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Localization of the complement-component-C3b-binding site and the cofactor activity for factor I in the 38 kDa tryptic fragment of factor H

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Trypsin treatment of human factor H (H160) [enzyme/substrate ratio 1:100 (w/w), 30 min, 37°C] generated a 38 kDa (H38) and a 142 kDa (H142) fragment linked by disulphide bonds (H38/142). The fragments were purified by reduction with 2-mercaptoethanol, gel filtration on a Sephadex G-200 column and affinity chromatography with monoclonal anti-(factor H) antibody coupled to Sepharose 4B. This monoclonal antibody bound to a site in the 38 kDa fragment. To localize the C3b binding site in factor H we used two enzyme-linked immunosorbent assays (e.l.i.s.a.). For the first test, e.l.i.s.a. plates were coated with C3b; H160, H38/142, H38 and H142 were added, and their binding was monitored by goat anti-(factor H) and peroxidase-labelled rabbit anti-goat antibodies. Only intact factor H bound to the C3b-coated plates. For the second test, e.l.i.s.a. plates were coated with comparable amounts of factor H or its fragments, and C3b was offered at several dilutions. In contrast with the results from the first assay, C3b bound to intact factor H, H38/142 and H38 but not to H142, thus characterizing H38 as the fragment carrying the C3b-binding site. To identify the fragment responsible for the cofactor activity of factor H (cleavage of fluid-phase C3b by factor I), ^{125}I-C3b was incubated with either H38 or H142 and factor I. H142 had no cofactor activity, whereas H38 had the same cofactor function as intact H. To further investigate the relationship between the C3b-binding site and the site of factor H essential for its cofactor activity, we made use of monoclonal antibodies directed against the H38. Those antibodies inhibiting the binding of C3b to H160 also inhibited the cofactor function, whereas those without effect on the C3b binding also did not interfere with the cofactor activity. This suggests that the C3b-binding site and the site essential for the cofactor activity of factor H are both localized in the 38 kDa tryptic fragment of factor H in close proximity or are identical.

Activation of the complement cascade by the classical (Müller-Eberhard, 1968) or the alternative pathway (Müller-Eberhard & Götze, 1972) results in cleavage of the α-chain of C3 into C3a anaphylatoxin and C3b. C3b bound to particles has numerous biological functions, e.g., promotes binding of other molecules by acting as a ligand to C3b-specific cell-surface receptors (Dierich *et al.*,

Abbreviations used: C3b, 180 kDa fragment of the third component in the complement system; factor H, a controlling factor of the complement system; e.l.i.s.a., enzyme-linked immunosorbent assay; ABTS, 2,2′-azino-bis-(3-ethylbenzthiazolinesulphonic acid); Tos-Phe-CH_2Cl ('TPCK'), tosylphenylalanylchloromethane; Dip-F, di-isopropyl fluorophosphate; factor I, factor I (C3b-inactivator) of the complement system; IgG, IgA, immunoglobulins of class G and A; SDS, sodium dodecyl sulphate; MAH, monoclonal anti-H antibodies; SP-, sulphopropyl-; phosphate-buffered saline, 10 mM-sodium phosphate/150 mM-NaCl, pH 7.3.

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1982) and excites continuation of the complement sequence when it is a subunit of the classical C5- or the alternative C3/C5-convertase (for reviews, see Reid & Porter, 1981; Müller-Eberhard & Schreiber, 1980).

The complement-activating activities of C3b are controlled by factor I (C3b-inactivator; KAF), factor H (β1H; C3b-inactivator accelerator), complement receptor type one and decay accelerating factor ('DAF') (Fearon, 1980; Nicholson-Weller *et al.*, 1981). Factor H is a single-chain glycoprotein of about 160 kDa. It was first described by Nilsson & Müller-Eberhard (1965), and its ability to act as a control protein of the complement system was first recognized by Whaley and colleagues (Whaley & Ruddy, 1976*a*,*b*; Whaley *et al.*, 1978). Factor H binds to C3b and prevents the interaction of C3b with factor B and C5, dislodges the Bb fragment of factor B from the active alternative-pathway C3-convertase (C3bBb) and C5-convertase (C3bBbC3b) (Weiler *et al.*, 1976; Pangburn & Müller-Eberhard, 1978; Nagaki *et al.*, 1978; Conrad *et al.*, 1978) and serves as a cofactor for the cleavage of C3b to iC3b by factor I (Pangburn *et al.*, 1977; Harrison & Lachmann, 1980). The proteolytic cleavage of the α-chain of C3b by factor I destroys most of the biological activities of C3b, e.g., the ability to form C3bBb complexes (Conrad *et al.*, 1978). In addition to its regulatory role, factor H binds to lymphocytes, granulocytes and monocytes by means of a receptor (Schmitt *et al.*, 1981; Lambris & Ross, 1982). Some authors have reported that preparations of human factor H contain a partially cleaved form of the factor in which all fragments are linked by disulphide bonds (Harrison & Lachmann, 1979; Gardener *et al.*, 1980). This cleavage has also been shown for guinea-pig factor H (Bitter-Suermann *et al.*, 1981). Trypsin digestion of native factor H mimicks this cleavage and produces fragments with comparable M_r values. The trypsin-treated factor H loses its ability to interact with surface-bound C3b, whereas the cofactor activity remains unchanged or even increases (Hong *et al.*, 1982; Sim & DiScipio, 1982). However, the precise binding site for C3b in factor H and the mechanisms that enable the factor to perform its regulatory functions are unknown. In the present study we treated human factor H with trypsin and purified the resulting fragments. The isolated fragments were tested for C3b binding and for factor I cofactor activity. In addition, monoclonal antibodies against factor H (MAH) were used to localize the fragment carrying the C3b-binding site and the cofactor activity. The present results show that the two functionally relevant activities are localized in the same tryptic fragment of H.

