of the above described approaches have implicated the NH2-terminal segment of C3 α-chain (residues 727–767) as contributing at least one contact site to the interaction of C3b with factor B, factor H, and CR1. In particular, an anti-peptide antibody directed against this segment recognizes a neopeptide in C3b and can block the interaction with factor B, factor H, and CR1 (24). Furthermore, the 727–767 peptide can compete with factor H and CR1 for binding to C3b (24, 25), and in one study (24), but not the other (25), was capable of inhibiting the interaction between C3b and factor B. Finally, a recombinant molecule in which the 727–767 segment was deleted lost the ability to interact with all three proteins (21).

Fishelson (19) had analyzed a series of overlapping hexameric and heptameric peptides spanning the 727–767 region for their ability to bind factor B and factor H. The results suggested that the segment 730DDIILAEENI contributed to factor B binding, whereas the segment 744EPFESWLNVNVE contributed to the binding of factor H. Site-directed mutagenesis work on intact C3 examined the role of the carboxylate side chains within the 730–739 segment and identified those of glutamic acid residues 736 and 737 as being important not only for the interaction of C3b with factor B but also for the ability of target-bound C3b and iC3b to bind, respectively, to CR1 and CR3 on phagocytes (22). In keeping with the predictions of the Fishelson work, these mutations did not alter the interaction with human factor H. In the present work, we have extended our previous mutagenesis study on human C3 to cover the remainder of the charged residues in the 727–768 segment, initially with the aim of identifying residues crucial for the interaction with factor H. Since like factor H, CR1 and MCP also act as cofactors for the factor I-mediated cleavages of C3, the same series of mutants were also examined for their ability to interact with soluble forms of CR1 (sCR1) and MCP (sMCP). We have identified Glu-744 and Glu-747 within the predicted “Fishelson” segment as residues whose carboxylate side chains contribute to the interaction with factor H. We have also found that the charged residue contacts with CR1, although partially overlapping with factor H, extend over a much larger portion of the segment. In contrast, none of the mutations examined had any effect on the interaction with MCP.

EXPERIMENTAL PROCEDURES

Purified Complement Components and Antibodies—C1s (26), C2 (27), C3 and C5 (28), C4 (29), factor B (30), factor H (31), and factor I (32) was generated from native C3 by incubation with KBr, 24 h at full concentration, at 37 °C for 4 h, followed by extensive dialysis against veronal-buffered saline (VBS, 4 mM veronal, pH 7.2, 0.15 M NaCl, 0.15 mM CaCl2, 0.5 mM MgCl2). A functionally pure human C1 reagent was obtained by gel filtration of serum (33), and a guinea pig C6–9 reagent was prepared from guinea pig serum (Life Technologies, Inc., Oakville, Ontario, Canada) as described (34). Soluble CR1 (sCR1) and soluble MCP (sMCP) were generously provided by Dr. Richard Smith of SmithKline Beecham (Harlow, UK) and Dr. Grace Yeh of CytoMed Inc. (Cambridge, MA), respectively.

Rabbit polyclonal IgG against human C3c (Sigma), goat antiseraum against human C3 (Quidel, San Diego), and alkaline phosphatase-conjugated antibody against goat IgG (Jackson ImmunoResearch, West Grove, PA) were purchased from the indicated suppliers. Mouse monoclonal IgG. 4C2 against human C3d was a gift from Dr. V. Koistinen (Helsinki, Finland). A mouse monoclonal IgG1 recognizing a determinant in human C3c was purified from protein A-agarose (35) from the supernatants of the hybridoma cell line P63-3A2, originally provided by Dominion Biologics Ltd. (Truro, Nova Scotia, Canada).

Cell Culture—All cell lines were maintained at 37 °C with 5% CO2 in a humidified tissue culture incubator. COS-1 cells were maintained in complete DMEM containing 0.6 mg/ml streptomycin (complete DMEM).

When rC3-containing culture supernatants were to be harvested for use in the cofactor-dependent factor I cleavage assay (see below), it was necessary to use FCS in which the bovine factor I had been irreversibly inactivated with K76-monocarboxylic acid-RK76-COOH, obtained from Dr. W. Miyazaki, Otsuka Pharmaceuticals, Tokushima, Japan). Our modification of the procedure for K76-COOH treatment of FCS has been described previously (22). One day before culture supernatants were to be harvested, the cells were washed in Hanks’ buffered salt solution (Life Technologies, Inc.) and then incubated in DMEM/K76, this medium being complete DMEM in which the 10% FCS is replaced with 4%...
K76-4COOH-treated FCS and supplemented with 1% Nutridoma-HU (Boehringer Mannheim, Montreal, Quebec, Canada).

