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Identification of the Second Heparin-Binding Domain in Human Complement Factor H¹

Timothy K. Blackmore,²* Jens Hellwage,† Tania A. Sadlon,* Naomi Higgs,* Peter F. Zipfel,† Helena M. Ward,* and David L. Gordon*¹

Complement factor H (fH) regulates activation of the alternative pathway of C, reducing the amount of C3b deposited on sialic acid-rich surfaces. Heparin binding has been used as a model for examining the sialic acid-binding characteristics of fH. We have previously shown that of the 20 short consensus repeat (SCR) modules of fH, SCR 7 contains an important heparin binding site, but other SCRs also play a role in heparin binding. To localize the other sites, we prepared recombinant truncated and SCR deletion mutants of fH and tested them by heparin-agarose affinity chromatography. The 5 C-terminal SCRs were found to contain a heparin binding site as an SCR 7 deletion mutant of the N terminal 15 SCRs did not bind heparin, but a construct consisting of SCRs 16–20 was shown to bind heparin. Double deletion of SCRs 7 and 20 from fH abrogated binding to heparin, indicating that SCR 20 contains a heparin binding site. This finding was confirmed with the observation that attachment of SCR 20 to a group of nonbinding SCRs produced a heparin-binding protein. A protein consisting of SCRs 19 and 20 did not bind heparin, whereas SCRs 18–20 did, indicating that, although SCR 20 contains a heparin binding site, at least two nonspecific adjacent SCRs are required. fH-related protein-3 (FHR-3) possesses an SCR homologous to SCR 7 of fH and bound heparin, whereas FHR-4, which lacks such an SCR, did not. Thus, fH contains two separate heparin binding sites, which are located in SCRs 7 and 20. The Journal of Immunology, 1998, 160: 3342–3348.

¹Department of Microbiology and Infectious Diseases, Flinders Medical Centre, Bedford Park, South Australia, Australia; and ²Department of Molecular Biology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany.

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² Address correspondence and reprint requests to Dr. Timothy K. Blackmore, at his current address, The Department of Microbiology and Infectious Diseases, Flinders Medical Centre, Bedford Park, South Australia 5042. E-mail address: tim.blackmore@flinders.edu.au

³ Abbreviations used in this paper: fH, factor H; CHO, Chinese hamster ovary; FHR-3 and -4, factor H-related proteins 3 and 4; H20, recombinant factor H; HT, N-terminal tryptic fragment of fH; RCA, regulators of complement activation; SCR, short consensus repeat.

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of great interest, as they differ in their possession of an SCR similar to SCR 7 of fH, yet both contain SCRs similar to fH SCRs 19 and 20. SCRs similar to SCRs 19 and 20 of fH are present in all of the FHR proteins, and it has been postulated that these SCRs may mediate important biologic functions (20).

In this paper, we identify the second heparin binding site in fH, using truncation and deletion mutants of fH, and determine the heparin-binding characteristics of recombinant FHR proteins. Attachment of SCR 20 to the first five SCRs of fH converted it from a nontobinding to a heparin-binding protein. The heparin-binding characteristics of FHR-3 and -4 proteins correlate with the possession of an SCR similar to SCR 7 of fH.

Materials and Methods

DNA constructs
cDNA encoding SCRs 1 through 15 (H15) and an SCR 7 deletion of full-length fH (H20Δ7) were prepared and cloned into the BSRaEn eukaryotic expression vector as previously described (18). XhoI and XbaI restriction sites were incorporated at the 5’ and 3’ termini, respectively.

