Analysis of the interaction between properdin and Factor B, components of the alternative-pathway C3 convertase of complement

Timothy C. FARRIES,* Peter J. LACHMANN and Richard A. HARRISON†

Mechanisms in Tumour Immunity Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

The interactions between Factor B (B), its activation products Ba and Bb, properdin (P) and C3i or C3b, components that together form the alternative-pathway C3 convertase enzyme of human complement, have been analysed. Fluid-phase complexes of the purified components C3i, B and P were probed with the homobifunctional cross-linking reagent disuccinimidyl tartarate, and efficient cross-linking of B to P was observed. The 140 kDa B–P conjugate formed was cleaved by Factor D to yield a single product of 85 kDa. This is consistent with a Ba–P heterodimer, and suggests that the initial interaction of B and P includes an interaction of P with the Ba domain of intact B. (The Ba fragment is not retained in the active P-stabilized complex, C3bBbP.) By contrast, no cross-linking of P to the Bb domain of B could be demonstrated. Binding studies on cellular intermediates also provided evidence for a site of interaction between B and P, with high concentrations of B inhibiting P binding to EAC3b (sheep erythrocytes coated with antibody and C3b). Neither isolated Ba nor Bb had any effect on the P–EAC3b interaction. High concentrations of B also accelerated the decay of the functional EAC3bBbP complex. These data indicate that the positive co-operativity of binding to C3i or to C3b between B and P is mediated, at least in part, through a direct interaction between B and P.

INTRODUCTION

The complement component C3 is activated either by proteolysis, generating C3b and C3a, or by slow hydrolysis by water of an internal thioester, generating C3i. C3i and C3b have similar functional properties (Isenman *et al.*, 1981; Pangburn *et al.*, 1981; von Zabern *et al.*, 1981), and both may form a bimolecular C3 convertase enzyme (C3iBb or C3bBb). The pathway by which these enzymes are generated, outlined below, forms the basis of the positive-feedback loop of the alternative pathway of complement. Either C3i, which comprises two disulphide-linked polypeptide chains, α and β , or C3b (α and β) combines with Factor B (B) in a reversible and Mg^{2+} -dependent manner to form the proenzyme C3iB (or C3bB). Factor D (D), which circulates in plasma as an active enzyme, cleaves C3i- or C3b-bound B into a 30 kDa fragment, Ba, which dissociates from the complex, and the enzymatically active fragment, Bb (63 kDa), which remains bound. The C3 convertase enzymes formed (C3iBb or C3bBb) act on C3 to generate further C3b molecules and thus, by recruitment of further B, additional C3 convertase enzymes (reviewed in Pangburn & Müller-Eberhard, 1984). Where activation results in C3b deposition on to a protected surface and thus evasion by the C3bBb

enzyme of regulation by Factors H and I (H and I), C3b generation is amplified and the pathway activated (Fearon & Austen, 1977a,b; Pangburn & Müller-Eberhard, 1978). C3i can also be generated by treatment of C3 with nitrogen nucleophiles such as methylamine (Janatova *et al.*, 1980). The product is essentially structurally and functionally indistinguishable from C3i generated by water hydrolysis.

Properdin (P) is a mixture of cyclic ('head-to-tail') dimers, trimers, tetramers and higher oligomers of identical 56 kDa subunits (Minta & Lepow, 1974; Reid & Gagnon, 1981; Smith *et al.*, 1984; Farries *et al.*, 1987). It binds to C3b in the presence of B (Schreiber *et al.*, 1975; Medicus *et al.*, 1976; DiScipio, 1981; Farries *et al.*, 1987, 1988), binds to, and stabilizes, the C3 convertase C3bBb (Fearon & Austen, 1975) and protects C3b from the action of H and I (Medicus *et al.*, 1976; Farries *et al.*, 1988). Two forms of P, 'activated' (aP) and 'native' (nP), have been described. We have recently shown that the aP form is an artefact of isolation (Farries *et al.*, 1987). It is present in variable concentrations in all preparations of the purified protein unless rigorous steps are taken to exclude it. A distinctive feature of aP, a conformationally and functionally distinct form of the protein, is that it binds to C3b-coated surfaces under physiological conditions, whereas the 'native' form only

