The C-terminus of factor H: monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins

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We have generated monoclonal antibodies (mAbs) specific for the C-terminus of factor H that can be used as inhibitory antibodies for heparin binding and for the specific detection of factor H and factor H-related proteins (FHRs) in plasma and triacylglycerol-rich lipoproteins. Four distinct mAbs were established: IXF9 (IgG1), VD3 (IgG2a), VIG8 (IgG1) and IIC5 (IgG1). Each reacts specifically with FHR-1 and factor H (and also with FHR-2 in the case of VIG8), but none binds to the related FHR-3 and FHR-4 proteins nor to factor H-like protein 1. By the use of deletion mutants of factor H and by comparing the reactivity with FHR-1 and FHR-2, the binding epitopes of the mAbs were identified and localized to different short consensus repeats (SCRs): mAbs IXF9 and VD3 bind to related or even identical sites within SCR 18 (factor H) and SCR 3 (FHR-1) respectively. mAbs VIG8 and IIC5 bind to different epitopes located within SCRs 19 to 20 of factor H and SCRs 4 to 5 of FHR-1 respectively. Only mAb VIG8 reacts with the corresponding SCRs 3 to 4 of FHR-2. These antibodies are useful for the detection of the corresponding proteins in biological specimens such as fractions of lipoproteins. In addition, mAb VIG8 has the unique feature of inhibiting binding of factor H to heparin. Given the recent identification of a heparin- and a C3b-binding domain within the C-terminus of factor H, these mAbs should provide useful tools for functional analysis and for the precise localization of the domain(s) required for this interaction.

INTRODUCTION

Complement factor H (fH), the four factor H-related proteins (FHR-1 to FHR-4) and factor H-like protein 1 (FHL-1) comprise a family of structurally and immunologically related proteins [1]. The plasma forms of these proteins are exclusively composed of individually folding protein domains termed short consensus repeat (SCR) elements or complement control protein modules [2,3]. SCR elements represent a common structural motif of about 60 amino acids and are found in several complement regulatory proteins that interact with and regulate C3b and C4b [4].

Among the members of the H protein family, complement regulatory functions have been described for fH [5] and FHL-1 [6,7], but not for any of the FHR proteins. fH and FHL-1 act as cofactor for factor-I-mediated degradation of C3b, and both proteins have decay-accelerating activity [6–9]. In addition, both proteins possess heparin-binding domains, and, for fH, additional binding sites for C3b and heparin are reported to be located at the C-terminus of the protein [10–13]. The potential of fH to bind to the polyanion heparin is thought to reflect the affinity of fH for negatively charged surface constituents such as sialic acid residues which determine the alternative-pathway-activating potential of a given particle or cell.

All members of the H gene family are composed of different numbers of SCR domains. fH has 20 SCRs [14], FHL-1 comprises seven SCRs, and the FHR proteins are either represented by four (FHR-2) or five SCRs (FHR-1, FHR-3 and FHR-4) [15–20]. It has been shown that the four N-terminal SCRs of fH and the FHL-1 protein are sufficient and essential for both cofactor and decay-accelerating activity [6,7,21]. A heparin-binding domain that is present in both fH and FHL-1 protein has been identified in SCR 7 [11]. In addition, two other heparin/glycosaminoglycan-binding sites have been mapped to SCR 13 and SCR 20 of fH [10,12]. Deletion studies have identified three unique C3b-binding sites within the 20 SCRs and these sites have been localized to SCR 1–4, SCR 6–10 and SCR 16–20 [7,13,21]. These functional analyses demonstrate that the C-terminal domain of fH has biological functions.

The identification of independent functional domains and their localization underlines the fact that fH and other members of this family represent multidomain proteins. Monoclonal antibodies (mAbs) with well-characterized binding epitopes are pivotal for the identification of functional protein domains and have advanced the understanding of the recognition mechanism of the alternative complement pathway [22]. The majority of mAbs that react with fH recognize epitopes located in the N-terminal domain of the protein [22–25]. However, such mAbs are of limited use for discrimination or specific identification of the various FHR proteins, as these proteins show extensive similarity to the C-terminus of fH. The C-terminus of these proteins may also play a role in the reported association of FHR-1 and FHR-2 with high-density lipoprotein (HDL) particles [26]. The two proteins were shown to be major components of a complex of phospholipids and proteins that facilitate the adhesive response of neutrophils to lipopolysaccharide (LPS). Additional components of this complex are apolipoprotein A-I, LPS-binding protein and other yet to be identified molecules. A similar

Abbreviations used: fH, factor H; FHL-1, factor H-like protein-1; FHR, factor H-related protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPS, lipopolysaccharide; mAb, monoclonal antibody; SCR, short consensus repeat; VLDL, very-low-density lipoprotein.

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association with triacylglycerol-rich lipoproteins has been demonstrated for FHR-4 [20].

