

Review

Recombinant generation of two fragments of the rat complement inhibitory factor H [FH(SCR1-7) and FH(SCR1-4)] and their structural and functional characterization in comparison to FH isolated from rat serum

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Summary. Factor H (FH) is the predominant soluble inhibitor of the complement system. With a concentration of 200-800 µg/ml in human and rat plasma it acts as a cofactor for the soluble factor I (FI)-mediated cleavage of the component C3b to iC3b. Furthermore it competes with factor B for binding to C3b and C3(H₂O) and promotes the dissociation of the C3bBb complex. FH is a monomer of about 155 kDa which comprises 20 short consensus repeats (SCR), each of which is composed of approximately 60 amino acid (aa) residues. Two functional fragments of FH comprising the SCR1-4 or SCR1-7 were generated using either the Baculovirus system or stably transfected human embryonal kidney cells, respectively. These fragments, as well as FH purified from rat serum, were first analyzed for their relative molecular weights (Mr) using non-reducing or reducing SDS-PAGE. The Mr of the FH variants differed by about 20 % depending on the experimental conditions employed. Only the Mr of proteins separated under reducing conditions were in accordance with the MW calculated from the aa sequence. Analyses of the glycosylation patterns using PAS-staining showed a lack of staining of the recombinant variants (SCR1-4 and SCR1-7) in contrast to FH(SCR1-20) from serum. Using a complement hemolysis assay (CH₅₀-assay) all three variants exhibited a molar complement inhibitory activity of FH(1-20)/FH(1-7)/FH(1-4) of about 3/1/1. These data support the postulated model of FH bearing three binding sites for its ligand C3b, from which one is located in the SCR1-4, whereas the other two are located in the SCR8-20.

Key words: Complement inhibitory factor H (FH), Complement hemolysis (CH₅₀), Recombinant expression, Short consensus repeats (SCR)

Introduction

The complement system is part of the innate immune system and mounts a first line of defence against invading bacteria. There exist three activation pathways of the system: (i) The classical pathway is generated by immune complexes containing immunoglobulin IgG or IgM, (ii) the alternative pathway may be directly initiated on surfaces of pathogens and (iii) the mannose-binding lectin (MBL) pathway is initiated by microbes bearing terminal mannose groups. Both the MBL and the alternative pathway are initiated independently of antibodies. The MBL pathway joins the classical pathway of complement activation at the level of the component C4 cleavage thereby indicating that the alternative in comparison to the classical and the MBL pathways generate C3/C5 convertases via different routes. This leads to the opsonization of pathogens, the recruitment of phagocytes and a direct killing by the insertion of pores into the outer membrane of pathogens. In addition, the protective functions of the complement system may induce an inflammatory response which leads to the damage of normal tissue (Hill et al., 1992; Mulligan et al., 1992; Smith et al., 1993; Piddlesden et al., 1994). This requires a strict regulation of the complement system at the level of the C3 and C4 components, which is mediated by several receptors and soluble regulatory proteins. With the exception of complement factor I (FI) these proteins form the family of "regulators of complement activation" (RCA)

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(Hourcade et al., 1989) including the decay accelerating factor (DAF/CD55), the membrane cofactor protein (MCP/CD46), the complement receptor 1 (CR1/CD35), and 2 (CR2/CD21) as well as the soluble factors I (FI) and H (FH). In man and mouse the genes of the RCA family are closely linked on the long arm of chromosome 1 (Hourcade et al., 1989), whereas the serine protease FI is located on chromosome 4 in humans (Goldberger et al., 1987) and on chromosome 3 in mice (Minta et al., 1996). The predominant soluble regulator FH represents a single chain protein of 155 kDa Mr exhibiting a concentration in human and rat serum ranging between 200-800 $\mu\text{g/ml}$ (Demberg et al., 2002). It has an important role for those host cell surfaces on which the membrane-bound complement inhibitory proteins are only poorly expressed. FH acts as a co-factor for the FI-mediated cleavage of C3b to iC3b. The affinity of human FI for its substrate C3b is 15-fold higher in the presence of FH than in the absence of any co-factor (DiScipio, 1992). Furthermore, FH competes with factor B for binding to C3b (Kazatchkine et al., 1979; DiScipio, 1981) and promotes the dissociation of the C3bBb complex (Whaley and Ruddy, 1976; Weiler et al., 1976).

FH consists of 1234 aa in mouse (Kristensen and Tack, 1986), 1231 aa in man (Ripoche et al., 1988a) and 1236 aa in the rat (Demberg et al., 2002). Recently, the primary structure of porcine FH has been published consisting of 1234 aa (Hegasy et al., 2003). The 42 kDa FH-like protein/reconectin (FHL-1) represents a truncated splicing form of the FH-specific mRNA and comprises the short consensus repeats (SCR) 1-7 (Schwaeble et al., 1987, 1991). It is present in serum at a concentration of 20-50 $\mu\text{g/ml}$. Similar to FH, FHL-1 acts as a co-factor of FI for cleaving of the C3b/C4b components (Misasi et al., 1989) and promotes the dissociation of the C3bBb complex (Kühn et al., 1995). However, FHL-1 also exhibits some unique biological functions in comparison to FH (Hellwage et al., 1997; Johnsson et al., 1998).

