INTERACTION OF FIBRONECTIN WITH C1q AND ITS COLLAGEN-LIKE FRAGMENT (CLF)

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Received 26 March 1981; revised version received 13 May 1981

1. Introduction

Fibronectin is a glycoprotein (M_r 440 000) present on cell surfaces and in the blood [1,2]. It forms complexes with collagen, gelatin and collagen-derived fragments [3–5]. A region has been identified in fibronectin that is responsible for its collagen-binding activity [6]. This region is located ~2/3rds of the linear distance from the NH_2-terminus of each chain in the dimeric molecule. Fibronectin evidently possesses, therefore, 2 independent binding sites for collagen, one on each chain, enabling it to bivalently interact with collagen and to form extended polymers.

Since C1q, a subcomponent of the first complement component C1, contains a collagen-like fragment [7] that competes functionally with collagen [8–13], we have investigated whether C1q and/or its collagen-like fragment is able to form complexes with fibronectin and whether such an interaction can be inhibited by collagen or by antifibronectin-antibody. Complex formation between collagen and fibronectin can be completely inhibited by the addition of an antiserum to fibronectin [14], which suggested that the antibodies to fibronectin were at least in part directed against the collagen-binding site of fibronectin and that the affinity of the antibody for fibronectin is much higher than that of collagen for fibronectin.

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2. Materials and methods

C1q was prepared from human serum according to [15] and further purified using con A–Sepharose [16]. C1q was labeled with ^{125}I using the chloramine-T method [17], 1.0 mCi $Na^{125}I$ was used/mg protein. Excess labeling reagent was eliminated by gel chromatography on Sephadex G-25. The collagen-like fragment of C1q (CLF) was obtained by limited peptic digestion [18]. Purity of the preparation was checked by M_r determination and amino acid analysis [18]. CLF was ^3H-labeled by acetylation: to 1 mg CLF in 0.1 M Tris–HCl buffer of pH 9.0 (1 mg CLF/ml) 50 µl [^3H]acetic anhydride in toluene (equivalent to 5 mCi; spec. act. 3.85 Ci/mmol; Amersham, batch 49) was added dropwise with vigorous stirring. After 30 min at room temperature, the solution was dialysed against 1.0 M NaCl/0.05 M Tris–HCl buffer (pH 7.5) and the [^3H]CLF was then chromatographed on a Sephacryl S-300 column (Pharmacia), equilibrated against the same buffer. For interaction studies, [^3H]CLF was dialysed against phosphate-buffered saline of pH 7.2 (PBS). Plasma fibronectin (cold-insoluble globulin, CIG) was purified by affinity chromatography on gelatin–Sepharose [3]. CIG was labeled with $Na^{125}I$ using 0.5 mCi/mg [14].

Human type I collagen was prepared by acidic extraction of infant dura mater [19]. Human collagen type II was obtained by limited pepsin digestion of infant cartilage after extraction of proteoglycans [20]. Human type III collagen was prepared by peptic digestion of infant skin [21]. Collagens were used in denaturated state (30 min, 60°C).

Antifibronectin antiserum from sheep was pur-

chased from Boehringer Mannheim. Rabbit antifibronectin serum was a gift. A commercial rabbit antiserum raised against human C1q was used (Behring-Werke, Marburg; batch 6304 B). Its anti-C1q and anti-CLF titer was determined using passive hemagglutination [13].

Interaction experiments: Labeled C1q or CLF was incubated with different amounts of fibronectin in glass tubes (30 min, 37°C), then antifibronectin was added (5 μl–0.2 ml of the incubation mixture) and incubation continued for 30 min at 37°C and 1 h at 4°C. Goat–anti-rabbit gammaglobulin (0.2 ml) diluted 1:4, was added (Calbiochem, Luzern) and incubation was done at 4°C for 20 h. As controls instead of antifibronectin and/or goat–anti-rabbit gammaglobulin (steps 2,3) PBS was added. The precipitates were centrifuged (5000 × *g*, 30 min, 4°C), washed 3 times with PBS and counted (for ^{3}H-labelled sediments after dissolution in NaOH). Alternatively, 1:10-diluted antifibronectin antiserum (0.2 ml) was incubated with increasing amounts of ^{125}I-labelled fibronectin for 60 min at 37°C followed by 20 h at 4°C. In parallel analogous experiments were performed in the presence of CLF. Precipitated radioactivity was determined.

