Human Factor H-related Protein 5 (FHR-5)

A NEW COMPLEMENT-ASSOCIATED PROTEIN*

Received for publication, August 17, 2000, and in revised form, October 23, 2000
Published, JBC Papers in Press, October 31, 2000, DOI 10.1074/jbc.M007495200

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A novel human plasma protein has been identified as a universal component of complement deposits, when complement is detected immunohistochemically in vivo. The protein is homologous to complement factor H and related proteins and has been designated factor H-related protein 5 (FHR-5). FHR-5 was identified by a monoclonal antibody raised using pathologic human glomerular preparations as the immunogen. FHR-5 was purified by affinity chromatography from complement-lysed erythrocytes, and the peptide sequence was obtained. The cDNA was cloned from a human liver library, and FHR-5 was deduced to be a protein containing 551 amino acids organized into nine short consensus repeat motifs. The short consensus repeats of FHR-5 show homology to Factor H and to other Factor H-related proteins, with some unique features demonstrated. Recombinant FHR-5, expressed in insect cells, was shown to bind C3b in vitro. The strong association of FHR-5 with tissue complement deposits in vivo suggests that this additional member of the Factor H family of proteins has a function in complement regulation.

In this paper we describe the characterization of the protein recognized by K2.254. The deduced amino acid sequence of the protein, obtained by partial peptide sequencing and cDNA cloning and sequencing, indicates that it is a member of the factor H-related family of proteins. In keeping with current nomenclature in this area, the new protein has been designated factor H-related protein 5 (FHR-5).†

EXPERIMENTAL PROCEDURES

Antibodies—The production and initial screening of monoclonal antibodies K2.254, K2.322 (anti-human C9), K1.115 (anti-human C6), and an isotype-matched control monoclonal antibody, K1.431, has previously been described (1). A rabbit polyclonal antibody against the surface of K562 cells was produced by repeated immunization with K562 cells in pertussis vaccine. Mouse anti-human Factor H and polyclonal rabbit-anti-human C3 antibodies were purchased from Serotec (Kidlington, Oxford, UK), and mouse anti-human serum albumin (HSA) antibody was from Sigma (St. Louis, MO). The secondary antibodies used were fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (Dako, Carpente- ria, CA), rabbit anti-mouse and donkey anti-goat immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, MD), which were 125I-labeled as described previously (2), horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins, horseradish peroxidase-conjugated swine anti-rabbit (Dako), and sheep anti-fluorescein-peroxidase Fab fragments (Roche Diagnostics, Mannheim, Germany).

A Tetra-His monoclonal antibody (Qiagen, Hilden, Germany) was used for detection of His-tagged recombinant protein expression.

Binding of K2.254 to Complement-exposed Cells—The human lymphoblast cell line K562 was obtained from American Type Culture Collection (Manassas, VA). 10⁶ K562 cells were incubated with 0.1 mg/ml anti-K562 antibody for 2 h at 37 °C, washed 3 times in phosphate-buffered saline (PBS), and incubated with normal human serum (NHS) or control heat-inactivated NHS, 1:2 dilution in PBS/4 h at 37 °C. After incubation with sera, the cells were washed 3 times in PBS, and the preparation was divided, spun onto aminovalkylsilane (Sigma)-treated microscope slides, fixed in acetone for 10 min, and air-dried. The slides were incubated with 10 μg/ml anti-K562, K2.322, or K1.431 for 1 h at room temperature (RT) and washed 3 times in PBS. Slides were finally incubated in rabbit fluorescein isothiocyanate-conjugated mouse immunoglobulin (1:20) for 30 min at RT, washed a further three times, and examined by epifluorescence microscopy.

In additional experiments, sensitized cells were incubated with NHS, which had been depleted of C9, C8, or C7 as described previously (3).

Preparation of Complement-lysed Guinea Pig Erythrocyte Ghosts—Guinea pig erythrocytes (GPE) were directly lysed using fresh NHS.

