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Complement Inactivation by Recombinant Human C3 Derivatives

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From the implications of the complement system in a large number of diseases, an urgent need for therapeutics effecting reduced complement activity in vivo has emerged. In this study we report the design of a novel class of enzymes of human origin that obliterate functional complement by a noninhibitory, catalytic mechanism. Combining the framework of human C3 and the enzymatic mechanism of cobra venom factor, a nontoxic snake venom protein, we established molecules capable of forming stable C3 convertase complexes. Although the half-life of naturally occurring C3 convertase complexes ranges between 1 and 2 min, these complexes exhibit a half-life of up to several hours. Because the overall identity to human C3 could be extended to >90%, the novel C3 derivatives can be assumed to exhibit low immunogenicity and, therefore, represent promising candidates for therapeutic reduction of complement activity in vivo. The Journal of Immunology, 2004, 173: 5540–5545.

The complement system, an integral component of the innate immune system, comprises >30 proteins that interact in a cascade of enzymatic reactions, culminating in the generation and release of proinflammatory peptides, opsonization of target cells, and formation of the membrane attack complex. In addition to its essential role in immune defense, the complement system contributes to tissue damage in many clinical conditions, including ischemia-reperfusion injuries after myocardial infarction, interventional activation of complement in patients undergoing cardiopulmonary bypass, and immune-mediated diseases (1–4). The therapeutic approaches for inhibition of complement developed to date use the blockade of essential cascade components using inhibitory proteins, small compound inhibitors, or solubilized receptor molecules (5–7). In this study we address the design of stable C3 convertase complexes of the alternative pathway of complement (AP),4 capable of obliterating functional complement via consumption of complement component C3.

The AP is continuously activated at a low rate in human plasma due to slow, spontaneous hydrolysis of plasma protein C3 to C3(H2O) (8). C3(H2O) provides a subunit for binding of factor B and formation of the initial AP C3 convertase, C3(H2O)Bb, which cleaves fluid phase C3, generating metastable C3b that can attach covalently to surfaces. After binding of factor B and cleavage of C3b-bound factor B by factor D, the actual AP C3 convertase, C3bBb, is formed, which initiates amplification of the complement cascade by increased deposition of C3b and subsequent formation of new C3 convertases (9). Because complement proteins can exert highly destructive effects, the complement cascade has to be tightly controlled. Regulation of convertase C3bBb, in addition to its intrinsic lability, is performed by a complex network of the regulators of complement activation proteins, which irreversibly dissociate the convertase subunits and serve as cofactors for the proteolytic cleavage of C3b by factor I (10).

A stabilized C3 convertase complex, active in fluid phase and escaping regulation by regulators of complement activation, is formed by the structural and functional C3b homologue cobra venom factor (CVF), a nontoxic 149-kDa snake venom protein (11, 12) that displays an overall sequence identity to human C3 (hC3) of 50% (13, 14). In serum, CVF forms the CVFBb convertase complex that exhibits a half-life of 7 h compared with 1.5 min for the C3bBb complex (12, 15). As a result of its stability, the CVFBb convertase continuously activates the AP and consumes all downstream complement components, thereby obliterating functional complement in experimental animals. Due to these properties, CVF has been shown to be highly effective in a variety of animal models for complement-mediated pathologies (reviewed in Ref. 16), but the therapeutic potential in man is severely limited by its high immunogenicity.

In this study we exploited the framework of hC3 for the design of derivatives displaying the complement-inactivating mechanism of CVF. These molecules putatively avoid the high immunogenicity of CVF and represent potentially valuable reagents for therapeutic intervention in complement-mediated pathologies. Furthermore, the derivatives will help to elucidate the mechanism(s) responsible for the formation of stable convertase complexes, a prerequisite for a rational design of future derivatives suitable for therapy.