Abbreviations used: PBS, phosphate-buffered saline (exact composition is given in the text); Na/K phosphate, buffers made using KH_2PO_4 solutions of the required phosphate molarity and adjusted to the correct pH with NaOH; CFD, complement-fixation diluent (exact composition is given in the text); BSA, bovine serum albumin; DST, disuccinimidyl tartarate; PAGE, polyacrylamide-gel electrophoresis; P, B, D, H, I and C3, complement components properdin, Factors B, D, H and I, and C3 respectively, aP and nP, 'activated' and 'native' properdin respectively; *P and *B, properdin or Factor B radiolabelled with ^{125}I ; Bb, the 63 kDa activation fragment of Factor B retained in the alternative-pathway C3 convertase enzyme; Ba, the 30 kDa activation fragment of Factor B released from the C3 convertase after cleavage of C3bB by Factor D; C3b, the major C3 convertase product of C3; C3i, haemolytically inactive C3 in which the internal thioester bond has been hydrolysed; E, sheep erythrocytes; EAC3b, sheep erythrocytes coated with antibody and C3b; SBTI, soybean trypsin inhibitor; DMSO, dimethyl sulphoxide.

* Present address: MRC Collaborative Centre, 1–3 Burtonhole Lane, Mill Hill, London NW7 1AD, U.K.

† To whom correspondence and reprint requests should be sent.

binds at low ionic strength (Medicus *et al.*, 1976; Farries *et al.*, 1987).

In the present paper, investigations of interactions within the C3iBP and C3iBbP complexes are reported. A weak interaction between P and B has previously been proposed as the basis for their mutual positive cooperativity in binding to C3b (DiScipio, 1981), and structural and functional evidence presented here strongly supports this hypothesis. Productive use was made of the homobifunctional amine-reactive cross-linking reagent disuccinimidyl tartarate (DST). DST reacts readily at physiological pH and has only a short bridging length of 0.6 nm (Smith *et al.*, 1978).

MATERIALS AND METHODS

Buffers and reagents

PBS is 9.6 mM-K/Na phosphate/0.14 M-NaCl, pH 7.2. CFD is 4 mM-sodium barbitone buffer/0.145 M-NaCl/0.83 mM-MgCl₂/0.25 mM-CaCl₂, pH 7.2, and is prepared by using 'CFD-tablets' (Oxoid, London E.C.4, U.K.). PBS-dextrose is PBS diluted 1:2 in 5% (w/v) D-glucose.

BSA, bovine pancreatic trypsin and SBTI were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Iodogen and DST were products of Pierce Chemicals (Rockford, IL, U.S.A.). Di-n-butyl phthalate and dinonyl phthalate were purchased from BDH Chemicals (Poole, Dorset, U.K.), and Sephadexes G-75 (superfine grade) and G-150 (superfine grade) were from Pharmacia Fine Chemicals (Uppsala, Sweden). The rat monoclonal anti-(human C3) antibodies, Clones 3, 4 and 9 (Lachmann *et al.*, 1980) were coupled to Sepharose 4B by using the CNBr procedure (March *et al.*, 1974).

Purified complement components C3, C3i, P, D, B and H were prepared as described previously (Farries *et al.*, 1987, 1988; Harrison & Lachmann, 1986). Unless otherwise specified, the low level of aP present in the purified protein was not specifically removed. A second B preparation (P33), used only where indicated, was prepared as described by Niemann *et al.* (1980). Ba and Bb fragments were generated by incubation (150 min, 37 °C) of 50 mg of B with 3 mg of C3 (containing some C3i) and 0.1 mg of D in 7 ml of 0.1 M-Na/K phosphate/0.1 M-NaCl/3 mM-MgCl₂, pH 7.0. The mixture was then dialysed against 20 mM-Na/K phosphate/1 M-NaCl, pH 7.0, and Ba and Bb were separated by gel filtration on Sephadex G-75 (superfine grade; 100 cm × 2.5 cm column) in the same buffer. The Bb pool was also passed through columns of Clone 3-Sepharose and Clone 9-Sepharose to remove residual C3 derivatives.

Radiolabelling of proteins with ¹²⁵I was accomplished using the solid-phase Iodogen procedure of Fraker & Speck (1978). Labelled proteins were stored at -70 °C in portions containing 10 mg of BSA/ml.

