Interactions between human complement components factor H, factor I and C3b

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Using a microtitre plate assay, direct binding between complement factors I and H was demonstrated, and ligand blotting indicated that factor H interacts with the heavy chain of factor I. Similarly, direct C3(NH_3)–factor I and C3(NH_3)–factor H binding was characterized [where C3(NH_3) is a form of C3 that is cleaved by factor I in the presence of factor H]. Both factor H and factor I interacted with both chains of C3(NH_3) in ligand blotting. Binding reactions between all three pairs of components were highly dependent on ionic strength, and showed similar pH optima. Binding assays with all three components present led to the following conclusions. (a) Binding sites for C3(NH_3) and factor I on factor H do not overlap, and binding of factor I and C3(NH_3) to soluble factor H promotes the weak factor I–C3(NH_3) interaction. (b) Anomalies arise with immobilized factor H, which may be artefactual or may reflect the physiological situation. (c) Similarly, binding sites on factor I for C3(NH_3) and for factor H do not overlap, and binding of factor H and C3(NH_3) to factor I promotes direct factor H–C3(NH_3) interactions. Based on these results, a model of the interactions between factor H, factor I and C3(NH_3) leading to the processing of C3(NH_3) is proposed.

INTRODUCTION

Factors H and I play a major role in the regulation of the alternative pathway of complement. Binding of factor H to C3b inhibits the interaction of C3b with factor B and C5, and displaces the Bb fragment of factor B from the active C3 and C5 convertases of the alternative pathway. Factor H also acts as a cofactor for the serine protease factor I, which cleaves the 108 kDa α' chain of C3b into fragments of 68 kDa, 43 kDa and 3 kDa, thus abrogating the action of C3b [1–5].

Factor H is a plasma glycoprotein comprising a single polypeptide chain that forms dimers under some circumstances [6]. The monomer has a molecular mass of 155 kDa, of which approx. 12 % is carbohydrate [5–8]. Each factor H molecule is organized into 20 modules or domains called complement control protein motifs (CPs) [7,9], each of which is approx. 60 amino acids long and contains four conserved cysteine residues, of which the first and third, and the second and fourth, are disulphide-linked together. Structural studies [10,11] have indicated that each CP is composed of a hydrophobic core comprising mainly β-strand structure, with protruding loops that may be involved in ligand binding. One large loop, comprising approximately residues 16–23 of each CP, is variable in length and sequence between CPs, and is a good candidate for the major site of ligand interaction. This is called the 'hypervariable loop'. X-ray and neutron solution scattering data [6] indicate that factor H is a long extended molecule of 77–87 nm in length in solution. The hypervariable loops of adjacent CPs are relatively far apart from one another ($\sim$ 2.5–3.5 nm).

The complete cDNA-derived amino acid sequence of factor I has also been established [12,13]. Factor I consists of a heavy chain (polypeptide molecular mass 35400 Da) disulphide-linked to a light chain (polypeptide molecular mass 27600 Da), each with three N-linked glycosylation sites. The light chain is a serine protease domain. The heavy chain is composed of a factor I module, a CD5-like module and two class A low-density lipoprotein receptor modules [12–14]. The maximum dimension of factor I has been calculated to be 14–15 nm using X-ray and neutron solution scattering data [15].

C3 is composed of two polypeptide chains, the α chain (molecular mass 116 kDa) and the β chain (molecular mass 68 kDa). It possesses approx. 1.5 % carbohydrate [16,17]. Solution scattering studies of C3 have indicated that it has a flat discoid shape and is 18 nm long [18,19]. Factor I inactivates C3b in the presence of factor H by cleaving the α' chain of C3b at the Arg–Ser bonds between residues 1281–1282 and 1298–1299, liberating a 3 kDa C3f fragment and yielding inactive C3b (iC3b) [20].

Previous work has investigated the interactions between factor H, factor I and C3b in the processing of C3b [21,22]. In the latter study, C3b was fixed to zymosan and the interaction with ^{125}I-radiolabelled factor H or factor I was studied under various conditions. The results indicated that factor H and factor I both interact with C3b directly. The interaction of factor I with C3b was, however, of very low affinity. No evidence for a direct interaction between factors H and I was provided [22].

Additionally, the interaction between factor H and C3b alone has been studied using C3b bound to zymosan or erythrocytes as a solid phase [23–25]. These methods have the disadvantage that the nature of the support provides a non-homogeneous surface, and also the attachment of C3b to the support is inefficient. Other workers have used C3b and factor H bound to microtitre plates to look at the interaction between them [26,27]. This method proved unreliable due to the weak nature of the interactions being studied.

In the present study, a direct binding assay using glutaraldehyde-cross-linked ^{125}I-radiolabelled soluble ligand, which effectively increases the sensitivity of detection of binding, was used in order to investigate the interactions between factor H, factor I and C3b.

Abbreviations used: CP, complement control protein motif; C3(NH_3), form of C3 that is cleaved by factor I in the presence of factor H; iC3b, inactive C3b; pfI, glutaraldehyde-polymerized factor I; pfH, glutaraldehyde-polymerized factor H; pC3(NH_3), glutaraldehyde-polymerized C3(NH_3).

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MATERIALS AND METHODS

Purification of complement components

All complement components were prepared from human plasma. C3 was purified using the method of Dodds [28]. Factor H and factor I were purified using the method of Sim et al. [29]. Trace contaminants were eliminated from C3 and factor H preparations by ion-exchange chromatography on FPLC Q-Sepharose. The running buffer was 10 mM potassium phosphate/0.5 mM EDTA, pH 7.2. Proteins were eluted with a linear gradient of NaCl (0.0–0.5 M) in the same buffer. Contaminants were removed from factor I preparations, in the same buffer, in a similar manner using FPLC on a Superose 12 column.

Inactivation of C3

Purified C3 was made 0.1 M NH_4HCO_3, pH 8.5. Incubation for 1 h at 37 °C resulted in cleavage of the thiolester [30], converting C3 into a form that is cleaved by factor I in the presence of factor H. This form of C3 is referred to C3(NH_3), and was used as a substrate in factor I cofactor assays, as it is functionally similar to C3b.

