Prediction from sequence comparisons of residues of factor H involved in the interaction with complement component C3b

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The amino acid sequence of the region of bovine factor H containing the C3b binding site has been derived from sequencing overlapping cDNA clones. A cDNA sequence encoding 669 amino acids was obtained. Like human and mouse factor H the sequence can be arranged into a number of internally homologous units (CPs), each of which is about 60 amino acids long and is based on a framework of four conserved cysteine residues. Bovine factor H is of the same molecular mass as human and mouse factor H, and is therefore likely to be composed of 20 contiguous CPs. Comparisons with human and mouse factor H indicate that the partial bovine sequence encodes CPs 2–12 inclusive of bovine factor H. Bovine factor H binds to human ammonia-treated C3 (causing thiolester cleavage) [C3(NH₃)] and promotes the cleavage of human C3(NH₃) in the presence of

bovine factor I. Other studies indicate that CPs 2–5 of human factor H encompass the C3b binding and factor I cofactor activity site. Multiple sequence alignments of human factor H, mouse factor H (which also interacts with human C3b) and bovine factor H with CP modules whose structures have been determined experimentally, have been used to predict residues in the hypervariable loops of CPs 2–5 and to identify residues of potential importance in human C3 binding and factor I cofactor activity. Leu-17 and Gly-20 of CP 2, Ser-17, Ala-19, Glu-21, Asp-23 and Glu-25 of CP 3 and Lys-18 of CP 4 are all conserved between the three species. It may be that CPs 3 and 4 interact with C3(NH₃) directly, whilst CPs 2 and 5 maintain the correct orientation for CPs 3 and 4 to interact.

INTRODUCTION

Factor H is an abundant plasma glycoprotein. Human factor H has a molecular mass of 155 kDa and contains about 12% carbohydrate [1,2]. A clearly defined role of factor H is the regulation of C3 turnover in the alternative complement pathway [3,4]. Binding of factor H to C3b prevents the interaction of C3b with factor B [5] and C5 [6], and displaces the Bb fragment of factor B from the active C3 convertase and C5 convertase of the alternative pathway [7]. Factor H also acts as a cofactor for the serine protease factor I which cleaves the 108 kDa alpha' chain of C3b into 68 kDa, 43 kDa and 3 kDa fragments, thus converting C3b into the major opsonic form, iC3b [8].

The structure of factor H from human and mouse is known at the cDNA and amino acid sequence level [2,9]. Factor H has been isolated from other species (rat, guinea pig, rabbit, pig), but no, or little sequence has been reported [10–13]. A factor H-like protein (130 kDa) has been characterized in sand bass and the cDNA sequence obtained [14]. Factor H is arranged into a number of contiguous homologous repeating units called CPs (complement control protein modules, also sometimes termed SCRs or sushi domains) [15]. In human and mouse factor H there are 20 and in the sand-bass protein 17 CPs. Each CP is approx. 60 amino acids long and has a framework of four conserved cysteines, disulphide linked together in the pattern Cys1–Cys3 and Cys2–Cys4.

Tertiary structure determinations using two-dimensional proton NMR have been used to solve the structure of human

factor H CPs 5, 15 and 16 (H–5, H–15, H–16), and the double domain of H 15+16 [16–19]. Results indicate that each module is composed mainly of beta strands and the N- and C-termini are at opposite ends of the long axis of the module. Three external loops protrude from the hydrophobic core. Variations in sequence length between CPs are accommodated within these loops. The loop most variable in sequence composition and length is known as the 'hypervariable' loop and it is believed to form the most likely site for interaction with ligands. The core tertiary structure between CPs is highly conserved, even though the extent of amino acid identity between them may be low. It is therefore feasible to model the core structures of all CP modules on the basis of existing experimental structures. This has been done for the fifth CP module of β_2 glycoprotein 1 [20] and for the N-terminal modules of complement receptor type 2 (CR2) [21].

More than 150 examples of CP modules are known, many occurring in complement proteins including the other control proteins CR1, C4bp, DAF, MCP and CR2, in C1r and C1s, C2, factor B, C6 and C7, and in non-complement proteins including the selectins, β_2 -glycoprotein 1 and the interleukin-2 receptor [22–24]. Ligand-binding sites spreading over several CP modules seem to be emerging as a common feature amongst proteins composed of CPs. In human CR1 for instance, 4 CP modules form the ligand-binding site [25] and in mouse C4bp, CPs 1–3 are required for C4b binding [26].

Previously the C3b binding and cofactor activity site on factor H was localized to the first 5^{1/2} CP modules [27]. More recently, enzymic fragmentation of factor H coupled with the use of

Abbreviations used: CP, complement control protein module; CR1(CR2), complement receptor type 1, type 2; C3(NH₃), ammonia-treated C3 causing thiolester cleavage, functionally equivalent to C3b; pC3(NH₃), glutaraldehyde-polymerized C3(NH₃); C4bp, C4b-binding protein; DAF, decay acceleration factor; LHR, long homologous repeat; MCP, membrane cofactor protein; PVDF, poly(vinylidene difluoride).

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monoclonal antibodies raised against factor H and analysis of recombinant modules of H have localized the C3b binding and cofactor activity site to modules 2–5 [28,29]. This is consistent with hydrodynamic data which indicate that factor H is a long extended molecule in solution [30], and therefore an extended binding site can readily be accommodated. Biophysical data indicate the length of C3b corresponds to the length of four CP modules [31]. Four binding sites on C3b for factor H, widely separated in the primary sequence of C3b, have been tentatively identified [32–35].