For solid phase interaction studies polystyrene tubes (11 × 70 mm, Nunc, Denmark) were coated with C1q by incubation with a PBS-solution containing 10 μg C1q/ml (1.0 ml/tube) for 3 days at 4°C. After washing with cold PBS and incubation with 0.1% bovine serum albumin in PBS for 2 h at room temperature tubes were reacted with ^{125}I-labelled fibronectin or with mixtures of collagen (CLF) and ^{125}I-labelled fibronectin, preincubated for 30 min at 37°C, for 1 h at 37°C and 30 min at 4°C. The final assay volume was 0.5 ml/tube. After incubation, the tubes were washed 3 times with PBS and adsorbed radioactivity was determined.

For affinity chromatography cold fibronectin was coupled to 25 mg BrCN-activated Sepharose (Pharmacia) by incubating with 250 μg protein in 0.25 ml buffer (0.5 M NaCl/0.1 M $NaHCO_3$ (pH 8.0)) for 2 h at room temperature. The gel was packed in a Pasteur pipette, stoppered with a small plug of cotton wool. C1q or [^{3}H]CLF solution (1 ml) in PBS was slowly passed at room temperature through the gel (5 min). In the eluate the remaining protein was determined. As control CNBr-activated Sepharose that had been reacted with ethanolamine was used.

3. Results

[^{3}H]CLF was freed of aggregates by gel chromatography on Sephacryl S-300. The monomeric fraction eluting between 190–200 ml was incubated at 10 μg/ml with unlabeled fibronectin in different molar ratios. The influence of adding rabbit antifibronectin serum and/or second antibody on the amount of precipitated [^{3}H]CLF was studied ([^{3}H]CLF spec. act. 30 × 10^{6} dpm/mg). Fig.1 indicates that maximal precipitation occurs at a molar ratio [^{3}H]CLF:fibronectin = 2:1, 47% of total added radioactivity precipitating at this molar ratio, if [^{3}H]CLF is allowed to interact with fibronectin without later additions. The addition of antifibronectin antibody (final dilution 1:80) nearly completely abolishes [^{3}H]CLF-precipitation due to complex formation with fibronectin, while addition of goat–anti-rabbit gammaglobulin alone only results in a reduction of [^{3}H]CLF precipitation by fibronectin (17.5% precipitated at 2:1 molar ratio). Addition of normal rabbit serum in the second incubation step also caused a reduction in precipitated radioactivity (24.7% at 2:1 ratio).

In the case of C1q–fibronectin interaction only a small fraction of ^{125}I-C1q was precipitated by fibronectin. Thus, with equimolar amounts of C1q and fibronectin 1.9% of total added ^{125}I-labelled C1q were

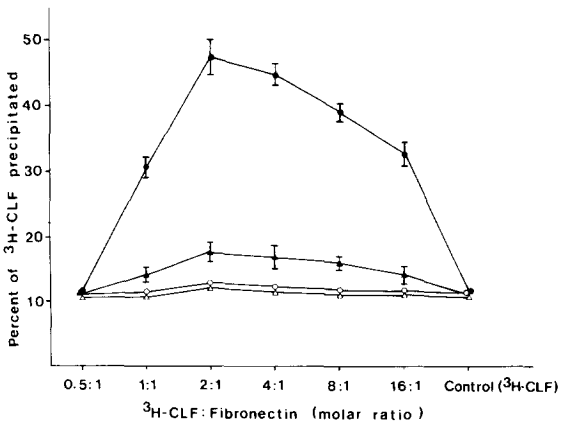

Fig.1. Precipitation behavior of different [^{3}H]CLF–fibronectin mixtures. (●) [^{3}H]CLF + fibronectin; at the second and third incubation step PBS was added; (○) step 2, + antifibronectin antibody; step 3, + goat–anti-rabbit gammaglobulin; (△) step 2, + antifibronectin antibody; step 3, + PBS; (▲) step 2, + PBS; step 3, + goat–anti-rabbit gammaglobulin; similar to this curve: step 2, + normal rabbit serum (not shown; maximal precipitation 24.7%).

precipitated (control incubation of ^{125}I-labelled C1q alone: 1.2%). This was increased to 2.7% by adding antifibronectin antibody in step 2. Addition of anti-Cq1 antiserum, however, completely abolished the small ^{125}I-labelled C1q–fibronectin precipitate formed with equimolar amounts of the two proteins. The anti-C1q serum used displayed a significant reactivity towards CLF (passive hemagglutination titer using C1q-coated erythrocytes 1:10 000, using CLF-coated erythrocytes 1:2500). The specific activity of the ^{125}I-labelled C1q used in our experiments was 330×10^6 dpm/mg.