Attempting to improve the detection of the FHR proteins in such biological specimens and trying to define the biological role of their conserved C-terminal domain more precisely, we set out to generate mAbs specific for epitopes shared between these proteins. FHR-1 purified from plasma was used as an immunogen, as it has a C-terminus almost identical with fH, but lacks all other domains. Here we report the generation of four mAbs that allow discrimination between fH, FHR-1 and FHR-2. The binding epitopes of these antibodies were mapped to three distinct epitopes in the three most C-terminal SCRs of fH.

EXPERIMENTAL

Antibodies

The binding site of mAb 3D11 [24] is located in the C-terminus of fH. This mAb also reacts with FHR-1 and detects a genetically determined polymorphism of FHR-1 [27]. Polyclonal goat anti-human fH was obtained from Atlantic Antibodies (Stillwater, ME, U.S.A.).

Purification of FHR-1 from human plasma

Fresh frozen citrated human plasma (500 ml) was obtained from the Central Blood Transfusion Unit, Innsbruck University Hospital. All steps were performed at 3 °C or under constant cold water cooling with buffers containing 0.02 % NaN_3 and 2 mM EDTA. After thawing at 37 °C, the plasma was diluted to a conductivity of 3.7 mS/cm, made 1 mM in PMSF and applied to an XK 50/50 column (Pharmacia, Uppsala, Sweden) containing 560 ml of Q-Sepharose FF (Pharmacia) equilibrated in Q-II buffer (40 mM NaCl, 20 mM potassium phosphate, pH 7.4) with a conductivity of 3.7 mS/cm. The column was eluted with a linear NaCl gradient up to 200 mM NaCl (conductivity = 10.52 mS/cm). FHR molecules were found in the break-through fraction which was brought to the conductivity of Q-III buffer (10 mM NaCl, 10 mM potassium phosphate, pH 7.4) of 1.12 mS/cm and applied again to the same column equilibrated with Q-III. After elution with a linear NaCl gradient in 1.8 litres of Q-III buffer with a final conductivity of 10.5 mS/cm, fractions showing FHR bands in immunoblots were pooled, concentrated by positive pressure ultrafiltration using a PM10 membrane (Amicon Corp., Danvers, MA, U.S.A.) and applied to a 100 ml BioRex-70 column (Bio-Rad, Richmond, CA, U.S.A.) equilibrated with Q-III buffer. The column was eluted with an NaCl gradient in 300 ml of Q-III buffer (final conductivity 25.1 mS/cm). Fractions containing FHR proteins were pooled and passed over a CNBr-activated Sepharose CL-4B column (Pharmacia) that had been coupled to purified mAb 3D11 at 3 mg/ml gel. FHR-1 was eluted with 0.1 M triethylamine, pH 11.7, immediately neutralized with 1 M sodium phosphate, pH 8.0, dialysed against PBS, concentrated and stored in aliquots at −80 °C. This procedure yielded 0.7 mg of FHR-1 (3.3 % of the starting material) with about 75 % purity, containing approx. 10 % of FHR-2, but no detectable fH.

Cloning of C-terminal recombinant fragments of fH

For amplification of DNA fragments coding for SCR 8–20 and SCR 19–20 of human fH, sequence-specific primers were synthesized: forward primer for SCR 8, *CTG CAG* AAA ACA TGT TCC AAA TCA AGT ATA GA; forward primer for SCR 19, *CTG CAG* GAT TCT ACA GGA AAA TGT GGG CCC; reverse primer for SCR 20, *GAA TTC* TCT TTT TGC ACA AGT TGG ATA CTC (restriction sites are shown in italics). Cloning into expression vector pBSV-8His was performed as described previously [6]. The other recombinant proteins representing selected domains of human FHL-1 and fH were also synthesized in the baculovirus expression system. Construction and expression of the various deletion mutants and FHRs has been described [6,7]. All recombinant proteins include a C-terminal His tag to allow direct purification from the culture medium by nickel chelate chromatography [28].

Production of polyclonal rabbit antisera to SCR 19–20 or FHR-4

A rabbit was immunized with 50 μg of purified SCR 19–20 protein, mixed with complete Freund's adjuvant and injected subcutaneously. The animal was boosted twice with 50 μg of protein together with incomplete Freund's adjuvants at 2-week intervals. Antiserum was collected 2 weeks after the last injection and used for Western blotting at a dilution of 1 : 2000. Production of antiserum raised against FHR-4 has been described recently [29].

Cell lines and culture

Sf 9 cells from *Spodoptera frugiperda* were used for infection with recombinant baculoviruses. Cells were grown at 27 °C at 95 % humidity in Grace's medium (BioWhittaker) containing 10 % fetal calf serum and antibiotics.