In this study it is shown that bovine factor H can directly bind to human C3(NH₃) and act as a cofactor in its breakdown by bovine factor I. It has previously been reported that mouse factor H and the sand-bass factor H-like protein can interact with human C3b [36, 37]. The cDNA and derived amino acid sequence of CP modules 1^{1/4}–12 inclusive, encompassing the C3b-binding site region of bovine factor H is presented. A multiple-sequence-alignment program has been used to compare the bovine factor H sequences with the sequences of CPs from human and mouse factor H and the factor H-like protein from sand bass. The results have permitted a prediction of residues likely to be involved in the interaction of human factor H with C3b.

MATERIALS AND METHODS

Purification of complement components

All human complement components were prepared from human plasma. C3 was purified using the procedure of Dodds [38]. Human factors H and I were purified as described by Sim et al. [39]. Trace contaminants were eliminated from C3 and factor H preparations (in 10 mM potassium phosphate, 0.5 mM EDTA, pH 7.2), using FPLC[®] on a Q Sepharose column, utilizing a gradient of increasing salt strength to elute the bound protein. Purified bovine factor H was a gift from Miss V. Perkins (MRC Immunochemistry Unit, Oxford).

Antibodies

Rabbit polyclonal antiserum was raised against factor H purified as described above. Immunoglobulins were purified from rabbit antiserum using a triple sodium sulphate precipitation [40].

Inactivation of C3

Purified C3 was made 0.1 M with respect to NH₄HCO₃, pH 8.5, and incubated for 1 h at 37 °C, which resulted in cleavage of the thiolester [41], converting C3 into a form which is cleaved by factor I in the presence of factor H. This form is referred to as C3(NH₃), and is functionally similar to C3b.

Chemical cross-linking and radioiodination

Cross-linking of C3(NH₃) was carried out in 5 mM potassium phosphate/0.5 mM EDTA, pH 7.2, using an 80-fold molar excess of glutaraldehyde over C3(NH₃) for 2 h at room temperature. The reaction was quenched by addition of 0.1 vol. of 1 M Tris/HCl, pH 7.5. Samples were subjected to gel filtration (PD-10: Sephadex G-25; Pharmacia) to remove Tris/HCl and excess glutaraldehyde. Radioiodination of both cross-linked and non-cross-linked C3(NH₃) and factor H with Na¹²⁵I (Amersham) was carried out using the Iodogen method [42]. Free iodine was removed using a gel filtration column (PD-10) which had been presaturated with BSA in 10 mM potassium phosphate, pH 7.2. The specific radioactivity of the radioiodinated proteins was between 1 × 10⁶ and 10 × 10⁶ c.p.m./μg. For the C3(NH₃) sample which had been cross-linked [pC3(NH₃)], the percentage of

protein in an oligomeric form was assessed by analysing the sample on SDS/PAGE, followed by autoradiography and quantification of radioactivity in the dried gel. In the case of cross-linked C3(NH₃), 70–80 % of the protein in the sample was in an oligomeric form with a molecular mass greater than 450 kDa, and no monomeric C3(NH₃) was seen. The proteins were made 2 mM with respect to iodoacetamide after iodination, in order to block any free SH groups.

SDS/PAGE and Western blotting

The Laemmli system [43] was used for SDS/PAGE analysis. Samples were analysed in both the alkylated, and reduced then alkylated forms [44]. For examples which were to undergo ligand blotting, no urea was added to the sample buffer. Gels were stained using Coomassie Blue [44]. Electrophoretic transfer of bovine factor H to Immobilon P membranes [poly(vinylidene difluoride) (PVDF), Millipore, Bedford, MA] wetted briefly with methanol, was carried out using an LKB horizontal blotter with 0.039 M glycine, 0.048 M Tris, 0.0375 % (w/v) SDS transfer buffer for 2.5 h at 0.8 mA/cm². After transfer, sites were blocked with PBS/0.1 % (w/v) Tween 20/20 mg/ml BSA for 40 min at room temperature. Purified rabbit polyclonal anti-(factor H) antibodies at a final concentration of 10 μg/ml in PBS and 0.1 % (w/v) Tween 20 were applied and incubated for 1 h at ambient temperature. After washing five times for 10 min with 150 ml of the same buffer, the secondary antibody (alkaline-phosphatase-conjugated anti-mouse or anti-rabbit IgG; Sigma) was applied. The incubation and washing steps were the same as for the primary antibody. Colour development was carried out using Sigma Fast NBT/BCIP tablets. Ovalbumin and molecular mass markers were used as non-specific binding controls. The molecular mass markers (Sigma) were: human α₂-macroglobulin (180 kDa); β-galactosidase from *Escherichia coli* (116 kDa); fructose-6-phosphokinase from rabbit muscle (84 kDa); pyruvate kinase from chicken muscle (58 kDa); fumarase from porcine heart (48 kDa); lactate dehydrogenase from rabbit muscle (36 kDa); triosephosphate isomerase from rabbit muscle (26.6 kDa).

Assay for breakdown of C3(NH₃)

Breakdown of C3(NH₃), which is dependent on the activities of factors H and I, was measured as described by Sim and Sim [45]. Cleavage of human C3(NH₃) in dilutions of whole human or bovine serum were analysed by the addition of 50000 c.p.m. of [¹²⁵I]C3(NH₃) per sample, starting at a dilution of serum in PBS of 1:8 for human serum and 1:10 for bovine serum. Soya-bean trypsin inhibitor (2 μg), was included to prevent non-specific binding of radioactivity to surfaces and to inhibit trace proteases. As a control, rabbit polyclonal anti-(human factor H) (purified IgG) at a 5-fold molar excess of specific antibody over the estimated quantity of factor H in each sample (assuming that approx. 1 % of the total antibodies present were specific) was preincubated with the serum prior to addition of [¹²⁵I]-labelled human C3(NH₃). Additionally, factor H with [¹²⁵I]-labelled human C3(NH₃) alone was used as a 'no cleavage' control.