C-terminal truncated proteins from which SCR 7 had been deleted were prepared utilizing a unique PpuMI site at nucleotide 2072 of the fH sequence. Digesting BSRaEn-H20Δ7 with PpuMI and XhoI released a fragment corresponding to SCRs 11–20. When this was replaced with truncated cDNA encoding SCRs 11–15, 11–18, or 11–19, cDNA encoding H15Δ7, H18Δ7 or H19Δ7, respectively, was produced. cDNA encoding SCRs 11–15 originated from BSRaEn-H15 digested with PpuMI and XhoI. cDNA encoding SCRs 11–18 and 11–19 was prepared by PCR using Vent polymerase (New England Biolabs, Beverly, MA) from the BSRaEn-H20 template. The forward primer was designed to anneal to nucleotides 2021–2040 in SCR 11, 5’ to the PpuMI site. Reverse primers annealed to the 3’ termini of SCRs 18 and 19 and were selected on the basis of the SCR junction analysis by Zipfel and Skerka (20). These primers also incorporated stop and XbaI sites. After digestion with PpuMI and XhoI, the PCR products were then cloned into BSRaEn-H20Δ7.

A mutant fH protein consisting of SCRs 16–20 (H16–20) was prepared by ligating cDNA encoding the fH signal peptide via a MluI restriction site. This was achieved by incorporating an XhoI site into the fH signal forward primer, a MluI site into the fH signal reverse primer, and an XbaI site in the PCR primers used to amplify full-length fH, containing XhoI and XbaI restriction sites (18). However, an SCR 7 deletion mutant of fH (H20Δ7) was prepared utilizing a unique PpuMI site at nucleotide 2072 of the fH sequence. Digesting BSRaEn-H20Δ7 with PpuMI and XhoI released a fragment corresponding to SCRs 11–20. When this was replaced with truncated cDNA encoding SCRs 11–15, 11–18, or 11–19, cDNA encoding H15Δ7, H18Δ7 or H19Δ7, respectively, was produced. cDNA encoding SCRs 11–15 originated from BSRaEn-H15 digested with PpuMI and XhoI. cDNA encoding SCRs 11–18 and 11–19 was prepared by PCR using Vent polymerase (New England Biolabs, Beverly, MA) from the BSRaEn-H20 template. The forward primer was designed to anneal to nucleotides 2021–2040 in SCR 11, 5’ to the PpuMI site. Reverse primers annealed to the 3’ termini of SCRs 18 and 19 and were selected on the basis of the SCR junction analysis by Zipfel and Skerka (20). These primers also incorporated stop and XbaI sites. After digestion with PpuMI and XhoI, the PCR products were then cloned into BSRaEn-H20Δ7.

An enterokinase site incorporated immediately N terminal to these His residues allowed their removal with EnterokinaseMax (Invitrogen, San Diego, CA). The His residues were removed because it was found that His tagging weakly affected heparin-binding characteristics (data not shown).

Western blotting

The correct identity of expressed proteins was confirmed by SDS-PAGE and Western blotting. Proteins were electrophoresed under nonreducing conditions and transferred to nitrocellulose. Polyclonal goat Ab to human fH (Calbiochem, San Diego, CA) followed by donkey anti-goat Ab conjugated to horseradish peroxidase (Silenus, Hawthorn, Australia) was used to detect most proteins. FHR-3 and FHR-4 were detected by polyclonal Ab produced in rabbits, followed by sheep anti-rabbit Ab conjugated to horseradish peroxidase (Silenus). Proteins were detected with the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, U.K.). All proteins migrated on SDS-PAGE according to their predicted m.w. and degree of glycosylation.

Heparin-agarose affinity chromatography

All heparin-binding experiments used 50 mM phosphate buffer, pH 7.4, for dialysis or dilution of samples and equilibration and washing of the columns (27). Ten to twenty milliliters of transfected CHO cell culture supernatant or 5 to 10 µg of diluted baculovirus-expressed protein was applied to 1 × 1 cm heparin-agarose columns (Pierce, Rockford, IL). The sample was passed over the column at least five times and a sample of the fall-through collected. The column was washed with >40 ml of buffer, and 2.5-ml fractions were collected for testing. Bound protein was then eluted by a linear salt gradient to 300 mM sodium chloride-phosphate-buffered, and 4-ml fractions were collected. An automated fraction collector and a flow rate of 1 ml/min was used for all experiments. Conductivity was measured at 24°C, using a portable meter (Actevon model 301, Sydney, Australia), and a consistent salt gradient was obtained for all experiments. Columns were reconstituted by washing afterward with 1 M NaCl, then stored in 50 mM phosphate buffer with 0.02 M sodium azide.