Cross-linking and radiolabelling of C3(NH_3), factor H and factor I

Cross-linking was carried out in 5 mM potassium phosphate/0.5 mM EDTA, pH 7.2, using an 80-fold molar excess over protein of glutaraldehyde (BDH, Poole, Dorset, U.K.) for 2 h at room temperature [31]. Samples consisted of 400 μg of protein in 1 ml of buffer. The reaction was quenched by the addition of 0.1 vol. of 1 M Tris/HCl, pH 7.5. Samples were desalted on a gel-filtration column (PD-10; Sephadex G-25; Pharmacia) in 10 mM potassium phosphate, pH 7.2, to remove Tris/HCl and excess glutaraldehyde. Samples (100 μg) were subsequently labelled with 0.5 mCi of $Na^{125}I$ (Amersham) using the Iodogen method [32]. Free iodide was removed by gel filtration as above. The PD-10 column was presaturated with 2 mg of BSA. The specific radioactivity of the cross-linked proteins was generally between 1×10^7 and 5×10^7 c.p.m./μg. The percentage of the protein that had been cross-linked in each sample was assessed by SDS/PAGE analysis and autoradiography of the dried gel followed by direct quantification of the radioactivity in the dried gel. In all cases, no monomeric protein appeared to remain after cross-linking. Dimers, trimers and higher oligomers were observed for all three proteins. Higher oligomers predominated in all three preparations, with $\sim 50\,\%$ of the total radioactivity being present in higher oligomers for factor I and ~ 80–$90\,\%$ of the radioactivity being present in trimers and higher oligomers for factor H and C3(NH_3). The C3 preparation was made 2 mM in iodoacetamide after iodination in order to block free -SH groups. Factor I polymerized in this manner was known as pfI, factor H polymerized in this way as pfH and polymerized C3(NH_3) as pC3(NH_3).

Antibodies

Rabbit polyclonal antibodies were raised against factor H, purified as described above. Immunoglobulins were purified from rabbit antiserum using triple sodium sulphate precipitation [29]. Purified rabbit polyclonal anti-(factor I) immunoglobulins were obtained from Dr. B. Teisner, University of Odense, Denmark.

SDS/PAGE and transfer to PVDF membranes

The Laemmli system [33] was used for SDS/PAGE analysis, but with sample preparation and sample buffer as described in [34]. Samples were analysed both alkylated and reduced then alkylated. For samples that were to undergo ligand blotting, no urea was used in the sample buffer. Gels were stained using Coomassie Blue [34]. Electrophoretic transfer of protein to PVDF membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.) was carried out using an LKB horizontal blotter with a transfer buffer of 0.039 M glycine, 0.048 M Tris and $0.0375\,\%$ (w/v) SDS. The transfer membrane was briefly wetted with methanol prior to transfer. A transfer time of 2.5 h was used, with a current of 0.8 mA/cm^2. After transfer, non-specific sites were blocked using PBS (Dulbecco A), $0.1\,\%$ (w/v) Tween 20 and 20 mg/ml BSA for 40 min at room temperature.

Microtitre plate binding assays using ^{125}I-labelled cross-linked complement proteins as soluble ligands

Factor H, factor I and ovalbumin were coated on to microtitre plates (Nunc, Copenhagen, Denmark) in 0.1 M sodium bicarbonate, pH 9.0, at a coating 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 and 5 mg/ml BSA. The plates were washed five times with 200 μl/well 5 mM potassium phosphate, pH 7.2. All binding experiments were carried out on ice to minimize cleavage of C3(NH_3) in the presence of factors H and I; cleavage has been shown to result in the loss of factor H and factor I binding sites on C3b [21]. In all experiments the incubation time with ^{125}I-radiolabelled ligand in 5 mM potassium phosphate and 10 mg/ml BSA, pH 7.2, was 30 min. After removing unbound ligand and washing five times with 200 μl/well 5 mM potassium phosphate, pH 7.2, bound radioactive ligand was removed by incubation with 4 M NaOH (120 μl/well) at room temperature for 30 min. Counting was performed on an LKB-minigamma counter (counting efficiency $68\,\%$). Binding assays with C3(NH_3) bound to microtitre plates were not performed, as this arrangement is thought to result in an altered conformation of C3(NH_3) [35], which may affect its interactions with some ligands; e.g. plate-bound C3b interacts very poorly with factor B. In all cases, binding of the ^{125}I-radiolabelled cross-linked protein to ovalbumin was used as a non-specific binding control. The radioactivity bound to ovalbumin was subtracted from any radioactivity bound to the test protein in order to obtain a final value for the radioactivity bound to the test protein. The radioactivity bound to ovalbumin was low, and in general did not exceed $5\,\%$ of the maximum radioactivity bound to factor H or factor I.

Saturation binding assays

Binding of ^{125}I-labelled cross-linked ligands to factor H and factor I coated on to microtitre plates was examined to determine whether binding was saturable. In each case the radioactivity bound was compared with that found with non-cross-linked ^{125}I-radiolabelled proteins, so that the effect of cross-linking on the sensitivity of detection of the interactions could be assessed. In all cases, 2-fold serial dilutions of ^{125}I-radiolabelled ligand, starting from 200000 c.p.m./well, were incubated with the proteins bound to the plate in 5 mM potassium phosphate, pH 7.2, for 30 min.