* This work was supported by the National Health and Medical Research Council of Australia and the Baxter Extramural Grant Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: FHR, Factor H-related protein; HSA, human serum albumin; NHS, normal human serum; PBS, phosphate-buffered saline; RT, room temperature; GPE, guinea pig erythrocytes; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis, HPLC, high performance liquid chromatography; GSP, gene-specific primers; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); ORF, open reading frame; SCR, short consensus repeat; ELISA, enzyme-linked immunosorbent assay; RCA, regulators of complement activation; FHL, Factor H-like.
Washed GPE were suspended 1:10 (v/v) in 0.9% sodium chloride solution, and 5 ml of this preparation was incubated with 20 ml of NHS overnight at 37 °C. The GPE ghosts were retrieved by centrifugation and washed three to five times in saline until the supernatant was clear.

To prepare negative control erythrocyte ghosts, 5 ml of the washed 1:10 GPE suspensions was osmotically lysed by the addition of 10 ml of distilled water and the erythrocyte ghosts were recovered and washed as above.

**Western Blotting**—NHS, erythrocyte ghosts, and affinity-purified K2.254 antigen were prepared and electrophoresed on 7.5% or 10% SDS-PAGE gels (4) and transferred to nitrocellulose (Trans-Blot transfer medium, Bio-Rad, Hercules, CA) using a Bio-Rad transblot cell (5). Nitrocellulose membranes were blocked with 5% dried milk in 0.1 M Tris-HCl (pH 7.0). Each 5-ml extract was passaged twice through the column, and the 5-ml digitonin extract was passed over the column and collected. The column had been coupled according to the manufacturer's instructions. The K2.254 antigen from NHS was precipitated with 50 mg of the K2.254 monoclonal antibody or rabbit anti-mouse-HRP (1:1000) for 1 h at RT. Following washing, radioactive bands were detected by autoradiography and the K2.254 antigen from NHS. 10 ml of NHS was passed 3 times over Amicon, Beverly, MA). The membrane was washed, incubated with enhanced chemiluminescence substrate (Kodak, Rochester, NY) for 40 cycles. 3

**Cloning and Sequencing of Full-length cDNA—** Full-length cDNA was synthesized from 2 µg of human liver poly(A)+ RNA using oligo(dT) or random primers and SuperScript II reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions.

**Affinity Purification**—Affinity-purified K2.254 antigens were cloned and sequenced. The cDNA was amplified from human liver poly(A)+ RNA as above using the Exonuclease Kit. PCR products were gel-purified (Qiagen gel extraction kit) and cloned into pGEM-T Easy (Promega, Madison, WI). Oligonucleotide primers were synthesized by Life Technologies, Inc.

Plasmid DNA was prepared from bacterial cultures using Qiagen Plasmid Mini Kits. The correct identity of the cloned fragments was confirmed by sequencing, PCR, and restriction enzyme analysis. DNA sequencing reactions were performed using the BIGDYE Terminator Cycle Sequencing Ready Reagents (PE Applied Biosystems, Foster City, CA) and electrophoresed by the Australian Genome Research Facility, Melbourne, Australia. Both strands of cDNA clones were sequenced using standard vector primers and several of the internal primers listed in Table 1. Sequence analysis was performed using MacVector (version 5.0.2, Oxford Molecular Group, UK).

**Northern Blot Analysis—** 10 µg of total RNA from human liver was
and contained a MluI restriction site at its 5'-end. The reverse primer (BAC-R2, see Table I) was designed to anneal immediately 3’ of the signal peptide-encoding region and contained a MluI restriction site at its 5’-end. The PCR product was digested with MluI/Sall (Promega, Madison, WI) and cloned into the pFastBac1-His10 vector, which was partially digested with MluI, gel-purified, then treated with XhoI. pFastBac1-His10, a modified version of pFastBac1 (Life Technologies, Inc.), encodes a gp67 signal peptide to ensure efficient secretion and a 10-histidine C-terminal epitope tag to facilitate detection and purification. The complete sequence of the insert and its junctions was confirmed by sequencing.