All experiments were performed at least three times, and reproducible results were obtained in all cases.

Results

Expression and binding of H20Δ7 and H15Δ7

SCR 7 was shown in a previous report to contain a heparin binding site (18). However, an SCR 7 deletion mutant of fH (H20Δ7) still bound to heparin and eluted in a linear salt gradient at approximately 150 mM (Fig. 1). As H15 also binds to heparin (18), we first prepared a deletion of SCR 7 from H15 (H15Δ7) to assess whether a second heparin binding site existed in SCRs 1–15.
H15Δ7 expressed well and migrated slightly faster on SDS-PAGE than H15 did (data not shown). H15Δ7 did not bind heparin, with a similar amount of protein seen in the start and fall-through fractions. Moreover, the small amount of protein remaining associated with the column was washed off in 50 mM of phosphate equilibration buffer. No protein eluted from the column in the linear salt gradient. Figure 2 shows the heparin-binding and elution profiles of H15 and H15Δ7, demonstrating the clear difference in behavior of the two proteins. These results show that SCR 7 contains the only heparin binding site within SCRs 1–15.

Binding of H16–20 to heparin

The binding of H20Δ7 to heparin and the lack of binding of H15Δ7 together indicate that a second heparin binding site is located in SCRs 16–20 of human fH. To investigate this possibility, we prepared a construct in which the fH signal sequence was attached to cDNA encoding SCRs 16–20. An ~40-kDa protein was identified by Western blot in supernatants of transfected CHO cells. Heparin binding analysis showed that H16–20 bound to immobilized heparin and was not washed off in 50 mM phosphate buffer. H16–20 eluted in a lower concentration of salt than H15Δ7 (Fig. 3). These results indicate that a second heparin binding site resides within SCRs 16–20, and that this site may be of lower affinity than that contained in SCR 7.

Localization of the second heparin binding site in fH

To further localize the second heparin binding site, we next produced SCR 7 deletion mutant proteins of SCRs 1–18 and 1–19 (H18Δ7 and H19Δ7).

Expression of the proteins was determined by Western blot with both proteins migrating on SDS-PAGE at their predicted m.w. In contrast to H20Δ7, neither H18Δ7 nor H19Δ7 demonstrated significant binding to heparin. A small amount of protein associated with the heparin-agarose affinity column, but was washed off in 50 mM of phosphate buffer (Fig. 4).

These results suggest that SCR 20 is the essential component of the second heparin binding site of human fH. To confirm this opinion, SCR 20 was attached to the N-terminal five SCRs of fH (H5 + 20) and its heparin-binding characteristics examined. The five N-terminal SCRs of fH do not contain a heparin binding site (18).
The addition of SCR 20 to SCRs 1–5 converted a non-heparin-binding protein to a heparin-binding protein, clearly demonstrating that the second heparin binding site is located in SCR 20 (Fig. 5).

**Assessment of the role of adjacent SCRs**

The above results indicate that SCR 20 is an essential component of the second heparin binding site, but do not address whether SCR 20 alone is sufficient for heparin binding. Recombinant proteins consisting of SCRs 19 and 20 (H19–20) and SCRs 18–20 (H18–20) were prepared. H19–20 did not bind heparin, because protein was present in the fall-through and wash fractions and none was eluted (Fig. 6). In contrast, H18–20 was not detected in the fall-through or wash fractions, and bound protein eluted in the salt gradient (Fig. 6). Similar results were found for a protein containing SCRs 17–20 (data not shown). Therefore, while SCR 20 is an essential component of the second heparin binding site, at least two adjacent SCRs are required. The adjacent SCRs may be nonspecific, since H5 + 20 also bound to heparin.