Expression and purification of recombinant fragments

Sf 9 cells (3×10^6) were grown in a 140 mm cell culture dish in 22 ml of Express medium (BioWhittaker), supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) and were infected with recombinant virus containing the domains of FHL-1 (SCR 1–7), SCR 8–20, SCR 19–20, FHR-3 or FHR-4 using a multiplicity of infection of 5. The culture supernatant was harvested after 10 days and the recombinant proteins were purified by affinity chromatography as described [28]. Purified recombinant proteins were dialysed against 50 mM Tris/HCl, pH 7.5, overnight and concentrated with an Ultrafree-15 Centrifugal Filter Device Biomax-5K (Millipore, Bedford, MA, U.S.A.). The protein concentration was measured by the method of Bradford [30].

SDS/PAGE and immunoblotting

Purified fH, FHR-1, FHR-2, recombinant fragments of fH, recombinant FHR-3, FHR-4, human sera or lipoproteins (Sigma) were analysed under non-reducing conditions and detected by either silver staining or Western blotting after transfer to nitrocellulose membranes [31]. After non-specific binding had been blocked with PBS containing 3 % BSA and 0.05 % Tween 20 for 1 h, the blots were incubated for 1 h with either a polyclonal goat antibody against fH in blocking buffer or hybridoma culture supernatant and developed with the respective peroxidase-conjugated secondary antibody. Proteins were visualized by incubation in 0.3 % (w/v) 4-chloro-1-naphthol in 10 % (v/v) methanol in PBS and 50 μl of H_2O_2.

Establishment of hybridoma clones

Female 8-week-old Balb/c mice were immunized with 10 μg of purified FHR-1 antigen emulsified in complete Freund's adjuvant. The mice were boosted three times with 10 μg of FHR-1 in incomplete Freund's adjuvant every 2 weeks and a fourth time

intraperitoneally with 25 μg of FHR-1 alone 3 days before being killed. Spleen cells were fused with the P3X63Ag8.653 [32] mouse myeloma cells in a ratio of 1:1 (spleen lymphoblasts to myeloma cells) by using PEG 4000 (Sigma) and following a modified procedure [33] initially described by Galfre et al. [34]. Hybridoma clones were screened by testing the supernatant in an ELISA as described below and eventually subcloned twice by limiting dilution using mouse peritoneal macrophages as feeder cells. Monoclonal antibody subtypes were determined by ELISA using subtype-specific antisera (Medac, Hamburg, Germany) according to the manufacturer's instructions.

Purification and labelling of mAbs

Immunoglobulin was purified from ascites fluid by affinity chromatography on Protein G–Sepharose (Pharmacia) and coupled to *N*-hydroxysuccinimidobiotin (Pierce) according to standard procedures [35].

ELISA

Maxisorp®-plates (Nunc, Copenhagen, Denmark) were coated with 50 μl/well purified FHR-1 (2 μg/ml) or fH (7 μg/ml) in 0.1 M $NaHCO_3$ buffer, pH 9.6, for 1 h at room temperature. After blocking with PBS containing 1 % BSA (100 μl/well), 50 μl of hybridoma culture supernatant was added and incubated for 1 h at room temperature. Plates were washed with PBS plus 0.05 % Tween 20 (PBST), incubated with 50 μl of peroxidase-conjugated anti-mouse immunoglobulin (Dako) for 1 h (room temperature) and developed with 15 mg of 2,2′-azinobis-(3-ethylbenzthiazolinesulphonic acid) (ABTS) obtained from Sigma (St. Louis, MO, U.S.A.) per ml of 0.01 M KH_2PO_4 containing 0.025 % H_2O_2. For competitive ELISA, wells were coated with purified fH or FHR-1 and saturated as above. Then mAbs were added at various concentrations for 45 min at room temperature, before a biotinylated mAb was added for a further 30 min at room temperature at an appropriate concentration to allow optimal detection of unblocked epitopes (0.1–1 μg/ml). After a wash with PBST, wells were incubated with peroxidase-labelled avidin (Dako) and subsequently developed with ABTS/H_2O_2 for 30 min.

For catch-ELISA, purified antibodies were coated as described above at 2 μg/ml, saturated with 1 % BSA in PBS, allowed to bind to purified fH (2.5 μg/ml) or FHR-1 (1 μg/ml) diluted in saturation buffer for 1 h at room temperature and washed with PBST. The percentage of binding of each biotinylated mAb to fH caught by the mAb coated to the ELISA plate was calculated in relation to binding to fH directly coated on to the ELISA plate.