Site-directed Mutagenesis—The construction of a full-length cDNA expression plasmid for human C3, pSV-C3, has been described previously (36). The same cDNA was also inserted as a HindIII fragment into pBluescript in both orientations, and the restriction sites were designated pBS-T-C3A (C3 coding strand is top strand of plasmid) and pBS-T-C3B (C3 coding strand is bottom strand of plasmid), respectively. pBS-T-C3B was used as a wild-type template and as an intermediate subcloning vector for the production of site-directed mutants by the overlap extension polymerase chain reaction method (37) using the procedure for both orientations, and the resulting plasmids were designated pBS-T-C3A (C3 coding strand is top strand of plasmid) and pBS-T-C3B (C3 coding strand is bottom strand of plasmid), respectively. Site-directed mutagenesis was performed with the QuickChange mutagenesis kit (Stratagene) using the primers described in Table I.

Circular Mutations—Circular mutations of C3 were made using the methods described for the construction of site-directed mutants.

Quantitative Measurement of Secreted Recombinant C3—The amount of recombinant C3 secreted by the transfectants, both as unlabeled and 35S-labeled proteins, was determined by a competitive solid-phase radioimmunoassay (42) using 125I-labeled purified human C3 as the probe and rabbit polyclonal IgG anti-human C3 as the capture antibody. A standard curve was obtained using known amounts of the probe and rabbit polyclonal IgG anti-human C3c as the capture antibody. A standard curve was obtained using known amounts of purine and rabbit polyclonal IgG anti-human C3c as the capture antibody. A standard curve was obtained using known amounts of rC3, as determined by RIA. The resulting EAC4b3b cells were washed in GVBE (VBS containing 0.1% gelatin and 10 mM EDTA) and then incubated with human C5 (1 mg) and guinea pig C6-C9 reagent (1/50 dilution in GVBE, 1 ml) at 37 °C for approximately 30 min until sufficient degree of lysis was reached in the plates containing the comparable concentration range of purified human C3. After spinning down unlysed cells, the degree of lysis in each tube was determined by measuring the absorbance of the supernatant at 412 nm. After correcting for background, the degree of specific lysis was converted to "Z" units where Z = (ln1 − fractional lysis) and corresponds to the number of hemolytically effective molecules of C3 per target cell (43). Comparisons were made on the basis of Z units per amount of C3 added within the linear portion of the dose-response curve.

ELISA—For the purpose of determining the conformational dependence of the reactivity of a pair of C3-specific monoclonal antibodies, polystyrene microtiter plates were first coated (2 h, 37 °C) with 200 μl of various dilutions of purified human C3 or C3(H2O) in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) that had either been treated or not treated with SDS (10% w/v). A starting concentration of 30 μg/ml was used for coating, and a 3-fold dilution series in PBS was established. Following blocking with Blotto/Tween (5% skim milk in PBS, 0.05% Tween 20) and three washes in PBS/Tween, mouse monoclonal antibodies against either a C3c epitope or a C3d epitope were then added (2 μg/ml in Blotto/Tween; 200 μg/ml). A polyclonal rabbit anti-human C3 IgG (10 μg/ml) was used as the secondary antibody. Following incubation, the wells were washed with alkaline phosphatase-conjugated donkey anti-rabbit IgG or alkaline phosphatase-conjugated goat anti-mouse IgG, as described below. For hemolytic assays and ELISA-based experiments, the culture supernatants were harvested 48 h after the change of the medium and then dialyzed extensively against PBS.