A modification of the Bac-to-Bac baculovirus expression systems protocol (Life Technologies, Inc.) was used to obtain purified recombinant baculovirus. Recombinant pFastBac1-His10 vector was transferred into DH10Bac-competent cells and cultured overnight in 4 ml of S.O.C. medium containing gentamicin, tetracycline, and kanamycin. To obtain a pure recombinant virus strain without the need for plaque purification, recombinant pFastBac1-His10 was transfected into DH10-competent cells (Life Technologies, Inc.) by electroporation (2.5 kV, 25 microfarads, 200 ohms). Transfected cells were cultured on Luria agar plates containing 5-bromo-4-chloro-3-indol β-D-galactoside (X-gal), isopropyl β-D-thiogalactoside, kanamycin, and gentamicin for 24 h. Colonies containing pure recombinant Bacmid DNA were selected by blue/white screening, and total DNA was prepared and used to transfect Spodoptera frugiperda (Sf9) cells, which were adapted to serum-free conditions using HyQ51 insect serum-

Electrophoresis of Recombinant Factor H-related 5 (rFHR-5)—A 1672-bp fragment was amplified from the full-length (3.0 kb) DNA by PCR (touchdown 56–50 °C) using Pfu DNA polymerase (Roche Diagnostics, Mannheim, Germany). The forward primer (BAC-F2, see Table I) was designed to anneal immediately 3’ of the open reading frame (ORF) and incorporated a Sall restriction site at its 5’-end. The PCR product was digested with MluI/Sall (Promega, Madison, WI) and cloned into the pFastBac1-His10 vector, which was partially digested with MluI, gel-purified, then treated with XhoI. pFastBac1-His10, a modified version of pFastBac1 (Life Technologies, Inc.), encodes a gp67 signal peptide to ensure efficient secretion and a 10-histidine C-terminal epitope tag to facilitate detection and purification. The complete sequence of the insert and its junctions was confirmed by sequencing.

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Electrophoresis on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane GenScreen Plus (PerkinElmer Life Sciences, Boston, MA) by standard procedures (7). RNA was cross-linked to the membranes using a UV Stratalinker 1800 (Stratagene, La Jolla, CA), and blots were prehybridized in Rapid-hyb buffer (Amer sham Pharmacia Biotech, Upsalla, Sweden) at 65 °C for 1 h. Hybridization with specific probes labeled with 32P using the Megaprime DNA labeling system (Amer sham Pharmacia Biotech) was performed at 65 °C for 2 h. Membranes were washed for 15 min in 2 x SSC + 0.1% SDS at 65 °C, 1 x SSC + 0.1% SDS at 65 °C, and 0.1 x SSC + 0.1% SDS at RT and filters exposed at −70 °C overnight.

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anti-K562 antibody and fresh human serum, however, the cells showed morphological evidence of complement damage and strong cell surface staining for the K2.254 antigen. Similar surface labeling with K2.254 monoclonal antibody was seen when cells were incubated with human sera deficient in C9, C8, or C7 (Table II). The surface staining was similar to that seen using the positive control anti-C9 antibody (K2.322). No staining was detected with the isotype-matched antibody (K1.431). When fresh human serum was substituted with guinea pig or rabbit serum, there was morphological damage to the K562 cells but no cell surface reactivity with K2.254 (Table II).

These data suggest that the epitope for K2.254 was expressed on an antigen derived from human serum upon activation of complement. The detection of the antigen in cells incubated with C7-deficient serum indicated that the antigen associates with the cell prior to assembly of the terminal attack complex of complement.

**Western Blotting**—Western blotting of NHS-lysed GPE with K2.254 identified a band of ~65 kDa under nonreducing conditions (lane 1, Fig. 1). This band was not observed in control osmotically lysed GPE (lane 2, Fig. 1). Blotting of NHS failed to reveal specific binding of K2.254 (lane 3, Fig. 1), even after inulin pretreatment (2) to activate complement. However, the 65-kDa band was observed in Western blots of the column eluate following affinity purification from NHS using a K2.254 affinity column (lane 5, Fig. 1). K2.254 did not detect a band in any preparations under reducing conditions, suggesting that the K2.254 epitope is sensitive to reduction.

In additional Western blotting studies, using purified human Factor H (Sigma), the K2.254 antibody showed no cross reactivity with Factor H.²

**Purification of the K2.254 Antigen and Peptide Sequence Analysis**—The 65-kDa K2.254 antigen, affinity-purified from NHS, was heavily contaminated by albumin. Therefore, complement-lysed GPE were used to purify sufficient protein for peptide sequencing. Digitonin-extracted GPE ghosts were passed through a K2.254 affinity column, and the eluate was analyzed by Western blotting. The 65-kDa K2.254 antigen was detected (lane 2, Fig. 2), but the eluate also contained C9 (lane 1, Fig. 2) and human serum albumin (HSA, lane 3, Fig. 2), although the amount of albumin was markedly less than that in preparations affinity-purified from NHS. Separation from HSA and C9 was achieved by reverse-phase HPLC. Fractions containing material reactive with K2.254 but not anti-human C9 or anti-HSA antibodies were pooled, lyophilized, and used for peptide sequencing.