Cross-linking studies

Cross-linking reactions were performed with the homobifunctional amino-reactive DST (Smith *et al.*, 1978). Reactions with DST were performed at 37 °C in PBS/myoglobin (1 mg/ml), pH 7.2, and, unless otherwise indicated, in the presence of 2 mM-MgCl₂. Myoglobin was added in order that each reaction might have essentially the same concentration of amino groups. Each reaction included one ¹²⁵I-labelled complement protein; the parallel use of a different radiolabelled component in otherwise identical incubation mixtures

Table 1. Apparent molecular mass of the component polypeptide chains of the alternative-pathway C3 convertases, C3iBbP, C3bBbP, and their precursors, C3iBP and C3bBP

Component	Chain	Apparent molecular mass (kDa)	
		Reduced	Unreduced
C3i	α	126	200
	β	75	
C3b	α'	117	192
	β	75	
B		93	63
Bb		63	63
(Ba)		30	30
P		56	56

demonstrated that cross-linking of myoglobin to complement proteins did not occur. The components in each reaction were premixed in 30 μl aliquots before adding 20 μl of DST (1.5 mg/ml in the coupling buffer with rapid mixing). This working solution of DST was itself diluted from a fresh stock solution (100 mg/ml in DMSO) immediately before use. D, when used, was added 30 s before DST. After 30 min incubation the reactions were quenched by the addition of glycine (to 50 mM) and a further 5 min incubation. The cross-linked products were analysed by SDS/PAGE in linear 5–10% (w/v)-polyacrylamide gradients using the buffer system of Laemmli (1970), followed by autoradiography.

Cellular intermediates and binding studies

EAC3b were made using sheep erythrocytes (E), rat monoclonal (IgM) anti-(sheep E) antibody (Antibody SO16), yeast-treated guinea-pig serum ('R3') and partially purified C3, as described previously (Harrison & Lachmann, 1986). Cellular intermediates were calibrated by measuring the uptake of ¹²⁵I-labelled monoclonal antibodies specific for C3 and its fragments (Lachmann *et al.*, 1980, 1982) and generally carried 10000–50000 C3b molecules/cell. Binding studies with radiolabelled components were performed as described previously (Cuatrecasas & Hollenberg, 1976; Iida *et al.*, 1982; Farries *et al.*, 1987).

RESULTS

Cross-linking of P, B and C3i

Cross-linking with DST was used to probe the structure of the C3iBP complex. The apparent molecular masses (on SDS/PAGE) of the polypeptide chains of proteins involved in complex formation are summarized in Table 1. Cross-linking reactions were performed as described in the Materials and methods section, and the products analysed by SDS/PAGE and autoradiography, as shown in Fig. 1 (tracks 1–13). A number of cross-linked species, representing only a proportion (generally < 10%) of the radiolabelled component present, were formed. These are summarized below and in Table 2.

(1) *P. Cross-linking of P with DST (track 2) generates a strong band at about 110 kDa (dimer) as well as a

Fig. 1. Initial analysis of products of cross-linking of components of the alternative pathway with DST

Cross-linking reactions were performed as described in the Materials and methods section. P and B were used at specific activities of about 2000 c.p.m./ μ g. The components in each reaction are indicated beneath the corresponding gel track, either '+' (non-radiolabelled) or '*' (radiolabelled). Samples were either reduced in 1% (v/v) 2-mercaptoethanol (tracks 1–10) or unreduced (tracks 11–13). The concentrations of components used in the cross-linking reactions were as follows: P, 0.1 mg/ml; B, 0.21 mg/ml; C3i, 0.5 mg/ml; D, 66 μ g/ml. An autoradiograph of the dried gel is shown.

fainter band at 170 kDa (trimer). On longer exposure a band at 220 kDa (tetramer) was also observed. These bands are not seen in the absence of DST (track 1).

(2) ***P + C3i, *P + C3iB.** In the absence of B, only a weak band at 180 kDa (track 3) and a diffuse unidentified area of radiolabel (also seen in track 7) of higher molecular mass are detected. As the affinity of P for fluid-phase C3b and C3i is low, failure to demonstrate efficient cross-linking of P to C3i is not unexpected. However, although the affinity of P for C3b is considerably enhanced in the presence of B (DiScipio, 1981; Farries *et al.*, 1987), the efficiency of generation of the 180 kDa band in the presence of Factor B (track 7) is not increased; indeed the intensity of the conjugate may well be diminished.

(3) ***B + C3i, *B + C3i + P.** 125 I-B cross-linked efficiently to C3i, giving a series of bands (B–C3i) at ~200 kDa when reduced (track 4) and at 250–300 kDa when unreduced (track 11). The strongest of the bands seen in the reduced sample is at 170 kDa. Its generation was

enhanced by the presence of P (track 8), supporting other data demonstrating a weak interaction between P and the fluid-phase C3iB complex (Medicus *et al.*, 1976; DiScipio, 1981; Farries *et al.*, 1988). The prominent lower-molecular-mass band, similar in size to fragment Bb and seen in tracks 4, 5 and 8, arises in 125 I-radiolabelled B on storage, presumably as a result of radiation damage, and is not seen in unlabelled B. The apparent degree of fragmentation is exaggerated by the overexposure of the autoradiograph required to demonstrate conjugates.