Materials and Methods

Materials

Native hC3 and factor B were purchased from Calbiochem (Schwalbach, Germany). Factor D and Boc-Leu-Gly-Arg-7-amido-4-methylcoumarin acetaldehyde were purchased from Sigma-Aldrich (Taufkirchen, Germany). Native CVF (nCVF) from Naja kaouthia was purified according to established protocols (17). Anti-C3 Ab was purchased from Cappel Laboratories (Eschwege, Germany), and polyclonal anti-CVF-Abs were raised in goat using purified nCVF. Streptactin was purchased from IBA (Gottingen, Germany).

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4 Abbreviations used in this paper: AP, alternative pathway; CHO, Chinese hamster ovary; CVF, cobra venom factor; hC3, human C3; nCVF, native CVF; rCVF, recombinant CVF.

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Generation of expression plasmids for CVF, hC3, and hC3 derivatives

Generation of full-length cDNA of CVF and hC3, providing a N-terminal strep-tag II affinity peptide (St) and an endorokinese cleavage site, was performed as described (18). For generation of hC3-DI and hC3-DIII, the cDNA of CVF in pUC18 as well as the cDNA of hC3 in pCDNA3 were used (18). In pUC18CVF, a BglII restriction site was generated by PCR at position 1793 (mutation A1797C) using the oligonucleotides S01 (GGATCCAGGTGCTCGGGTTGG), AS24 (TTGTGCTCCGATGCTTAGCTTGGGC), S26 (TTTGGGTTGTTAAGGATACAG-CC), and AS04 (CATAAATCTCCTGTTAACGTTTGGC). After joining these fragments, the product obtained was inserted into the cDNA of CVF via the restriction sites of Aval and Hpal. For generation of the constructs hC3-DI and hC3-DIII, the plasmid pUC18CVFBglII was digested with EcoI36I and BglII, and the fragment containing the pUC18 vector as well as the 3’ terminus of CVF was isolated and ligated with a fragment containing the 5’ terminus of hC3, which was generated from the cDNA of hC3 in pCDNA3 using EcoRII, mung bean nuclease, and BglII. The plasmid pUC18CVFBglII was digested with BglII, and the resulting 2.3-kb fragment of CVF was inserted into the vector pUC18DI9A to generate the derivative hC3-DI via the corresponding restriction site. Generation of the derivative hC3-DIII was performed in an analogous manner by inserting the 2.3-kb fragment of hC3 via BglII. Finally, the cDNA of the derivatives hC3-DI and hC3-DIII were transferred to pCDNA3 via EcoRII restriction sites. For generation of the derivative hC3-DIII, pUC18CVFBglII as well as pCDNA3hC3 were digested with BglII, and the CVF cDNA fragment obtained was inserted into the digested cDNA of hC3. Thereafter, the cDNA of hC3-DIII was transferred to pCDNA3 using XhoI, mung bean nuclease, and NorI. Insertion of an N-terminal strep-tag and an endorokinese cleavage site was performed as previously described (18).

Transfection of Chinese hamster ovary (CHO) cells

CHO cells cultivated according to standard protocols (19) were transfected with DNA of the expression vector using Geneporter (Peqlab, Erlangen, Germany). Transfection and expression were performed in roller bottles using OptiMEM (Invitrogen Life Technologies, Karlsruhe, Germany) without phenol red supplemented with 4% (v/v) heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Purification of hC3-DIII from supernatant of stably transfected CHO cells