Ligand blotting with [¹²⁵I]-radiolabelled cross-linked human C3(NH₃) [pC3(NH₃)]

The [¹²⁵I]pC3(NH₃) was diluted to 400000 c.p.m./ml in 5 mM potassium phosphate/0.1 % Tween 20/20 mg/ml BSA, pH 7.2, and incubated (1 ml/5 cm² of membrane) with the PVDF-bound bovine factor H, both alkylated and reduced then alkylated, for 1 h at room temperature. After washing five times with 150 ml of 5 mM potassium phosphate/0.1 % Tween 20, pH 7.2, the blots

Figure 1 The effect of bovine factor H on the cleavage of human C3(NH₃)

(a) A twofold serial dilution of bovine serum, 50 000 c.p.m. of [¹²⁵I]C3(NH₃) was added to each reaction vessel with 2 µg of soya-bean trypsin inhibitor to prevent non-specific binding of radioactivity to surfaces. In each experiment, 10 µl of diluted serum in PBS was added to 10 mM potassium phosphate/0.5 mM EDTA, pH 7.2, final volume 50 µl and incubated for 2.5 h. Lanes 1–6, twofold dilutions of bovine serum starting with a dilution of 1:10 in lane 1; lane 7, rabbit polyclonal anti-(human factor H) antibodies preincubated with serum prior to the addition of C3(NH₃). (b) Human serum treated similarly, starting with a serial dilution of 1:8 in lane 1; lane 7, rabbit polyclonal anti-(human factor H) antibodies preincubated with serum prior to the addition of C3(NH₃); lane 8, C3 + I alone. (c) A comparison of the results obtained in (a) and (b). The results are means for two experiments. Note: kD = kDa.

were dried and subjected to autoradiography. Molecular-mass markers and chicken ovalbumin were used as non-specific binding controls.

Microtitre plate binding assays using ¹²⁵I-labelled human PC3(NH₃)

Human and bovine factor H and ovalbumin were coated on to microtitre plates (NUNC, Copenhagen, Denmark) in 0.1 M sodium bicarbonate, pH 9.0, at a concentration of 50 µg/ml and 100 µl/well, overnight at 4 °C. Unbound protein was removed by aspiration and the plates were blocked for 1 h at room temperature using PBS/0.1% (w/v) Tween 20/5 mg/ml BSA. There was no significant difference between human and bovine factor H binding to the plates as judged by binding of ¹²⁵I-labelled samples of bovine and human factor H. The plates were washed five times with 5 mM potassium phosphate, pH 7.2 (200 µl/well) at room temperature and incubated with [¹²⁵I]pC3(NH₃) in 5 mM potassium phosphate/10 mg/ml BSA, pH 7.2, for 30 min. After removing unbound pC3(NH₃) and washing with 5 mM potassium

Figure 2 pC3(NH₃) binding to factor H

(a) Incubation of [¹²⁵I]pC3(NH₃) with bovine and human factor H attached to PVDF membranes. A total of 400 000 c.p.m./ml of [¹²⁵I]pC3(NH₃) was incubated with membrane-attached bovine and human factor H for 1 h at ambient temperature. Lanes 1 and 4, human factor H reduced and non-reduced respectively; lanes 2 and 5, bovine factor H reduced and non-reduced respectively; lane 3, ovalbumin and molecular-mass markers as non-specific binding controls. Note: kD = kDa. (b) Binding of serial dilutions of pC3(NH₃) to bovine (□) and human (◆) factor H attached to microtitre plate wells. The experiment was performed in triplicate and the mean of the results is shown. Binding to ovalbumin was subtracted in order to obtain a value for specific binding.

phosphate, pH 7.2, bound pC3(NH₃) was removed by incubating with 4 M NaOH (120 µl/well) at room temperature for 30 min and radioactivity was measured (LKB-minigamma counter; counting efficiency 68%). In all cases, binding of [¹²⁵I]pC3(NH₃) to ovalbumin was used as a non-specific binding control. A final value for the radioactivity bound to factor H was derived by subtracting the radioactivity bound to ovalbumin from the initial counts. The radioactivity due to non-specific binding did not exceed 5% of the total radioactivity.

Library screening and selection of cDNA clones

A bovine liver cDNA library in λgt11 (Clontech, Palo Alto, U.S.A.; Cat. no. BL1024b) was plated on to agar, 1 × 10⁷ plaques in all, and screened using the human factor H cDNA clone B38-1 as the probe [2]. B38-1 is 1644 nucleotides in length and contains sequences corresponding to the first seven CPs of human factor H. The B38-1 cDNA insert was excised from the pAT 153 vector using *Bam*H1/*Cla*I double-restriction digestion, was separated from the plasmid by electrophoresis on low-gelling-temperature agarose and eluted from the gel. The probe was labelled with ³²P using a random-prime-labelling method. Hybridization was performed at 42 °C for 16 h, followed by washing at 55 °C. Nine positive plaques were replated on to agar

(a)