Materials and methods

Complement components

All complement components were prepared from fresh human plasma. C3 was purified as described by Hammer *et al.* (1981) and Nilsson & Müller-Eberhard (1965). In addition, C3 was purified from residual H, C5 and IgG by using anti-(factor H, C5, IgG, IgA)–Sepharose 4B (antibodies coupled to CNBr-activated Sepharose 4B). A modified form of the method described by Sim & DiScipio (1982) was used for the purification of factor H. Factor H pools resulting from DEAE-Sephadex A-50 chromatography were further purified by ion-exchange chromatography on SP-Sephadex C-50 (5 cm × 90 cm column equilibrated in 20 mM-sodium phosphate/0.05% NaN_3, pH 5.9), gel filtration on Sephacryl S-200 (1.6 cm × 70 cm column equilibrated in 10 mM-sodium phosphate/150 mM-NaCl buffer, pH 7.3) and affinity chromatography on anti-(C3, C5, IgG, IgA)–Sepharose 4B (equilibrated in 10 mM-sodium phosphate/150 mM-NaCl buffer, pH 7.3). Factor I was purified as described by Lambris *et al.* (1980). C3b was obtained by tryptic cleavage of C3 as described by Bokisch *et al.* (1969). After stopping the reaction with Dip-F (1 mM), C3b was purified by gel filtration on Sephadex G-100 (equilibrated in 10 mM-sodium phosphate/150 mM-NaCl buffer, pH 7.3). ^{125}I-C3b had a specific radioactivity of (5–5.5) × 10^6 c.p.m./μg of C3b and was prepared with Iodogen (Pierce Chemical Co., Rockford, IL, U.S.A.) by the method of Fraker & Speck (1978) with 1 mCi of $Na^{125}I$ (Amersham Buchler, Braunschweig, Germany)/50 μg of C3b.

Trypsin treatment of factor H and purification of factor H fragments

In all experiments, factor H was digested with Tos-Phe-CH_2Cl-treated trypsin (Serva, Heidelberg, Germany) at an enzyme/substrate ratio of 1:100 (w/w) in 10 mM-sodium phosphate/150 mM-NaCl buffer, pH 7.3 at 37°C. The reaction was stopped by the addition of 1 mM-Dip-F (Sigma, München, Germany). Factor H trypsin-treated for 30 min was used for the purification of factor H fragments. SDS (Serva) was added to a concentration of 0.02% and the mixture was adjusted to 3 M by adding solid urea. Afterwards, 50% 2-mercaptoethanol (Serva, Heidelberg, Germany) was added until its concentration was 0.2% of the total and incubation was continued for 15 min at 37°C. The trypsin-treated factor H was loaded on to a column (90 cm × 2.5 cm diam.) of Sephadex G-200 (equilibrated in 100 mM-NH_4HCO_3/3 M-urea/0.05% NaN_3 buffer, pH 7.0). The column was run at room temperature (average flow rate 20 ml/h) and fractions (3.3 ml each) were collected. The column had

been calibrated previously, under the same conditions, with aldolase (158kDa), bovine serum albumin (66kDa), ovalbumin (45kDa) and chymotrypsinogen A (25kDa) as molecular-mass marker proteins. Binding of MAH1 and C3b to the fractions was then determined by e.l.i.s.a. Each resulting pool was dialysed against 2 × 5 litres of 10mM-sodium phosphate/150mM-NaCl/0.05%-NaN_3 buffer, pH 7.3, and was passed through 6ml of MAH1–Sepharose 4B (2mg of MAH1/ml of Sepharose 4B, equilibrated in the dialysis buffer). The column was washed with 5vol. of the equilibration buffer and bound protein was eluted with phosphate-buffered saline/3M-KSCN (fraction size 1ml; average flow rate 40ml/h). Again the fractions were tested for MAH1 and C3b binding. The resulting pools were dialysed against 2 × 5 litres of 10mM-NH_4HCO_3 buffer, pH 7.0, concentrated 10-fold by freeze-drying and redissolving the protein in 0.1 (original) vol.

Antibodies

Polyclonal anti-H and anti-C3 antibodies were prepared as described by Schulz *et al.* (1984). Peroxidase-conjugated rabbit anti-(goat IgG) and peroxidase-conjugated rabbit antibodies to mouse IgG were purchased from E*Y Laboratories, San Marcos, CA, U.S.A., and Dako, Copenhagen, Denmark. MAH were prepared as described by Schulz *et al.* (1984) from ascites or culture supernatants. In addition they were purified by affinity chromatography on factor H-coated Sepharose 4B (1.6mg of factor H/ml). Bound antibodies were eluted with 3M-KSCN (MAH1, 2 and 3) or with 10mM-sodium phosphate/150mM-NaCl buffer, pH 4.0 (MAH4) and dialysed against 10mM-sodium phosphate/150mM-NaCl buffer, pH 7.3. The monoclonal antibody 89A11, which recognized an unknown antigen, was prepared as described by Schulz *et al.* (1984). Mouse IgG was commercially available (Sigma, München, Germany).

E.l.i.s.a.