Five hundred milliliters of supernatant obtained from stably transfected cells was adjusted to pH 7.5, passed through a 0.45-μm pore size cellulose acetate membrane, and loaded onto a Poros HQ/M anion exchange column equilibrated with 50 mM Tris (pH 7.5) using ΔKTA purifier (Amersham Bioscience, Freiburg, Germany). The recombinant protein was eluted using a linear (0–500 mM) NaCl gradient. Fractions (2 ml) were analyzed using 7.5% SDS-PAGE and Western blotting, pooled, and dialyzed against PBS. The pooled sample was diluted (1/9) in 50 mM sodium phosphate, 0.55 M sodium sulfate buffer (pH 7.0), filtered (0.2 μm), and applied to a thiolipid resin (1.5 ml; BD Bioscience, Heidelberg, Germany) equilibrated with 50 mM sodium phosphate and 0.5 M sodium sulfate buffer (pH 7.0). After extensive washing of nonadsorbed proteins with the equilibration buffer (>30 column volumes), elution was performed using 50 mM sodium phosphate buffer, pH 7.0. Fractions (1.5 ml) were analyzed by 7.5% SDS-PAGE and Western blotting. Fractions containing hC3-DIII were pooled, dialyzed against 100 mM Tris and 150 mM NaCl (pH 8.0; buffer W), loaded onto Strep-Tactin-Sepharose (2 ml; IBA, Göttingen, Germany) equilibrated with buffer W, washed with 10 ml buffer W, and eluted with buffer W containing 2.5 mM desthiobiotin. Protein concentration and purity of the fractions were analyzed by 7.5% SDS-PAGE. Pooled fractions were dialyzed against PBS and used for further characterization.

Complement consumption assay

The complement-consuming activity was determined according to established protocols (20) with slight modifications as previously described (18). Briefly, 20 μl of protein sample or GVBS1+2 (2.5 mM sodium/5,5-diethylbarbituric acid, 143 mM NaCl, 0.75 mM MgCl2, 0.15 mM CaCl2, and 0.1% gelatin, pH 7.4; serum control) was mixed with 20 μl of normal human serum (diluted 1/2 in GVBS1+2) and incubated for 3 h at 37°C. After addition of 100 μl of GVBS1+2 and 30 μl of sensitized sheep erythrocytes (5 × 107 cells/ml) generating hC3-DIII (3% lysis) was determined using an excitation filter of 485 nm and an emission filter of 530 nm in a microplate reader (Genios, Tecan, Creiselsheim, Germany). Values after 60 min of fluorophore release were defined as 100%. The slope of the graph was used to determine the enzymatic activity of the sample.

Other methods

SDS-PAGE, Western blotting, as well as standard procedures in molecular biology were performed according to established protocols (19).

Results

Recombinant expression of hC3, recombinant CVF (rCVF), and hC3 derivatives

In this study we used hC3 as framework for the design of derivatives containing extended CVF domains on the basis of several control values reached ~80% of maximal lysis, all samples were processed as described above. GVBS1+2 control containing only erythrocytes was performed parallel. The percentage of lysis was determined according to formula (sample – GVBS1+2 control)/(serum control – GVBS1+2 control) × 100.

Solid phase complement consumption assay

The assay was performed as previously described (18). Briefly, strep-tag II-derivated proteins were immobilized onto polystyrene supports coated with streptactin. Subsequently, 40 μl of GVBS1+2 containing 10 μl of normal human serum were added and incubated for 3 h at 37°C. Subsequently, the supernatants were assayed for complement consumption as described above.

Complement consumption assay of immunoprecipitated proteins

The assay was performed as previously described (18). Briefly, His-tag-derivated Ab fragments (scFv) with specificity for the protein to be analyzed were immobilized onto Ni-NTA-agarose and incubated with the protein sample overnight at 4°C. The Ab fragments used for precipitation of the recombinant proteins were isolated by selection of an scFv library against human native C3 or CVF. After centrifugation, bound proteins were eluted by 300 mM imidazole, centrifuged, dialyzed, and assayed for complement-consumption activity as described above.

Bystander lysis assay

The bystander lysis assay for analysis of fluid phase C5 convertase activity was conducted according to established protocols (17) with slight modifications. Briefly, the sample (20 μl) was mixed with 20 μl of GVBS1+2, 20 μl of guinea pig serum and 20 μl of guinea pig erythrocytes (5 × 107 cells/ml) and incubated for 3 h at 37°C. Samples containing no protein (serum control) or no serum (water control) were used as controls. After addition of 1 ml of ice-cold GVBS1+2 or water, respectively, the samples were analyzed as described above. The percentage of lysis was determined according to formula (sample – serum control)/(water control – serum control) × 100.