The primary synthesis of FH occurs in the liver (Zipfel and Skerka, 1994; Schlaf et al., 2001, 2002). High constitutive FH expression levels were found in hepatocytes (HC) as well as in Kupffer cells (KC) (Schlaf et al., 2002). However, extrahepatic cell types such as human umbilical vein endothelial cells (HUVEC) (Brooimans et al., 1989, 1990; Ripoche et al., 1988b), peripheral blood monocytes (Whaley, 1980), cells of the monocyte-macrophage series (De Ceulaer et al., 1980), primary skin fibroblasts (Katz and Strunk, 1988), fibroblast-like L-cells (Munoz-Canoves et al., 1989; Vik, 1999), primary myoblasts and rhabdomyosarcoma cell lines (Legoedec et al., 1995), glioma cell lines (Gasque et al., 1992), and glomerular mesangial cells (van den Dobbelen et al., 1994) also constitutively express FH. These cell types represent local sources of FH and may reduce tissue damage which is caused by local complement activation. Since the cell-specific expression and regulation of FH and FHL-1 as well as the attachment of FH to the surface of self-cells with pathophysiological consequences for several human diseases have been recently reviewed by Friese et al. (1999) and Jozsi and coworkers (2004), respectively, we focussed this review on the recombinant expression of the FH(SCR1-4) and (SCR1-7) as well as their structural and functional analyses in comparison to FH(SCR1-20) isolated from rat serum by immunoaffinity chromatography.

Purification and identification of three FH variants

The human embryonal kidney cells HEK293 were stably transfected with rat FH(SCR1-7). Consecutively, FH(SCR1-7) expressing clones were generated, subcloned and expanded to purify larger amounts of FH(SCR1-7) using the Strep-Tag affinity chromatography system (IBA limited, Goettingen, Germany). 500 μl and 100 μl from the elution fractions were precipitated using TCA, separated using SDS-PAGE and either stained with Coomassie Blue or

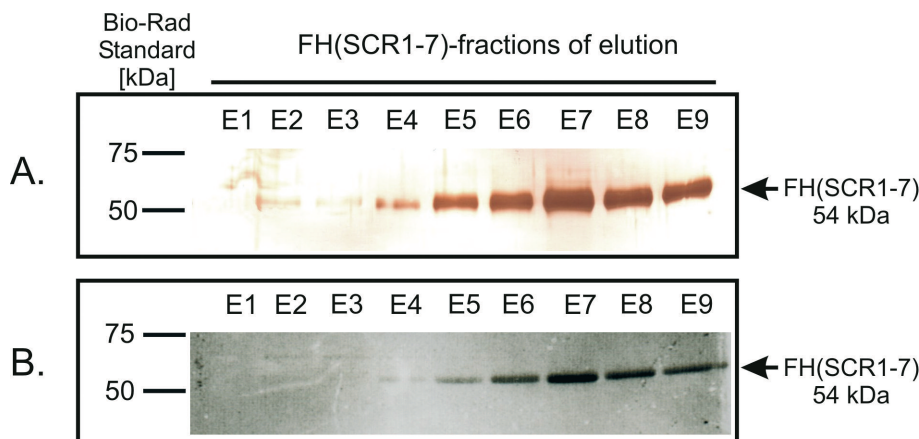


Fig. 1. Elution fractions of the recombinant FH(SCR1-7) derived from HEK 293 cell supernatants after Strep Tag chromatography (IBA limited, Göttingen, Germany) as detected by immunoblotting (A) and by corresponding Coomassie Blue staining (B). A. Immunoblot analysis of 100 μl supernatant each of which had been precipitated with TCA and was afterwards separated in SDS-PAGE under reducing conditions. Detection was performed using a rabbit anti-rat FH pAb followed by a POD-conjugated donkey anti-rabbit pAb. B. Corresponding elution fractions as stained with Coomassie Blue (volumes of 500 μl supernatant each of which had been precipitated with TCA).

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identified by immunoblot analyses using an anti-rat FH pAb. As shown in Figure 1, a protein band of 54 kDa is visible in the Coomassie Blue stained gel as well as in the corresponding immunoblot analysis which is in accordance with the predicted Mr of the recombinantly expressed FH(SCR1-7). In contrast to the first seven domains containing FH(SCR1-7) the FH(SCR1-4) was expressed in SF21 cells using the Baculovirus system which resulted in an increased expression level of the FH variant when compared to the mammalian HEK293 cells. For secretion of FH from both SF21 and HEK293 cells the normal leader sequence of rat FH (MRLSARIWLILWTVCA) was employed (Demberg et al., 2002). After its purification by Ni-chelate

chromatography using a carboxyterminal tail of 6 histidine aa residues 10 μ g of FH(SCR1-4) were separated by PAGE for staining with Coomassie Blue (Fig. 2), whereas 0.5 μ g, 1 μ g or 2.5 μ g of FH (SCR1-4) was subjected to the corresponding immunoblot analysis (Fig. 2) using a polyclonal rabbit anti-FH Ab. As shown in Figure 2 a monomer was detectable at an Mr of approximately 28 kDa, whereas a dimer appeared as the dominating protein band at approximately 56 kDa. However, neither the monomer nor the dimer represented the predicted Mr of about 36 kDa. The observed differences in the Mr might depend on the experimental conditions employed (section 4). The high level of dimerization is caused by the HIS-Tag of the recombinant proteins and was observed to increase after prolonged storage. It sometimes results in a complete loss of protein function. In contrast, such an effect was not visible with proteins bearing a Strep-Tag (Fig. 1).

The complete FH protein (SCR1-20) was not recombinantly expressed, but was purified by immunoaffinity chromatography from rat serum (Fig. 3) as formerly described by Demberg and coworkers (2002) using the anti-rat FH mAb 4-7D (Schlaf et al., 2002). The elution fractions of FH (SCR1-20) in SDS-PAGE under reducing conditions were size fractionated. Under these conditions the FH(SCR1-20) protein migrates as a single band of the predicted Mr of 155 kDa (Fig. 3).