Binding of fluid-phase C3b to surface-attached factor H was assayed as follows. (1) Factor H, factor H fragments or fractions containing factor H (20μl/well) were adsorbed to microtitre plates (NUNC, Copenhagen, Denmark) for 2h or overnight at 4°C. (2) The plates were saturated twice with 50μl of phosphate-buffered saline/1% bovine serum albumin (fraction V powder, Sigma)/1% ovalbumin (grade V, Sigma) per well for 15min. (3) A 20μl portion of serially diluted C3b was added and incubated for 30min. (4) The wells were washed twice with 200μl of phosphate-buffered saline/0.05% Tween 20 (Serva), pH 7.5, and saturated again with 50μl of phosphate-buffered saline/bovine serum albumin/ovalbumin for 5min. (5) A 20μl portion of a polyclonal goat anti-C3 antibody (5μg/ml) was added. (6) After 30min, step 4 was repeated, and (7) bound antibody was detected by incubation with 20μl of a 1:500 dilution of peroxidase-conjugated rabbit anti-goat antibody for 30min. (8) The plate was washed twice with 200μl of phosphate-buffered saline/Tween, and once with 200μl of substrate buffer (10mM-potassium phosphate buffer, pH 6.0) and was then developed by the addition of 20μl of the substrate [27.43mg of ABTS (Sigma)/ml of substrate buffer containing 0.025% H_2O_2 (Merck, Darmstadt, Germany)]. Steps (2)–(8) were carried out at room temperature. C3b and the antibodies were diluted in phosphate-buffered saline/bovine serum albumin/ovalbumin.

Inhibition studies with monoclonal antibodies were performed as follows. Factor H-coated plates were saturated (steps 1 and 2) and preincubated with 20μl of the monoclonal antibody for 30min. The plate was washed twice with 200μl of phosphate-buffered saline/Tween and saturated with 20μl of phosphate-buffered saline/bovine serum albumin ovalbumin. The assay was continued as described above, starting with step (3). The interaction of factor H with C3b fixed to an e.l.i.s.a. plate was determined in a modified form of the e.l.i.s.a. described above. The plate was coated with 20μl of C3b (20μg/ml) per well instead of factor H. A 20μl portion of serially diluted factor H was offered (in step 3) and its binding to C3b was detected by a polyclonal goat anti-(factor H) antibody (10μg/ml) (in step 5). Phosphate-buffered saline/bovine serum albumin/ovalbumin was replaced in this assay by phosphate-buffered-saline/bovine serum albumin, pH 7.3; all other conditions and steps were as described above.

Chromatography

All columns and chromatographic materials were purchased from Pharmacia, Freiburg, Germany. Factor H and monoclonal antibodies were coupled to CNBr-activated Sepharose 4B as recommended by the manufacturer.

SDS/polyacrylamide-gel electrophoresis and Western blotting

SDS/polyacrylamide-gel electrophoresis with 5–20% or 9–20% (w/v) gradient gels was applied generally as described by Laemmli (1970). Coomassie Blue was used for staining. The non-reducing sample buffer consisted of 5% (w/v) SDS and 50% (v/v) glycerol (Merck) in 200mM-Tris/HCl (Merck) buffer, pH 7.3. The reducing sample buffer additionally contained 5% 2-mercaptoethanol (Serva). Sample buffer and sample were mixed at a ratio of 1:5 (v/v) and heated up

to 100°C for 2min. M_r standards were purchased from Bio-Rad. Electrophoretic transfer of protein to nitrocellulose membranes (Bio-Rad) was carried out as described by Towbin *et al.* (1979), with a slightly modified buffer [0.2M-glycine/0.025M-Tris/HCl/20% (v/v) methanol/0.01M-$MgCl_2$/0.1% Triton X-100, pH 8.3] and a current of 0.8A was used. The blots were stained by the immunoperoxidase technique (Towbin *et al.*, 1979) with monoclonal antibodies (5 μg/ml of phosphate-buffered saline/bovine serum albumin) or with polyclonal antibody (2 μg/ml of phosphate-buffered saline/bovine serum albumin) as primary antibody and a substrate solution containing 15 mg of orthodianisidine (Sigma) and 30 μl of 30% (v/v) H_2O_2/100 ml of 50 mM-Tris/HCl buffer, pH 7.2. All other chemicals were of analytical grade, purchased from Merck and Serva.

Results

Breakdown of factor H by trypsin

After exposure to Tos-Phe-CH_2Cl-treated trypsin for 1–60 min, unreduced factor H on SDS/polyacrylamide-gel electrophoresis had an apparent M_r of 148000 and remained unchanged for 60 min (Fig. 1*a*). When reduced with 2-mercaptoethanol, factor H had an apparent M_r of 160000 (Fig. 1*b*). The factor H band disappeared within 30 min of trypsin treatment, and the gel revealed two bands with apparent M_r values of 142000 and 38000. The 142 kDa fragment that seemed to be generated via a slightly larger intermediate was subsequently degraded to 93 kDa and 52 kDa fragments. Incubation for a longer time (3h or more) or with more trypsin led to cleavage of the 93kDa fragment into 63 kDa and 32 kDa fragments, and the 38 kDa fragment was cleaved into two fragments with apparent M_r values of 14000–16000 (result not shown).