(4) ***P + B + C3i, P + *B + C3i.** No cross-linking between B and P occurred in the absence of C3i (tracks 5 and 6). In contrast, an interaction between B and P in the trimolecular complex, C3iBP, was clearly evident. 125 I-P gave a distinct band (track 7), not seen in the absence of C3i (track 6) and B (track 3). 125 I-F B also gave a specific band, with identical mobility (track 8), again dependent on C3i (track 5) and P (track 4). The apparent molecular mass of these (140 kDa) is consistent with a B–P conjugate. This identification was confirmed by com-

Table 2. Summary of the conjugates formed by cross-linking of components of the alternative-pathway C3 convertase, C3iBbP, and its precursor, C3iBP

Components in reaction	Molecular mass (kDa)		Deduced composition
	Reduced	Unreduced	
*P	110 170 (220)	– – –	P–P dimer P–P–P trimer (P–P–P–P tetramer)
*P + C3i, *P + C3i + B	180	–	P–C3 α
*B + C3i, *B + P + C3i	(Several ~ 200) 170	250–300 –	– B–C3 β (?)
*P + B + C3i, *B + P + C3i	140	140	B–P
*P + B + C3i, *B + P + C3i, then incubated with D	86	–	Ba–P

parison with the unreduced samples (tracks 11–13), where the same specific bands at approx. 140 kDa are evident (tracks 12 and 13). Complexes containing C3i would have molecular masses greater than 220 kDa in unreduced samples.

(5) C3i + *Bb + P, C3i + Bb + *P. Samples in which the C3iB was cleaved by D to C3iBb + Ba before DST was added are analysed in tracks 9 and 10. Under these conditions there was no detectable cross-linking of P to Bb (i.e. no 119 kDa band in tracks 9 or 10) nor of ¹²⁵I-Bb to C3i [i.e. no ¹²⁵I-Bb–C3 α (189 kDa) or ¹²⁵I-Bb–C3- β (138 kDa)-chain conjugates in track 10]. These results should be compared with those obtained by cross-linking of C3i, P and uncleaved B (tracks 7 and 8), where both P–B and B–C3 conjugates are seen.

Additional experiments were performed in order to define further the conjugates and the conditions under which they can be formed. These are illustrated in Fig. 2 and summarized below.

(6) *B + P + C3i; B + *P + C3i. Formation of the 170 kDa conjugate between Factor B and C3i (track 14, Fig. 2; Fig. 2 covers tracks 14–26), again enhanced by P (track 16) and the 140 kDa conjugate of B and P, dependent on the presence of C3i (tracks 16 and 17), are both evident. None of these conjugates were formed in the absence of DST (tracks 15 and 18) or of Mg²⁺ (tracks 22 and 23).

(7) C3i-bound Factor B is cleaved by Factor D, generating Ba, which is released, and Bb, which remains bound to C3i. This reaction was used in analysis of the B–P conjugate. Tracks 19 and 20 contain samples of incubations of B, P and C3i that, after termination of the cross-linking, were incubated with D. Both track 19 and track 20 show that the 140 kDa B–P conjugate has been cleaved. In track 19 this can be correlated with the appearance of a new band at 85 kDa; surprisingly, this molecular mass corresponds to that expected of a P–Ba, and not a P–Bb, conjugate. In track 20 no new bands [other than *Ba (30 kDa) and *Bb (63 kDa)] can be distinguished. This is not surprising, since the broad band of *B at 93 kDa would obscure any fainter band at 85 kDa. No new species at 119 kDa, corresponding to a

P–Bb conjugate, can be seen either in track 19 or in track 20. Although in track 19 this might, if it had been generated, be obscured by P–P dimers (110 kDa), it should be visible in track 20.

(8) Absence of a conjugate of P and Bb in C3iBbP incubations (tracks 9, 10 and 21). This is further evidence for the contention that the 85 kDa band is a P–Ba, and not a P–Bb, conjugate, and supports the conclusion that, in the C3iBP complex, P cross-links to the Ba and not to the Bb domain of B.

(9) The B–C3i conjugates were also split by Factor D, but the products, which should be apparent in track 20, are not distinct. There is a weak band, possibly a Bb–C3 α conjugate, at 180 kDa. There is no 138 kDa band (Bb–C3 β) even though a 170 kDa band, tentatively identified as a B–C3 β conjugate, was the most prominent band seen on cross-linking of the C3i*B complex (tracks 8, 14 and 16). Similarly, Ba–C3 α - or β -chain conjugates (156 kDa or 105 kDa) are not seen. [The latter, if present, might possibly be obscured by intact B (93 kDa).]