In most of the elutions of FH(SCR1-7) from Strep-Tag columns employed for the purification of the supernatants of HEK293 cells the recombinant FH(SCR1-7) migrated as a double band in the range of Mr 36 kDa which was detectable after Coomassie Blue staining (Fig. 4A) of FH(SCR1-7) as well as after its immunoblot detection (Fig. 4B) using peroxidase-conjugated streptactin (IBA limited, Göttingen, Germany). Both bands were directly subjected to N-terminal protein sequencing resulting in the identification of the first 12 amino acid residues of SCR1 [bold letters: EDCKGPPPRENS; (Fig. 4C)]. Thus, the identity of the purified FH(SCR1-7) protein and its correct processing by removal of the leader sequence was ensured. Since the recombinant expression of the complement factor H in SF21 cells is possible (Sharma and Pangburn, 1994; Kühn and Zipfel, 1995;

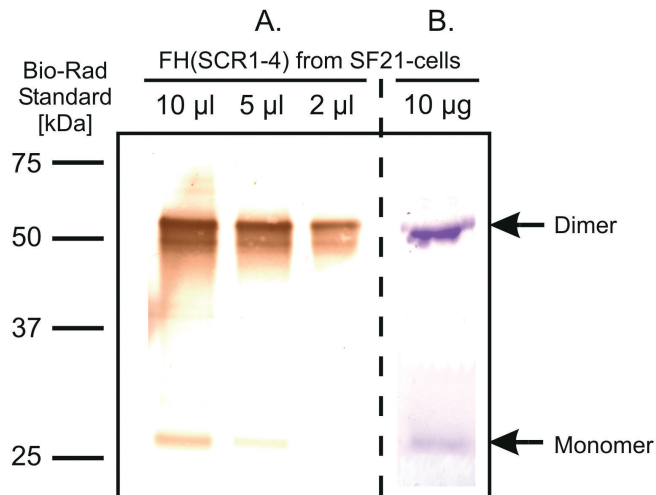


Fig. 2. Identification of recombinant FH(SCR1-4) derived from SF21-cells by immunoblot analysis (A) and by Coomassie Blue staining (B). A. 10 μ l, 5 μ l or 2 μ l of FH(SCR1-4) eluate which had been purified by Ni²⁺-chelate chromatography were applied onto SDS-PAGE under non-reducing conditions. Detection was performed with a polyclonal anti-rat FH antibody from rabbit. As is visible the dimer dominates (upper arrow) whereas only with a volume of 10 μ l the monomer is clearly visible in the immunoblot analysis. B. 10 μ g of purified FH(SCR1-4) were separated in SDS-PAGE and afterwards stained with Coomassie Blue. In accordance with the immunoblot analysis the dimer of 56 kDa clearly dominates whereas the monomer of 28 kDa is hardly visible.

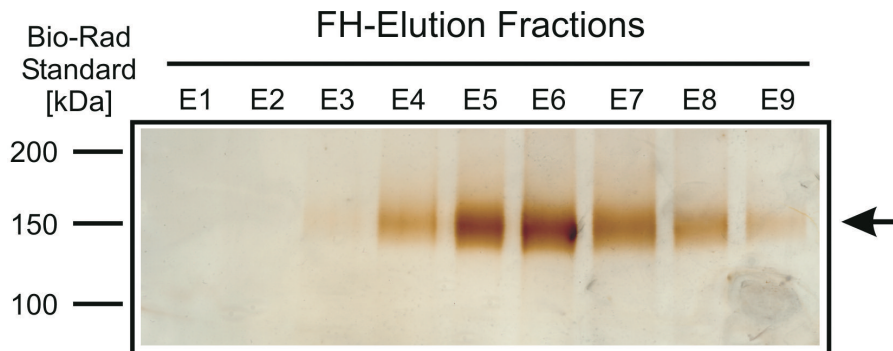


Fig. 3. Silver-staining of the FH(SCR1-20) protein which is contained in the elution fractions of the immunoaffinity column (using the immobilized anti-rat FH mAb 4-7D). Volumes of 10 μ l of each elution fraction were applied onto SDS-PAGE. As is visible, FH (Mr = 155 kDa under reducing conditions) was eluted in the fractions E3-E9 with a maximum in E5-E6.

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Pangburn et al., 2000; Hellwage et al., 2002) the baculovirus system was used several times to produce FH variants which contained deletions for functional investigations. However, the generation of recombinant FH and its truncated variants using mammalian cells has so far only been described in COS cells upon transient transfection (Estaller et al., 1991). Unfortunately, no information concerning the yield was given in that study. Based on the intensities of FH-specific immunoblot signals from COS cell supernatants in comparison to signals which had been obtained with FH from 3 µl of

serum as a reference the FH concentration in the COS cell supernatant was calculated as 500-600 ng/ml. This concentration is similar to that of FH(SCR1-7) produced by transfected HEK293 cells (~600ng/ml).

Analyses of the glycosylation patterns of FH(SCR1-20) and recombinant FH variants FH(SCR1-7) and FH(SCR1-4)

Based on the sequence N-Xaa-S/T both recombinant variants were screened for potential N-glycosylation