To further investigate the relative affinity of antifibronectin antibody and CLF to fibronectin, antifibronectin antiserum was incubated with increasing amounts of ^{125}I-labelled fibronectin in the presence or absence of CLF (final conc. 25 μg/ml). The specific activity of our ^{125}I-labelled fibronectin was 45×10^6 dpm/mg. Fig.2 (rabbit anti-fibronectin serum) demonstrates that the characteristic precipitation behavior of the antiserum towards labeled fibronectin is not essentially altered in the presence of constant amounts of CLF. A reduction of specifically precipitated ^{125}I-labelled fibronectin is, however, observed, especially at high antibody excess. Analogous results were obtained with commercial sheep antifibronectin antiserum.

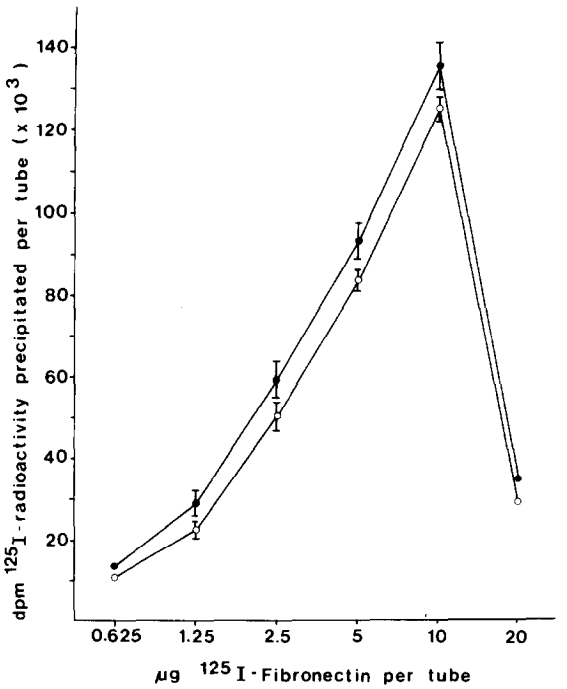

Fig.2. Precipitation characteristics of a rabbit–anti-fibronectin antiserum (1:10), influence of CLF (25 μg/ml); 0.2 ml antiserum were incubated with 0.2 ml fibronectin solution in PBS (+0.05% bovine serum albumin): (•) without CLF; (○) with CLF.

The precipitation experiments using labeled C1q and cold fibronectin provide no conclusive evidence of any significant complex formation between the two proteins, therefore an attempt was made to demonstrate such an interaction by affinity chromatography. Sepharose-bound fibronectin was incubated with C1q and [^{3}H]CLF to compare the reactivity of native C1q and its collagen-like fragment with the matrix-bound protein. The results of these experiments are listed in table 1, showing significant binding both of C1q and [^{3}H]CLF to the insolubilized fibronectin, while adsorption to the control Sepharose was negligible. It could be demonstrated that the bound [^{3}H]CLF was not eluted by native C1q.

Finally, we wanted to compare the relative affinity of collagens type I, II and III to fibronectin with that of CLF and C1q. We used solid phase interaction experiments. We incubated C1q-coated polystyrene tubes with ^{125}I-labelled fibronectin in the presence of increasing amounts of CLF or denatured collagens of human origin (table 2). Our results clearly indicate that fibronectin–C1q interaction may be significantly inhibited by CLF and denaturated collagens, most efficiently by type III collagen. The high affinity of this collagen to fibronectin was also proven in a precipitation experiment, in which even very low relative collagen concentrations (molar ratio collagen:CLF = 1:20) nearly completely inhibited the characteristic fibronectin precipitation by CLF (fig.3).

Table 1
Interaction of C1q ([^{3}H]CLF) with Sepharose-bound fibronectin

Parameters determined	Fibronectin-column	Control column
Fibronectin coupled (μg)	200	–
Coupling efficiency (%)	80	–
% C1q [a] retained by column (240 μg added)	48	3
% [^{3}H]CLF[a] retained (100 μg added)	62	3
[^{3}H]CLF eluted by 1 ml C1q (240 μg/ml)	–	–

[a] Original concentration was 0.6 μM

Table 2
Interaction of labeled fibronectin with C1q-coated polystyrene tubes and its inhibition by CLF and denaturated collagens

Inhibitor (μg/ml)	% inhibition by			
	CLF[a]	Collagen I	Collagen II	Collagen III
1.0	–	–	12.4	12.3
10.0	12.4	20.2	27.3	32.6