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V S G S P H L A E G N Q F E Y G A K V V
GTTTCTGGTTCTCTCATCTTGGCGAAGAAATCAGTTGAATATGGTGCAAGGTTGTT
10      20      30      40      50      60
Y T C D E G Y Q M V G E M N F R E C D T
TATACGTGTGATGAGGGGTATCAGATGGTGGTGAGATGAATTTCCGTGAATGTGACACA
70      80      90      100     110     120
N G W T N D I P I C E V V K C L P V T E
AATGGATGGACCAATGATATTTCCCATATGTGAAGTTGTTAAGTGTATACAGTGACAGAA
130     140     150     160     170     180
P E N G K I F S D A L E P D Q E Y T Y G
CCAGAGAATGGAAAATTTTCAGTGATGCCCTGGAACAGACCAAGAATATACCTATGGA
190     200     210     220     230     240
Q V V Q F E C N S G Y M L D G P K Q I H
CAAGTGGTACAGTTTGAGTGTAATTCAGGTACATGCTGGATGGACCAACAAATACAT
250     260     270     280     290     300
C S A G G V W S A E T P K C V E I F C K
TGCTCAGCAGGTGGTGTGGAGTGCAGAAACCAAAATGTGTAGAAATTTCTGCAAA
310     320     330     340     350     360
P P V I L N G Q A V L P K A T Y K Q N E
CCACCAAGTCATTTAAATGGACAAGCTGTACTTCCAAAGGCCACTTATAAGCAAAATGAA
370     380     390     400     410     420
R V Q Y R C A A G F E Y G Q R G D T V C
CGAGTTCATACAGATGTGCGACCGGTTTGAATATGGTCAGAGAGGAGATACAGTATGC
430     440     450     460     470     480
T K S G W T P A P T C I E I T C D P P R
ACTAAATCTGGATGGACTCCAGCTCTACTTGTATAGAAATAACATGTGATCCTCCTCGT
490     500     510     520     530     540
I P N G V Y R P E L S K Y R G Q D K I T
ATTCCAAACGGTGTGTACAGACCTGAATTAAGCAAAATACAGAGGTCAAGATAAAATCACT
550     560     570     580     590     600
Y E C K K G G F F P E I R G T D A T C T R
TATGAGTGTAAAAAGGTTTTCCTGTAGATCCGTGGAAGTGTGCAACGTGTACACAGA
610     620     630     640     650     660
D G W V P V P R C A W K P C S Y P V I K
GATGGCTGGTACCTGTTCCGAGATGTGCTGGAACCTTGTAGTTTACAGTAATAAAA
670     680     690     700     710     720
H G R L Y Y S Y R G Y F P A R V N Q Q F
CATGGACGGCTATATTATTCATACAGAGGATCTTCCAGCACGTGTAATCAACAGTTT
730     740     750     760     770     780
V Y S C D H H F V P P S Q R S W D H L A
GTCTATAGCTGTGATCATCATTTGTACCTCCTTCAACGTTCTGGGACCACTAGCT
790     800     810     820     830     840
C T A E G W S P E E P C L R Q C I F N Y
TGCACAGCAGAAGGATGGTCTCCAGAAAGCAATGCCTCAGACAATGATTTTCAATTAT
850     860     870     880     890     900
L E N G H N Q H R E E K Y L Q G E T V R
TTGGAATGGACATAACCAACATCGTGAAGAAAAATATTACAGGTTGAACTGTGAAGA
910     920     930     940     950     960
V H C Y E G Y S L Q N D Q N T M T C T E
GTTCACTGCTATGAAGGCTACAGTCTTCAAAATGATCAGAACCAATGACCTGCACAGAG
970     980     990     1000    1010    1020
S G W S P P P R C I R V K T C S K S N I
AGTGGCTGGTCTCTCTCCAGATGCATCCGTGCAAAACATGTTCAAAATCCAATATA
1030    1040    1050    1060    1070    1080
R I E N G F L S E S T F T Y P L N K Q T
AGGATTGGAATGGATTTTGTCTGAATCTACCTTTACGTATCCCTAAATAACAAACA
1090    1100    1110    1120    1130    1140
E Y K C K P G Y V T A D G K T S G L I T
GAATATAAGTGAACACAGGATATGTAACAGCAGATGGTAAGACATCAGGGTTAATTACA
1150    1160    1170    1180    1190    1200
C L K N G W S A O P V C I K S C D R P V