Metabolic Labeling, Biosynthetic Characterization, and Immunoprecipitation—The transfected cells were depleted of endogenous methionine and cysteine by incubating for 1 h in methionine- and cysteine-free DMEM (ICN Biochemicals, Costa Mesa, CA) containing 4% K76-treated calf serum and 1% Nutridoma (1 ml per 35-mm plate). The cells were then labeled with 200 μCi of Tran35S-label (ICN Biochemicals) for 6 h at which time an equal volume of methionine- and cysteine-sufficient DMEM/K76 was added, and the incubation was continued overnight. To assess the biosynthetic processing and C3 convertase cleavability of the recombinant molecules, metabolically labeled supernatants were immunoprecipitated with anti-C3c and analyzed by SDS-PAGE as described previously (40, 41). Quantitative densitometry of band intensities on pre-flashed film was performed by scanning the film on an Epson-ES-1000C color scanner and quantifying band intensities using NIH Image version 1.56.

Quantitative Measurement of Secreted Recombinant C3—The amount of recombinant C3 secreted by the transfectants, both as unlabeled and 35S-labeled proteins, was determined by a competitive solid-phase radioimmunoassay (42) using 125I-labeled purified human C3 as the probe and rabbit polyclonal IgG anti-human C3 as the capture antibody. A standard curve was obtained using known amounts of purified human C3 as the competitor. The assay was performed in opaque polystyrene microtiter plates (Packard Instruments, Meridan, CT), and bound radioactivity was measured by liquid scintillation counting directly in the plates using a TopCount instrument (Packard Instruments).

Classical Pathway-dependent Hemolytic Assays—Antibody and C3-converting sheep erythrocytes, EAC4b3b oxy2a (1.5 μl), were added to the wells, followed by incubation for 2 h at 37 °C with a single amount of purified human factor I and with variable amounts of rC3, as determined by RIA. The resulting EAC4b3b cells were washed in GVBE (VBS containing 0.1% gelatin and 10 mM EDTA) and then incubated with human C5 (1 mg) and guinea pig C6-C9 reagent (1/50 dilution in GVBE, 1 ml) at 37 °C for approximately 30 min until sufficient degree of lysis was reached in the plates containing the comparable concentration range of purified human C3. After spinning down unlysed cells, the degree of lysis in each tube was determined by measuring the absorbance of the supernatant at 412 nm. After correcting for background, the degree of specific lysis was converted to "Z" units where Z = (ln1 − fractional lysis) and corresponds to the number of hemolytically effective molecules of C3 per target cell (43). Comparisons were
Fig. 1 lists the mutant proteins examined in this study for their interaction with the I cofactors H, CR1, and MCP. In addition to the new mutants engineered within the 742–767 target region, we have also re-examined three mutant C3 molecules that were engineered for our previous study (22) and that span the NH2-terminal-most charged cluster of C3 α-chain. When transiently expressed in COS-1 cells, all of the mutant molecules were secreted at levels comparable to wild type. Furthermore, as deduced from metabolic labeling experiments, the ratio of mature 2 chain C3 to pro-C3 present in the secretions was in each case also comparable to the ratio seen in the wild-type protein (see for example zero cofactor lanes of Figs. 3–6). Both of these pieces of data point toward the general conformational integrity of the various mutant molecules having been maintained in a native-like state. This was further assessed by measuring the binding of the various rC3(H2O) molecules (i.e. thioester-hydrolyzed C3 produced by KBr treatment) to two monoclonal antibodies, each of which recognized an epitope in native C3 or C3(H2O), but which in preliminary experiments (detailed under “Experimental Procedures”) showed greatly diminished binding to the equivalent SDS-denatured molecules. One of these monoclonals (F63–3A2) recognizes an as yet uncharacterized epitope in C3c. The other monoclonal (4C2) is C3d-specific and has previously been shown to be a blocking antibody for the interactions of C3b with factor H, CR1, and factor B (49). When we compared the amount of wild-type or mutant rC3(H2O) antigen captured by the respective monoclonal antibodies to the amount captured in each case by a polyclonal anti-C3c, we found that for each of the two monoclonal antibodies this ratio was invariant for all of the rC3(H2O) molecules tested. We conclude that all of the mutant rC3(H2O) molecules engineered for this study exhibited wild-type reactivity with respect to the conformational epitopes recognized by these two monoclonal antibodies.