[a] % inhibition obtained at CLF levels of 20.0, 40.0 and 80.0 μg/ml: 39.0, 59.8 and 69.5, respectively. Amount of ^{125}I-labelled fibronectin bound/tube in the absence of an inhibitor: 36 ng (uncoated tube: 17 ng)

4. Discussion

Complex formation between fibronectin and the collagen-like fragment of C1q results in the formation of precipitates, suggesting generation of extended lattice structures as a consequence of bivalent (antibody-like) interaction of fibronectin. Addition of small amounts of anti-fibronectin antibody even after preincubation of fibronectin with CLF caused complete inhibition of this precipitation. An identical amount of non-immune rabbit serum or an amount of second antibody equivalent to the first serum added, although significantly reducing fibronectin precipitation by CLF, did not completely inhibit it. While the effect of the non-antifibronectin sera may be attributed to the competitive action of C1q present in these sera, the much stronger interference caused by antifibronectin serum can be interpreted by assuming that the antifibronectin antibodies are at least partially directed against the CLF-binding site of fibronectin and that they have higher affinity to it than CLF.

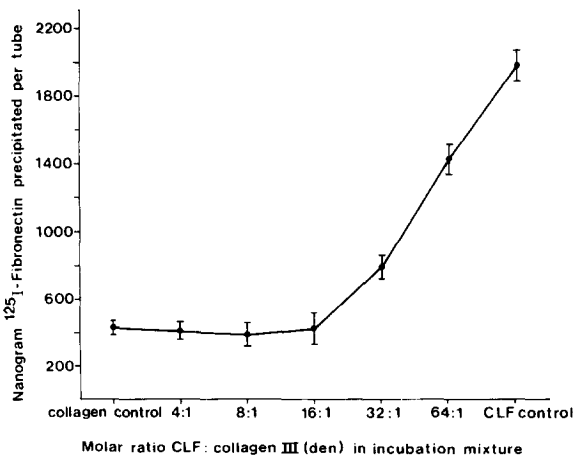

Fig.3. Inhibition of ^{125}I-labelled fibronectin precipitation by CLF due to addition of denaturated collagen type III. CLF (0.1 ml) (100 μg/ml) + 0.1 ml collagen + 0.2 ml ^{125}I-labelled fibronectin (50 μg/ml) were incubated for 60 min at 37°C. Buffer: PBS + 0.05% bovine serum albumin.

C1q was found less prone to precipitate with fibronectin. Due to the more bulky structure of C1q the formation of extended lattice structures could be less favoured in this system. The fact that anti-fibronectin enhanced ^{125}I-labelled C1q precipitation may be explained by less complete shielding of determinants in the CLF-binding region and/or by additional incorporation of ^{125}I-labelled C1q into the antigen–antibody complex via conventional complement activation. That C1q, while not precipitating with fibronectin, significantly interacts with this protein, was demonstrated in our affinity chromatography experiments using Sepharose-bound fibronectin. This interaction occurs via the CLF of C1q, since it may be inhibited by CLF.

The portion of the fibronectin molecule involved in complex formation with CLF is likely to be at, or near, the collagen-binding region, since the C1q–fibronectin interaction was inhibited by denaturated collagens. Thus, in the precipitation experiment shown in fig.3, we see that small amounts of denatured collagen type III are sufficient to abolish the characteristic precipitation of fibronectin by much larger amounts of CLF (up to 20-fold molar excess of CLF). This correponds quantitatively to the results obtained in [22], showing that denatured collagen type III is very efficient in 'saturating' fibronectin by forming soluble complexes.

Since C1q, fibronectin and collagens coexist in some in vivo and in vitro situations (fibroblast culture

supernatants and fibroblast extracts [14,23]; in plasma and other physiological fluids), it is probable that the interactions between these molecules observed by us have some physiological implications. Thus, C1q–fibronectin interaction could be of major importance in reticuloendothelial clearance of C1q-coated immune complexes from blood, fibronectin acting as 'opsonic protein' facilitating ingestion by macrophages. It has been shown that plasma fibronectin (CIG) is involved in the RES elimination of collagen-coated particles [24]. In this context, it may be significant that the collagen-like region of C1q only appears to be exposed in serum after activation of the C1 complex and removal of the activated $C\bar{1}r$ and $C\bar{1}s$ by $C\bar{1}$-inhibitor [25].

Acknowledgements

We thank Mrs Sabine Al-Farr, Ms Grazyna Sobal and Mrs Margarethe Karanitsch for skilled technical assistance. This work was supported in part by Fonds zur Förderung der wissenschaftlichen Forschung, projekt 4148.

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