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TGTCGAAAAATGGATGGTCAGCTCAACCTGTATGCATTAATCTCTGTACAGGCCAGTA
1210    1220    1230    1240    1250    1260
F E K A R V K S D G T W F R L N D R L D
TTTGAGAAGGCCAGAGTCAAAAGTGATGGCAGCTGGTTTCAAGGTGAACGACAGGCTGGAC
1270    1280    1290    1300    1310    1320
Y E C V D G Y E N R D G R T T G S I V C
TACGAATGTGTTGATGGGTATGAGAACCAGAGATGACGACCAACAGGCTCCATAGTATGT
1330    1340    1350    1360    1370    1380
G Q D G W S D K A A C Y E R E C S I P E
GGTCAAGACGGCTGGTCTGATAAAGCTGCATGCTATGAAAGAGAATGCAGTATTCCTGAA
1390    1400    1410    1420    1430    1440
M D P Y L N A Y P R K E T Y K V G D V L
ATGGATCCATCTTAATGCTTATCCAGGAAAGAAACATATAAAGTTGGAGATGTGCTG
1450    1460    1470    1480    1490    1500
K F S C S Q G R I M V G A D S V Q C Y H
AAATTTCTCTCGAGTCAAGGCCGTATTATGGTTGGAGCAGATTCAAGTTCAGTGCTACCAC
1510    1520    1530    1540    1550    1560
F G W S P K L P T C K V K K V K S C A L
TTTGGATGGTCCCCCTAACTTCCAACTGTAAAGTAAAGTAAAGTCTCTGTCTCTA
1570    1580    1590    1600    1610    1620
P P E L P N G K R K E I H K E E Y A H N
CCTCTGAACTTCTTAATGGGAAAGGAAAGAAATACACAAAGAAAGATGCACACAAT
1630    1640    1650    1660    1670    1680
E V V E Y A C N P K F L M K G S H K I Q
GAGGTCTGGAATATGCTTGAACCCCAATTTCTGATGAAGGGTTCTCATATAAATTCAG
1690    1700    1710    1720    1730    1740
C V D G E S T A L P V C I E E E R T C E
TGTTGTGATGGAGAATCCACAGCTTTACCTGTGTATTGAGGAGGAGCGTACCTGTGAA
1750    1760    1770    1780    1790    1800
I S D L D H G D V K P S V P P I H H G D
ATATCTGACCTTGATCAGGCGATGTCAAGCCTTCTGTCCCTCCCATTCACATGGAGAC
1810    1820    1830    1840    1850    1860
S V E F S C R E A F T M I G P R F I T C
TCGGTTGATGTCAGTTCAGAGAGAAGCATTAACATGATTGGACCAAGATTATACAGTGT
1870    1880    1890    1900    1910    1920
I S G E W T Q P P Q C I A T D E L R K C
ATTAGTGGAGAGTGGACCCAGCTCCTCAGTGCATTGCAACAGATGAACATAGGAAGTGT
1930    1940    1950    1960    1970    1980
K G S T L F F L L
AAAGGGTCAACATTATTTTCTCTCTGG
1990    2000

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(b)

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CP 2      VSGSPHLAE GNQ E EYQAKVYVTCDEGQXOMVGMENFRE CDING WINDIRICE
CP 3      VVKQLPV TEPENGKIFSDA LEPDOEX TYGOVVOEENSGXIM LDGPKQI H QSAGGVMSAETKCV
CP 4      EIF C KP EVLINGQAVLPK A T Y KNERVQYRGAAGEYGO RGDV V CTXSG NI PAETCI
CP 5      EIT C DP ERIPNGVYRPEL S K Y RQDKITYETCKKGE FPEIRGTD ATCTRDG HV PVERCA
CP 6      WKPCSY EVLKHRLYY SYRG XFPARVNOQFVSGDHHEVPPSQRSWDHQLACTARQ NS PEEPQ
CP 7      LRQCIF NYLENGHNOHRE E K Y LOGETVYRHHVCPGYSLONDNTM T CTESG NS PPERCI
CP 8      RVKTSKSNIRIENGFLSEST F T Y PLNKQTEYKPKGVYADNTGKSLITLKNK NS AQEVCI
CP 9      KSCDRP V FEKA RVKSD G T WE RINDRLDQECVQXENRDGRTTGSIVCGODG NS DKAQCY
CP 10     ER E C SI REMDPYLNAYPR KET Y KVEDVLKESCSQGR I MVGADSV Q CYHFG NSPKLETCKV
CP 11     KVKSKALP RELPNGKRKEIH KEE Y ANNEVVEYACNPKEI MKGSHKI Q QVD GEST ALEVCI
CP 12     EEERTC EI SDLDHGVKPSV PP I HHGDSVEFSCREAT MIGPRFI T CIS GENT OPROCI
CP 13     ATDELAKCGSTLFFLL

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-----P-I-NG-----Y-----GE-V-Y-C--CY-----G-----C--WS---P-C
V F ND L F F T

Figure 3 cDNA and the derived amino acid sequence

(a) The sequence derived from the two overlapping clones. The continuous sequence of 2008 bp and the derived amino acid sequence is shown. The sequence has no potential N-linked glycosylation sites. (b) The partial sequence arranged in homologous units, each about 60 amino acids long. Gaps are inserted to maximize the alignment. Highly conserved residues are underlined, including conservative replacements, and a consensus sequence is shown at the bottom. Only the cysteine residues are absolutely conserved in all repeat units.

and rescreened. Two further rounds of screening using the same conditions led to the isolation of three positive cDNA clones. Lambda-phage DNA was prepared [46] and inserts excised using *Eco*R1 restriction digestion. Inserts were separated by electroelution and subcloned into *Eco*R1-cut Bluescript vector (Stratagene, La Jolla, CA, U.S.A.) in TG1 cells.

Sequence analysis

The complete sequence of the cDNA clones was obtained using a T7 sequencing kit (Pharmacia), and the Sanger dideoxy method. Internal sequencing primers were used to obtain the full sequence of both unique clones. Sequencing data were processed using the programs described by Staden [47]. Sequence com-

parison analyses were performed using the AMPS program [48]. A bias of 6 was added to each term of the mutation data matrix and a break penalty of 6 was used. One hundred random runs were performed for each pairwise comparison to allow calculation of mean random scores. The distances, in S.D. units, of pairwise scores from mean random scores for a particular comparison were used to order the sequences.