Interaction of trypsin-treated factor H with surface-bound C3b

On the basis of experiments in which Hong *et al.* (1982) found that trypsin-treated ^{125}I-factor H lost its ability to interact with cell-bound C3b, and our confirmation with the highly sensitive and specific assay developed by Schmitt *et al.* (1981) using agglutination of C3b-coated erythrocytes loaded with factor H (results not shown), we have now achieved the same effect without cells. We developed an e.l.i.s.a. in which polystyrene microtitre plates were coated with C3b (20 μl/well, 20 μg of C3b/ml in 10 mM-sodium phosphate/150 mM-NaCl buffer, pH 7.3). Factor H (25 μg/ml) was treated with trypsin for 30 or 120 min under the conditions described above. The control was factor H similarly incubated with Dip-F-inactivated trypsin (trypsin solution made 1 mM with respect to Dip-F). After 20 μl samples of factor H were serially diluted and offered to the C3b-coated plates, the binding of factor H to C3b was monitored with polyclonal goat anti-(factor H) antibody and peroxidase-labelled anti-(goat-IgG) antibody. After 30 min of

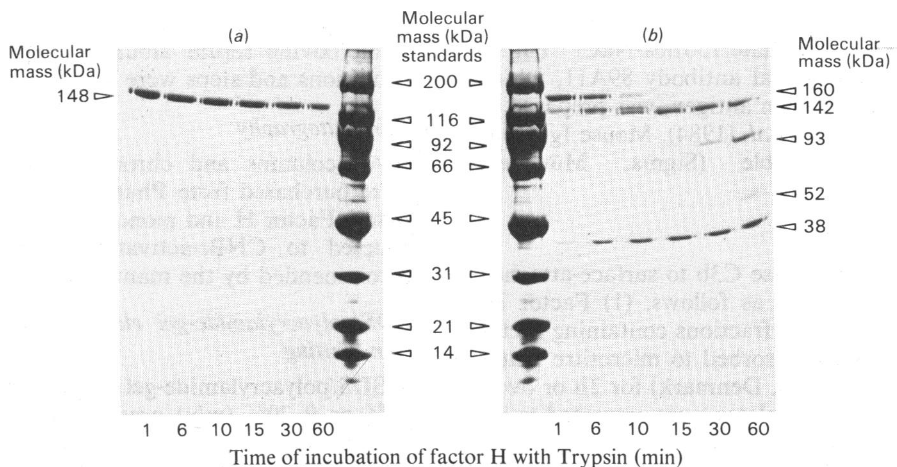

Fig. 1. *Breakdown of factor H by trypsin: analysis of Tos-Phe-CH_2Cl-treated-trypsin digestion [enzyme/substrate 1:100 (w/w)] of factor H on SDS/5–20%-polyacrylamide gradient gels*

Each track contained 20 μg of factor H. (*a*) Samples treated with 1% SDS for 5 min at 100°C; (*b*) samples reduced with 1% 2-mercaptoethanol. The molecular-mass standards were: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase, 92 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soya-bean trypsin inhibitor, 21 kDa; lysozyme, 14 kDa.

trypsin treatment, about 10–15 times more factor H was necessary to obtain the same absorbance as in the control. No factor H bound after 120min of tryptic digestion (Fig. 2*a*).

Interaction of fluid-phase C3b with surface-fixed factor H

The samples of factor H prepared for the e.l.i.s.a. described above were adjusted to 20 μg/ml (Lowry assay; A_{280}). E.l.i.s.a. plates were coated with 20 μl of factor H per well and serially diluted C3b was offered. C3b binding to factor H was detected with polyclonal goat anti-C3b antibody and peroxidase-labelled anti-(goat IgG) antibody. Compared with the rapidly decreasing activity in the foregoing e.l.i.s.a., C3b now bound strongly to the surface-attached trypsin-treated factor H, even when factor H was applied after 120min of tryptic digestion (Fig. 2*b*).

Purification of tryptic factor H fragments

All fractions resulting from the Sephadex G-200 purification of tryptic factor H fragments (see the Materials and methods section) (Fig. 3*a*) were tested in an e.l.i.s.a. with a polyclonal anti-(factor H) antibody and with MAH1, which is directed against the 38kDa fragment of factor H. The binding of MAH1 revealed two peaks corresponding to those in the C3b-binding e.l.i.s.a. (Fig. 3*b*). Next, the fractions within these peaks were pooled (as indicated by horizontal bars), dialysed and were further purified by affinity chromatography with MAH1 (see the Materials and methods section). All resulting pools were analysed by SDS/polyacrylamide-gel electrophoresis on 5–20% gels under non-reducing (Fig. 4*a*) and reducing (Fig. 4*b*) conditions. A comparison of the molecular masses obtained under these conditions showed that the 160kDa band corresponded to 148kDa, the 142kDa to 134kDa, the 93kDa to 88kDa, the 52kDa to 44kDa and the 38kDa to 32kDa in the reduced and non-reduced gels respectively. The molecular masses determined from the reduced gel are used below. Under the conditions described above, tryptic cleavage of factor H resulted in four bands: 142, 93, 52 and 38kDa (track 1). Gel filtration on Sephadex G-200 separated the remaining intact factor H, the 142kDa and the 93kDa fragments (=pool 1; track 2) from the 52kDa and 38kDa fragments (=pool 2; track 5). Affinity chromatography on MAH1–Sepharose 4B retained the uncleaved factor H in pool 1 (track 3) and the 38kDa fragment in pool 2 (track 7), whereas the 142kDa and 93kDa fragments (track 4) and the 52kDa fragment (track 6) passed through. Pool 2 showed an additional 64kDa band in the non-reduced gel (Fig. 4*a*, track 5) that was shown by the immunoblotting technique to be a dimer of the 38kDa fragment and that disappeared under reducing conditions (Fig. 4*b*, track 5).