The classical pathway-dependent hemolytic activities of the various mutant molecules were also examined. As indicated in Fig. 1, all of the mutants show some level of defect, with hemolytic activities ranging from approximately 50 to 70% of wild-type activity. The level of hemolytic activity generally correlates with the extent to which the molecule is cleaved by a limiting concentration of fluid-phase classical pathway C3 convertase (Fig. 1). A corollary to this observation is that events downstream of C3 convertase cleavage, such as thioester-mediated transacylation efficiency and C5 convertase subunit activity, are essentially normal in the various mutant molecules. Collectively, these data suggest that the mutations have not introduced conformational alterations that extend beyond the target segment. The partial defect observed on cleavability by the classical pathway C3 convertase in virtually all of the mutants is in keeping with a previous study (50) showing that susceptibility of mouse C3 to cleavage by the alternative pathway C3 convertase was quite sensitive to sequence alterations in the segment immediately downstream of the cleavage site.

Optimization of Cofactor-dependent Factor I Cleavage Assay—The respective abilities of the various rC3(H2O) molecules to interact with the human SCR-containing regulatory molecules factor H, soluble CR1 (sCR1), or soluble membrane cofactor protein (sMCP) were assessed by their cofactor binding-dependent susceptibility to cleavage by human factor I. The rC3(H2O) molecules were present as metabolically labeled entities in the KBr-treated COS-1 cell transfection supernatants. The fetal bovine serum employed in the culture medium had been treated with K76-COOH in order to inactivate bovine

2 In Fig. 1 and throughout the text, the standard convention for naming single and multiple mutations is employed. However, in order to be able to annotate subsequent figures within the restricted space available, it was necessary to adopt a non-standard but nevertheless unambiguous nomenclature system.
factor I and thereby reduce the background cleavage of rC3(H2O) by bovine H and I (22). In the case of the interactions with factor H and sMCP, rC3(H2O) is cleaved by factor I to the iC3b-like species iC3(H2O). Upon SDS-PAGE analysis of the immunoprecipitated material, this cleavage is detected by the conversion of α-chain into two major fragments, α-75, that co-migrate with the β-chain and α-40. By using sCR1 as the cofactor, in addition to the above products, one can get a further cleavage of α-75 COOH-terminal to residue 932, thereby producing an NH2-terminal 37-kDa fragment (α-37) and a COOH-terminal 38-kDa fragment corresponding to C3dg. Since in our experiments immunoprecipitation was with an anti-C3c reagent, only the α-37 fragment would be visualized on SDS-PAGE in cases where the factor I cleavage COOH-terminal to residue 932 had occurred.

At least for the case of factor H and CR1 binding to C3b, there is evidence in the literature that in addition to a binding site within the NH2-terminal α′-chain segment, there is a minimum another binding site located in the C3d region of the molecule (24, 49). Thus a mutation that affects only the NH2-terminal segment might only partially impair the binding and might even be missed in our cofactor-dependent cleavage assay if saturating concentrations of the cofactor were used. To establish a concentration range over which H was subsaturating in the assay, metabolically labeled wild-type C3(H2O) was treated with a constant amount of factor I and a range of H concentrations. It can be seen in Fig. 2 that the extent of α-chain conversion to α-75 and α-40 was dependent upon the concentration of H up to 100 ng/ml, after which the assay was saturated with respect to cofactor. Also shown are the concentration-dependent cleavage profiles using sCR1 and sMCP as the I cofactors. For sMCP the assay is subsaturating with respect to cofactor up to 400 ng/ml. For sCR1, by 200 ng/ml there is essentially quantitative conversion of α-chain into α-75 and α-40. However, the further cleavage of α-75 into the 38-kDa C3dg fragment and the NH2-terminal 37-kDa fragment only became apparent at the 400 ng/ml concentration of sCR1. Since it was desirable to simultaneously assay all of the mutants, there was a practical need to restrict the number of samples to be handled. Consequently, in addition to the zero cofactor control, each rC3(H2O) molecule was analyzed at two cofactor concentrations, one of these concentrations giving an intermediate level of cleavage of wild-type rC3(H2O) and one giving near total cleavage of this molecule. These concentrations corresponded to 100 and 200 ng/ml for each of factor H and sMCP. For sCR1, the two concentrations chosen were 100 and 400 ng/ml, the latter being chosen in order to see whether the mutations had an effect on the cleavage of α-75 to C3dg and α-37.