RESULTS

Bovine factor H functional analysis

There was no cleavage of human [125 I]C3(NH₃) in a mixture of purified bovine factor H and purified human factor I, indicating

Figure 4 Overlapping cDNA clones

The extent of overlap of the clones BH1 and BH2 is shown. Overall they span 2008 bp. The dark-shaded box represents DNA thought to have arisen from a cloning artefact. Short solid lines with arrows indicate the extent and direction of DNA sequence determined from individual sequencing reactions. The long solid lines with arrows represent the total extent and direction of DNA sequence determination.

an incompatibility between the bovine and human factors in C3(NH₃) cleavage (results not shown). Therefore human [¹²⁵I]C3(NH₃) cleavage was tested in bovine serum which contains bovine factor I and cofactors (factor H and C4bp). Bovine serum broke down human [¹²⁵I]C3(NH₃) (Figure 1a) and the pattern was the same as that of C3(NH₃) breakdown in human serum (Figure 1b). In the presence of both human and bovine serum the 116 kDa α chain of C3(NH₃) was cleaved to 76 and 43 kDa products. This suggested that factor I in the presence of a cofactor was responsible for cleavage, as is the case in human serum. A quantitative comparison of the cleavage of human C3(NH₃) by human and bovine serum (Figure 1c) indicated that the extent of breakdown of human C3(NH₃) by bovine serum was approx. 25% greater than by human serum. It may be that bovine factor H is more abundant in serum than human factor H. Rabbit polyclonal anti-(human factor H) antibodies significantly inhibited C3(NH₃) cleavage by both human and bovine sera (approx. 85–90%) (Figures 1a and 1b, lane 7), again indicating that factor H was the predominant cofactor involved, although weak cross-reactivity of anti-factor H with bovine C4bp could not be ruled out. More conclusive evidence that bovine factor H bound to human C3(NH₃) was obtained from ligand blotting (Figure 2a). Experimental details are given in the figure legend. Radiolabelled pC3(NH₃) clearly binds to both human and bovine factor H, either reduced or unreduced. Bovine factor H contains an additional band of 100 kDa which binds pC3(NH₃). Examination of bovine factor H by SDS/PAGE followed by Coomassie Blue staining revealed that approx. 30% of the bovine H was cleaved to a form containing two disulphide-linked fragments of apparently 100 and 60 kDa. N-terminal sequencing of the 100 kDa band was kindly performed by Mr. A. C. Willis (MRC Immunochemistry Unit). This fragment corresponded to the N-terminus of bovine factor H, as judged by its similarity to human factor H. The N-terminal 16 amino acids of bovine factor H are:

EDCKEPPPRKETEILS

Bovine factor H attached to microtitre wells exhibited approx. 50 % of the binding of an equivalent amount of human factor H when incubated with fluid-phase human [^{125}I]pC3(NH_2) (Figure 2b).

[illegible]

Figure 5 Homology comparisons of individual CPs of human, mouse and bovine H

(a) Homology comparisons between CPs 2–12 of human, mouse and bovine factor H. Gaps are inserted to maximize the alignment. Identities are marked *; conservative replacements are marked with a full stop and underlined; % identity (%) and % identity including conservative replacements (+ CR) for each CP are indicated at the end of each line. Abbreviations used for the proteins are: H, human; M, mouse; B, bovine. Data was compared using the AMPS multiple-sequence-alignment program [48].

cDNA sequence

Two unique clones, BH1 and BH2, encoding 11 CPs (Figure 3) of bovine factor H, were isolated and sequenced. A third clone, BH3, was identical in size and 5' and 3' sequence to BH2 and was therefore not sequenced further. Figure 4 shows the extent of overlap of the two clones, BH1 and BH2, and the extent to which the entire coding region was sequenced in both directions.

Clone BH1 contained 912 nucleotides and BH2 1506 nucleotides. There was a 241-nucleotide overlap between the two clones; nucleotides 75–314 of BH2 overlapped with nucleotides 673–912 of BH1 and the regions of overlap between the two clones corresponded exactly. The first 74 nucleotides at the 5' end of BH2 did not overlap with BH1, and this segment may have arisen from a cloning artefact. Likewise, the first 96 nucleotides at the 5' end of BH1 showed no sequence identity with known factor H sequences from other species and are likely to have arisen from a cloning artefact. No sequence polymorphisms were detected during sequencing.

Amino acid sequence

The complete cDNA sequence and derived amino acid sequence from the two overlapping clones is shown in Figure 3(a). Together they encode 669 amino acids of bovine factor H sequence. Figure

Figure 6 Multiple sequence alignments

(a) Multiple sequence comparison of human, mouse and bovine factor H CPs 2–12 inclusive and their alignment with H–5, H–15 and H–16 sequence. Sand-bass factor H-like protein and human and bovine CPs 14–20 are not shown. The abbreviations used for the proteins are: HR, human repeat (CP); BOVR, bovine repeat (CP); MR, mouse repeat (CP). The sequences in the hypervariable loops of H–5, H–15 and H–16 are underlined as are the sequences predicted to be in the hypervariable loops of CPs 2–12 of human, mouse and bovine factor H. The sequences are in order of those showing the greatest homology to those showing the least. Missing numbers are from sand-bass H-like protein and human CPs 14–20. The sequences of the partial CPs 2 and 13 have

3(b) shows the sequence arranged in units showing internal homology, each of about 60 amino acids long, based on four conserved cysteines and highly conserved asparagine, glycine, proline, phenylalanine and tryptophan residues. Gaps are inserted to maximize alignment. A consensus sequence of highly conserved residues is shown at the bottom of Figure 3(b) and this arrangement of homologous repeating units is also found in factor H from other species [2,9,14].