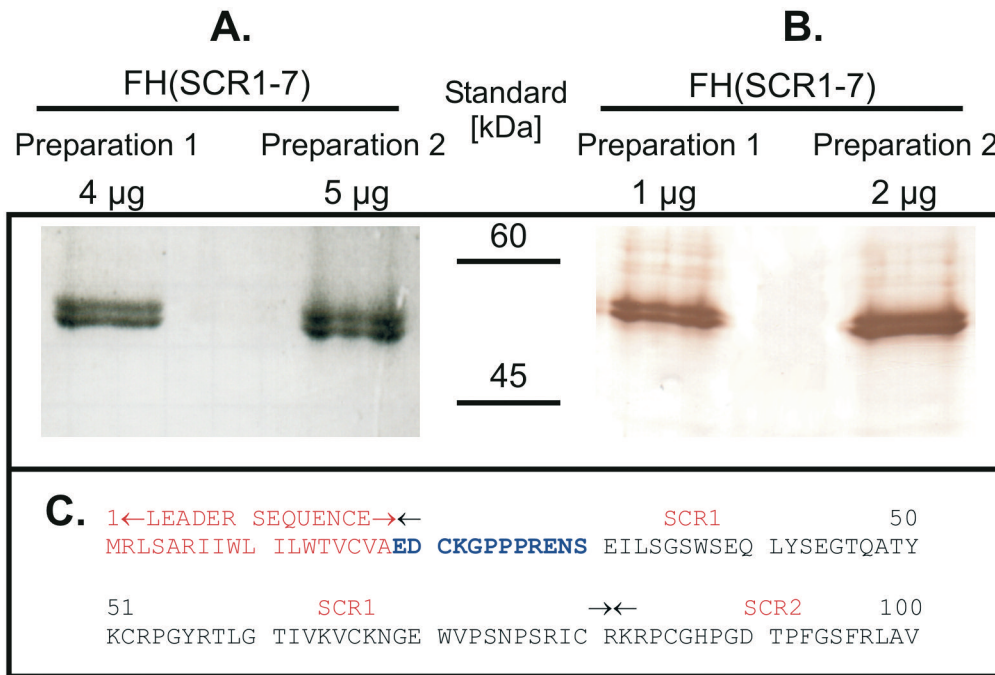


Fig. 4. Coomassie-Blue-staining (A) and corresponding immunoblot analysis (B) of the FH(SCR1-7) preparations which were used for N-terminal peptide sequencing (C) A. 5 µg of FH(SCR1-7) were applied onto SDS-PAGE and stained with Coomassie Blue. B. 1 µg of FH(SCR1-7) was identified in the corresponding immunoblot analysis with peroxidase-conjugated streptactin (IBA limited, Göttingen, Germany). C. The identified amino acid sequence EDCKGPPPRENS (blue bold letters) consists of 12 amino acid residues which follow the Leader sequence (signal peptide). This indicates that after the removal of the Leader sequence (first 18 aa residues in red colour) the FH(SCR1-7) is processed correctly. The same amino acids were identified from both protein bands.

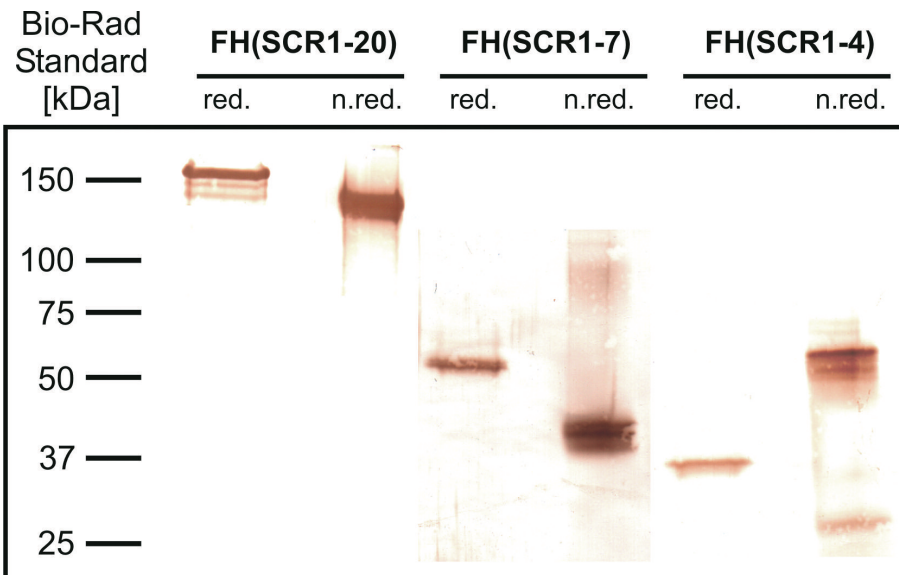


Fig. 5. Comparison of the Mr of FH(SCR1-20), FH(SCR1-7) and FH(SCR1-4) under reducing and non-reducing conditions, respectively, by immunoblot analysis. 1 µg each of FH(SCR1-20), FH(SCR1-7) and FH(SCR1-4) was applied onto a PAG and afterwards transferred onto NC-membrane. Detection was performed using a rabbit anti-rat FH pAb followed by a secondary peroxidase-conjugated anti-rabbit IgG antibody. The Mr of FH(SCR1-20) was 155 kDa under reducing and 125 kDa under non-reducing conditions; the Mr of FH(SCR1-7) was 54 kDa under reducing and 43 kDa under non-reducing conditions; the Mr of FH(SCR1-4) was 36 kDa under reducing and 28 kDa under non-reducing conditions, under which, in addition, the molecule showed a strong tendency to dimerize (Mr of the dimer about 56 kDa). The dimerization was completely inhibited when reducing conditions were chosen for the separation of FH(SCR1-4).

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sites. According to the cDNA-derived rat primary structure no potential N-glycosylation sites were found in the first 8 SCR (Demberg et al., 2002). In order to identify other potential glycosylation patterns such as O-glycosylation, the Periodic Acid Schiff (PAS) staining procedure was chosen since the difference between both bands of FH(SCR1-7) was in the range of 1-2 kDa (Fig. 4) and thus might be caused by differences in the glycosylation pattern. However, in contrast to FH(SCR1-20) both of the recombinant variants FH(SCR1-4) and the double band of FH(SCR1-7) did not show any PAS staining (data not shown). These data suggest that the PAS staining procedure confirmed the prediction of potential glycosylation sites according to the rat-specific cDNA-derived primary structure (Demberg et al., 2002). Horseradish peroxidase with a carbohydrate moiety of 18% and a Mr of 47 kDa served as a control and exhibited a significant PAS staining (data not shown). Therefore, the existence of a carbohydrate portion in both of the recombinant FH fragments (SCR1-4 and SCR1-7) might have no impact on their functional potency. These data are in accordance with other authors who described no functional relevance of the FH glycosylation pattern (Kazatchkine et al., 1979; Sim and DiScipio, 1982; Demberg et al., 2002).