**Analysis of heparin binding by FHR-3 and FHR-4**

Human FHR-3 and -4 are both composed of five SCRs, with each SCR similar to certain SCRs of fH. SCRs 1–3 of FHR-3 and FHR-4 share partial identity with fH SCRs 6, 7, and 8 and SCRs 6, 8, and 9, respectively. SCRs 4 and 5 of both FHR-3 and FHR-4 are almost identical to each other and have partial similarity with SCRs 19 and 20 of fH (20, 24). Therefore, we used these proteins as “natural mutants” of fH and examined their heparin-binding characteristics. These results were then correlated with the amino acid sequences of SCRs 7 and 20 in an attempt to identify potential heparin binding sites. FHR-3 was expected to bind heparin because its third SCR is so similar to SCR 7 of fH. Binding of FHR-4 to heparin would most likely depend on whether there is sufficient similarity between SCR 5 of FHR-4 and SCR 20 of fH to preserve the heparin binding site.

The heparin-binding characteristics of FHR-3 and FHR-4 are shown in Figure 7. As expected, FHR-3 binds to heparin. FHR-4 does not bind, indicating that its SCR 5 is not sufficiently similar to SCR 20 of fH to result in heparin binding.

**Summary of recombinant proteins and their heparin-binding characteristics**

A schematic representation of the proteins produced and analyzed in this study is shown in Figure 8. SCRs are numbered in the figure on the basis of similarity to native human fH. The heparin-binding properties of each construct are also shown.

**Discussion**

The heparin-binding characteristics of fH are of great interest because of the central role that fH plays in controlling the alternative pathway (28) and the influence that polyanions have on fH binding to surfaces (16). Our previously reported experiments demonstrated the presence of a heparin binding site in SCR 7 of human fH, but it was apparent that at least one other site was present in SCRs 10–20 (18). The purpose of the present study was to determine the location of the additional site(s). Recombinant DNA techniques allow construction of truncated proteins and deletion mutants and have been used by several groups to examine the functional domains of fH (8–11, 18, 29) and other SCR containing proteins (30–32). These techniques were used, therefore, to determine the location of the second heparin binding site in human fH.

A series of recombinant mutant proteins was initially expressed in transfected CHO cells. Recombinant fH (H20) produced in this system has full cofactor activity and migrates on SDS-PAGE in a fashion identical to the native protein (18). This suggests that transfected CHO cells produce correctly glycosylated recombinant proteins.

Deleting SCR 7 from H15 (H15Δ7) resulted in the loss of heparin binding, while H20Δ7 retained its binding to heparin (Figs. 1 and 2). These findings identified a second heparin-binding domain and localized it to SCRs 16–20. H16–20 was produced by attaching the fH signal sequence to cDNA encoding SCRs 16–20 in the

![Figure 5](image_url)
expression vector BSRαEN. This approach has been used previously in our laboratory to produce recombinant N-terminal truncations of fH (8), domains of the HIV gp120 glycoprotein, and recombinant rubella virus proteins (unpublished data). H16–20 bound heparin, but eluted earlier in a salt gradient (Fig. 3). Therefore, it is possible that the binding site in this region is of lower affinity.

We next prepared truncations of the C-terminal SCRs from H20D7 to determine the location of the second heparin binding site. In contrast to H20Δ7, neither H18Δ7 nor H19Δ7 bound to heparin (Fig. 4). These findings indicate that SCR 20 contains an essential component of the second heparin binding site of fH. To confirm that it contains a heparin binding site, SCR 20 was attached to a non-heparin-binding protein. The addition of SCR 20 to the N-terminal five SCRs (H5+20) generated a heparin-binding protein (Fig. 5), conclusively demonstrating that SCR 20 contains a heparin binding site.

These results indicate that SCR 20 is an essential part of the second heparin binding site, but the role of adjacent SCRs remained to be determined. Therefore, the heparin-binding characteristics of SCRs 19 and 20 construct (H19–20) and SCRs 18–20 (H18–20) were examined (Fig. 6). H19–20 did not bind heparin, whereas H18–20 did. Therefore, adjacent SCRs in spacer or structural roles are required for heparin binding. SCR 20 will mediate heparin binding even when attached to nonspecific SCRs, as shown by the binding of H5 + 20. This indicates that the second heparin binding site consists of SCR 20 and at least another two nonspecific SCRs. Three to four SCRs are also necessary for maximal C3 regulatory activity of RCA proteins including CR1 (31) and fH (8, 9). Within the three- to four-SCR unit, one to two SCRs are essential, with the flanking SCRs required for full functional activity.