(10) C3iB*aP, C3iB*nP and C3iBb*nP. All the above reactions were performed with unfractionated P. Although this was largely composed of nP, a minor population of aP was also present. nP and aP were therefore separated by gel filtration (Farries *et al.*, 1987) and compared in cross-linking experiments involving C3iB*aP and C3iBb*aP (tracks 24 and 25). B cross-linked more efficiently to nP (track 25), generating the same 140 kDa conjugate as was seen with unfractionated P (track 7). Again, as with the experiments using unfractionated P (track 19), this was clearly split by D (track 26) to generate a product of 85 kDa (P–Ba) and not of 119 kDa (P–Bb).

Inhibition of nP binding to EAC3b by B

Others have shown that P and B bind co-operatively to C3b and C3i (Medicus *et al.*, 1976; DiScipio, 1981). The cross-linking experiments described here show that B and P are in close proximity in the trimolecular complex C3iBP. Together, these observations suggest that a direct interaction between B and P might exist. This was investigated by analysing the effect of B on P binding, at low ionic strength and in EDTA, to C3b. (EDTA inhibits B binding to C3b and hence prevents the otherwise enhanced binding of P to EAC3b.) As Fig. 3 shows, high B concentrations inhibited nP binding to EAC3b; this indicates that there is a weak association of B and P in free solution. At high concentrations, free B can therefore compete with C3b for P binding, diminishing association of P with EAC3b.

As the cross-linking data suggested an interaction between the Ba domain of B and P, the cleavage products, Bb and Ba, were compared with B in the above assay. Neither Bb, Ba nor an equimolar combination of the two had any effect on the binding of ¹²⁵I-P to EAC3b (Fig. 3).

Contamination of B with low concentrations of P, C3 derivatives or with proteinases would also produce a B-concentration-dependent inhibition of P binding. However, samples of purified B further chromatographed on both Clone-3- and Clone-4-Sepharose affinity columns (to remove any contaminating C3 fragments) or on

Fig. 2. Further analysis of products of cross-linking of components of the alternative pathway with DST

Cross-linking reactions were performed as described in the Materials and methods section. The concentrations of components used were as follows: P, 0.1 mg/ml; B, 0.21 mg/ml; C3i, 0.5 mg/ml; D, 33 μ g/ml. All samples were reduced before analysis. After quenching the cross-linking reaction with glycine, the samples in tracks 19 and 20 were treated with D (at 33 μ g/ml; 37 °C, 10 min). nP and aP were analysed using fractions of ¹²⁵I-P chromatographed on Fractogel TSK HW-55(S) (Farries *et al.*, 1987) and mixed with non-radiolabelled unfractionated P to give the same protein concentration as in other incubations. ‘+’ and ‘*’ have the same meaning as in Fig. 1; ‘↓’ indicates that D was added after completion of the cross-linking reaction.

Sephadex G-150 (superfine grade), which would resolve it from P and most plasma proteinases, as well as a preparation of B (p33) isolated by a different procedure, all had similar inhibitory activities. By contrast, BSA, at an equimolar concentration to that of P, did not inhibit P binding of EAC3b, and H, which binds to C3b, may even have enhanced P binding (Table 3). These results exclude contamination of B with C3 derivatives or with P as the basis for its inhibitory activity.

Proteolytic inactivation of EAC3b or of P at high B concentrations during the reaction was also considered as a possible cause of inhibition. To investigate this, EAC3b (0.2 ml; 6.25 × 10⁸ cells/ml) were incubated either with B (9 μ M) or with buffer, both containing

EDTA, for 30 min at 37 °C. The cells were then washed with incubation buffer before the addition of ¹²⁵I-P. In this experiment, washing decreased the inhibition of P binding from 31 to 5% (i.e. P binding was almost fully restored by the removal of B), indicating that the C3b ligand had not been destroyed by proteolysis during the initial incubation. In addition, no cleavage of ¹²⁵I-P by EAC3b (6.25 × 10⁸ cells/ml), both with or without added B (9 μ M), could be detected by SDS/PAGE analysis (under reducing conditions) and autoradiography (results not shown). These results argue against inhibition of binding being caused by contamination of either the B preparation or the EAC3b with extraneous proteolytic activity.