| Peptide | Sequence | Homology coordinates | Homology |
|---------|----------|----------------------|----------|
| H1113   | WNPEDCDETKE | Factor H 416–423     | 50 %     |
| H1114   | GEHVPEIAEDVAQPK | Factor H 1205–1211  | 57 %     |
| H1115   | FEYPIE     | Factor H 629–651     | 70 %     |
| H1116   | EGEYHVMEVYDNPNFINGPK | Factor H 453–462     | 87 %     |
| H1117   | IVCQDGENTLFPCV | FHR1/2 115–123      | 100 %    |
| H1118   | G68TP      | FHR1/2 115–119      | 92 %     |
| H1119   | SFWRITCTTEEG | FHR 1/2 68–72       | 60 %     |
| H1120   | MCSFP      | Unassigned           | <30 %    |
| H1121   | AMISSPFRAICQEGK | Factor H 1175–1184  | 70 %     |
| H1122   | TGDAVEFQCK | Factor H 837–845     | 88 %     |
| H1123   | DGRWQSLPR  | Factor H 817–822     | 67 %     |
| H1124   | VALVCK     | Factor H 823–831     | 56 %     |
| H1125   | ENYLLPEAK  |                      |          |

² J. L. McRae, unpublished data.

After in-gel proteolysis and HPLC separation of peptides, the sequence of 13 purified peptides was obtained. The majority of peptides showed some homology with members of the Factor H family of proteins (11). Table III shows the peptide sequences and lists the peptides to which they show strongest homology.

**Cloning of Full-length cDNA—Alignment of the peptides with human Factor H was used to design primers for cDNA isolation. Human liver was chosen as the source of RNA, because members of the Factor H gene family are expressed in liver. Degenerate primers, 254-F1 and 254-R1, were used to amplify a 709-bp product. Sequencing of this PCR product revealed an incomplete ORF encoding a protein with 55.5% identity to amino acids 549–785 of human Factor H. The deduced protein sequence contained perfect matches with peptides H1113, H1114, H1116, and H1117 (Table III). Because the new protein was similar but not identical to Factor H and was distinct from Factor H-related proteins 1–4, it was designated Factor H-related protein 5 (FHR-5).

We then performed 3'- and 5'-RACE, which yielded 1072- and 2248-bp products, respectively. Sequencing of these clones enabled the amplification of a single full-length FHR-5 cDNA from liver RNA. Fig. 3 shows the position and overlap of these clones to produce the 2823-bp cDNA. To minimize the possibility of PCR-generated errors, the sequence of both strands was obtained from clones generated from at least two independent PCRs.

cDNA, Amino Acid, and Structural Analysis—The complete FHR-5 cDNA sequence and deduced amino acid sequence are shown in Fig. 4 (GenBank™ data base accession number AF295327). There was an ORF from bases 94 to 1800, with the region of the start codon displaying good agreement with the Kozak consensus sequence for translation initiation (12). The ORF encoded an 18-amino acid signal sequence and a mature protein of 551 amino acids. The ORF was followed by 1000 bp
of 3′-untranslated sequence, including a consensus polyadenylation signal at positions 2705–2710 and a poly(A)“ tail. Mature FHR-5 has a predicted molecular mass (nonglycosylated) of ~62,405 Da, and two potential N-linked glycosylation sites at positions 108 and 382 (Fig. 4).

There are nine short consensus repeat (SCR) domains, each containing the four characteristic cysteine (C) residues (boxed in Fig. 5) and additional conserved amino acids (aligned in Fig. 5). All of the peptide fragments isolated could be assigned to the mature protein.

SCRs of FHR-5 display varying homology to members of the Factor H family (Fig. 6). SCRs 1 and 2 of FHR-5 show greatest homology to SCRs 1 and 2 of FHR-1 (X56210) and FHR-2 (X86564–X86565). SCRs 3–7 display homology to SCRs 10–14 of Factor H (Y00716) and SCRs 8 and 9 display homology to the two C-terminal SCRs of Factor H and all FHR proteins (FHR-3, X68679; FHR-4, NM006684).