We took advantage of the similarity of the FHR proteins FHR-3 and FHR-4, both of which were cloned and expressed in a baculovirus system. FHR-3 and FHR-4 contain SCRs similar to particular SCRs of fH. They differ in that only FHR-3 contains an SCR similar to SCR 7 of fH; SCR 2 of FHR-3 has 88% amino acid identity to SCR 7 of fH. SCRs 4 and 5 of FHR-3 are almost identical to the comparable SCRs of FHR-4, with a total of only four amino acids different between the four SCRs (24). Therefore, finding that FHR-3 binds to heparin and that FHR-4 does not (Fig. 7) suggests that SCR 2 of FHR-3 is likely to be responsible for the binding of FHR-3. The highly conserved amino acid sequences of SCR 7 of human fH and SCR 3 of human FHR-3 make it difficult to identify putative heparin-binding domains within these SCRs. Using two-dimensional nuclear magnetic resonance (2D NMR), a “hypervariable loop” within each SCR was postulated to be the
region responsible for specific ligand binding (4, 33–35). The hypervariable loop of SCR 7 of fH consists of the sequence (H/Y)GRK (the first amino acid is the site of H/Y polymorphism) (36, 37) with YGRK in SCR 3 of human FHR-3 (20). Despite the fact that this site contains two or three basic amino acids, it would be surprising if it alone is responsible for heparin binding, because in other proteins the heparin-binding domain may be spread over a discontinuous sequence of many amino acids (38). It would also be interesting to examine whether a heparin-binding domain exists in SCR 7 of murine and bovine fH, as each shares only 57% identity with the human counterpart. It may then be possible to identify likely amino acid sequences responsible for heparin binding and to determine the functional significance of the hypervariable loop of SCR 7. The removal of SCRs 7 and 20 from fH completely abrogates its capacity to bind heparin, which rules out a significant contribution from SCR 13 in our assay system. It is surprising that no heparin-binding activity was observed for SCR 13 of fH, because based on the model of Barlow et al. (3), the hypervariable loop of SCR 13 contains many basic amino acids (HLKNNKKEF). This suggests that the hypervariable loop model may not be applicable to every SCR.

As demonstrated by the lack of binding of FHR-4, the heparin binding site in SCR 20 of fH is not conserved in SCR 5 of FHR-4. The amino acid sequence of SCR 20 of fH (37) was analyzed for clusters of basic residues not present in SCR 5 of FHR-4 (24). No such areas were apparent, making it difficult to identify putative heparin binding sites within SCR 20 of fH.

We plan to use H19Δ7 to establish the role that fH plays in regulating C activation by sialic acid-rich bacteria and cells. Important pathogens that may utilize fH binding as a way of avoiding alternative pathway activation include Streptococcus agalactiae (39, 40), Neisseria meningitidis, Neisseria gonorhoeae (41–44), and Escherichia coli K1 (45). By comparing the protective effects against C deposition of H19Δ7 and H20, it will be possible to test the hypothesis that the heparin/sialic acid-binding capacity of fH is essential to self/nonself recognition by the alternative pathway. C activation is controlled on host cells to which fH is bound (17), and the degree of fH binding to cell surfaces is related to the amount of membrane-associated sialic acid (12–14). Sheep erythrocytes, which are rich in sialic acid, may be converted by neuraminidase treatment from nonactivators to activators of the alternative pathway, accompanied by a reduction in fH binding (15). Therefore, it would be expected that H19Δ7 added to an alternative pathway lacking fH would not protect sheep erythrocytes from C-mediated lysis. Such an approach would examine the possibility that other regulators, such as those found on sheep erythrocytes (46), are also playing an important C-regulatory role.

In summary, we have identified the second heparin binding site of human fH and produced a mutant fH containing all SCRs except SCR 7 added to an alternative pathway by b1H for cell-bound C3b. J. Immunol. 122:75.