C3 reconstitution assay

To determine the ability of proteins of forming complexes that exert both fluid phase C3 and C5 convertase activity, a slightly modified complement consumption assay was performed. In this assay each sample was supplemented with 10 μg of purified hC3 after incubation for 3 h at 37°C. Proteins capable of forming complexes with both fluid phase C3 and C5 convertase activity consume serum C3 and C5 upon addition to serum. As a result, complement activity cannot be restored by the addition of C3 only, and lysis of sensitized sheep erythrocytes is impaired.

Determination of the stability of C3 convertase

Following established protocols (21) with slight modifications, 500 ng of each nCVF, hC3, and the derivative hC3-DIII were mixed with 950 ng of factor B and 8 ng of factor D in a volume of 60 μl of veronal-buffered saline (2.5 mM sodium-5,5-diethylbarbituric acid and 143 mM NaCl, pH 7.4). After addition of MgCl2 to a final concentration of 10 mM, the samples were incubated for 2 h at 37°C to allow for convertase formation. Subsequently, all samples were supplemented with EDTA to a final concentration of 10 mM to inhibit further formation of convertases. Thereafter, the samples were incubated at 37°C, and after different periods of incubation 20-μl aliquots were added to 150 μl Boc-Leu-Gly-Arg-7-amido-4-methylcoumarin acetate in 180 μl of veronal-buffered saline. The time course of fluorophore release was determined in black FIA-Plates (96 K; Greiner Bio-One, Frickenhausen, Germany) using an excitation filter of 370 nm and an emission filter of 465 nm in a microplate reader (Genios, Tecan, Creiselsheim, Germany). Values after 60 min of fluorophore release were defined as 100%. The slope of the graph was used to determine the enzymatic activity of the sample.

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studies that have provided evidence for the implication of the N terminus of the α-chain and the C3d region in the binding of several regulatory proteins (22–29) (Fig. 1A). As shown in Fig. 1B, the domains corresponding to the γ- and β-chains in CVF (hC3-DI), to the γ-chain in CVF (hC3-DII), and to the terminal 275 aa of the CVF β-chain (hC3-DIII) were substituted. Resulting constructs display overall sequence identity to hC3 of 68, 77, and 91%, respectively.

After transient transfection of CHO cells, all recombinant proteins were expressed at a level of 1–2 μg/ml. Although natural CVF is composed of three disulfide-bridged chains, rCVF displays a two-chain structure, which resembles the structure of hC3 (Fig. 1A). Under nonreducing conditions, rCVF, hC3, and its derivatives exhibited an identical apparent m.w. in the range of 200 kDa, similar to that of hC3 (Fig. 1C).

Functional analysis of transiently expressed proteins

The complement-consuming activity of the recombinant proteins was analyzed by two different affinity-based procedures, a fluid phase assay of immunoprecipitated derivatives and a solid phase complement consumption assay (18). For the solid phase complement consumption assay, the recombinant proteins were derivatized with the affinity octapeptide strep-tag II (30) at the C terminus of the leader sequence and expressed in CHO cells. The strep-tag II-derivatized proteins exhibited identical molecular masses as the nonderivatized proteins, suggesting analogous processing. Because in both assays the derivative hC3-DI exhibited CVF-like complement-consuming activity (Fig. 2), structural domains of the CVF γ- or β-chains appeared to be important for conferring stability to the C3 convertase complex. Similar to derivative hC3-DI, hC3-DIII displayed also CVF-like complement-consuming activity (Fig. 2). In contrast to these two derivatives, hC3-DII did not exhibit any complement-consuming activity. The presence of each recombinant protein in the supernatants of transiently transfected cells and in the immunoprecipitates was verified by Western blot analysis. Based on the intensity of the Western blot bands, the concentrations of the recombinant protein were adjusted to comparable values. Furthermore, the presence of immobilized strep-tag II-derivatized proteins was verified by ELISA techniques. These data revealed that only the C-terminal domain of the CVF β-chain is required for the formation of a stable hC3-dependent C3 convertase complex. Restricting the substitution to the CVF γ-chain did not confer complement-consuming activity to the molecule. The data in Fig. 2 clearly indicate that removal of domains responsible for regulatory dissociation of the convertase complex is not sufficient for conferring stability to the C3 convertase complex.