**Interaction of Factor H with the Series of Mutant C3(H2O) Molecules**—Shown in Fig. 3A is the H cofactor-dependent cleavage assay for wild-type C3 and for the 9 charged residue alanine-scan mutants constructed within the 742–767 target region. Visual inspection of the autoradiogram reveals that mutants E744A and E747A show a significant impairment in H-dependent cleavage by factor I that is apparent at both 100 and 200 ng/ml of the cofactor. At least an equal degree of impairment was also observed in E744A/E747A double mutant. However, mutations at any of the other 7 charged residues in the 742–767 target segment were without effect in this assay. In order to determine whether the negatively charged side chains of Glu-744 and Glu-747 were required, isosteric amide substitutions were engineered, and the functional activities of these molecules were determined. It can be seen in Fig. 3A that the E744Q and E747Q mutants showed the same degree of impairment as did the equivalent alanine substituents, thereby implying that the negative charge per se is important for mediating binding of factor H.

Although the three mutants within the 727–737 segment (see Fig. 1) had previously been examined for their interaction with human factor H in both cofactor-dependent cleavage assay and were reported to be unimpaired in this binding interaction (22), it is now clear that the assay had been carried out using saturating concentrations of factor H. When reexamined under the current subsaturating assay conditions, only the tetramer D730N/E731Q/E736Q/E737Q showed a degree of impairment that was similar to that seen with either E744Q or E747Q alone.

In order to assess the reproducibility of the assay, and to determine whether the effects of the mutations at Glu-744 and Glu-747 were cumulative, this assay, as well as two other independent replicates, were subjected to densitometric analyses. For each lane the intensity of the α-chain was expressed as a fraction of the summed intensities arising from α-chain, α-75, β-chain, and α-40. The means and standard deviations of fractional α-chain intensity for each recombinant at the two factor H concentrations are shown in bar graph form in Fig. 3B. The bar graph presentation of the data, especially for the 200 ng/ml factor H concentration, readily identifies molecules having substitutions at either or both of Glu-744 or Glu-747 as being impaired with respect to factor H binding. There is also some suggestion that the effects on H binding of the individual mutations at residues 744 and 747 are cumulative, although not strictly additive.

As a second approach to assessing subtle defects in H binding activity produced by the various mutations, a version of the cofactor-dependent cleavage assay was employed in which 10% non-K76-COOH-treated fetal calf serum was used as a source of heterologous factors H and I. We reasoned that the interaction between the human C3(H2O) derivatives and the bovine H would be less strong than with its autologous counterpart and therefore that subtle defects or cumulative effects that were difficult to detect when using the autologous components may be observed using the heterologous components. The results of this experiment are shown in Fig. 4, and they essentially mirror those obtained using the autologous components under
non-saturating conditions. In this case, however, the cumulative effect of having residues Glu-744 and Glu-747 both mutated to alanine is more readily apparent.

Interaction of sCR1 with the Series of Mutant C3(H2O) Molecules—
The results from a representative CR1-dependent I-cleavage experiment are shown in Fig. 5 A, and 5 B depicts results from replicate experiments as a bar graph. Our previous work had shown that isosteric amide substitutions at Glu-736 and Glu-737, but not at Asp-730 and Glu-731, had resulted in a marked loss in the ability of the mutant red cell-bound C3b to mediate rosette formation with CR1 on neutrophils (22). The present analysis of these mutants in the CR1-dependent I-cleavage assay therefore provided the opportunity to validate this assay as a surrogate CR1 binding assay. In accordance with the CR1-dependent rosette assay results, mutant proteins containing the E736Q/E737Q substitutions show markedly impaired conversion of C3(H2O) to iC3(H2O) and no evidence of the third cleavage converting a-75 to C3dg and a-37. Similarly, as was the case for the rosette assay, mutation of residues 730 and 731 on their own resulted in unimpaired activity in the CR1-dependent I-cleavage assay. These results therefore confirm that the cofactor-dependent I-cleavage assay faithfully reports on the relative strength of the binding interaction between the SCR-containing I cofactor and the rC3(H2O) molecule.

