Characterization of binding of human β_2 -glycoprotein I to cardiolipin

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β_2 -Glycoprotein I–cardiolipin complexes are reported to be a target antigen for the binding of a subset of anti-phospholipid antibodies. The characteristics of binding of β_2 -glycoprotein I to cardiolipin are reported in this paper. Binding at neutral pH is specific, saturable, dependent on ionic strength and independent of bivalent cation. Binding at low pH is qualitatively different from that at neutral pH, and is not dependent on ionic strength. Denaturation of β_2 -glycoprotein I by heat inactivation and reduction/alkylation indicates that β_2 -glycoprotein I–cardiolipin interaction does not require the native three-dimensional structure of β_2 -glycoprotein I, implying that a linear sequence motif

may be responsible. Modification of amino acid residues by KCNO treatment completely destroys binding capacity, indicating crucial involvement of lysine residues in binding of β_2 -glycoprotein I to cardiolipin. Complement factor H, which has some similar highly charged linear sequence motifs to β_2 -glycoprotein I and is composed of the same type of protein module, was found to bind to cardiolipin and inhibit the binding of β_2 -glycoprotein I to cardiolipin. Three different lysine-rich segments of the fifth domain of β_2 -glycoprotein I may be involved in binding to cardiolipin.

INTRODUCTION

Human β_2 -glycoprotein I (β_2 I), also called apolipoprotein H, was first described by Schultze et al. [1]. It is composed of 326 amino acids and has a molecular mass of approx. 50 kDa. The concentration of β_2 I in plasma, where it is partially associated with lipoproteins [2], is 100–200 μ g/ml. The amino acid sequence was originally determined using protein sequencing techniques [3], and minor errors in this sequence were corrected by cDNA cloning and sequencing [4,5]. β_2 I consists of five contiguous homologous repeating units of about 60 amino acid residues, which belong to the complement control protein (CCP) superfamily [6]. The CCP repeat is present in proteins involved in the regulation of the complement system (e.g. factor H, complement receptor type 1, complement receptor type 2, C4b-binding protein etc.), and in several non-complement proteins, including interleukin 2 receptor, blood clotting factor XIII b-chain and the selectin family of leucocyte adhesion molecules [7]. The major site of biosynthesis for this protein is hepatocytes [4,8]. The physiological function of β_2 I is uncertain, but it has been shown to bind to anionic phospholipids [9], platelets [10], heparin [11], DNA [12] and mitochondria [13]. β_2 I may have a regulatory role in the pathway of blood coagulation, because it inhibits prothrombinase activity of resting and activated platelets [14] as well as ADP-mediated platelet aggregation [15].

Anti-phospholipid antibodies are found in the plasma of patients with certain autoimmune diseases (e.g. systemic lupus erythematosus) or other immunological, neoplastic or infective disorders as well as in normal people without evidence of disease. McNeil et al. [16], Galli et al. [17] and Matsuura et al. [18] have shown that at least a subset of such antibodies are directed not against phospholipid alone, but against an antigen consisting of a complex of β_2 I and negatively charged phospholipids. Harris and Pierangeli [19], however, suggest that anionic phospholipids are the true antigen for anti-phospholipid antibodies. β_2 I was first

shown to bind to cardiolipin (CL) by Schousboe [13], but no detailed characteristics of the binding have been reported. Studies on β_2 I interaction with other negatively charged phospholipids, including phosphatidylserine and phosphatidylinositol, have been published [9].

In a previous paper [4] the hypothesis was put forward that a highly charged sequence (KNKEKK) in the fifth CCP domain of β_2 I may be responsible for binding to negatively charged phospholipids. This sequence is also found in rat [20], mouse [21], canine [22] and bovine β_2 I [23,24]. From the model of the three-dimensional structure of the fifth CCP domain of human β_2 I it appears that this sequence is exposed on the surface of the molecule [25]. Moreover, the fifth domain of human β_2 I was produced as a fusion protein in an *Escherichia coli* expression system and was shown to bind CL almost as well as the intact protein [25]. Lauer et al. [26] suggested that an adjacent sequence in the fifth domain of human β_2 I was able to mediate both binding to CL and binding of anti-CL antibodies.

In order to understand the molecular basis of the interaction of β_2 I with CL, we set out to determine the characteristics of this interaction. The aim of the work was to clarify the mode of interaction of β_2 I with CL, and obtain further information on the site of interaction.