Binding assays involving three components

Based on the results of ligand blotting (see below), a number of binding assays were carried out in which C3(NH_3), factor H and factor I were all present, and the concentration of one of these was varied. These were as follows: (1) ^{125}I-labelled pC3(NH_3)

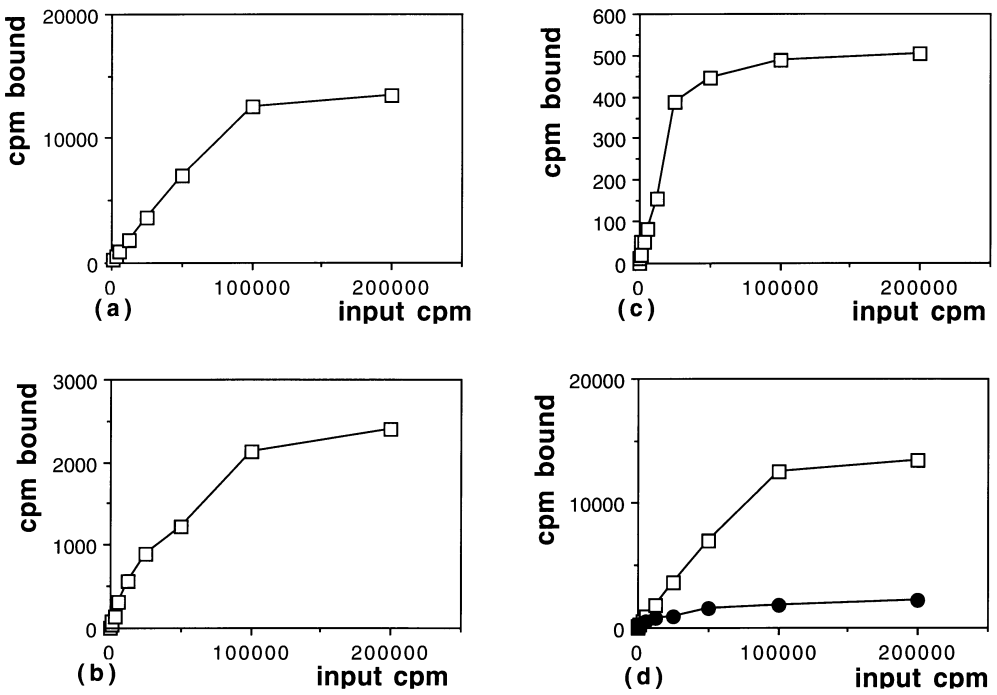

Figure 1 Binding of fluid-phase ^{125}I-labelled complement components to complement proteins attached to microtitre wells

Complement proteins were coated on to microtitre wells at a coating concentration of 50 μg/ml in 0.1 M sodium bicarbonate, pH 9.0. Serial dilutions of ^{125}I-labelled complement proteins in 5 mM potassium phosphate, pH 7.2, and 10 mg/ml BSA were applied (starting concentration 200000 c.p.m./ml) and left to incubate for 30 min on ice. The specific radioactivity of the components was (1–5) $\times 10^7$ c.p.m./μg. The radioactivity bound to ovalbumin attached to microtitre wells was subtracted from any radioactivity bound to the test protein to obtain a final value for the radioactivity bound to the test protein. (**a**) Binding of ^{125}I-labelled pC3(NH_3) to immobilized factor H. Background binding of ^{125}I-labelled pC3(NH_3) to ovalbumin never exceeded 110 c.p.m. (**b**) Binding of ^{125}I-labelled pfI to immobilized factor H. Background binding of ^{125}I-labelled pfI to ovalbumin never exceeded 100 c.p.m. (**c**) Binding of ^{125}I-labelled pC3(NH_3) to immobilized factor I. Background binding of ^{125}I-labelled pC3(NH_3) to ovalbumin never exceeded 80 c.p.m. (**d**) Comparision of binding of ^{125}I-labelled pC3(NH_3) (□) and C3(NH_3) (●) to immobilized factor H. Background binding of ^{125}I-labelled pC3(NH_3) to ovalbumin never exceeded 100 c.p.m.

binding to a plate coated with factor I, with factor H as the variable; (2) ^{125}I-labelled pC3(NH_3) binding to a plate coated with factor H, with factor I as the variable; (3) ^{125}I-labelled pfI binding to a plate coated with factor H, with C3(NH_3) as the variable; and (4) ^{125}I-labelled pfH binding to factor I bound to the plate, with C3(NH_3) as the variable. In all cases a constant amount of radioactivity (100000 c.p.m. in 100 μl of 5 mM potassium phosphate, pH 7.2) was preincubated for 10 min at 37 °C with a serial dilution of the variable component before the mixture was applied to each well. A 2-fold serial dilution of the variable component starting from a maximum of 2 μg/ml was employed. All experiments were carried out in triplicate. An irrelevant protein (ovalbumin) was also used as the variable component, in order to check whether the effects seen were due to the bulk addition of any protein. The radioactivity bound to wells coated with ovalbumin was subtracted from the total radioactivity bound to wells containing the test protein, in order to obtain a final value for the radioactivity bound to the test protein.

Dependence on ionic strength of interactions between C3(NH_3), factor H and factor I

Binding assays were carried out with 5 mM potassium phosphate, pH 7.2, adjusted to different salt strengths with NaCl (0–160 mM). All washings were carried out with buffer of the appropriate ionic strength.

pH-dependence of interactions between C3(NH_3), factor H and factor I

Binding assays were carried out in a number of buffers made up of 25 mM Mes + 25 mM Hepes + 25 mM Tris + 25 mM glycine, adjusted to various pH values. These were diluted 20-fold in distilled water. The range of pH values used was 4.8–8.6, determined after dilution. Again, all washing steps were carried out using buffer of the appropriate pH. The soluble ligand was also added in buffer of the appropriate pH.

Dependence on bivalent metal ions of interactions between C3(NH_3), factor H and factor I

The effects of $MgCl_2$ (0–2 mM) and $ZnSO_4$ (0–0.2 mM) on the ability of ^{125}I-labelled pC3(NH_3) to bind to factor I in the presence of 2 μg/ml factor H were investigated. Binding in the presence of metal ions was compared with that in the presence of 2 mM EDTA.