In examining the 742–767 series of mutants in this assay, it became apparent that the mutants showing impaired cleavage fell into two categories: those affecting the first I-mediated cleavage at residue 1281 and those affecting only the third cleavage at residue 932. For example, whereas mutant E747A showed significantly impaired CR1-dependent conversion of C3(H2O) to iC3(H2O), and no evidence of the third cleavage at the high concentration of sCR1, mutant E744A showed no impairment in C3(H2O) to iC3(H2O) conversion but nevertheless was resistant to the third cleavage. An impairment in C3(H2O) to iC3(H2O) conversion was also seen with the E754A/D755A mutant. Although there was no impairment in this conversion for neighboring K757A/E758A mutant, there was impairment of the third cleavage. Combination mutants showed the phenotype of the individual mutations, and at least within this range of sCR1 concentrations, there was no obvious indication of a cumulative effect. Finally, as was the case for the interaction with factor H, isosteric amide substitutions at residues 744 and 747 showed the same degree of impairment as did their alanine-substituent counterparts. Thus for these two residues at least, one can conclude that the negative charge per se is important to the binding with CR1. In contrast, mutation to alanine of the positively charged residues Arg-742, Lys-761, and Lys-767 were each without effect on interaction between rC3(H2O) and CR1.

Interaction of sMCP with the Series of Mutant C3(H2O) Molecules—Like factor H and CR1, MCP is also a cofactor for the factor I-mediated cleavage of C3b to iC3b (or C3(H2O) to iC3(H2O)), and furthermore since no binding site for MCP has been identified, the results of these experiments provide additional information on the relative significance of the different residues in the C3(H2O) binding site.
been delineated, the complete series of 727–767 segment mutants were analyzed in a factor I cleavage assay in which a soluble form of MCP acted as the I cofactor. The results of a representative experiment are shown in Fig. 6A and as a bar graph from replicate experiments in Fig. 6B. In contrast to what was observed with both factors H and sCR1, when sMCP was used as the I cofactor, there appears to be no impairment in the interaction of this molecule with any of the mutant rC3(H₂O) molecules examined.

**DISCUSSION**

As summarized in Table I, the observations in this study have identified critical residues within the hydrophilic 42 amino acid segment at the NH₂ terminus of C3b α’-chain that contribute to the binding interaction with factor H and CR1. In contrast, we were unable to show any defect in the association of MCP with any of mutant proteins examined. With respect to a number of criteria including level of expression, biosynthetic processing, reaction with two conformationally sensitive monoclonal antibodies and hemolytic activity, the mutant molecules examined can be considered to be native-like with respect to their conformational state. Although the extent of hemolytic activity, which largely reflected the extent to which the C3 convertase cleavage site was affected, ranged from 73 to 46% of wild-type levels, it is important to note that there was no correlation between the extent of the hemolytic defect and whether or not an I cofactor binding site was affected.

Although the factor H and CR1 sites of interaction within the 727–767 segment partially overlap, the charged residue contacts required by CR1 extend over a wider portion of the segment than is the case for factor H. In particular, whereas the negatively charged side chains of Glu-744 and Glu-747 appear to be most important for the interaction with factor H, for CR1 there were 3 clusters of charged residues, namely Glu-736/Glu-737, Glu-747, and Glu-754/Asp-755 that had approximately equal effects on the CR1-dependent cleavage of C3(H₂O) to iC3(H₂O) upon replacement of the negative charge by a neutral residue. Additional contacts with Glu-744 and the Lys-757/Glu-758 pair appeared to be required for CR1 to act as a cofactor for the third factor I-mediated cleavage. This additional requirement is reminiscent of previous results using factor H as the I cofactor for this cleavage at physiologic ionic strength, it becomes one at low ionic strength (7), suggesting a need for additional contacts facilitated by relatively weak ionic bonds in order to alter either the conformation of iC3b/C3(H₂O) or more likely to position the factor I appropriately to enable the third cleavage of C3 α’-chain. The recent observation that factors H and I directly interact with one another (6) would be consistent with the
The observation that residues Glu-744 and Glu-747 are crucial for the binding of factor H to C3(H2O) is fully consistent with the data of the Fishelson (19) overlapping hexa/heptapeptide study which suggested that C3 residues 744–754 contribute to the binding of factor H. The only other mutant in which the interaction with H appeared to be compromised was the tetra mutant D730N/E731Q/E736Q/E737Q. Since the magnitude of impairment is the same as that seen with either E744Q or E747Q alone, it would suggest that either the contributions of the Asp-730/Glu-731 and Glu-736/Glu-737 charged pairs on their own to the binding of factor H were too small to be detectable in our assay or, more likely, that there is sufficient local conformational distortion caused by the loss of four negative charges over a space of seven amino acids to have had an effect on the nearby segment which now by two independent approaches has been shown to make a measurable contribution to H binding.