Binding of fH to heparin

Human plasma fH was purified by standard methods [35] and stored in PBS at −80 °C. For the heparin assay, 50 μg of fH was diluted in equilibration buffer (70 mM NaCl, 10 mM sodium phosphate pH 7.4, 0.05 % NaN_3), subjected to affinity chromatography on 1 ml of agarose coupled to 10 mg of porcine heparin (HiTrap Heparin; Pharmacia). Samples were passed over the column five times at a flow rate of 1 ml/min at room temperature and the fall-through fraction was collected. After a wash with 5 ml of equilibration buffer, elution was performed by using a linear 70–500 mM NaCl gradient at a flow rate of 1 ml/min over 20 min. Fractions were collected every minute and the column was regenerated with 5 ml of 2 M NaCl followed by 15 ml of equilibration buffer. The amount of fH eluted was determined by ELISA using purified fH (Quidel) as standard.

To test the effect of the newly generated mAbs on fH binding to heparin, 20 μg of fH was preincubated with a 5-fold molar excess of the respective mAb for 30 min at 4 °C and the mixture was then subjected to heparin chromatography. fH in elution fractions was determined as described above, and the presence of mAbs in the fractions was confirmed by ELISA with mouse IgG-specific antisera.

RESULTS

We used purified FHR-1 to establish mAbs specific for C-terminal epitopes shared by fH and other members of the fH protein family. Four stable hybridoma cell lines producing IXF9 (IgG1), VD3 (IgG2a), VIG8 (IgG1) and IIC5 (IgG1) were obtained.

Epitope mapping of the four mAbs

To identify whether the mAbs detect overlapping or unique epitopes, we analysed their binding sites by direct and competitive ELISA techniques. With fH as antigen for competitive ELISA experiments, two mAbs, IXF9 and VD3, were found to compete for binding to fH (Figures 1A and 1B). In addition, the well-characterized mAb 3D11, which recognizes an epitope within the C-terminal domain of fH, influenced binding of the two mAbs IXF9 and VD3. This indicates that the epitopes recognized by the three mAbs IXF9, VD3 and 3D11 are overlapping or even identical.

Specific binding of the other two mAbs VIG8 and IIC5 was directed to different epitopes. A dose-dependent inhibition was detected with the mAb itself, but not with any of the other mAbs (Figures 1C and 1D). mAb IIC5 showed weaker staining of coated fH (note the different scale in Figure 1D). Identical results were obtained when purified FHR-1 was used as antigen (results not shown), indicating that the four mAbs recognize epitopes that are shared between FHR-1 and fH.

Characterization of the binding epitopes by catch-ELISA

The binding epitopes were further characterized in a catch-ELISA, using one mAb fixed to the ELISA plate. The addition of fH (or FHR-1; not shown) followed by biotinylated mAbs confirmed binding of each of the three mAbs, IXF9, VD3 and 3D11, to highly related or even identical epitopes (Table 1). mAb 3D11 was relatively inefficient as a catching antibody.

As observed before (see Figure 1), in this assay also VIG8 and IIC5 exhibited binding to unique epitopes, as binding of fH (Table 1) and FHR-1 (results not shown) was only inhibited by the same mAb, whereas the other mAbs did not significantly affect their binding.

Presence of binding epitopes in truncated fragments of fH and in FHR proteins

Given the similarity of the C-termini of fH to the four FHR proteins, we tested the new mAbs with recombinant peptides that represent either the C-terminal 13 SCRs (i.e. SCR 8–20) or the two most C-terminal SCRs of fH (SCR 19–20) and with recombinant FHR-3 and FHR-4. A representative purification of the His-tagged recombinant proteins by nickel chelate chromatography is shown for the SCR 19–20 construct (Figure 2). The purified proteins and recombinant peptides used for mapping analyses were separated by SDS/PAGE and detected by silver staining (Figure 3).

In dot-blot experiments the four mAbs reacted with normal

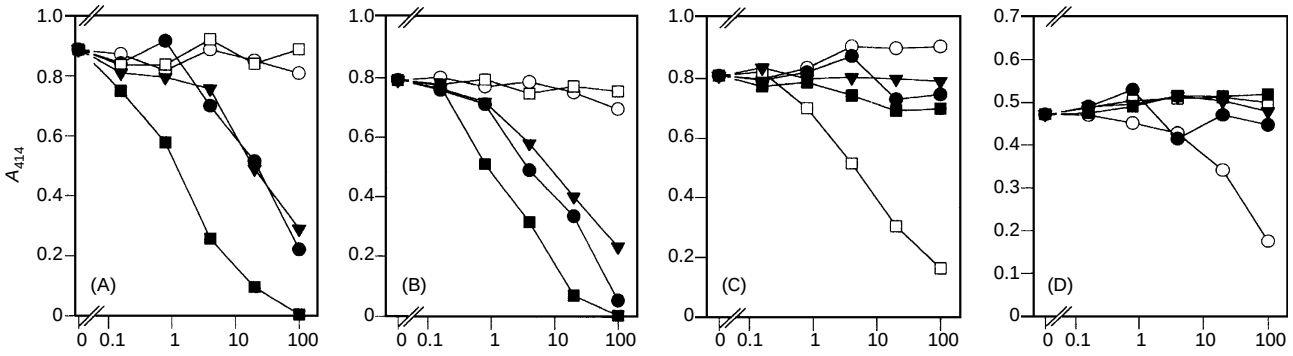

Figure 1 Identification of binding epitopes of fH- and FHR-detecting mAbs by competitive ELISA