Ligand blotting with ^{125}I-radiolabelled cross-linked proteins

The ^{125}I-radiolabelled cross-linked proteins were diluted to 400000 c.p.m./ml in 5 mM potassium phosphate, 0.1% Tween 20 and 20 mg/ml BSA, pH 7.2, and incubated with the PVDF-bound complement proteins, both alkylated and reduced and alkylated (1 ml/5 cm^2 of membrane), 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 were dried and

subjected to autoradiography. In a set of control experiments ^{125}I-labelled pfH and ^{125}I-labelled pfI were preincubated with a final concentration of 2 mg/ml rabbit polyclonal anti-(factor H) antibodies and rabbit polyclonal anti-(factor I) antibodies respectively at 37 °C for 30 min prior to incubation with the blots. This provided a method of checking the specificity of the interactions of the ligands with the samples on the blots. An additional control, in which ovalbumin at a final concentration of 2 mg/ml was preincubated with the same ^{125}I-labelled proteins in the absence of immunoglobulins and then applied to the membranes, was used to check that any inhibition of binding in the presence of anti-(factor H) and anti-(factor I) immunoglobulins was not due to the bulk addition of protein. In all experiments, prestained molecular mass markers and chicken ovalbumin, on the PVDF membranes, were also used as non-specific binding controls.

RESULTS

Saturation binding assays

Apparent saturation was observed for the binding of ^{125}I-labelled pC3(NH$_3$) and ^{125}I-labelled pfI to immobilized factor H (Figures 1a and 1b). Low but saturable binding of ^{125}I-labelled pfH (results not shown) and ^{125}I-labelled pC3(NH$_3$) (Figure 1c) to immobilized factor I was detected using this assay. Binding to solid-phase C3(NH$_3$) was not examined, as previous studies had indicated that C3b immobilized to microtitre plates does not interact satisfactorily with factor B [35] and that conformational alterations of C3b can occur [36] that may affect its interactions with several ligands.

In order to assess the effect of cross-linking on the sensitivity of detection of binding, saturation binding assays were also carried out as above, but using non-cross-linked radioiodinated proteins at the same total protein concentration and specific radioactivity. In the case of binding of ^{125}I-labelled C3(NH$_3$) to immobilized factor H, the amount of radioactivity bound was 5–7-fold greater across the range of C3(NH$_3$) input when ^{125}I-labelled pC3(NH$_3$) was used rather than ^{125}I-labelled C3(NH$_3$) (Figure 1d). In the case of binding of ^{125}I-labelled factor I to immobilized factor H, the sensitivity of detection was 3–4-fold greater when pfI was used as opposed to factor I (results not shown). For binding of ^{125}I-labelled C3(NH$_3$) to immobilized factor I, binding was not detectable, in contrast with the results in Figure 1(c).

Binding assays involving three components

In order to gain further insight into the interactions between C3(NH$_3$), factor H and factor I, binding assays were performed in which all three components were present (Figure 2). With

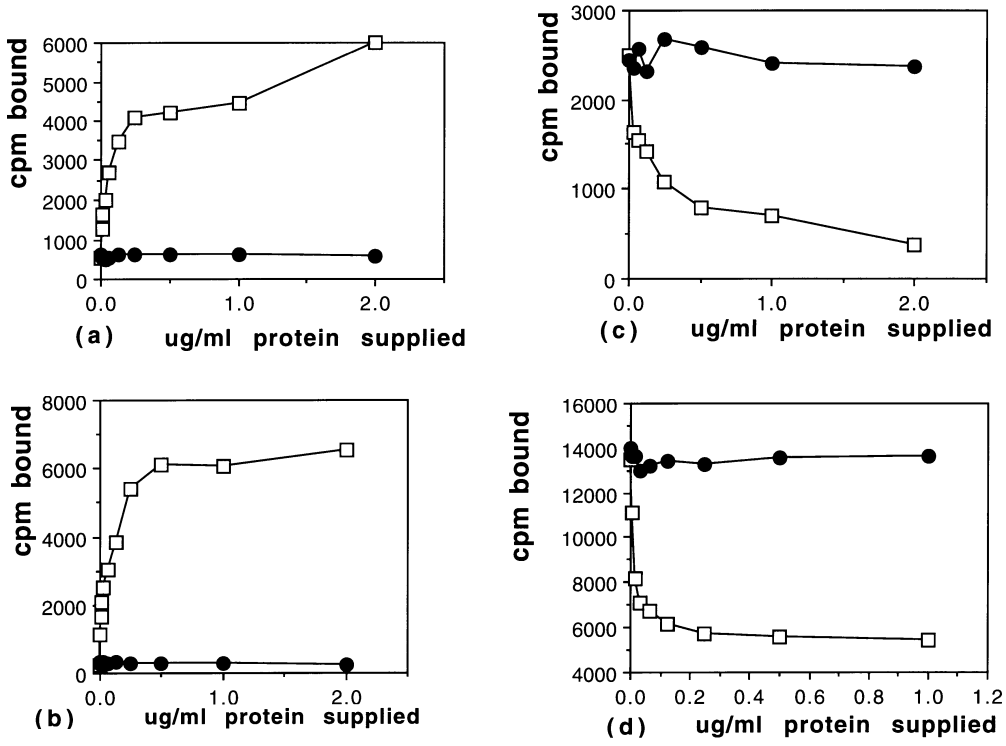

Figure 2 Influence of a third component on the binding of fluid-phase ^{125}I-labelled complement components to complement proteins attached to microtitre wells

In all experiments, 2-fold serial dilutions of the variable third component (starting at a concentration of 2 μg/ml) in 5 mM potassium phosphate, pH 7.2, and 10 mg/ml BSA was preincubated with 100000 c.p.m. of ^{125}I-labelled polymerized complement protein in the same buffer. The mixture was then added to each well. All incubations were for 30 min on ice. In all cases, 2-fold serial dilutions of ovalbumin (starting at 2 μg/ml) as the variable component were employed as a control to check that the effects seen were not attributable to the bulk addition of any protein. Additionally, the radioactivity bound to microtitre wells alone was subtracted from any radioactivity bound to the test protein to obtain a final value for the radioactivity bound to the test protein. (**a**) Binding of fluid-phase ^{125}I-labelled pC3(NH$_3$) to factor I attached to microtitre wells, with factor H (□) or ovalbumin (●) as the variable component. (**b**) Binding of fluid-phase ^{125}I-labelled pfH to factor I attached to microtitre wells, with C3(NH$_3$) (□) or ovalbumin (●) as the variable component. (**c**) Binding of fluid-phase ^{125}I-labelled pfI to factor H attached to microtitre wells, with C3(NH$_3$) (□) or ovalbumin (●) as the variable component. (**d**) Binding of fluid-phase ^{125}I-labelled pC3(NH$_3$) to factor H attached to microtitre wells, with factor I (□) or ovalbumin (●) as the variable component.