Distinct molecular weights (Mr) of FH variants under reducing and non-reducing conditions

Depending on reducing or non-reducing SDS-PAGE conditions, the Mr of all three variants showed a significant difference. To systematically investigate this issue the three proteins were simultaneously applied onto a 10% SDS-PAGE under reducing or non-reducing conditions and analyzed by immunoblotting (Fig. 5). Under non-reducing conditions FH(SCR1-4) had a Mr of 28 kDa (monomer) and 56 kDa (dimer), whereas under reducing conditions this variant exhibited the predicted Mr of 36 kDa. Similar results were obtained with FH(SCR1-7) and FH(SCR1-20). Under non-reducing conditions FH(SCR1-7) protein had a Mr of 43 kDa, under reducing conditions it showed the predicted Mr of 54 kDa. Furthermore, the FH(SCR1-20) protein had a Mr of 155 kDa only under reducing conditions and a Mr of 125 kDa under non-reducing conditions. Thus, the differences in Mr of all three variants separated under reducing or non-reducing conditions were approximately 20% (Fig. 5).

According to the coefficients of sedimentation and diffusion the Mr of FH(SCR1-20) was calculated to 150 kDa (Whaley and Ruddy, 1976) and to 155 kDa (Sim and DiScipio, 1982), whereas the Mr of the recombinant FH(SCR1-7) consisting of seven SCR is calculated to 52.5 kDa [if FH(SCR1-20)=150 kDa] or 54.3 kDa [if FH(SCR1-20)=155 kDa], respectively. The rat FH(SCR1-7) after its separation in SDS-PAGE under reducing conditions had a Mr of 54 kDa. This suggests that the molecular weight of 43 kDa primarily proposed for the FHL-1 splicing variant (Schwaeble et al., 1987) also consisting of SCR1-7 is a Mr-value due to its

migration only under non-reducing conditions. This hypothesis is also supported by several investigators (Ripoche et al., 1988a; Zipfel and Skerka, 1999), who calculated a molecular weight for FHL-1 of 49 kDa representing 431 amino acid residues after the cleavage of the 18 aa leader sequence. Their calculations were performed for unglycosylated FHL-1 proteins without any glycosylation residues at the potential N-glycosylation site of human SCR4 (Ripoche et al., 1988a). The theoretical molecular weight of the recombinant fragment generated in our group was 51.7 kDa which is in accordance with a Mr of 54 kDa obtained by separation under reducing conditions. Furthermore, the purified human FH(SCR1-20) protein applied onto SDS-PAGE under reducing conditions showed an increased Mr of approximately 20% in comparison to that applied under non-reducing conditions (Sim and DiScipio, 1982). Although this phenomenon was experimentally shown for FH purified from human serum and followed by separation in SDS-PAGE (Sim and DiScipio, 1982) it could not primarily be explained. The specific structure of SCR4 under non-reducing conditions is a result of two disulphide bonds between two pairs of highly conserved cysteine residues (Fig. 6) (Zipfel and Skerka, 1999). This model had originally been developed for the C4b-binding protein that consists of eight SCR (Janatova et al., 1989) and was afterwards assigned to complement factor H or its

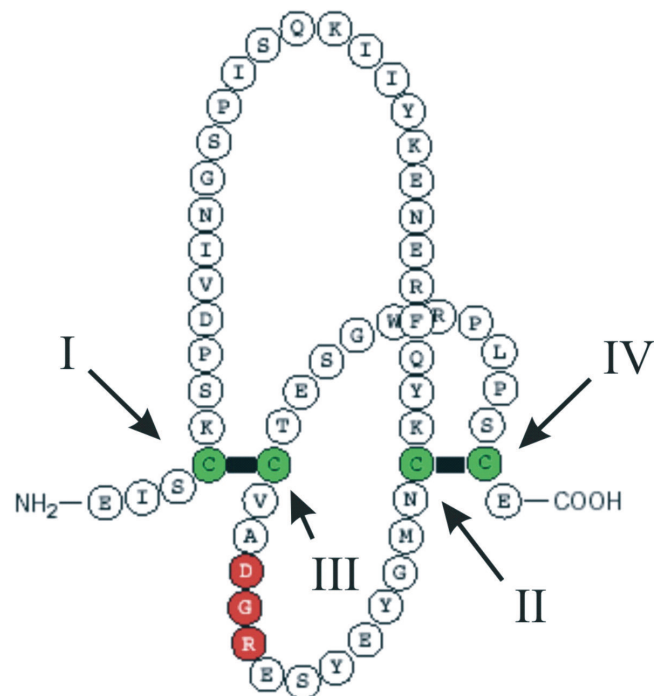


Fig. 6. Model of SCR4 of FH/FHL-1 as developed by Zipfel and Skerka (1999). The size differences in the Mr under reducing and non-reducing conditions are the result of the structure of the SCR (disulphide bonds between the highly conserved cysteine residues I/III and II/IV).

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splicing variant FHL-1 (Zipfel and Skerka, 1999). Under reducing conditions the proteins have a broader three-dimensional conformation causing an increased resistance on their migration behaviour in the SDS-PAGE, whereas under non-reducing conditions the FH(SCR1-20) protein and its recombinant variants exhibit a condensed conformation resulting in 20 % reduced relative molecular weights. The experiments described within this paper support the results of Sim and DiScipio (1982), but are in contrast to the data of Daha and van Es (1982) who detected no difference in the Mr of purified FH under reducing or non-reducing conditions. So far, there exists no explanation for these discrepant results by Daha and van Es (1982).

Functional characterization of FH variants using complement-mediated hemolysis (CH_{50} -assay) assay

Using deletion mutants of FH(SCR1-20) experimental evidence was provided that the cofactor activity for factor I is determined by SCR1-4 (Gordon et al., 1995; Sharma and Pangburn, 1996; Pangburn et al., 2000). In addition, these four SCRs contain the first binding region for the complement component C3b, whereas the remaining SCR5-20 bear two additional C3b-binding domains (SCR8-15 and SCR19-20) (Fig. 7). These additional domains, which lack cofactor activity, bind C3b and therefore prevent complement activation on the surfaces of self cells. Deletions of the SCR1-5, SCR6-10 and SCR16-20 each resulted in an approximately 6-fold reduced binding of C3b (Sharma and Pangburn, 1996) in comparison to FH(SCR1-20) suggesting the existence of three equal binding sites for C3b in FH(SCR1-20).