Based on the sequence Asn-Xaa-Ser/Thr there are no potential sites for asparagine-linked glycosylation in the region sequenced (Figure 3). Human factor H possesses three potential sites of N-linked glycosylation in the homologous region (CP 2–12 inclusive) (2); one is on CP 4 and another on CP 12 which are unoccupied; the third is on CP 9, which is occupied. Mouse factor H has two glycosylation sites in the homologous region, one on CP 11 and one on CP 12. Only the glycosylation site on CP 12 is conserved between mouse and human, and it is not too surprising that no glycosylation sites were found in the partial bovine sequence.

Bovine, mouse and human factor H, taken together, share 50 % identity, or 58 % identity if conservative replacements are taken into account. A comparison of individual CPs can be seen in Figure 5. Over this region of sequence, mouse and human factor H are 61 % identical (68 % if conservative replacements are included). The corresponding values for identity, including conservative replacement, are 67 % for bovine–mouse and 66 % for bovine–human.

Sequence comparison analyses

Figure 6(a) shows a section of the multiple sequence alignment of human, mouse and the partial bovine factor H sequence from CPs 2–12 inclusive. Sand-bass factor H-like sequence and the sequence encoding CPs 14–20 of human and mouse factor H were also compared (results not shown); thus a total of 69 CP sequences were compared. The regions of human and mouse factor H which show most identity with the partial bovine sequence are CPs 2–12 inclusive. As bovine factor H has a similar molecular mass (158 kDa, when reduced, by SDS/PAGE) to human factor H (155, when reduced, by SDS/PAGE) bovine factor H would be expected, like human and mouse factor H, to be composed of 20 CPs. The conservation of core tertiary structure between CPs [19] indicates that it is feasible to model the core structures of all CP domains on the basis of the existing experimental structures. In this study the sequences of human, mouse and bovine factor H CPs 2–12 were compared with those of H–16, H–15 and H–5, whose structures have been solved by two-dimensional proton NMR [16–19]. Figure 6(b) shows a ribbon diagram of the tertiary structure of human factor H CP 16 (H–16). The upper hypervariable loop shows little structural organization and most variation in sequence composition and length when compared with other CPs, and is thus thought to be involved in the activities of proteins containing CP modules. For H–16, the hypervariable loop stretches over residues 17–22. In the case of H–15, a 3 amino acid insertion occurs over the hypervariable loop region; residues 17–25 form the loop. In the case of H–5, residues 17–20 comprise the hypervariable loop.

As it has been shown that both bovine (Figures 1 and 2) and mouse factor H interact with human C3(NH₃) [36] and that the

Table 1 Conserved residues in the hypervariable loops of CPs 2–12 inclusive of human, mouse and bovine H

H, human; M, mouse; B, bovine H.

CP	Estimated residues in hypervariable loop	Identities (I) or conservative replacements (CR)*
2	17–22	Leu-17, Gly-20
3	17–25	Ser-17, Ala-19, Glu-21, Asp-23, Glu-25
4	17–20	Lys-18
5	17–20	—
6	17–22	Arg-21
7	16–19	—
8	17–21	Glu-17, Ser-18,
9	15–19	Thr-18, Try-19
10	17–21	(Arg-18 M,B; Lys H)*, Lys-19, (Glu-20 M,B; Asp-20 H)
11	18–24	Lys-22, Glu-24
12	17–22	Ser-19, Pro-21, Pro-22

C3(NH₃) binding and cofactor activity site on human factor H was comprised of CPs 2–5 [28,29], cross-species sequence comparisons between the hypervariable loop regions of CPs 2–5 of human, mouse and bovine factor H were carried out in order to predict residues of potential importance in C3(NH₃) binding and factor I cofactor activity. In predicting the residues in the hypervariable loops, residue 17 was taken as the N-terminal boundary of the loop, except where it was outside the boundary created by residue 17 of CP 5 (the smallest loop) and CP 15 (the largest loop). In this case the 5' boundary of each loop was defined as being the residue aligning with residue 17 of CP 5 or CP 15, whichever was the closer to residue 17 in the sequence being modelled. Sequences in the hypervariable loop regions of sand-bass factor H were not used for cross-species sequence comparisons, as sand-bass plasma possessed only 2 % of the human C3b cleavage activity of human plasma and no further quantification of the interaction between human C3 and sand-bass H was reported. The results of loop sequence comparisons are summarized in Table 1.

Half of the conserved residues in the hypervariable loops of CPs 2–5 of human, mouse and bovine factor H were of a charged nature, which is consistent with experimental evidence showing that the factor I cofactor activity [45] and factor H binding to C3b attached to zymosan [49] exhibit strong pH and ionic-strength dependencies.

Conserved residues in the hypervariable loop region of CP 2 are Leu-17 and Gly-20; no charged residues are conserved. For CP 3, five of the nine residues in the hypervariable loop, namely Ser-17, Ala-19, Glu-21, Asp-23 and Glu-25, are conserved between human, mouse and bovine factor H. Any or all of these residues are candidates for the interaction of factor H with C3(NH₃). These results are consistent with a recent study carried out in this laboratory which indicated that CP 3 forms an essential part of the C3(NH₃) binding site on factor H [23]. The only residue conserved in the hypervariable loop region of CP 4 between the three species factor H proteins is Lys-18 and it seems

been aligned visually, as the AMPs program can only compare whole CP sequences. (b) A ribbon diagram of the tertiary structure of H–16 along with the amino acid sequences of H–16, H–15 and H–5 whose structures have been determined experimentally [22]. The N-terminus is on the left, and the C-terminus is on the right. Broad arrows represent beta strands, narrow sections indicate loops and areas of non-standard secondary structure. The upper loop is the hypervariable loop. The remaining human factor H sequence and mouse and bovine factor H sequences were aligned to H–5, H–15 and H–16 using multiple sequence alignment as noted above. The hypervariable loops of H–5, H–15 and H–16 are underlined with a filled rectangle, and the regions of primary sequence of H–16 which correspond to tertiary structural elements on the ribbon diagram are indicated.

likely that this charged residue is involved in the interaction with C3(NH₃).