Table 1 Summary of the interactions between C3(NH_3), factor H and factor I

The results for C3b as solid phase (zymosan) are taken from [22]. Factor I–C3b interactions are weak in the absence of factor H.

Interaction	Effect of third partner
Factor I–C3b	Enhanced by factor H with either factor I or C3b as solid phase
Factor I–factor H	Enhanced by C3b with factor I as solid phase; inhibited by C3b with factor H as solid phase
Factor H–C3b	Inhibited by factor I with factor H as solid phase; no effect of factor I with C3b as solid phase

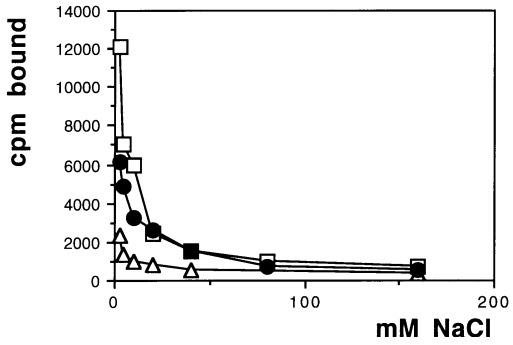

Figure 3 Binding of fluid-phase ^{125}I-labelled polymerized complement components to complement proteins attached to microtitre wells, as a function of ionic strength

Each well contained 100000 c.p.m. of ^{125}I-labelled polymerized complement protein in 5 mM potassium phosphate, pH 7.2, and 10 mg/ml BSA. The radioactivity bound to ovalbumin attached to microtitre wells was subtracted from any radioactivity bound to the test protein to obtain a final value for the radioactivity bound to the test protein. □, Binding of ^{125}I-labelled pC3(NH_3) to factor H attached to microtitre wells. Background binding of ^{125}I-labelled pC3(NH_3) to ovalbumin never exceeded 120 c.p.m. ●, Binding of ^{125}I-labelled pC3(NH_3) to factor I attached to microtitre wells, in the presence of 2 μg/ml factor H. Background binding of ^{125}I-labelled pC3(NH_3) to ovalbumin in the presence of 2 μg/ml factor H never exceeded 130 c.p.m. △, Binding of ^{125}I-labelled pfI to factor H attached to microtitre wells. Background binding of ^{125}I-labelled pfI to ovalbumin never exceeded 130 c.p.m.

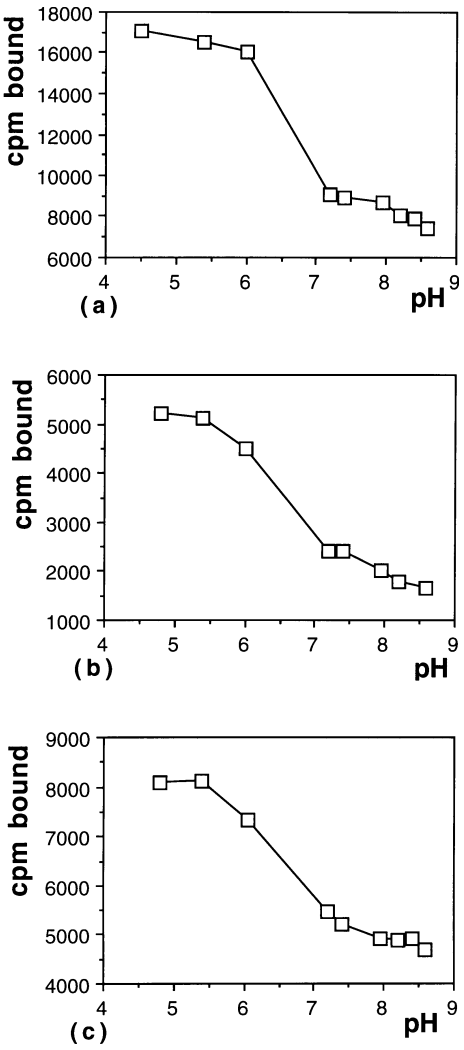

Figure 4 Binding of fluid-phase ^{125}I-labelled polymerized complement components to complement proteins attached to microtitre wells, as a function of pH

Preadjusted buffers made up of 25 mM Mes + 25 mM Hepes + 25 mM Tris + 25 mM glycine were diluted 20-fold in distilled water to give a range of pH values between 4.8 and 8.6. Each well contained 100000 c.p.m. of ^{125}I-labelled polymerized complement protein in a defined pH buffer. The radioactivity bound to ovalbumin attached to microtitre wells was subtracted from any radioactivity bound to the test protein to obtain a final value for the radioactivity bound to the test protein. (**a**) Binding of ^{125}I-labelled pC3(NH_3) to factor H attached to microtitre wells. (**b**) Binding of ^{125}I-labelled pfI to factor H attached to microtitre wells. (**c**) Binding of ^{125}I-labelled pC3(NH_3) to factor I attached to microtitre wells, in the presence of 2 μg/ml factor H. In all cases the background binding of the radiolabelled component to ovalbumin never exceeded 140 c.p.m.

immobilized factor I, soluble factor H enhanced the binding of ^{125}I-labelled pC3(NH_3). The binding was greatly enhanced over the concentration range 0–0.3 μg/ml factor H, and appeared to reach a plateau at values greater than 0.3 μg/ml (Figure 2a). Likewise, when C3(NH_3) was present when measuring the binding of ^{125}I-labelled pfH to immobilized factor I, binding was also greatly enhanced (Figure 2b). It seems that high-affinity binding to immobilized factor I requires all three components to be present. Based on these results, it was expected that the use of pairs of soluble components would enhance binding to factor H immobilized on microtitre wells, but the opposite was observed: C3(NH_3) inhibited the binding of ^{125}I-labelled pfI (Figure 2c) and likewise factor I inhibited the binding of ^{125}I-labelled pC3(NH_3) (Figure 2d). A possible explanation for this is that surface-bound factor H interacts differently with C3(NH_3) and factor I than does fluid-phase factor H; this may be due to spatial restriction or conformational alteration of factor H on the polystyrene plates that prevents both factor I and C3(NH_3) interacting simultaneously with factor H attached to microtitre wells. Reports have been published indicating that surface-bound and fluid-phase C3b molecules interact differently with factor H [26,37]: our results suggest that the reverse phenomenon also occurs. The three-component binding assays are summarized in Table 1.