Fig. 3. Effect of B and its isolated fragments, Ba and Bb, on the binding of nP to EAC3b in EDTA

Binding of ^{125}I -nP ($1.5 \mu\text{g/ml}$) to EAC3b (2.5×10^8 cells/ml) was measured after incubation for 30 min at 37°C in PBS-dextrose containing 5 mM- NaN_3 , 10 mM-EDTA and BSA (2 mg/ml). B (■), its isolated fragments, Ba (▲) and Bb (△), and an equimolar mix of Ba and Bb (□), were assayed at the ligand concentrations shown.

Finally, mild treatment of Factor B with trypsin generates Bb and Ba-like fragments (Curman *et al.*, 1977). Samples taken at various times during tryptic digestion were therefore analysed by SDS/PAGE and assayed for inhibition of P binding to EAC3b. As Fig. 4 shows, and in agreement with the results obtained with purified Ba and Bb fragments, cleavage of the intact 93 kDa polypeptide chain of B correlated with loss of its ability to inhibit binding. The requirement for the intact protein demonstrated in these experiments also further emphasizes the specific nature of the reaction.

Effect of high concentrations of B on the decay of stabilized alternative-pathway convertases

The preceding experiments show that, at low ionic strength, B diminishes P binding to EAC3b. We have also investigated the ability of B to displace P specifically bound to EAC3bBb. The decay rates of the surface-bound C3 convertase EAC3b ^{125}I -Bb stabilized either by P or by Ni^{2+} (Fishelson & Müller-Eberhard, 1982) were measured by release of radiolabelled Bb (Table 4). B produced at least a 2-fold decrease in the half-life, at 37°C , of EAC3bBbP. By contrast, the effect of B on the Ni^{2+} -stabilized EAC3bBb complex was negligible. As EDTA alone had little effect on the half-lives of the convertases, this result cannot be explained by B-dependent displacement of Bb from EAC3bBb. Taken together, these data imply that the effect of B is a direct effect on P, with B binding to P being competitive with P binding to EAC3bBb. P binding to this complex must therefore be reversible, with release in the absence of dissociation of Bb.

Table 3. Comparison of the effects of equimolar concentrations of H, BSA and various preparations of B on nP binding to EAC3b, at low ionic strength, in EDTA

Binding of ^{125}I -radiolabelled nP to EAC3b (2.5×10^8 cells/ml) was measured in PBS/dextrose containing 5 mM- NaN_3 , 10 mM-EDTA, BSA (5 mg/ml) and the protein under test (at $9 \mu\text{M}$). The concentration of nP was 150 ng/ml, and binding was measured from duplicate samples taken after 30 min at 37°C . Each data point is the average from three or six replica reaction mixtures, and is expressed as the percentage of P bound in buffer alone [mean $\pm$ s.d. (n)]. Portions of purified B were further chromatographed on columns of either Sephadex G-150 (superfine; 50 cm $\times$ 1 cm column; flow rate 1.4 ml/h in 20 mM-Na/K phosphate/0.5 M-NaCl, pH 7.0) to give B (G150sf), or of Clone 3- and Clone 4-Sepharoses, to give B (anti-C3). The B peaks from these were re-concentrated, dialysed against the assay buffer and then compared with both the parent B preparation and with a different B preparation (p33). H and BSA were included as negative controls.

Sample	P bound in buffer alone (%)
Buffer	100 $\pm$ 6 (6)
B (parent)	45 $\pm$ 12 (3)
B (G150sf)	58 $\pm$ 2 (3)
B (Anti-C3)	56 $\pm$ 8 (3)
B (p33)	54 $\pm$ 10 (3)
H	144 $\pm$ 4 (3)
BSA	101 $\pm$ 8 (3)

DISCUSSION

In the present paper we describe a combination of structural and functional approaches that have been used to analyse a putative interaction between P and B. The results of these confirm that such an interaction does exist, both in association with C3i in the C3iBP complex and in the absence of other ligands.