Northern Blot Analysis—A Northern blot of human liver RNA was probed with the 709-bp FHR-5 PCR fragment (Fig. 3). The 709-bp fragment hybridized to a single mRNA species with an estimated size of 3.0-kb (lane 1, Fig. 7). When the same blot was reprobed with a full-length FHR-5 probe, the 3.0-kb band was the predominant transcript observed (lane 2, Fig. 7), with a minor band at 4.4 kb and a diffuse band at 1.4 kb, the latter probably representing transcripts of the FHR proteins 2–4 (13–16). The 4.4-kb band is likely to represent Factor H (17), because it (but not the 3.0-kb band) was also observed when the blot was hybridized with an internal Factor H probe.2

Expression and Analysis of Recombinant FHR-5—A recombinant version of FHR-5 incorporating a polyhistidine epitope tag at the C terminus was expressed in insect cells using a baculovirus expression system. Western blotting, under nonreducing conditions, using the Tetra-His monoclonal antibody or the K2.254 monoclonal antibody detected a band of similar size (65 kDa) to the native protein.

Several members of the Factor H family have been shown to bind C3b (18–20), and FHR-5 has SCRs homologous to a C3b binding domain in Factor H. To determine whether FHR-5 demonstrates C3b binding, a quantitative ELISA was performed using the recombinant FHR-5 protein and compared to purified Factor H, as a positive control, and human C5b-6 as a negative control. Because of the copurification of FHR-5 with HSA, specific binding of FHR-5 to HSA was also tested in the ELISA system. Like Factor H (Fig. 8B), C3b bound to FHR-5 (Fig. 8A) in a dose-dependent and saturable manner. Neither human C5b-6 complexes nor HSA demonstrated any binding to FHR-5 or to Factor H (Fig. 8).

DISCUSSION

We describe here the identification and initial characterization of FHR-5, a new member of the human factor H family of proteins. Factor H (21) is a fluid phase regulator of the alternative pathway of complement, which functions to prevent amplification of the alternative pathway by accelerating the decay of C3 and C5 convertases and by acting as a cofactor for factor I-mediated cleavage of surface bound C3b. Factor H consists of 20 repeats of a characteristic structural domain called the short consensus repeat (SCR). Factor H is a member of the regulators of complement activation (RCA) family and is encoded by a gene ~7 megabase pairs from the main RCA gene cluster on chromosome 1q32 (22). A number of the functional properties of Factor H have been mapped to particular SCR domains. SCRs 1–10 and 16–20 of Factor H are believed to contain the binding site(s) for C3b, and decay-accelerating and cofactor activities have been localized to SCR 1–5 (Fig. 9) (20, 23–28).

More recently a number of smaller proteins structurally related to Factor H and also consisting entirely of SCR domains, have been identified. These are Factor H-like protein 1 (FHL-1), and four Factor H-related proteins, FHR 1–4. FHL-1 is a...
truncated version of Factor H consisting of SCRs 1–7 of Factor H plus an additional four C-terminal amino acids and is generated by alternative splicing of the Factor H gene (29). The four FHR proteins (FHR 1–4) are separate gene products and contain four or five SCRs. Some of the SCRs in the FHR proteins can be aligned with Factor H based on homology (Fig. 9). For example, the two C-terminal SCRs in all FHR proteins show strong homology to the corresponding SCRs of Factor H. In contrast, the two N-terminal SCRs of FHR-1 and FHR-2 (Fig. 9, A and B) appear to be unrelated to the SCRs of Factor H. The functional properties of FHL-1 and FHR1–4 are not fully defined. FHL-1 demonstrates the complement regulatory properties of Factor H and also has an active RGD integrin binding site. The four FHR proteins contain SCRs homologous to those in Factor H, which are implicated in C3b binding but do not have SCRs homologous to the Factor H domains involved in decay acceleration and Factor I cofactor activity. No complement regulatory properties have yet been demonstrated in the FHR proteins. FHR-1, FHR-2, and FHR-4 have been shown to associate in plasma with lipoproteins (16, 31, 32). Other complement-related proteins (C4-binding protein, CD59, and clusterin) have shown lipoprotein association (33–36), but the biological significance of this interaction is unclear.