The binding of the indicated biotinylated mAb (**A**, IXF9; **B**, VD3; **C**, VIG8, **D**, IIC5) to fH was tested in the presence of increasing concentrations of unlabelled mAbs IXF9 (■), VD3 (●), 3D11 (▼), VIG8 (□), IIC5 (○) as described in the Experimental section. Please note the different scale in (**D**). Values represent the means of duplicated assays of one representative experiment out of a total of five.

Table 1 Identification of overlapping binding epitopes by catch-ELISA

Catch-ELISA with IXF9, VD3, 3D11, VIG8 and IIC5 as catching antibodies was followed by the addition of fH and detection by biotinylated mAbs as indicated. Specific binding of the biotinylated mAb to captured fH is shown as percentage of specific binding (binding to directly coated fH was calculated as 100% of specific binding). The values represent means ± S.E.M. obtained from four independent experiments.

	Specific binding (%)			
	Catching mAb			
Detecting mAb	IXF9	VD3	VIG8	IIC5
IXF9	3.3 ± 0.9	6.5 ± 2.5	96.7 ± 2.8	90.7 ± 8.8
VD3	2.3 ± 0.3	3.5 ± 0.5	96.3 ± 3.7	90.7 ± 8.8
3D11	1.3 ± 0.9	3.5 ± 0.5	30.0 ± 6.5	21.0 ± 5.1
VIG8	91.7 ± 8.3	100 ± 0.0	7.3 ± 1.2	38.7 ± 12.2
IIC5	59.3 ± 21.7	78.1 ± 18.6	55.6 ± 2.6	2.7 ± 2.7

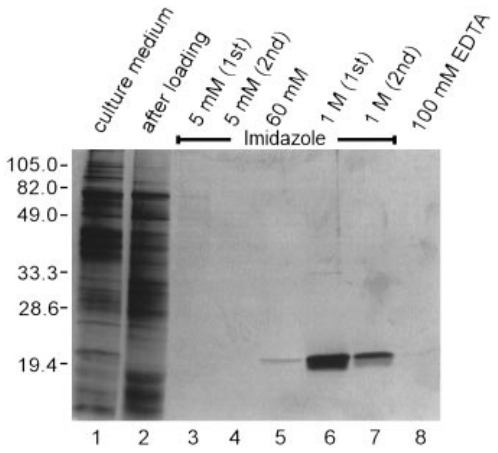

Figure 2 Purification of recombinant fH mutant consisting of the C-terminal SCRs (SCR 19–20)

The cDNA representing SCR 19–20 of complement fH was cloned into expression vector pBSV-8His and recombinant baculovirus was generated by homologous recombination. Culture medium was isolated from infected *Sf*9 cells, and equal volumes (10 μl) of individual fractions obtained after Ni^{2+}-chelate chromatography were separated by SDS/PAGE. The lanes represent: culture medium (lane 1), flow-through (lane 2), first and second low stringency wash (5 mM imidazole) (lanes 3 and 4), high stringency wash (60 mM imidazole) (lane 5), eluted protein (1 M imidazole) (lanes 6 and 7) and EDTA fraction (100 mM EDTA) (lane 8). Proteins were visualized by silver staining.

human serum and purified fH (Figure 4, lanes 1 and 2), whereas none stained the FHL-1 protein which is composed of SCR 1–7 of fH (Figure 4, lane 3). All four mAbs specifically interacted with a fragment of fH that consists of SCR 8–20 (Figure 4, lane 4). Peptide SCR 19–20 allowed further discrimination of the binding epitopes (Figure 4, lane 5): mAb IXF9 (like VD3; not shown) did not bind to SCR 19–20, whereas VIG8 and IIC5 did. Furthermore none of the four mAbs reacted with the FHR-3 or FHR-4 protein (Figure 4, lanes 6 and 7), demonstrating that the binding epitopes are independent of structural motifs common to these SCRs.

Use of mAbs for detection of FHR-1, FHR-2 and fH in human serum

After generating mAbs that bind to three unique domains located in the C-terminus of FHR-1, FHR-2 and fH, we tested their potential for specific detection of the corresponding proteins in human serum and lipoproteins. Human serum was separated by SDS/PAGE under non-reducing conditions, and Western-blot experiments were performed (Figure 5). All four mAbs reacted with FHR-1 and detected the two differently glycosylated forms of molecular mass 43 and 37 kDa. In addition, all detected the 150 kDa fH protein. mAb VIG8 also stained the 29 and 24 kDa forms of FHR-2 (Figure 5, lane 3). In contrast, mAbs IXF9, VD3 and IIC5 reacted with fH and FHR-1 alone (Figure 5, lanes 1, 2 and 4). Thus, to our knowledge, VIG8 is the only mAb available to date that can be used for detection of FHR-2. All four mAbs were unreactive with proteins separated under reducing conditions (not shown).