The inhibitory activity of FH(SCR1-20) appears to be due to its influence on the alternative complement pathway. There it prevents the generation of the active C3 convertase (C3bBb) by binding to the component Bb (Weiler et al., 1976; Whaley and Ruddy, 1976). At the

level of the C5 convertase of the alternative pathway [(C3b)₂Bb] FH acts by its “decay accelerating activity”, i.e. it induces the decay of this complex also by binding to C3b. For the third function i.e. the co-factor activity for the FI-mediated cleavage of C3b, both the alternative and the classical pathways are involved. However, the function of FH to inhibit the generation of the C5 convertase of the classical pathway (C4b2b3b) as well as by its binding to the C3b component was only rarely documented (Liszewski et al., 1996; Giannakis et al., 2001). In contrast, our experiments only demonstrate the effect of FH on the inhibition of the CH_{50} -value of the classical activation pathway, whereas we were not able to establish the CH_{50} -assay of the alternative activation pathway (AP CH_{50} -assay) by using rat serum. An underlying reason for this discrepancy might be the evolutionary distance between rodents and humans. The complement system of rodents but not of humans contains e.g. the complement receptor 1-related gene/protein y (Crry) which has important functions in the regulation of the complement system of rodents (Aegerter-Shaw et al., 1987; Parks et al., 1987). It exhibits many properties in common with the “decay accelerating factor“ (DAF). Both, the Crry as well as the DAF protein is found in mouse and rat in a soluble as well as in a membrane-bound form. Like most complement inhibitors Crry has a modular structure, i.e. in rat it consists of 7 SCR with the first 4 exhibiting a high degree of homology with the first SCR of the human complement receptor 1 (CR1). Investigations of the murine species showed that the DAF protein has a higher potency to inhibit the C3 convertase of the classical (C4b2b) and the MBL pathways (C4b2b), whereas the Crry protein is more potent in inhibiting the C3 convertase of the alternative pathway (C3bBb). These considerations may serve as an explanation for the phenomenon that the AP CH_{50} -assay developed for diagnostics of the human complement system is not necessarily suitable for investigations of the rat

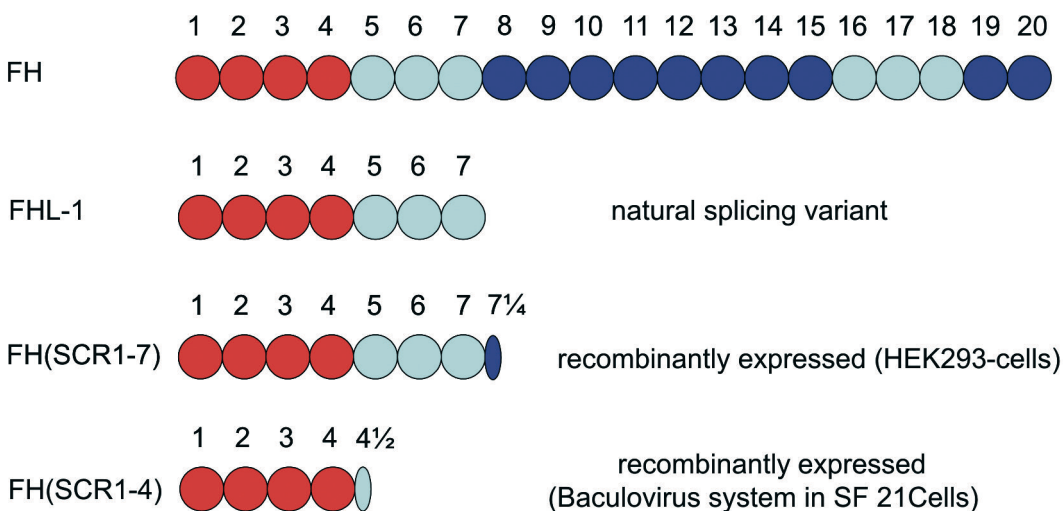


Fig. 7. Sizes and SCR-structures of FH and FHL-1 in comparison with the recombinantly expressed proteins FH(SCR1-7) and FH(SCR1-4). The SCR1-4 (red circles) bear the cofactor activity for factor I (FI), decay-accelerating activity and C3b-binding site I. SCR8-15 (dark blue circles) contain the C3b-binding site II, SCR19-20 (dark blue circles) form the C3b-binding site III.

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complement system.

In order to implement an assay for the functional characterization of recombinant FH-fragments as well as of FH(SCR1-20) from rat serum we consequently used only the classical pathway of complement activation. In a first approach five sera from rats were pooled and titrated to reach their half-maximal lysis value (CH_{50} -value) in the CH_{50} -assay. The corresponding volume of rat serum was used in all further experiments to adjust the initial basic CH_{50} -value [relative degree of hemolysis (OD405/650)] which had to be reduced by adding increasing amounts of FH or its truncated variants. The calculated FH concentration in the pooled sera of rats was approximately 700 $\mu\text{g}/\text{ml}$ as determined by an established ELISA (Demberg et al., 2002). As demonstrated in Figure 8A, the CH_{50} -value (OD 405/650) in the absence of FH(SCR1-20) was 0.15. A 50% inhibition was achieved after the addition of 1.7 μg FH(SCR1-20), a 70% inhibition after the addition of 3.5