We believe it noteworthy that all of the mutant proteins for which the I cofactor assay suggested a diminished interaction with either factor H or CR1 had lost at least one negative charge, whereas none of the single positive charge substitutions, representing one-third of the non-overlapping mutations examined, showed any defect. The only ambiguity involves the K757A/E758A mutant which showed resistance to only the third factor I cleavage obtainable with CR1 as the cofactor. It is thus possible that Lys-757 is not involved at all in the interaction with CR1 or that at most it contributes only to the additional cofactor binding site required to mediate the third factor I cleavage. The dominance of negatively charged residues as putative contacts on the C3 side of the interactions with CR1 and factor H, together with the documented ionic strength dependence of these interactions (44, 45), strongly suggests that ionic bonds to positively charged residues on the cofactor side of the interaction form an essential component of the binding interface. Indeed, with respect to the extensively studied C3b- and C4b-binding sites within human CR1, Krych et al. (11) noted that all loss-of-function mutations either resulted from the loss of a positive charge or the addition of a negative charge. These observations are therefore in keeping with the hypothesis that the ionic interface is formed between positively charged residues of CR1 and negatively charged residues contributed by C3b and C4b. Similar point mutagenesis studies of factor H SCR domains have yet to be done. Based upon other

![Figure 5](http://www.jbc.org/)

**Figure 5.** Factor I-mediated cleavage of recombinant C3(H2O) molecules using sCR1 as the I cofactor. A, culture supernatants from COS-1 cell transfections containing 100 ng/ml of wild-type or mutant [35S]-labeled rC3(H2O) (as determined by RIA) were digested for 2 h at 37 °C with the concentrations of sCR1 (ng/ml) indicated above each lane and 500 ng/ml factor I. Digestion products were immunoprecipitated using rabbit anti-human C3c and resolved on 9% SDS-PAGE under reducing conditions to quantify the major bands. The ratio of the α-chain band pixel intensity over the combined signals from the α-chain, α-75, α-40, and β-chains (total) was calculated and plotted as a bar graph as a function of cofactor concentration. Error bars represent the S.D. of the mean. Note, α-37 represents the NH-terminal half of α-75 that is produced as result of the third factor I-mediated cleavage of C3(H2O). The COOH-terminal half corresponds to the similar mass C3dg fragment; however, this fragment is not immunoprecipitated by the anti-C3c antibody. Therefore in cases where this cleavage occurs, the denominator of the ratio will be somewhat underestimated. The order of the bars (left to right) is the same as the order in the legend (top to bottom).
known structures of protein-protein interfaces, it is likely that there is also a hydrophobic component to the interactions of C3b with CR1 and factor H (51–53). Indeed within the peptide segment suggested by the Fishelson study (19) to contain a binding site for factor H, there is a fairly hydrophobic stretch of residues,749WLWNV, some of which may contribute a hydrophobic patch to the binding interfaces for factor H and CR1.

Lambris et al. (21) have examined the contribution of the NH2-terminal α9-chain segment of human C3 to the interactions with factor H, factor B, sCR1, and sMCP, all of human origin, by either deleting most of the segment or by replacing it with the homologous segments from Xenopus and trout C3 and from the C3-related molecule cobra venom factor (CVF). As summarized in Fig. 7, these molecules differ in their abilities to bind to the human ligands (20), although it does not necessarily follow that the NH2-terminal α-chain segment on its own dictates the inter-species compatibilities. The recombinant molecule in which the 727–767 segment was deleted lost the ability to interact with human H, B, and sCR1 but not sMCP (21). Notwithstanding the conformational integrity caveat about the interpretation of loss-of-function data from a molecule containing a 36-residue deletion, there is general agreement between the major conclusions reached from the deletion study and those from our point mutation study. There are, however, some inconsistencies with respect to the results ob-

![Table I](https://www.jbc.org/)