Dependence on ionic strength of interactions between C3(NH_3), factor H and factor I

In order to establish whether charged groups are important in binding, the dependence on ionic strength was investigated of the interactions of (1) ^{125}I-labelled pC3(NH_3) with factor H attached to microtitre wells; (2) ^{125}I-labelled pfI with factor H bound to microtitre wells; and (3) ^{125}I-labelled pC3(NH_3) with factor I

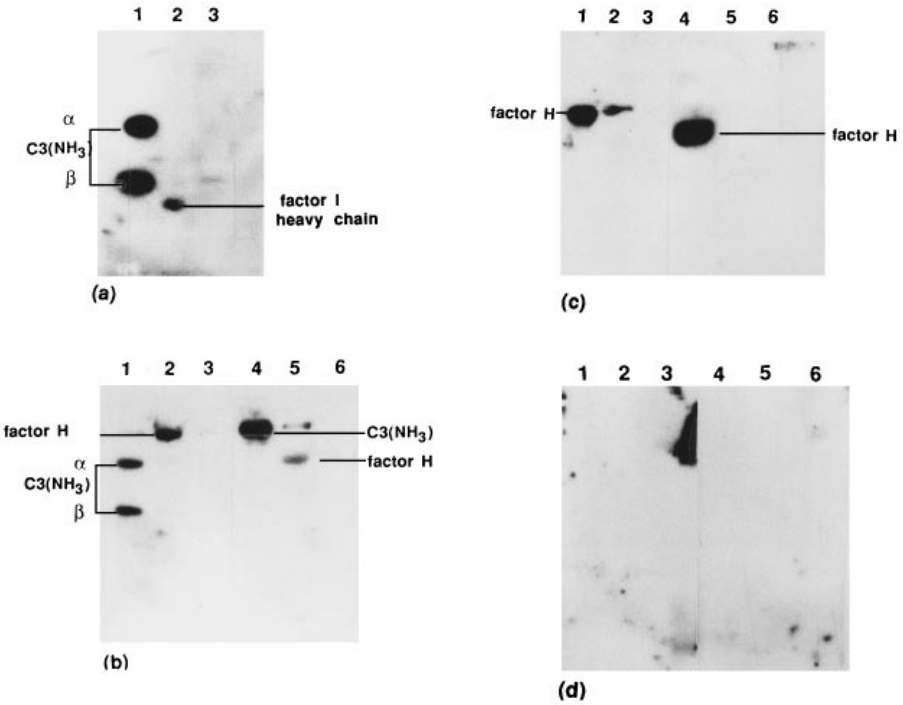

Figure 5 Binding of fluid-phase ^{125}I-labelled polymerized complement components to complement proteins attached to PVDF membranes

SDS/PAGE analysis (11% gels) was used throughout. ^{125}I-labelled polymerized complement component (400 000 c.p.m./ml) in 5 mM potassium phosphate, pH 7.2, and 20 mg/ml BSA was applied to each membrane. Incubation was carried out for 1 h at room temperature. In all cases, chicken ovalbumin and molecular mass markers were used as non-specific binding controls. (**a**) Binding of ^{125}I-labelled pfH to membrane-bound C3(NH_3) (lane 1), factor I (lane 2) and ovalbumin + molecular mass markers (lane 3). All samples were reduced. (**b**) Binding of ^{125}I-labelled pfI to membrane-bound C3(NH_3) (lane 1, reduced; lane 4, alkylated), factor H (lane 2, reduced; lane 5, alkylated) and ovalbumin + molecular mass markers (lane 3, reduced; lane 6, alkylated). Note that, for all samples, there is a small amount of spill-over from one track to the next. (**c**) Binding of ^{125}I-labelled pC3(NH_3) to membrane-bound factor H (lane 1, reduced; lane 4, alkylated), factor I (lane 2, reduced; lane 5, alkylated) and ovalbumin + molecular mass markers (lane 3, reduced; lane 6, alkylated). Note that again there is small amount of spill-over from lane 1 to lane 2. This is difficult to avoid, as the sensitivity of detection of binding using this system is extremely high. (**d**) ^{125}I-labelled pfI and ^{125}I-labelled pfH were preincubated with polyclonal anti-(factor I) (lanes 1–3) and polyclonal anti-(factor H) (lanes 4–6) antibodies respectively, and then bound to membrane-bound complement components and to ovalbumin + molecular mass markers as a control. All samples were reduced.

attached to microtitre wells, in the presence of 2 μg/ml factor H (Figure 3). In all three cases, binding was maximal at low salt strength, showing a sharp decrease in binding up to 40 mM NaCl. The dependences on salt strength observed were similar to those observed for the overall conversion of C3b into iC3b in the presence of factors H and I [38]. DiScipio [22] has previously observed similar dependence on ionic strength of the binding of factor I to zymosan–C3b.

pH-dependence

Further information regarding the types of charged groups involved in protein–protein binding was sought from assessing the pH-dependence of the binding of ^{125}I-labelled pC3(NH_3) to factor H (Figure 4a), ^{125}I-labelled pfI to factor H (Figure 4b) and ^{125}I-labelled pC3(NH_3) to immobilized factor I in the presence of 2 μg/ml factor H (Figure 4c). All the interactions studied exhibited similar pH-dependence: maximal binding was observed at low pH values between 4.0 and 5.5. A sharp decrease in binding was seen between pH 6.0 and 7.0, followed by a small decrease between pH values 7.0 and 8.5. Again, the results were similar to the pH-dependence of the overall cleavage reaction of C3b into iC3b in the presence of factors H and I [38]. Scott and Fothergill [39] reported binding of C3b to factor H bound to Sepharose; they observed a similar dependence on ionic strength to that reported above, but found a sharp pH optimum around 7.6.