In CP 5 there are no conserved residues in the hypervariable loops of human, mouse and bovine factor H. It may be that CP 5 merely orientates CP 4 so that CP 4 can interact with C3(NH₃) via Lys-18.

It was desirable to compare residues in another region within each of CPs 1–5. Site-directed mutagenesis experiments using a soluble secreted recombinant form of CR1 (CR1–4) implicated the C-terminus of CP 2 of LHR B as being of importance in conferring C3b binding specificity on CR1 [50]. The amino acids in question span the C-terminal amino acids downstream of the last conserved tryptophan of each CP module. It is apparent from Figure 6(a) that the C-terminal amino acids of each CP downstream of the final tryptophan are highly conserved when human, mouse and bovine factor H are compared. It is extremely unlikely that all the conserved residues within this region are required for ligand binding and identification of functional residues was not possible from sequence comparisons.

DISCUSSION

In this study it has been shown that bovine factor H can bind directly to human C3(NH₃) (Figure 2). A mixture of bovine factor H and human factor I could not cleave human C3(NH₃), although bovine serum could (Figures 1a and 1c). These results indicate that bovine factors H and I can interact with human C3(NH₃) and suggest that there is an incompatibility between bovine factor H and human factor I which prevents human C3(NH₃) cleavage. This in turn suggests that there may be a direct interaction between factors H and I. Recent studies carried out in this laboratory have demonstrated direct binding of human factor H to human factor I and vice versa using microtitre plate binding assays and ligand blotting [51]. The conclusion is also in agreement with work carried out on mouse allotypes of factor H which showed differences in their ability to cleave human methylamine-treated C3 in the presence of human factor I, but no difference in their ability to cleave human C3 in the presence of mouse factor I. The conclusion was that the human factor I recognized differences in the mouse factor H allotypes, and therefore there was an interaction between factor H and factor I [36]. Factor H, factor I and C3b bind in a complex with a conformational change occurring in C3b when it binds to factor H [52], which may facilitate factor I binding to C3b. Another conformational change has been suggested to occur in factor I on contact with C3b [53]. It is likely that, in heterologous systems, one or a number of the interactions may not be identical with those in homologous systems, with the consequence that C3b may not be cleaved. A previous study has examined bovine factor H functional activity [54]. It was reported that bovine factor H supported cleavage of the α' chain of bovine C3b by human factor I, indicating that human factor I is compatible with bovine factor H in the cleavage of bovine C3b. This is interesting in light of our findings that bovine factor H and human factor I appear not to be able to co-operate in the cleavage of human C3(NH₃). Cross-species incompatibilities among complement regulatory proteins seem to be commonplace [36,54,55].

A partial cDNA derived amino acid sequence encoding 11 CPs of bovine factor H has been determined (Figure 3) and a multiple-sequence-alignment program has been used to assess the identity with human, mouse and sandbass factor H (Figure 6). Based on the molecular mass of bovine factor H it is likely to be comprised of 20 CPs. From multiple sequence alignments the partial sequence encodes a region from CP 2 to CP 12 inclusive.

Like other proteins which possess CPs, each CP is approx. 60 amino acids long, based on a framework of four conserved cysteine residues (Figure 3b). The partial sequence shows 50 % identity with human and mouse H, or 58 % if conservative replacements are taken into consideration (Figure 5). It is interesting that CP 3 shows the highest degree of conservation between the three species and that the same CP plays a predominant role in C3 binding in human and probably mouse and bovine factor H. Homology to the sandbass factor H-like protein amino acid sequence is much lower. Sand-bass factor H-like activity is contained within a 115.2 kDa protein possessing only 17 CPs.

Sequence comparisons between the hypervariable loop regions of CPs 2–5 (the proposed C3b binding and cofactor activity site on factor H) of human, mouse and bovine factor H have permitted a refinement of the prediction of residues of importance in C3(NH₃) binding and cofactor activity. In CP 3 the residues Ser-17, Ala-19, Glu-21, Asp-23 and Glu-25 are all conserved. These residues are therefore of potential importance in binding human C3 and possibly in factor I cofactor activity. Likewise, Lys-18 in CP 4 is of potential importance. No conserved charged residues exist in the hypervariable loops of CPs 2 and 5. It may be that CPs 2 and 5 serve to orientate CPs 3 and 4 and it is these proteins which interact directly with C3(NH₃). This is interesting in light of the report that in human CR1, CPs 2 and 3 of each LHR alone can bind to C3b. CPs 1 and 4 contribute to, but are not essential, for the interaction [25].

These predictions agree with chemical modification experiments carried out in this laboratory which have indicated the importance of carboxyl and lysine, but not arginine residues in the cofactor activity of human factor H [56]. Arginine residues are highly conserved in a number of CPs, for instance CP 6 (Figure 5). These modules are, however, outside the putative cofactor activity site. It may be that they are important in other factor H functions.

Sequencing of bovine factor H and use of tertiary-structure data have permitted meaningful cross-species sequence comparisons to be made for factor H. The mouse and human sequences alone are too similar to be informative. The identification of potentially important residues in C3b binding can now be tested by mutagenesis.

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