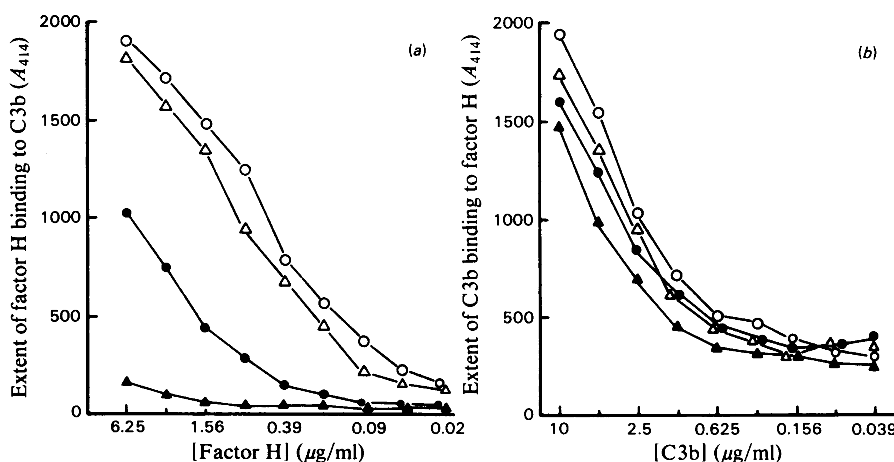

Fig. 2. *Effect of trypsin on the C3b-H interaction*
(*a*) Demonstrates the binding of serially diluted trypsin-treated factor H to constant amounts of C3b fixed to e.l.i.s.a. plates. (*b*) Shows the reverse experiment in which serially diluted C3b was offered to plates coated with constant amounts of trypsin-treated factor H. Factor H was cleaved with trypsin [enzyme/substrate ratio 1:100 (w/w)] for 30 (●) or 120 min (▲) at 37°C. Binding of factor H to C3b (*a*) and C3b to H (*b*) was quantified by using polyclonal antisera, peroxidase-conjugated anti-IgG antibodies and substrate. Factor H incubated with Dip-F-treated trypsin for 30min (○) and 120min (△) at 37°C served as a control.

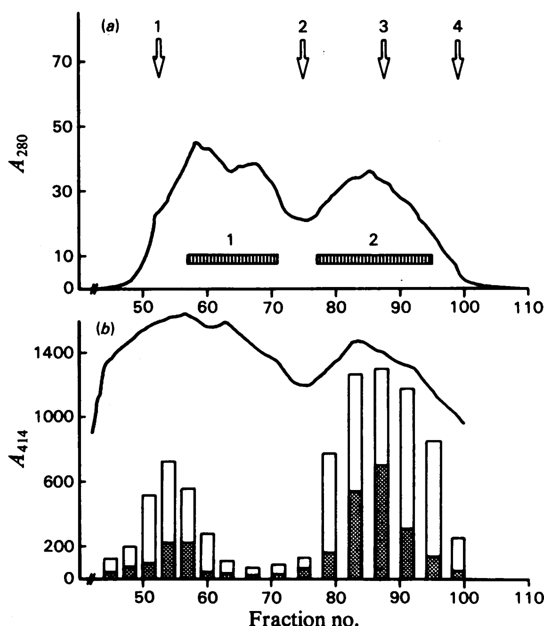

Fig. 3. *Purification of factor H fragments on Sephadex G-200*

(*a*) Gel filtration of trypsin-treated and reduced factor H on Sephadex G-200. Fractions (3.3ml) were collected. The column was calibrated with: 1, aldolase; 2, bovine serum albumin; 3, ovalbumin; 4, chymotrypsinogen A; their elution positions are marked by arrows. Fractions taken for further purification are indicated by horizontal bars (pools 1 and 2). (*b*) Binding of MAH1 (open bars), C3b (shaded bars) and a polyclonal anti-(factor H) antibody (——) to tryptic fragments of factor H eluted from the Sephadex G-200 column (e.l.i.s.a.)

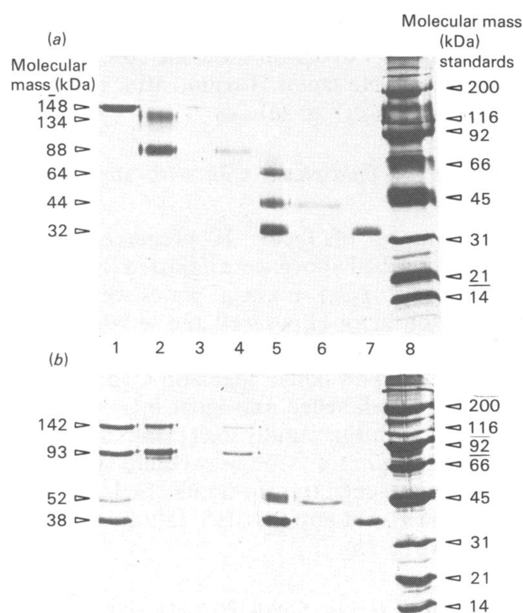

Fig. 4. *Purification of trypsin-generated factor H fragments analysed by SDS/polyacrylamide-gel electrophoresis (5–20% gradient gels) in the absence (a) or presence (b) of 1% 2-mercaptoethanol*

Track 1 contains factor H treated with trypsin at an enzyme substrate ratio of 1:100 for 30min at 37°C. The pools resulting from the Sephadex G-200 gel filtration (see Fig. 3) are shown in track 2 (pool 1) and track 5 (pool 2). Track 3 contains the 3M-KSCN eluate and track 4 the breakthrough of pool 1 after affinity chromatography. Track 6 shows the breakthrough, and track 7 the corresponding 3M-KSCN eluate of pool 2 from MAH1–Sepharose 4B. The same molecular-mass standards as those used in Fig. 1 were run in track 8.