Identification of fH, FHR-1 and FHR-2 in human lipoproteins

It has recently been reported that FHR-1, FHR-2 and FHR-4 are constituents of human lipoproteins [20,26]. Therefore, we used this panel of mAbs to demonstrate the relative amounts of fH and the FHR proteins present in fractions of human lipo-

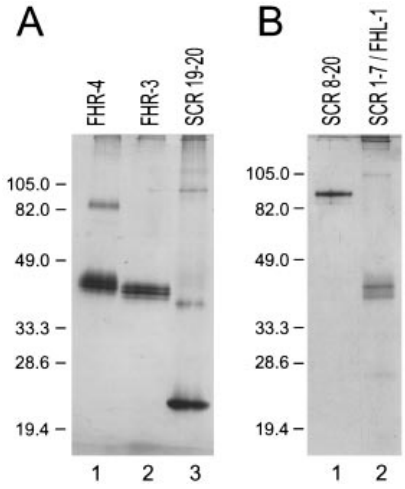

Figure 3 Identification of fH, purified recombinant proteins and peptides

Recombinantly expressed FHR-3 and FHR-4 and SCR 19–20 of fH (**A**), as well as recombinant FHL-1 (SCR 1–7) and SCR 8–20 of fH (**B**), were separated by SDS/PAGE and detected by silver staining. The mobility of the size markers is indicated in kDa.

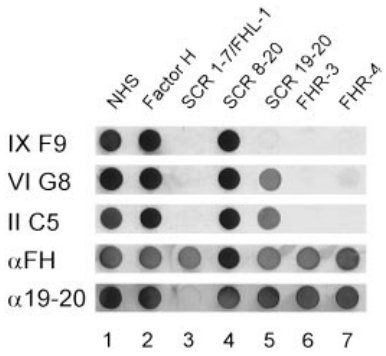

Figure 4 Presence of three distinct epitopes recognized by the mAbs in fH, FHL-1 (SCR 1–7), truncated mutants of fH (SCR 8–20), (SCR 19–20), FHR-3 and FHR-4

Dot-blot reactivity of mAbs IXF9, VIG8, IIC5 and polyclonal sera against fH (anti-fH) or SCR 19–20 of fH with normal human serum (NHS), purified fH, FHL-1 (SCR 1–7), SCR 8–20, SCR 19–20, FHR-3 and FHR-4.

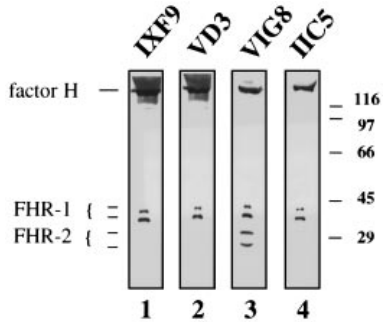

Figure 5 Reactivity of mAbs with human serum proteins

Western blot of human serum separated by SDS/PAGE on a 5–12% gel under non-reducing conditions and stained with mAbs IXF9 (lane 1), VD3 (lane 2), VIG8 (lane 3) and IIC5 (lane 4) as described in the Experimental section. The positions of marker proteins are given in kDa on the right.

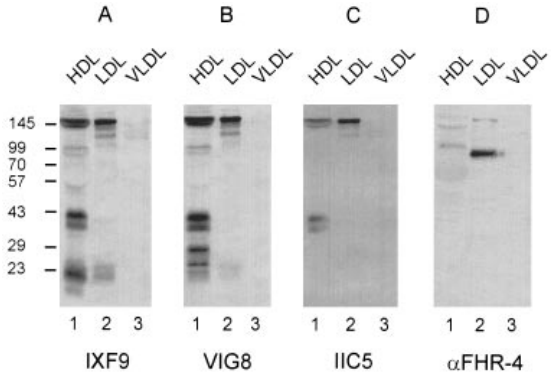

Figure 6 Use of mAbs for the identification of fH, FHR-1 and FHR-2 in human lipoproteins

Human lipoproteins (HDL, lane 1; low-density lipoprotein (LDL), lane 2; very-low-density lipoprotein (VLDL), lane 3) were separated by SDS/PAGE. A total of 22 μg of lipoprotein was loaded per lane. The indicated mAbs or the polyclonal anti-FHR-4 serum were used for Western blotting. In addition to fH, all three mAbs detected the two forms of the FHR-1 protein (the 43 kDa FHR-1β and the 37 kDa FHR-1α protein). VIG8 also detected the 29 kDa FHR-2α and the 24 kDa FHR-2 proteins. An as yet unidentified additional band of about 21–22 kDa was detected by IXF9 and VIG8. The positions of marker proteins (kDa) are indicated on the left.