FIGURE 1. Design and recombinant expression of hC3, CVF, and hC3 derivatives. A. Primary structures of hC3 ( ), CVF ( ), and the derivatives hC3-DI ( ), hC3-DII ( ), and hC3-DIII ( ). H, N-terminal strep-tag II). C. Western blot analysis of the transiently expressed proteins. The nCVF, hC3, and culture supernatants of transiently transfected CHO cells containing rCVF, rhC3, or one of the three hC3 derivatives were separated by SDS-PAGE (7.5%) under nondenaturing conditions, transferred to a nitrocellulose membrane, and detected with polyclonal anti-CVF Abs (nCVF and rCVF) or anti-hC3 Abs (hC3, rhC3, hC3-DI, hC3-DII, and hC3-DIII) as described in Materials and Methods.

FIGURE 2. Initial characterization of hC3, CVF, and hC3 derivatives. A. Solid phase complement consumption assay. Strep-tag II-derivatized proteins were immobilized to streptactin-coated supports and assayed for complement consuming activity as described in Materials and Methods. Strep-tag II-derivatized hC3 was used as a negative control. B. Complement consumption assay of immunoprecipitated recombinant proteins. The recombinant proteins were immunoprecipitated from supernatants of transient expressions using an anti-CVF scFv (rCVF) and an anti-C3 scFv (rhC3, hC3-DI, hC3-DII, hC3-DIII) and were assayed for complement-consuming activity as described in Materials and Methods. Data represent the mean ± SD obtained from at least three independent experiments.
Functional analysis of purified hC3 derivative hC3-DIII

To analyze the functional activity of derivative hC3-DIII in more detail, we established a stably transfected CHO cell line. SDS-PAGE analysis of the purified derivative (purity, ~90%) under reducing conditions revealed two bands, one of them corresponding to the α-chain and the other to the β-chain of hC3, an observation that supports a C3-analogue processing of derivative hC3-DIII to a two-chain structure (Fig. 3A).

A detailed analysis of the complement-consuming activity of hC3-DIII over a broad concentration range confirmed the CVF-like activity observed in analyses of transiently expressed hC3-DIII. As evident from Fig. 3B, hC3-DIII exhibits ~85% of the complement-consuming activity of purified native CVF. The higher m.w. derivative hC3-DIII exhibits an activity in the range of ~90%.

Because CVFBB also exerts C5 convertase activity in fluid phase (31), the hC3-DIII-dependent convertase complex was analyzed for potential fluid phase C5 convertase activity using two different approaches (Fig. 4). Both of them indicated fluid phase C5 convertase activity of derivative hC3-DIII, although only to an extent ~20% that of the CVF-dependent C5 convertase. Apparently, a CVF-like C5 convertase activity requires a more extended C-terminal domain of CVF than that present in derivative hC3-DIII.

To elucidate the molecular basis of the CVF-like complement-consuming activity, we analyzed the formation and stability of the convertase generated by the derivative hC3-DIII. As shown in Fig. 5A, the recombinant protein activates factor B by producing Bb and Ba in the presence of factor D and Mg²⁺ in an identical manner as C3(H₂O) and CVF. The time-dependent reduction in the release of 7-amido-4-methylcoumarin from a fluorogenic substrate analog by the action of the convertase (Fig. 5, B–D) revealed a half-life of the hC3-DIII-dependent and the CVF-dependent convertase of ~5–6 h (Fig. 5E), which is close to the reported 7-h half-life of the CVF-dependent convertase (12). In contrast, the C3bBb convertase complex exhibited no activity, thereby confirming the extremely short half-life in the range of 1–2 min (15).