μg FH(SCR1-20). Employing $\geq 8 \mu\text{g}$ FH(SCR1-20) the relative hemolysis was 90% reduced in comparison to hemolysis without inhibition which could not be diminished further. Similarly, the recombinant FH (SCR1-7 and SCR1-4) fragments were applied to this assay. A 50% inhibition was achieved after the addition of 2 μg FH(SCR1-7) which was further inhibited (70%) using 4.5 μg FH(SCR1-7) (Fig. 8B). The amounts of FH(SCR1-4) to achieve corresponding inhibition values were comparable (inhibition of 50%: 1.7 μg / inhibition of 70%: 4.5 μg) (Fig. 8C). As summarized in Table 1, the absolute amounts of the recombinant FH(SCR1-7) and (SCR1-4) fragments and of FH(SCR1-20) from serum and their corresponding inhibition values were similar and their functional activity comparable. On average, a 50% inhibition was obtained after the addition of 1.8 μg , and complete inhibition by 8.5 μg , respectively. Since three C3b-binding sites on FH(SCR1-20) were proposed to simultaneously bind

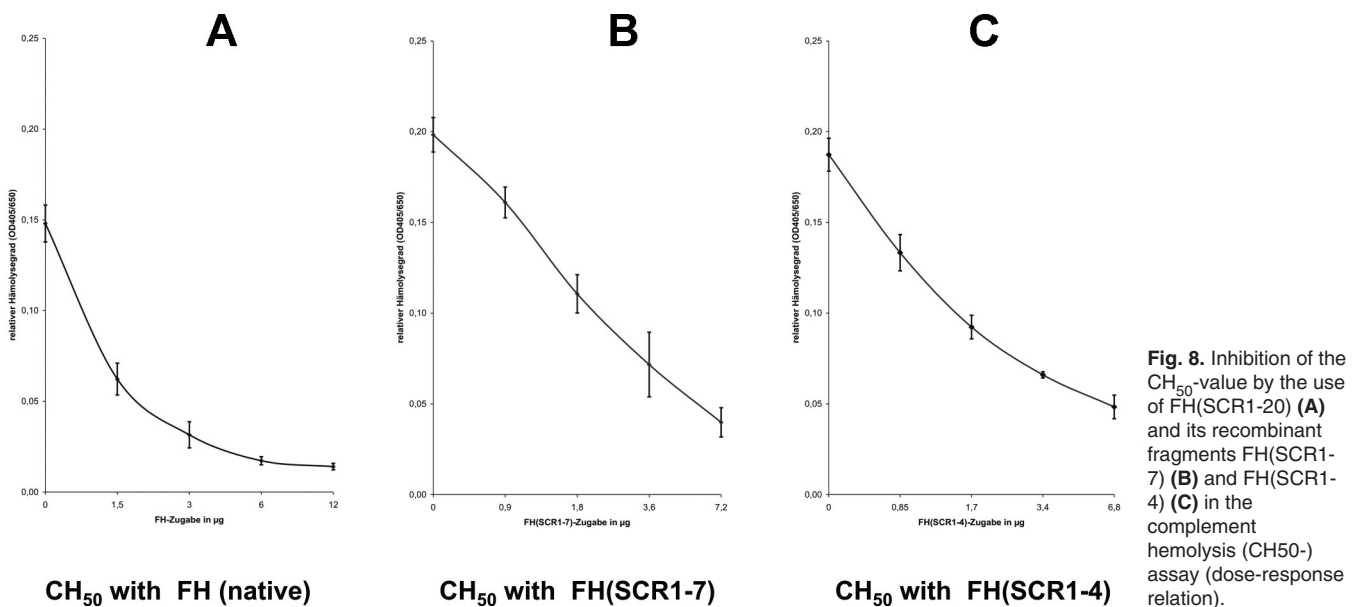


Fig. 8. Inhibition of the CH_{50} -value by the use of FH(SCR1-20) (A) and its recombinant fragments FH(SCR1-7) (B) and FH(SCR1-4) (C) in the complement hemolysis (CH_{50} -) assay (dose-response relation).

Table 1. Addition of FH(SCR1-20) from serum or its recombinant variants FH(SCR1-7/SCR1-4) [absolute amounts/corresponding pmol] which result in a 50% or a maximal inhibition of the CH_{50} -value.

VARIANT OF FH	ADDITION OF VARIANT (abs and pmol) FOR INHIBITION OF 50%	MOLAR INCREASE (BY FACTOR)*	ADDITION OF VARIANT (abs. and pmol) FOR MAXIMAL INHIBITION	MOLAR INCREASE (BY FACTOR)*
FH (SCR1-20) from serum	1.7 μg /11 pmol	2.4 x	8 μg /51.7 pmol	11.5 x
FH (SCR1-7) from HEK-293 cells	2 μg /35.7 pmol	7.9 x	9.5 μg /170 pmol ^a	37.8 x
FH (SCR1-4) from SF21 cells	1.7 μg /48 pmol	10.6x	8 μg /228 pmol ^a	50.7 x

*: with reference to a basal FH-concentration of 700 $\mu\text{g}/\text{ml}$ in the pooled rat serum samples [i.e. 700 ng (4.5 pmol) were initially used for the assay].

^a: extrapolated value

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C3b (Sharma and Pangburn, 1996; Pangburn et al., 2000) the absolute amounts of FH and of its fragments were additionally correlated in a stoichiometric way by calculating the corresponding molar amounts. Due to the fact that the Mr of the recombinant variants FH(SCR1-7) and FH(SCR1-4) comprise only about 1/3rd or 1/5th of the Mr of FH(SCR1-20), respectively, the molar amounts of FH(SCR1-20) and its variants required for 50% or complete suppression were calculated (Table 1). 35.7 pmol of FH(SCR1-7) and 48 pmol FH(SCR1-4) were needed for 50% inhibition, whereas 170 pmol of FH(SCR1-7) and 228 pmol of FH(SCR1-4) were required for a complete functional inhibition. In contrast, only 11 pmol or 51.7 pmol of FH(SCR1-20) were employed to cause 50% or complete inhibition in the CH₅₀-assay, respectively. Table 2 demonstrates the quotients of the respective fragments [i.e. FH(SCR1-4) and FH(SCR1-7)] and of FH(1-20) for either a 50% or a complete inhibition of the complement hemolysis assay with a mean value of the four individual quotients of 3.8. This value represents a more than three-fold enriched molar activity of FH(SCR1-20) comprising three C3b binding sites in comparison to FH(SCR1-4) or FH(SCR1-7) which bear only one binding site for this complement component. These functional data are in accordance with former results proposing an FH molecule with three independent sites for the binding of the component C3b, the first located in the SCR1-4, the second in the SCR8-15 and the third in SCR19-20 (Fig. 7) (Sharma and Pangburn, 1996; Pangburn et al., 2000).