Dependence on bivalent cations of the binding of ^{125}I-labelled pC3b to factor I in the presence of factor H

Previous reports have indicated that magnesium ions have little effect on factor I cofactor activity, whereas zinc ions strongly inhibit C3b breakdown in the presence of factors H and I [40,41]. The effects of these metal ions on the interactions between factor H, factor I and C3b in a three-component system comprising binding of ^{125}I-labelled pC3(NH_3) to factor I attached to microtitre wells in the presence of 2 μg/ml factor H were examined, by comparing with binding in the presence of 2 mM EDTA. The presence of these metal ions had no significant effect on the binding of ^{125}I-labelled pC3b to factor I in the presence of factor H (results not shown).

Ligand blotting with ^{125}I-labelled cross-linked complement proteins

Ligand blotting was carried out as an alternative method for the detection of protein–protein binding and in an attempt to identify which polypeptide chains [in the cases of factor I and C3(NH_3)]

are involved in interactions. ^{125}I-labelled pfH bound to both the α and β chains of C3(NH_3) when the latter was both reduced and alkylated, and to alkylated C3(NH_3) (results not shown), and to the heavy chain but not the light chain of factor I (Figure 5a). No binding of ^{125}I-labelled pfH to alkylated factor I could be detected (results not shown), although ^{125}I-labelled pfI could bind to both chains of C3(NH_3), and to alkylated C3(NH_3). ^{125}I-labelled pfI also bound to factor H when the latter was both reduced and alkylated, and alkylated alone (Figure 5b). Although binding of ^{125}I-labelled pfI to membrane-bound C3(NH_3) (Figure 5b) could readily be detected, the reverse arrangement, i.e. binding of ^{125}I-labelled pC3(NH_3) to membrane-bound factor I, could not, when factor I was either reduced and alkylated, or just alkylated (Figure 5c). Binding of ^{125}I-labelled pC3(NH_3) to membrane-bound factor H was detected both when factor H was reduced and alkylated, and when it was alkylated (Figure 5c). It seems likely that binding of factor I to a solid surface (PVDF membranes, and possibly polystyrene plates) affects its interaction with fluid-phase factor H and C3(NH_3).

In order to assess the specificity of binding, controls were carried out in which ^{125}I-labelled pfH and ^{125}I-labelled pfI were preincubated with rabbit polyclonal anti-(factor H) and anti-(factor I) immunoglobulins respectively, to a final concentration of 2 mg/ml, prior to binding to the membranes (Figure 5d). In both cases, all binding was abolished. An additional control in which ovalbumin, at a final concentration of 2 mg/ml, was preincubated with the same ^{125}I-labelled proteins in the absence of immunoglobulins and then applied to the membranes demonstrated that the inhibition of binding in the presence of anti-(factor H) and anti-(factor I) immunoglobulins was not due to the bulk addition of protein (results not shown). In all cases, binding to ovalbumin and to molecular mass markers was assessed as additional non-specific binding controls.

DISCUSSION

Previous studies have investigated the interactions between C3b, factor H and factor I. Many of these studies were performed using C3b bound to zymosan or erythrocytes as a support, and ^{125}I-radiolabelled factors H and I [22–25]. These methods have disadvantages, as the supports are laborious to make and the C3b often binds to the supports in clusters [25], which may affect the interaction with some ligands. Additionally, zymosan and erythrocytes provide a complex and non-homogeneous surface. Microtitre plate binding assays have also been performed in order to study the interaction between factor H and C3b only [26,27].

In the present study a microtitre plate binding assay was established that used glutaraldehyde cross-linked complement components in order to study directly the interactions between factor H, factor I and C3(NH_3). Similar use of cross-linked components has been reported in studying the C3b–CR1 interaction [42]. Cross-linking of the proteins resulted in a significant increase in the sensitivity of detection of binding (Figure 1d). A number of observations could therefore be made regarding the interactions between factor H, factor I and C3(NH_3).

Binding of factor I to factor H was demonstrated using microtitre plate binding assays (Figure 1) and ligand blotting (Figure 5). This is the first report of a direct interaction between factors H and I. The binding of pC3(NH_3) to factor I in the absence of factor H was low in the microtitre plate binding assays. An affinity constant for the interaction between C3b and factor I in water of 5.7×10^5 M^{-1} has been calculated [22]. Values for the interaction between C3b and factor H of $\sim 4.4 \times 10^6$ M^{-1}–2.1×10^7 M^{-1} have been reported [21,25]. Additionally, it has been reported previously that factor H can enhance the binding of factor I to C3b [1,22,43]; our results are consistent with these findings (Figure 2). This indicates that binding of factor H to C3b may cause a conformational change in C3b that facilitates the binding of factor I to C3b or the C3b–factor H complex. Evidence for a conformational change has also been reported [22]. More simply, since it is now clear that factors H and I interact directly with one another, the factor I–factor H and factor H–C3b interactions may stabilize the weak factor I–C3b interaction without the need for conformational changes.

The results from the three-component binding assays reported here and by DiScipio [22] support the following conclusions. (a) The factor I–C3b interaction is weak, but soluble factor H enhances this interaction whether factor I or C3b is on the solid phase. Therefore the C3b and factor I binding sites on factor H do not overlap, and binding of factor I and C3b to soluble factor H promotes a direct factor I–C3b interaction. As noted above, interactions of two proteins may also enhance the binding of a third protein due to a conformational change. (b) When factor H is bound to the solid phase, factor I and C3b cannot bind to it independently, in contrast with the situation with soluble factor H. This suggests that binding of factor H to microtitre plates alters the relative orientation/distance apart of the factor I and C3b binding sites, so that factor I and C3b may sterically hinder each other. This may be an artefact caused by the use of microtitre plates; it may, however, have physiological relevance and merits further investigation. (c) When C3b is bound to the solid phase [22] (zymosan rather than plastic), factor H enhances the binding of factor I, consistent with (a) above. Factor I, however, did not have a strong effect on the binding of factor H; this is anomalous, but insufficient information was given in [22] to calculate the relative ratios of factor H and factor I in the two situations, or to compare the order of addition of components to the binding assays. (d) When factor I is bound to the solid phase, binding of factor H and C3b is cross-enhanced, consistent with (a) above. This implies that the binding sites on factor I for C3b and factor H do not overlap, and that binding of factor H and C3b to solid-phase factor I promotes a direct factor H–C3b interaction. Again, conformational changes on binding might be involved.