Binding of C3b to factor H fragments

The pools were adjusted to 50μg/ml, and 20μl was used in an e.l.i.s.a. to test C3b binding. C3b did not bind to the isolated 142/93kDa or 52kDa fragments, but bound strongly to the 38kDa fragment, thus characterizing the 38kDa fragment as carrying the C3b-binding site in factor H (Fig. 5). As trypsin cleaved factor H, the 38kDa fragment failed to interact with bound C3b (result not shown).

Cofactor activity of tryptic H fragments in the cleavage of fluid-phase C3b by factor I

As shown in Fig. 6, ^{125}I-C3b contained two bands: the α-chain (115kDa) and the β-chain (75kDa) (track 1). Under the conditions reported by Crossly & Porter (1980) (pH6.5; conductivity 4mS), incubation of ^{125}I-C3b with factor H (track 2) or factor I (track 3) did not change this pattern. Treatment of C3b with both factors H and I caused cleavage of the α-chain into the 68kDa and the 46kDa fragments, then partial cleavage of the 46kDa fragment to a 43kDa fragment (track 4). ^{125}I-C3b treated with the 142/93kDa tryptic fragments of factor H in the absence (track 5) or presence (track 6) of factor I closely resembled the negative controls (tracks 1–3). In addition, incubation of ^{125}I-C3b with the 38kDa fragment and factor I (track 8) produced the same cleavage of the α-chain as in the positive control (track 4). The control without factor I was negative (track 7). These results indicated that the 38kDa tryptic fragment of factor H carried not only the C3b-binding site but also the cofactor activity for the cleavage of fluid-phase C3b by factor I.

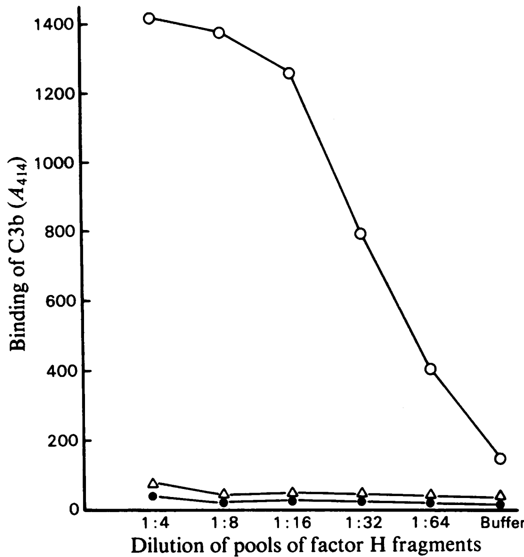

Fig. 5. *Binding of fluid-phase C3b to purified factor H fragments attached to a microtitre plate*

The 38kDa fragment (○) (see Fig. 4, track 7), 142/93kDa fragments (△) (see Fig. 4, track 4) or 52kDa fragment (●) (see Fig. 4, track 6) were attached to an e.l.i.s.a. plate. Serially diluted C3b was offered and its binding was monitored.

Monoclonal anti-(factor H) antibodies: effect on the binding of factor H to C3b and its cofactor activity for factor I

Our foregoing conclusions were confirmed by the use of four MAH (MAH1, 2, 3 and 4) (Schulz *et al.*, 1984). They were tested for binding to factor H fragments with the Western-blotting technique and shown to react only with the 38kDa fragment or its degradation products, but not with the 142kDa fragment (result not shown). The influence of MAH on the binding of C3b to factor H was then tested in an e.l.i.s.a. Compared with the buffer (Fig. 7*a*), neither unrelated monoclonal antibody nor mouse IgG interfered with the binding of C3b. However MAH1, 2 and 4 strongly inhibited the interaction of C3b and factor H, although MAH3 showed only weak inhibition. In the same assay with isolated 38kDa fragment (50 μg/ml, 20 μl/well) used instead of intact factor H, the inhibition pattern resembled that obtained with native factor H. In fact the inhibitory effect of the MAH was even stronger (result not shown).

The influence of MAH on the cofactor activity of factor H was also tested (Fig. 7*b*). Maximal cofactor activity was achieved in the controls when factor H was preincubated with buffer (track 2) or

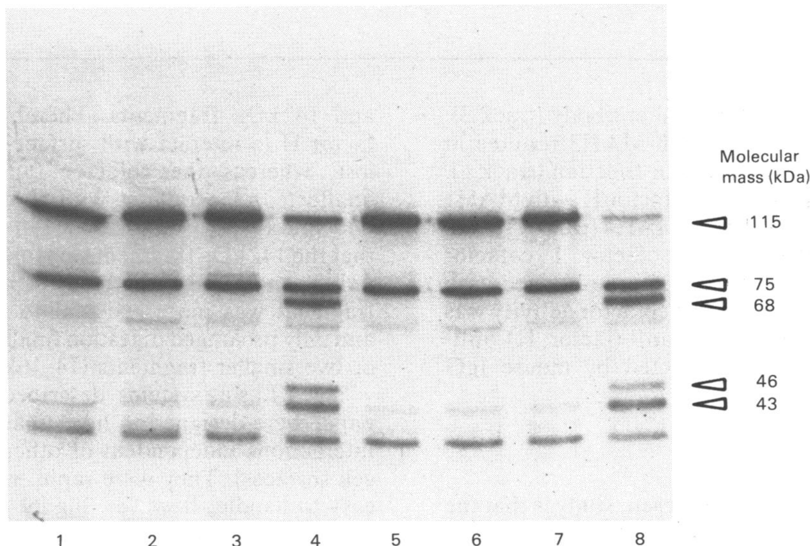

Fig. 6. *SDS/polyacrylamide-gel electrophoresis and autoradiography of fluid-phase ^{125}I-C3b treated with factor I and H or factor H fragments*