MATERIALS AND METHODS

Materials

CL (C-1649, from bovine heart), phosphatidylcholine (PC) (P-4279, from frozen egg yolk), cholesterol (Ch) (C-8667), BSA, chicken ovalbumin, soya-bean trypsin inhibitor (SBTI), dithiothreitol, iodoacetamide, Mops, Mes, Tris, Hepes, Tween 20 (P-1379), poly(ethylene glycol) (average molecular mass 3350 Da), TEMED (*N,N,N',N'*-tetramethylethylenediamine), EDTA, SIGMA Fast BCIP/NBT buffered substrate tablets (B-5655)

Abbreviations used: β_2 I, β_2 -glycoprotein I; CL, cardiolipin; PC, phosphatidylcholine; Ch, cholesterol; CCP, complement control protein; SBTI, soya-bean trypsin inhibitor; α_2 M, α_2 -macroglobulin; KP/SBTI, 10 mM potassium phosphate buffer containing 1 mg/ml soya-bean trypsin inhibitor, pH 7.

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and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (A-8025) were all purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). PBS tablets (BR14a) were obtained from Oxoid (Unipath Ltd.). Glycine, SDS and ammonium persulphate were obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.). Rabbit anti-(human factor H) was from Serotec (Kidlington, Oxon, U.K.). Protogel [30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide stock solution] was purchased from National Diagnostics (Aylesbury, Bucks., U.K.). Nitrocellulose (Immobilon P) was obtained from Millipore. Iodobeads were from Pierce and Warriner (Chester, Cheshire, U.K.). Na¹²⁵I was purchased from Amersham International (Aylesbury, Bucks., U.K.). Microtitre 96-well plates were obtained from Flow Laboratories (Irving, Ayrshire, Scotland), and 24-well plates were purchased from Nunc (Kamstrup, Roskilde, Denmark). Chromatography materials were from Pharmacia (Milton Keynes, Beds., U.K.).

Protein isolation

Human β_2 I was isolated by modifications of the method of Schultze et al. [1]. Further batches were isolated from human plasma as follows. The pellet from 18% (w/v) poly(ethylene glycol) precipitation of plasma was redissolved in 10 mM potassium phosphate/0.5 mM EDTA, pH 7.5, and run on a DEAE-Sephacel column, equilibrated in the same buffer. 95% of the applied protein bound to the column, but β_2 I and IgG did not and were eluted in the starting buffer. The β_2 I pool from this column was dialysed against 10 mM sodium phosphate, pH 6, and run on a column of S-Sepharose, equilibrated in the same buffer. β_2 I was eluted, at the trailing edge of the IgG peak, using a linear NaCl gradient, up to 0.5 M, in the same buffer. The β_2 I pool was then concentrated by freeze-drying, and separated from remaining traces of IgG by gel filtration on FPLC Superose 12 [27].

Human α_2 -macroglobulin (α_2 M) and human complement factor H were isolated from human plasma as described by Sim and Discipio [28].

Binding of ¹²⁵I-labelled β_2 I to CL

Microtitre plates were coated with 100 μ l of CL/well at 50 μ g/ml in ethanol. PC and Ch at the same concentration were used as controls. Wells without phospholipid coating were also included. Alcohol was evaporated and non-specific binding sites were blocked with 200 μ l of α_2 M/well at 250 μ g/ml for 2 h at room temperature (18–22 °C). α_2 M was chosen arbitrarily as a blocking protein in which there was no risk of contamination with β_2 I. Human β_2 I was radiolabelled with Na¹²⁵I by the Iodogen method [29] using Iodobeads. The specific radioactivity of the iodinated protein was 3.8×10^4 – 3.0×10^6 c.p.m./ μ g. Plates were incubated (100 μ l/well) with various amounts of ¹²⁵I-labelled β_2 I in 10 mM potassium phosphate, pH 7, containing 1 mg/ml soya-bean trypsin inhibitor (KP/SBTI) for 2 h at room temperature. SBTI was present as a carrier protein to reduce non-specific binding of β_2 I. In some experiments, BSA was used for this purpose. After the plates had been washed five times in the same buffer, bound radioactivity was eluted with 100 μ l of 0.5 M NaOH, and was measured in an LKB 1275 Minigamma counter.