The interactions of ^{125}I-labelled pC3b with factor H, of ^{125}I-labelled pC3b with factor I in the presence of 2 μg/ml factor H and of ^{125}I-labelled pfI with factor H exhibited similar dependence on ionic strength and pH (Figures 3 and 4). Previous studies have reported similar pH and ionic strength dependencies for the interaction of ^{125}I-labelled factor H with C3b bound to erythrocytes or to zymosan [23,25]. It is noteworthy that ionization of histidine residues occurs between pH 6.0 and 7.0, where the most significant decrease in binding occurs. This is consistent with chemical modification data [44], which showed that modification of the histidine residues of factor H completely abolished factor I cofactor activity. The same report, however, indicated that modification of carboxy groups and lysine residues resulted in the inhibition of factor I cofactor activity. Only a small decrease in binding occurs above pH 7.0. It may be that the essential lysine residues are in a protected environment and that their pK values are not in the usual range. The importance of carboxy groups could not be assessed in the present assay, as the pH values used were not low enough.

Our results indicate that the interactions between C3(NH_3), factor H and factor I were unaffected by the presence of either magnesium or zinc ions (results not shown). Previous studies have also indicated that magnesium ions have no effect on the ability of factor H to bind to C3b [23,40]. $ZnCl_2$, however, was reported to inhibit C3b breakdown in the presence of factors H

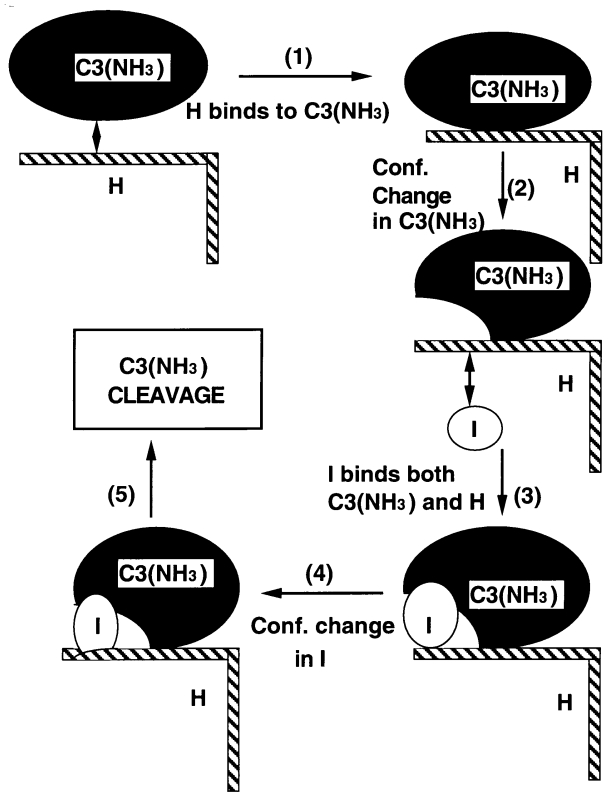

Figure 6 Model for the association between factor H, factor I and C3(NH_3), leading to the processing of C3(NH_3)

The model is discussed in the text. Abbreviation: Conf., conformational.

and I [40,41]. As the formation of the C3(NH_3)–factor H–factor I complex does not appear to be affected by the presence of zinc ions, it may be that the dissociation of the iC3b–factor H–factor I complex is inhibited.

Ligand blotting permitted potential ligand binding sites to be localized to specific polypeptide chains within the proteins. It should be noted, however, that the PVDF-membrane-bound proteins, particularly in the reduced form, would not be in their native conformations, and thus potential sites of interaction can only be identified tentatively. It appears that factor I can bind to both the α and β chains of C3(NH_3) and to factor H attached to a membrane (Figure 5). No previous studies have investigated where on C3 factor I binds, although, clearly, it must have at least a weak interaction with factor I cleavage sites on the α chain. Likewise factor H can bind to both the α and β chains of C3(NH_3). Previous studies using monoclonal antibodies directed against C3b and peptides derived from C3 as inhibitors of factor H binding to C3b only identified binding sites on the α chain; in these studies the β chain was not extensively investigated [27,45–47]. This is the first report of a potential binding site for factor H on the β chain of C3(NH_3). Factor H bound to the heavy chain of membrane-bound factor I. Previous studies have indicated that the light chain of factor I comprises a serine protease domain, whereas the heavy chain is composed of a number of protein modules believed to be involved in ligand binding [29]. This is the first report that the heavy chain of factor I can bind factor H, albeit under non-physiological conditions.

The results presented here are consistent with the model presented in Figure 6 for the interaction between C3(NH_3) and factors H and I. The pairs of proteins [factor I and C3(NH_3), factors H and I, and C3(NH_3) and factor H] can interact in the absence of the third member of the complex. When all three components are present, it can be envisaged that factor H binds to C3(NH_3) α and β chains. On binding C3(NH_3), factor H causes a conformational change in this molecule [22]. This may facilitate the binding of factor I to C3(NH_3) α and β chains, or it may be irrelevant to the binding of factor I. Factor I binds to both factor H and C3(NH_3). As noted above, the binding sites on factor I for factor H and C3b do not overlap. Similarly the binding sites on factor H for factor I and C3b do not overlap. There is evidence that a conformational change occurs in factor I on binding to C3b [48]. This may be irrelevant, or it may facilitate the cleavage of C3(NH_3).

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