By analogy with the structure of Factor H and the other FHR
proteins, it could be predicted that FHR-5 would bind C3b and heparin but probably lacks decay-accelerating and cofactor activity. We have confirmed C3b binding in vitro, and the widespread in vivo association of FHR-5 with complement deposits suggests that its C3b binding is functionally significant.

The cDNA for FHR-5 was isolated from human liver, the principle site of synthesis of many plasma proteins, and FHR-5 was able to be isolated from human plasma by affinity purification. However, detection of FHR-5 in human plasma was hampered by its comigration with albumin on SDS-PAGE gels, and FHR-5 affinity purified from human plasma was heavily contaminated with albumin. It was therefore simpler to initially purify FHR-5 from using human complement-lysed GPE membranes. The SDS-PAGE molecular weights of FHR-5 and albumin are similar, and it is possible that the small amount of FHR-5 in the substantial albumin component of human plasma on SDS-PAGE gels was masked from detection by Western blot analysis. This hypothesis was supported by experiments in which trace amounts of FHR-5 were added to HSA or human IgG (each 20 g/liter); the FHR-5 was detectable by Western blot only in the IgG solution. There was, however, no evidence, in vitro, of specific binding of FHR-5 to HSA in the ELISA studies.

FHR-5 is, so far, unique in the FHR family in that it has been widely detected in vivo in association with complement complexes in both normal and pathological human tissue (1). In a

![Figure 7](http://www.jbc.org/)

**FIG. 7. Northern blot analysis of human liver RNA.** Total cellular RNA was isolated from human liver tissue. 10 μg of total RNA was separated on a denaturing agarose gel and transferred onto nitrocellulose membranes. A 32P-labeled 709-bp cDNA probe specific for an internal fragment of FHR-5, hybridized to an RNA species of an estimated size of 3.0 kb (lane 1, arrow), is shown. The same membrane was stripped and probed with a full-length 32P-labeled 1711-bp FHR-5 cDNA probe, which also detected a major band at 3.0 kb (lane 2) and two additional bands at 1.4 and 4.4 kb (arrow heads), which probably represent transcripts for FHR proteins 2–4 and Factor H, respectively.

![Figure 8](http://www.jbc.org/)

**FIG. 8. Binding of recombinant FHR-5 in vitro.** Binding of C3b, C5b-6, and HSA to recombinant FHR-5 was determined by ELISA. Recombinant FHR-5 (A) or Factor H (B) was adsorbed to the ELISA plate at the concentration indicated and binding of C3b (○), C5b-6 (●), and HSA (▲) was analyzed. Only C3b binds to FHR-5 and Factor H and does so in a saturable and dose-dependent manner.

![Table 1](http://www.jbc.org/)

| Protein | Lipoprotein association | C3b binding |
|---------|------------------------|-------------|
| FH | + | nd |
| FHL-1 | + | + |
| FHR-1 | + | + |
| FHR-2 | + | + |
| FHR-3 | + | + |
| FHR-4 | + | + |
| FHR-5 | + | + |
prospective series of 90 human renal biopsies, K2.254 is a sensitive marker of complement deposition. FHR-5 appears to be a stable component of complement deposits in vivo, because it is antigenically detectable in postmortem material removed several hours after death. In tissue deposits, the intensity and distribution of FHR-5 closely matches that of C5b-9, rather than C3, which is less widespread (1). This, as with C5b-9 (37), may be an indication of the stability of FHR-5 in these tissue deposits. In vitro studies and the molecular structure of FHR-5 would suggest a primary interaction in the complement system deposits.

In vivo studies using recombinant FHR-5 with the C3/C5 convertases, but this awaits confirmation by studies using recombinant FHR-5.

We have identified a novel factor H-related protein, FHR-5. Based on the human histological studies and in vitro data, it appears that FHR-5 has a function associated with complement activation and could potentially play a significant role in human biology.

Acknowledgments—We would like to thank Dr. Brett Cromer (St. Vincent’s Institute of Medical Research (SVIMR) for providing the pFastBac1-His10 vector and for his assistance with baculovirus expression and Dr. William McKinstry (SVIMR) for providing C3b for these experiments.

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J. Biol. Chem. 2001, 276:6747-6754. doi: 10.1074/jbc.M007495200 originally published online October 31, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007495200

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