A 5 μl portion of ^{125}I-C3b (10^5 c.p.m.) was treated for 2h at 37°C (in 10mM-sodium phosphate/150mM-NaCl buffer, pH 6.5, adjusted to a conductivity of 4mS) with 50 μl of factor H (10 μg/ml) (4), 50 μl of 142/93kDa (6) or 50 μl of 38kDa (8) fragment (200 μg/ml) and 5 μl of factor I (10 μg/ml). Incubation of ^{125}I-C3b with factor H (2), 142/93kDa (5) or 38kDa fragment (7) without factor I served as controls. Track 1 contained only ^{125}I-C3b, and track 3 shows ^{125}I-C3b treated with factor I. Afterwards, the samples were analysed under reducing conditions by SDS/polyacrylamide-gel electrophoresis (9–20% gradient gel) and the gel was exposed to a Kodak X-ray film for 24h.

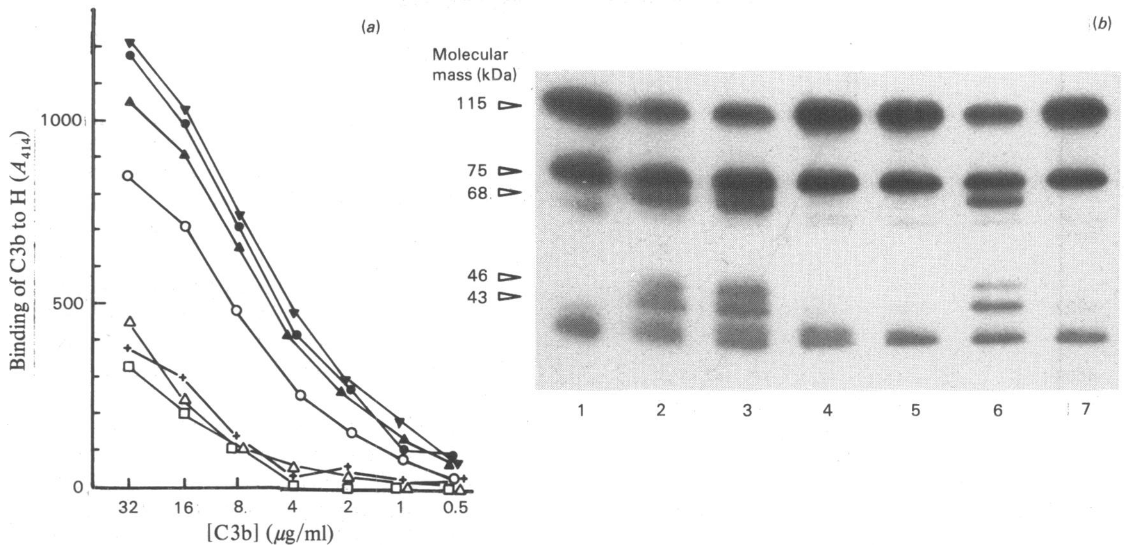

Fig. 7. *Effect of monoclonal anti-(factor H) antibodies on the binding of C3b to factor H and on the cofactor activity of factor H*

(*a*) Influence of MAH on the binding of C3b to factor H. A 20 μl portion of factor H (20 μg/ml) was attached to microtitre plates and preincubated with MAH1 (□), MAH2 (△) MAH3 (○) or MAH4 (+) (50 μg/ml). Buffer (▼), mouse IgG (50 μg/ml) (●) and an unrelated monoclonal antibody (50 μg/ml) (▲) served as controls. After washing with phosphate-buffered saline/Tween, various dilutions of C3b were offered and its binding evaluated by e.l.i.s.a. (*b*) Interaction of different MAHs with the C3b-inactivator cofactor function of factor H. A 5 μl portion of factor H (1 mg/ml) was preincubated for 30 min with 5 μl of buffer (2), unrelated monoclonal antibody (3), MAH1 (4), MAH2 (5), MAH3 (6) or MAH4 (7) (4 mg/ml). Afterwards, 10 μl of ^{125}I-C3b (10^5 c.p.m.) and 10 μl of factor I (10 μg/ml) were added and incubated for 2 h at 37°C (in 10 mM-sodium phosphate/150 mM-NaCl buffer, pH 6.5, conductivity 4 mS). ^{125}I-C3b alone served as a control (1). The samples were analysed by SDS/polyacrylamide gel electrophoresis and autoradiography (see Fig. 6).

with the unrelated monoclonal antibody (track 3). Pretreatment of factor H with MAH3 resulted in weak inhibition of the cofactor function (track 6). Conversely, preincubation of factor H with MAH1 (track 4), MAH2 (track 5) or MAH4 (track 7) led to complete inhibition of the factor I cofactor function, yielding the same pattern obtained with ^{125}I-C3b alone (track 1). The cofactor activity was also blocked by polyclonal anti-(factor H) antiserum, but remained unaffected by mouse IgG (result not shown).