The generation of recombinant FH which can be used to down-regulate the complement system is generally possible. The activities of the recombinantly expressed FH fragments were demonstrated using the complement hemolysis assay (CH₅₀-assay) which simulates the lytic activity of this system for pathogens by the lysis of erythrocytes i.e. by the measurement of released hemoglobin (OD 405/650). However, the molar increases of FH or their recombinant variants clearly show that high amounts of FH or its fragments are required to completely inhibit the complement system. As shown in Table 1 between 8 µg [FH(SCR1-20) or FH(SCR1-4)] and 9.5 µg [FH(SCR1-7)] had to be added

to the basal amount of 700 ng contained in 10 µl of rat serum (after a 1:10 predilution) which had been applied to the assay to adjust the initial CH₅₀-value. This is an approximately 11-fold increase in the concentrations of all three variants, but a 11.5-fold molar increase in FH(SCR1-20), a 37.8-fold molar increase in FH(SCR1-7) and a 50.7-fold molar increase in FH(SCR1-4) for the maximal (i.e. 90%) inhibition of the CH₅₀ value (Table 1). Whether the molar increases or the increases in the concentrations of FH or its recombinant variants for suppression of the complement system are considered, a large scale expression of FH or its recombinant fragments is required referring to the high basal concentration of FH in serum of man which has been determined to range between 300 µg/ml (Sim and DiScipio, 1982) and 600 µg/ml (Weiler et al., 1976) up to 800 µg/ml (Charlesworth et al., 1979). Similar concentrations have been determined in rat serum (Demberg et al., 2002). In accordance with those concentrations the FH concentration in the sample of pooled rat sera used for the complement inhibition assay was 700 µg/ml. Provided that the dose-response relation of the CH₅₀-*in vitro* assays can be assigned to *in vivo* experiments of complement inhibition, a 10-fold increase in the concentration of FH to completely inhibit the complement system in rat would require a total amount of 30 mg FH applied to an animal with 200 g of weight, a serum volume of 6 ml and an FH concentration of about 500 µg/ml. As a consequence of these calculations a strategy was developed to implement the complement inhibitory factors H and I in combination in order to obtain potential synergistic effects of both components which had been described formerly (DiScipio, 1992). Experiments in which the initial CH₅₀-value was at first decreased by 50% using 1.6 µg of FH(SCR1-20) and followed by the addition of increasing FI amounts purified by immunoaffinity chromatography did not exhibit significant differences in comparison to experiments using FH alone to completely decrease the CH₅₀-value to the basal levels (data not shown). The corresponding experiments employing 2 µg FI for reducing the value by 50% followed by the addition of FH(SCR1-20) for a reduction to the basal value did not show significant differences in comparison with the inhibition of the CH₅₀-value achieved only by FI (data not shown). Since the amounts of both factors used to further inhibit the lysis value (OD405/650) were comparable in both experiments, a lack of synergism of both factors was suggested by the experiments which contradicted our primary assumption. In addition, the hypothesis that inhibitory FI found in serum at a ten-fold lower concentration than FH might have a stronger effect with minor increases in its concentration was not supported by our experiments. Investigations in which only FI was used to inhibit the CH₅₀-value lead to a 50% inhibition of the CH₅₀-value by a 66-fold increase in the concentration of FI, whereas a reduction to the basal value was achieved with a 250-fold increase in the concentration of FI [with a presumed FI-concentration of 40 µg/ml in rat serum] (data not shown). Thus, there

Table 2. Molar quotients [FH(SCR1-7)/FH(SCR1-20)] and [FH(SCR1-4)/FH(SCR1-20)] of a CH₅₀-value-inhibition of 50% or an 90% inhibition to the basal value (maximal inhibition).

	50% INHIBITION	MAXIMAL INHIBITION (basal value)
Quotient pmol FH(SCR1-7)/ pmol FH(SCR1-20)	3.2 (35.7/11)	3.3 (170/51.7)
Quotient pmol FH(SCR1-4)/ pmol FH(SCR1-20)	4.3 (48/11)	4.4 (228/51.7)

average quotient 3.8

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exist hardly any differences whether isolated FI, FH or its recombinant fragments are implemented for the inhibition of the complement system. However, the purification of FH to obtain the required amounts from rat serum is easier to assess than that of FI due to its 10-fold higher concentration. Although the molar activity of FH(SCR1-4) derived from SF 21 cells (Baculovirus system) is lower than that of FH(SCR1-7) from HEK 293 cells (Table 2) the recombinant expression of the FH variant SCR1-4 using the Baculovirus system lead to a higher yield in comparison to the production in HEK293-cells. So far, many attempts and different strategies of others and our group to recombinantly express FI were unsuccessful. We used hepatoma cell lines for our approaches since hepatocytes are the main source of FI (Schlaf et al., 1999, 2001) and the Paired Amino Acid Cleaving Enzyme (PACE) expressed in these cells is required to correctly process the serine protease FI by cleaving the linker peptide RRKR (Goldberger et al., 1987; Minta et al., 1996; Schlaf et al., 1999). This cleavage forms the active FI consisting of two chains (Goldberger et al., 1984). Thus, for the reasons mentioned above there exists no advantage to use a combination of FH and FI for inhibition of the complement system. However, further work is required to upscale the production of recombinant FH fragments in order to yield them in sufficient amounts for *in vivo* studies.

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