Discussion

In this study we have generated derivatives of the human complement component C3 to identify structural domains that are critical for the design of a stable C3 convertase of the alternative pathway (reviewed in Ref. 25). Because in a previous study replacement of short motifs in the CVF molecule did not affect the functional characteristics of the CVF-dependent convertase (18), we now replaced entire domains in the hC3. Several approaches have provided strong evidence for the implication of the N terminus of the α-chain and the C3d region of hC3 in the binding of several regulatory proteins, including factor B, factor H, and CR1 (22–29). Accordingly, substitution of the α-chain of hC3 in hC3-DI by the corresponding γ- and β-chains of CVF resulted in drastic enhancement of the complement-consuming activity. However, restriction of the substitution in derivative hC3-DII to the domain corresponding to the CVF γ-chain did not affect any detectable enhancement of complement-consuming activity. Apparently, elimination of binding sites for regulatory proteins is of minor importance, in contrast to studies postulating the resistance against regulation by factor H and factor I as an important mechanism of convertase stabilization (32, 33).

The establishment of CVF-like, complement-consuming activity in hC3-DIII indicates that the C-terminal domain of CVF alone is sufficient for conferring an inherent molecular stability to the
C3-dependent C3 convertase complex. Although it cannot be excluded that the C-terminal domain resides in conformational vicinity to binding sites of regulatory proteins, thereby potentially impairing their function, it seems to be more convincing that the C-terminal domain itself contributes to stabilization of the binding of Bb. This could be the result of an increase in affinity due to the exposure of additional interaction points. Alternatively, the molecular framework of C3 could have been modified by fusion of the C-terminal CVF sequence, as described for C3 upon adsorption to polymer surfaces (34). Immobilized C3 has been shown to form a C3 convertase of the AP that is not cleaved by factors I and H, possibly the result of conformational changes in the molecule causing disruption of the thioester bond (35).

Substitution of the relatively short C-terminal domain of hC3 by the corresponding CVF sequence in hC3-DIII also conferred fluid phase C5 convertase activity. Although unexpected, the potential relevance of the C-terminal domain for this activity was suggested by the observation that the C5 convertase activity of CVF could be eliminated by introduction of a C-terminal His tag (36). The C5 convertase activity of hC3-DIII, however, is marginal compared with that of the CVF-dependent convertase. Because deposition of the anaphylatoxin C5a is known to cause tissue damage more efficiently than C3a (37, 38), the significantly reduced C5 convertase activity of hC3-DIII is favorable for therapeutic applications.

Due to their catalytic mode of action, hC3 derivatives represent potentially valuable therapeutics. Recombinant expression of the
hC3 derivatives in mammalian cells provides the advantage of a defined glycosylation that is analogous to native hC3. Especially hC3-DIII is a promising candidate due to its low percentage of foreign protein sequences. Substitution of the 275-aa C-terminal region provides an hC3 derivative with an overall length of 1640 aa, corresponding to an identity with the hC3 derivative and hC3 of 91%. Because therapeutic Abs provide between 65 and 95% human origin (39–41) the immunogenicity of the derivative hC3-DIII with an overall identity of 91% to hC3 can be assumed to be low. Moreover, the derivative provides only slight C5 convertase activity, reducing harmful deposition of anaphylatoxin C5a. Confinement of nonhuman sequences in future hC3 derivatives will help to further reduce potential immunogenicity and to eliminate C5 convertase activity in the fluid phase. Preliminary results, obtained with a construct containing <4% foreign protein sequences, underline the feasibility of this approach. Currently, the structural characteristics of the C-terminal domain are being investigated in more detail to elucidate the precise molecular mechanism underlying the stabilizing effect of this domain.

In summary, hC3 derivatives capable of forming stable convertases represent a milestone in the generation of novel therapeutics applicable for catalytic reduction of complement activity in vivo in different areas of pathology.

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