The homobifunctional cross-linking reagent DST proved to be an effective probe of the fluid-phase alternative-pathway C3 convertase. Possibly because it reacts rapidly at physiological pH, it revealed the polymeric nature of P, with conjugates up to the tetramer being detected, whereas the longer homofunctional reagent, dimethyl suberimidate, which also cross-links between amino groups but must be used at a higher pH, did not (DiScipio, 1982). The experiments and observations reported here also illustrate the limitations of analysis by chemical cross-linking. Firstly, both inter- and intra-chain cross-links are formed; the multiplicity and complexities of these are believed to be the reason for band broadening and the generation of multiple closely-spaced bands on SDS/polyacrylamide gels, both of which inhibit definitive interpretation. Secondly, only a small proportion of molecules form inter-chain cross-links, yet it is the appearance of this minor population that is of interest. Thirdly, modification (both univalent and bivalent) with DST may well limit or block the subsequent binding and activity of other components. Most important of all, failure to generate a conjugate between these two components cannot be taken as evidence for a lack of interaction, as the absence of

Fig. 4. Effect of tryptic digestion of B on its inhibition of P binding to EAC3b

B (4 mg/ml in 0.3 ml of PBS-dextrose containing 5 mM- NaN_3) was incubated at 37 °C with trypsin (16 $\mu\text{g}/\text{ml}$), and 50 μl samples were taken after 0, 2, 5, 10 and 30 min. These were immediately added to 20 μl of soybean trypsin inhibitor (1 mg/ml) in the same buffer, mixed, and left for a further 5 min at 37 °C. Aliquots (5 μl) of each were then analysed, under reducing conditions, by SDS/PAGE. A 12%-(w/v)-polyacrylamide gel was used, and the gel stained with Coomassie Blue R250 (inset). The remaining samples of SBTI-inhibited digested B were mixed in 0.2 ml of PBS-dextrose containing 5 mM- NaN_3 , 10 mM-EDTA and BSA (2 mg/ml) with EAC3b (2×10^8 cells/ml) and ^{125}I -nP. The final concentration of B (or its derived fragments) was 9 μM . After 30 min incubation at 37 °C, duplicate samples were assayed for P-binding to EAC3b. '0%' inhibition was measured by using a sample of buffer containing SBTI-inhibited trypsin. The EAC3b used carried approx. 50000 C3b molecules/cell.

suitably positioned and reactive amino groups within the complex will give a result indistinguishable from that given by non-interacting components. In spite of these limitations, a number of instructive observations can be made.

Several distinct conjugates of B and C3i were detected. As they were not seen in the absence of Mg^{2+} , a complex between the two proteins must exist for cross-linking to occur. The most prominent migrates, when reduced, at

Table 4. Effect of 9 μM -B on the decay of stabilized alternative pathway convertases

The stabilized alternative-pathway convertases (SAPC), EAC3bBbP and EAC3bBbNi $^{2+}$, were made by incubating (5 min, 37 °C) EAC3b (10^8 cells/ml) with D (17 $\mu\text{g}/\text{ml}$), ^{125}I -B (0.45 mCi/mg; 870 ng/ml) and either nP (6 $\mu\text{g}/\text{ml}$) or NiCl_2 (0.4 mM) in 0.42 ml of CFD containing BSA (2 mg/ml). After incubation, the cells were rapidly washed in CFD (three times at 4 °C) and resuspended, at 2.1×10^9 cells/ml, in CFD containing BSA (2 mg/ml) BSA. Portions (40 μl) were then added to prewarmed (37 °C) 0.2 ml reaction solutions containing either 10 mM-EDTA and/or 9 μM -B in the same buffer. Decay of the complexes was monitored by taking 50 μl samples at selected times and determining the amount of B still bound.

Treatment	Half-life (min)	
	SAPC ...	EAC3bBbNi $^{2+}$
CFD/BSA		12.1
+ 10 mM-EDTA	11.3	12.8
+ 9 μM B	9.5	9.8
+ 9 μM -B and 10 mM-EDTA	5.7	10.0

about 170 kDa, and may therefore be a B-C3 β conjugate. The lower mobility conjugates are more likely to be C3 α -chain-derived, consistent with the observation of Ganu & Müller-Eberhard (1985), who identified a site of interaction between B and C3 in the 27 kDa polypeptide of C3c. Although Factor B contained in B-C3i conjugates was split by D, the products were not sufficiently distinct to be unequivocally identified. However, others (Prydzial & Isenman, 1987) have recently reported cross-linking of Ba to C3b. This is of interest, since Ba contains a repeat sequence motif demonstrated in a number of C3b-binding proteins, and supports the idea that, although it is not retained in the active C3bBb complex, the Ba domain may be involved in the initial interaction of B with C3b (Morley & Campbell, 1984; Chung *et al.*, 1985; Holers *et al.*, 1985; Klickstein *et al.*, 1985; Kristensen *et al.*, 1986).

Direct cross-linking of P to C3i was barely detectable, with a faint band at about 180 kDa (reduced), tentatively identified as P-C3 α (theoretical molecular mass 181 kDa), being the only conjugate observed. B may obstruct the formation of this cross-link, since, in the C3iBP complex, its generation appears to be diminished rather than enhanced. Although generation of a P-C3i conjugate was inefficient, the data obtained are consistent with other results (Lambris *et al.*, 1984) that have identified a binding site for P on C3 to the C-terminal region of the C3 α -chain.