**Table I**

*Summary of cofactor binding activities of mutant C3 molecules examined in this study*

| Recombinant       | Factor H | sCR1 | sMCP |
|-------------------|----------|------|------|
| Wild-type         | + + + a  | + + + | + + + |
| R742A             | + + +    | + + + | + + + |
| E744A             | + + + b  | + + + | + + + |
| K761A             | + + + +  | + + + | + + + |
| K767A             | + + + +  | + + + | + + + |
| E744A/E747A       | + + + +  | + + + | + + + |
| E754A/D755A       | + + + +  | + + + | + + + |
| K757A/E758A       | + + + +  | + + + | + + + |
| E744A/E747A/E754A/D755A | + + + + b | + + + | + + + |
| E744Q             | + + + +  | + + + | + + + |
| E747Q             | + + + +  | + + + | + + + |
| D730N/E731Q       | + + + +  | + + + | + + + |
| E736Q/E737Q       | + + + +  | + + + | + + + |
| D730N/E731Q/E736Q/E737Q | + + + + | + + + | + + + |

a Activity relative to wild-type based on disappearance of α-chain in the cofactor-dependent factor I cleavage assay using the indicated cofactor.

b Normal with respect to I cofactor activity for the C3(H2O) to iC3(H2O) conversion but no cleavage by I of iC3(H2O) to C3dg and a C3c-like molecule.
tained with the homologous replacement mutants as the only deleterious effects seen were on the third factor I-mediated cleavage with sCR1 as the cofactor. In all other respects, the chimeric molecules displayed wild-type behavior. As a point of reference, a minimum gap alignment of the relevant peptide segments from the various species, along with a denotation of point mutants determined in our study to affect the interaction with human H and sCR1, is shown in Fig. 7. One can easily rationalize the lack of effect of the interaction with factor H of the Hu/Xe chimera, since residues Glu-744 and Glu-747 are conserved in Xenopus C3. However, in the case of the Hu/Tr and Hu/CVF chimeras, one of the two crucial acidic residues is replaced by either its isosteric amide (Glu-744 in Tr C3) or a positively charged residue (Lys-747 in CVF), and based on our current findings, these changes would have been expected to decrease the extent of cleavage in the H-cofactor assay. One possible technical reason why this was not seen is that the assay performed by Lambris and colleagues (21) may not have been performed under limiting conditions of cofactor. We would have also expected compromised CR1-mediated cleavages in the case of the Hu/Tr and Hu/CVF chimeras that were not limited solely to the third factor I cleavage site because of the presence of Lys at residue 747 in the Hu/CVF chimera and the replacement of Glu-744/Asp-755 by a neutral TN pairing in the Hu/Tr chimera. However, in the case of the latter change, the presence of an ED pairing at residues 752 and 753 may compensate for the loss of negative charge at residues 754 and 755. Similarly, it is perhaps not surprising that substitutions of the human Glu-736/Glu-737 pairing in the various chimeric molecules were without effect in the CR1-dependent conversion of C3(H2O) to iC3(H2O) because in each case a triplet composed of two negative side chains and one neutral side chain (EEN) in human C3 is replaced by a similarly composed triplet (Xe and CVF have DSD and Tr has SED). At least with respect to factor B binding activity, we have previously shown that the human EEN triplet can be replaced by the CVF-like DSD triplet without effect (22).

There is now a congruence of evidence from synthetic peptide, blocking antibody, and protein engineering approaches that the acidic residue-rich segment at the amino terminus of C3 α’-chain contributes a ligand-binding site for factor H, CR1, and factor B (19, 21, 22, 24, 25, 54, and the present work). The protein engineering approach has also implicated this region in contributing a binding site for CR3 (22). Nevertheless, there is also considerable evidence that this is not the only binding site in the C3 molecule for these ligands. Blocking monoclonal antibody studies (49), as well as binding and inhibition studies with proteolytic fragments (24, 55, 56) and synthetic peptides (55) all implicate the C3d fragment as contributing to these binding interactions. Given the functional homology among the three I cofactors, together with the fact that CR1, factor H, and MCP are competitive ligands of C3b (46), one might expect that they might share a common, or at least partially overlapping, binding site in C3d. The three-dimensional structure of human C3d has recently been solved (57) and can now serve as a platform for a structure-guided mutagenesis analysis that will hopefully further define the C3d-resident interactions sites for the various protein ligands of C3.

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Identification of Residues within the 727–767 Segment of Human Complement Component C3 Important for Its Interaction with Factor H and with Complement Receptor 1 (CR1, CD35)

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