proteins. fH, the two forms of FHR-1 (FHR-1α and FHR-1β) as well as the two forms of FHR-2 (FHR-2α and FHR-2) are present in high concentrations in HDL, as detected by staining with mAb VIG8 (Figure 6B). In contrast, fH, but not FHR-1 or FHR-2, was observed in LDL. VLDLs apparently lack all three proteins. FHR-4 was seen mainly in the LDL fraction. None of the mAbs reacted with FHR-4 nor did the anti-FHR-4 serum recognize FHR-1 or FHR-2 (Figure 6D). Interestingly, mAb IXF9 selectively identified a 21–22 kDa band in the HDL and LDL fractions which was clearly different from the FHR-2 protein at 24 kDa (Figure 6A). The identity of this protein has to be determined by further experiments.

mAb VIG8 interferes with binding of fH to heparin

As the C-terminus of fH contains a heparin-binding site, we tested whether the mAbs described here have any impact on this interaction. To this end, affinity chromatography of purified fH on heparin-coated agarose was performed. fH bound to the heparin matrix and was eluted at approx. 300 mM NaCl, which is consistent with the report of Blackmore et al. [11]. When purified mAbs were preincubated with fH in a 5-fold molar excess, only VIG8 affected binding of fH to heparin (Figure 7C). VIG8 completely abrogated binding, and all fH was detected in the fall-through fraction. mAbs VD3 (representative of the IXF9-VD3-3D11 epitope) and IIC5 did not alter the binding or the elution profile. However, VD3 and IIC5 did bind to fH in this assay, as mouse IgG–fH complexes were detected in the fractions that contained fH (Figures 7B and 7D).

DISCUSSION

We have generated mAbs in order to detect FHR-1, FHR-2 and fH in biological samples and to obtain further insights into the function of the conserved C-terminal domains of these proteins. Four distinct hybridomas were established. The corresponding mAbs specifically react with FHR-1 and fH (and also with FHR-2 in the case of VIG8), but do not bind to the proteins FHR-3, FHR-4 and FHL-1. Characterization of the binding epitopes

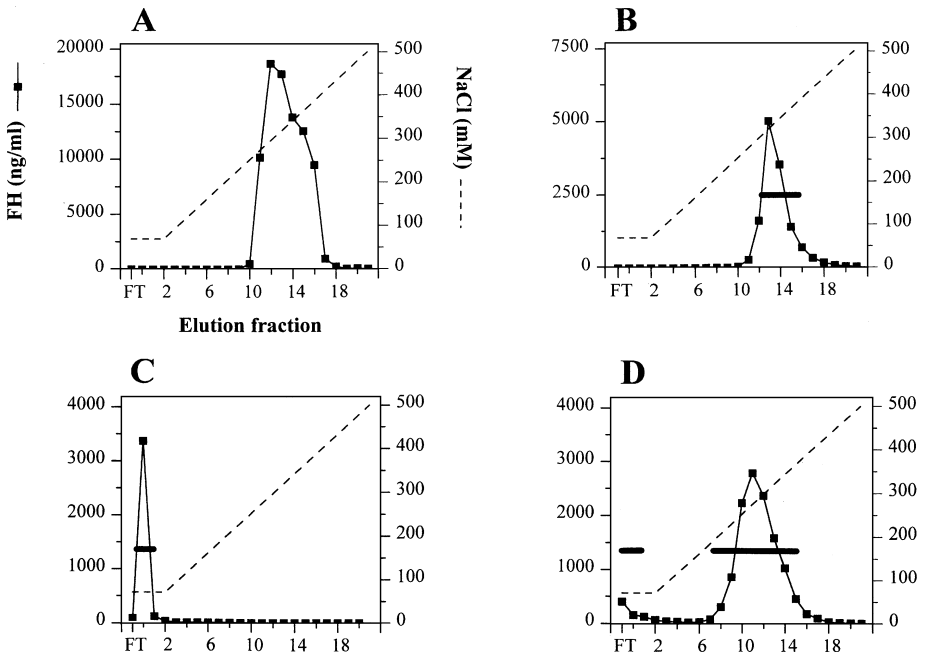

Figure 7 mAb VIG8 abrogates binding of fH to heparin

(**A**) fH (50 μg) was applied to a heparin–agarose column and eluted by a linear salt gradient (– – – –). (**B**–**D**) Before binding to heparin–agarose, fH (20 μg) was preincubated with mAbs VD3 (**B**), VIG8 (**C**) or IIC5 (**D**) for 30 min at 4 °C. Fractions were collected and fH concentration (■) and the presence of IgG bound to fH (bars) were determined by ELISA. FT, fall-through.