Discussion

Our conclusion from the present study is that the isolated 38 kDa tryptic fragment of factor H carries both the C3b-binding site and the cofactor function for the cleavage of fluid-phase C3b by factor I. These findings were ascertained by the use of monoclonal anti-(factor H) antibodies directed against the 38 kDa fragment of factor H.

In accordance with Hong *et al.* (1982) and Sim & DiScipio (1982) we found that tryptic cleavage of factor H produces first disulphide-linked 38 kDa and 142 kDa fragments. Thereby the ability of factor H to interact with surface-bound C3b was lost, whereas the cofactor function remained unaffected. In contrast with the data of Sim & DiScipio (1982), we and Hong *et al.* (1982) found that the 142 kDa fragment was further cleaved into 93 kDa and 52 kDa fragments, whereas the 38 kDa fragment was more resistant to tryptic cleavage and only prolonged digestion finally resulted in one or two smaller fragments (14–16 kDa).

The e.l.i.s.a. systems described in the present paper were designed to investigate C3b–factor H interactions independent of other structures (e.g. cell surfaces). They were rapid, reproducible and easy to handle; however, highly purified complement components and antisera were absolute requirements. It is noteworthy that a similar technology can be used for the description of C3b–properdin and C3b–factor B interactions (Lambris *et al.*, 1984). With these e.l.i.s.a. techniques we could demonstrate that tryptic cleavage of factor H destroyed only the ability of factor H to interact with surface-bound C3b but not its ability to bind fluid-phase C3b (Fig. 2). This phenomenon might

be due to a conformational change of the factor H molecule that occurs concomitantly with the cleavage of the peptide bond and prevents interaction between the cleaved factor H and C3b attached to a surface. Since the purified 38kDa fragment reacted in a similar way, such a conformational change must occur within the 38kDa fragment.

However, this does not explain why fluid-phase C3b could still bind to surface-attached trypsin-treated factor H or 38kDa fragment. One possibility is that the supposed trypsin-caused conformational change was reversed when cleaved factor H or 38kDa fragment was bound to the microtitre plate. Another possibility could be that there are two different binding sites in factor H. Since C3b bound to trypsin-treated factor H or 38kDa fragment only when offered in its fluid-phase form, we might have found only the binding site that attracts fluid-phase C3b. Tryptic digestion would then destroy the binding site or the additional structure that is responsible for the binding of factor H to surface-attached C3b while the 'fluid-phase C3b-binding site' remains unaffected and detectable. A third explanation could be that factor H binds to fluid-phase C3b in a way other than to C3b attached to a surface. In Fig. 7 we showed that those MAH inhibiting the binding of fluid-phase C3b to surface-bound factor H (MAH1, 2 and 4) also inhibited the cofactor function in solution, whereas MAH3 with low effect on C3b binding also left the cofactor function undisturbed. Furthermore we showed (Schulz *et al.*, 1984) that MAH1 and 2 inhibited the binding of factor H to C3b-coated red blood cells, but MAH3 did not. In addition, the epitopes recognized by MAH1 and 2 were not accessible in preformed C3b-factor H complexes, while the determinant recognized by MAH3 remained available. These data suggest that there is only one C3b-binding site in factor H for fluid-phase and surface-bound C3b. Under such a condition, and since the trypsin-treated factor H binds differently to surface-bound C3b (Fig. 2*a*) and to fluid-phase C3b (Fig. 2*b*; cofactor assay), an additional difference must exist between bound and fluid-phase C3b, probably in conformation.

A critical step of the factor H-fragment purification was the reduction of the disulphide bonds in the trypsin-treated factor H without loss of activity. In 1982, DiScipio & Hugli reported that the functions of H (C3b binding, cofactor activity) are very susceptible to agents that can rupture disulphide bonds and that the integrity of the original disulphide bridges is required for a functionally active molecule. In accordance with their data we found that even under fairly mild conditions (10mM-dithiothreitol in 0.1M-Tris/HCl buffer, pH 8.6, 2h at 37°C) there is a total loss of all factor H activities that are not restored by subsequent alkylation with iodacetamide. In an attempt to reduce the interchain disulphide bonds in the trypsin-treated factor H without loss of function, we found that a mixture of 0.2% 2-mercaptoethanol, 3M-urea and 0.02% SDS in 10mM-sodium phosphate/150mM-NaCl buffer, pH 7.3 (incubated for 15min at 37°C) was optimal for the separation of the trypsin-generated fragments. Under these conditions only the interchain disulphide bridges were cleaved, whereas the intrachain disulphide bridges, which appear to be important for the functional activity of the molecule, seem to remain undisturbed. A decrease in the amount of one of the added reagents (SDS, urea or 2-mercaptoethanol) led to an aggregation of the trypsin-treated factor H, whereas an increase of the amount of 2-mercaptoethanol destroyed the functional activities of the isolated fragments.

Since the 38kDa tryptic fragment of factor H was shown in the present study to carry the C3b binding site and the cofactor activity, it would be of interest now to look for functional and structural similarities between the other cofactors for factor I, the C4b-binding protein and the human erythrocyte C3b receptor, which also manifested disulphide-linked 35–38kDa fragments after tryptic digestion (Reid & Gagnon, 1982; Sim & DiScipio, 1982).

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