DST was particularly effective in generating a specific, unambiguous, C3i-dependent cross-link between B and P. Although the aggregate-like structure of aP might be expected to favour non-specific close proximity and hence cross-linking of P with other components of the complex, B cross-linked more efficiently to nP than to aP, demonstrating specificity in the cross-linking reaction for an interaction within the physiological nP-stabilized complex. In contrast with non-P-conjugated B, the B-P (and B-C3i) conjugates were efficiently cleaved by D.

Cross-linking to P may therefore stabilize B in the conformation it adopts upon binding to C3i or C3iP, enhancing D action. The product of D cleavage of the B-P conjugate had a molecular mass of 85 kDa. This is consistent with a Ba-P (86 kDa), but not a BP-P (119 kDa), heterodimer, and suggests that, in the C3iBP complex, P is positioned very close to, and probably in direct contact with, the Ba portion of B. After cleavage by D, the complex loses the Ba domain of B, and with it are lost the sites of cross-linking with P.

As P binds more avidly to EAC3bBb than to EAC3bB (Farries *et al.*, 1988), and also increases the half-life at 30 °C of EAC3bBb from about 4 min to at least 30 min (Fearon & Austen, 1975), our inability to detect cross-linked species of P and the Bb domain of B, both with DST cross-linking of C3iBP and C3iBbP, was surprising. However, the similar lack of cross-linked species formed between C3i and Bb in the C3iBb and C3iBbP complexes is clearly not due to a lack of interaction between them and must reflect an absence of lysine side-chains suitably reactive and positioned for cross-linking to occur. It is likely that the same explanation obtains for the lack of P-Bb conjugates. The alternative explanation, that there is no direct interaction between the Bb domain and P in the C3iBbP complex seems less probable, as it would require that the effect of P on the stability of the complex is mediated through an effect on the C3i conformation, and is thus independent of the initial mutual enhancement of binding to C3i.

Further evidence for an interaction between P and B was obtained from the ability of high concentrations of B to inhibit P binding, at low ionic strength in EDTA, to EAC3b. This inhibition demonstrates direct competition between free B and surface-bound C3b for P. However, binding was never totally inhibited, even at the highest inputs of B used, indicating that the affinity of P for B is considerably lower than that for bound C3b, which is itself low (Farries *et al.*, 1988). An apparent affinity of 'unfractionated' P for B of $3 \times 10^4 \text{ M}^{-1}$ was estimated. This is too weak for a B-P complex to be detected by direct methods such as the sucrose-density-gradient one used by Chaptis & Lepow (1976).

Because of the high concentrations of B required for inhibition of P binding to EAC3b, the possibility that inhibition of P binding to EAC3b was attributable to a trace contaminant in B had to be rigorously excluded. This was done for the most likely interfering contaminants: P, C3 products or proteolytic enzymes. In addition, B competed effectively with EAC3bBb for P binding, destabilizing the EAC3bBbP but not the EAC3bBbNi²⁺ complex. This activity cannot be explained by contamination of the B with P, or by blocking of weak or non-specific P-binding sites. Interestingly, neither Ba nor Bb nor an equimolar mixture of the two fragments, at equivalent concentrations to those effective for intact B, inhibited P binding to C3b. Thus either one or both of the fragments has an altered conformation, resulting in loss of the binding site for P, or that sites of interaction exist on both the Ba and the Bb domains but that neither alone is of sufficient affinity for binding to be detected. In this context, it should be noted that, on activation of C3iB, the Bb domain of B is initially retained by C3i, but that decay of the complex is irreversible. By contrast, C3i generated by decay of C3iBb is able to bind further B and reform C3iBb complexes. This is best explained by assuming that Bb is maintained in a metastable con-

formation in C3iBb until it is released, at which point it assumes a stable, but non-interacting, configuration.

The importance of P in the amplification of activation of the alternative pathway is well established. In the present paper we have shown that the positive cooperativity between B and P in binding to C3b, proposed by DiScipio (1981), can be attributed to a weak, but specific, interaction between them. Although this direct interaction is likely to be further stabilized by independent sites of interaction of both components with C3b (possibly, since it is polymeric, involving more than one subunit of P), factors that influence contacts between P and B could have a significant effect on the formation of the C3bBbP amplification convertase and hence influence the level of alternative-pathway activation.

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