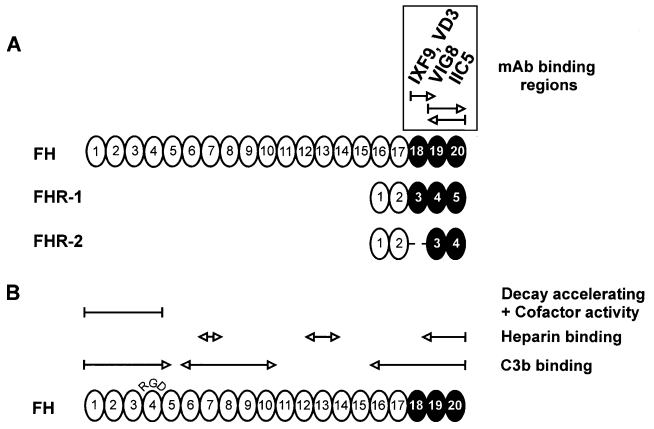

Figure 8 Schematic localization of the epitopes of the four newly generated mAbs in fH, FHR-1 and FHR-2

(**A**) fH and FHRs are shown in their SCR domain structure, and SCRs are numbered consecutively. Potential binding regions of the newly generated mAb are indicated. (**B**) Domain structure of fH and localization of the known functional domains.

Table 2 Reactivity of the mAbs with proteins of the fH family and truncated mutants of fH

For a more detailed identification of the binding epitopes see Figure 8.

	IXF9, VD3	VIG8	IIC5
fH(SCR 1–20)	+	+	+
SCR 8–20 (fH)	+	+	+
SCR 19–20 (fH)	–	+	+
FHL-1 (SCR 1–7)	–	–	–
FHR-1	+	+	+
FHR-2	–	+	–
FHR-3	–	–	–
FHR-4	–	–	–

showed that two mAbs (IXF9 and VD3) bind to overlapping or even identical epitopes, while the two others (VIG8 and IIC5) detect unique epitopes. Characterization of the epitopes of the mAbs is summarized in Figure 8 and Table 2.

By the use of recombinant fragments of fH and considering the sequence homology between fH, FHR-1 and FHR-2, we propose that mAbs IXF9 and VD3 bind to SCR 18 (fH) and SCR 3 (FHR-1) respectively, as they fail to recognize both the FHR-2 protein and the SCR 19–20 construct. An alternative explanation is that a conformational epitope comprises parts of the adjacent SCRs 18 and 19 of fH, i.e. that it is located at the junction of two globular domains. In this alternative model, the absence of the part located within SCR 18 should then account for the inability to react with FHR-2. The binding sites of mAbs VIG8 and IIC5 reside in SCR 19 to 20 of fH, as both react with the recombinant SCR 19–20 fragment. SCRs 19 and 20 of fH show homology of 100 and 97 % to the corresponding SCRs 4 and 5 of FHR-1, but only 88.3 and 61.2 % to the respective SCRs 3 and 4 in FHR-2. Thus the selective staining of FHR-2 by mAb VIG8 suggests that the VIG8 epitope is located in the parts conserved throughout the sequence of fH, FHR-1 and FHR-2. In contrast, the IIC5 epitope would be expected to be found in the, smaller, part conserved between fH and FHR-1 only, but diverging from FHR-2. As there are potential candidates for both epitopes in both SCRs, a final assignment of the epitopes to SCR 19, SCR 20, or a domain involving both SCRs at their

junction has to be based on further experiments, e.g. mutational analysis of the SCR 19–20 construct.

At the moment, the set of mAbs described is unique in its potential to detect and discriminate between members of the fH protein family in biological specimens, as shown here for plasma and triacylglycerol-rich lipoproteins. All four new mAbs detect all known allelic forms of FHR-1 but mAb 3D11 detects the FHR-1 protein only in 55% of a Caucasian population [27]. Furthermore mAb VIG8 is at present the only mAb that identifies the FHR-2 protein. In Western blots of human lipoprotein fractions, mAb VIG8 clearly detected the FHR-1 and FHR-2 proteins. In agreement with the report of Park and Wright [26], these proteins are found predominantly in the HDL fraction.

Recently a heparin- and a C3b-binding domain have been localized within the C-terminal SCRs of fH [12,13]. This points to an important role for the conserved C-termini of fH and FHRs. In particular, mAb VIG8 will be useful for making a precise functional characterization of the C-terminal heparin-binding domain, as, to date, VIG8 is the only mAb able to block heparin binding completely. The role of this mAb in fH binding to cell or particle surfaces needs to be investigated.

Note added in proof (received 9 February 1998)

The characterization and localization of the second heparin binding site within the C-terminal region of fH is described by Blackmore et al. [37].

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