Binding of total human serum proteins to CL

A 24-well Nunc plate was coated with 2 ml of CL/well (50 μ g/ml in ethanol), and non-specific binding sites were blocked with 3 ml of α_2 M (250 μ g/ml in 10 mM potassium phosphate buffer, pH 7). Human serum (2 ml/well) was added and incubated for 2 h at

room temperature. After the wells had been washed five times in the same buffer, bound proteins were eluted with 100 μ l of 1 M NaCl and analysed by SDS/PAGE [30]. Proteins on the gel were detected with Coomassie Brilliant Blue [31]. Western blotting was also performed to confirm the presence of complement factor H (as described in the legend to Figure 3b).

Effect of bivalent cations on binding

Plates were coated with CL, PC or Ch, and non-specific binding sites were blocked with α_2 M as above. ¹²⁵I-labelled β_2 I (100 μ l; 1.6×10^6 c.p.m.; 0.55 μ g of protein) in 10 mM Mops buffer, pH 7, containing 1 mg/ml SBTI plus different concentrations of CaCl₂ or MgCl₂ (0–10 mM final concentration) was added to the wells. To test the effect of EDTA, 100 mM Mops buffer, pH 7, containing physiological concentrations of CaCl₂ and MgCl₂ (1.5 mM and 0.5 mM respectively [32]) was used. ¹²⁵I-labelled β_2 I (100 μ l; 1.2×10^6 c.p.m.; 0.4 μ g of protein) containing various concentrations of EDTA (0–10 mM) in the Mops buffer was added to the wells. After incubation (2 h; room temperature) and washing, bound radioactivity was measured as above.

Effect of pH on binding

CL-, PC- and Ch-coated plates were incubated with 50 μ l/well ¹²⁵I-labelled β_2 I (2.9×10^5 c.p.m.; 2 μ g of protein) in 10 mM NaCl containing 3 mM CaCl₂ and 1 mM MgCl₂ plus 1 mg/ml SBTI, and pH was adjusted by adding 50 μ l of 25 mM Mes/25 mM Hepes/25 mM Tris/25 mM glycine buffer (from pH 4.34 to pH 10.32). Plates were incubated and washed as above, and bound radioactivity was measured.

Effect of ionic strength on binding

¹²⁵I-labelled β_2 I (100 μ l; 1×10^6 c.p.m.; 2.4 μ g of protein) in KP/SBTI containing different concentrations of NaCl (0–200 mM) was added to the wells. In a separate test to study the effect of ionic strength at different pH values, ¹²⁵I-labelled β_2 I was dialysed against water overnight and the pH was adjusted to 3.0 or 7.0 with 10 mM citric acid/NaH₂PO₄ buffer. Then 100 μ l of buffered labelled material containing different concentrations of NaCl was added to the wells. After incubation (2 h; room temperature) and washing, bound radioactivity was measured as above.

Competition binding assays

A fixed amount of ¹²⁵I-labelled β_2 I was preincubated with different concentrations of potential binding competitors, such as unlabelled β_2 I, ovalbumin, BSA and complement factor H, for 15 min at room temperature in KP/SBTI plus 1.5 mM CaCl₂ and 0.5 mM MgCl₂. Controls without competitor were included. Mixtures were then transferred to CL-, PC- or Ch-coated microtitre plates and left for 2 h at room temperature. After washing, bound ¹²⁵I-labelled β_2 I was measured as above.

Effect of various treatments of β_2 I on binding

Heat inactivation

Aliquots of ¹²⁵I-labelled β_2 I (1500 μ l; 8×10^6 c.p.m.; 8 μ g of protein) in 10 mM potassium phosphate buffer, pH 7, containing 20 μ g/ml BSA, 1.5 mM CaCl₂ and 0.5 mM MgCl₂ were incubated for 30 min at different temperatures (56–100 °C), then placed on ice. Then 100 μ l aliquots of each sample were loaded on to CL-, PC- and Ch-coated plates and binding was measured as above, and compared with a control kept at 4 °C.

Reduction/alkylation

^{125}I -labelled $\beta_2\text{I}$ (1500 μl ; 5.7×10^6 c.p.m.; 38 μg of protein) in 10 mM potassium phosphate buffer, pH 7, containing 20 $\mu\text{g}/\text{ml}$ BSA, 1.5 mM CaCl_2 and 0.5 mM MgCl_2 was denatured in a final concentration of 4 M guanidinium chloride/0.1 M Tris/HCl, pH 8. Reduction was performed by adding 40 mM (final concentration) dithiothreitol and incubating for 2 h at 37 °C. Samples were then alkylated with 82 mM iodoacetamide. A control without dithiothreitol or iodoacetamide was included. Samples were dialysed against 10 mM potassium phosphate buffer, pH 7, containing 1.5 mM CaCl_2 and 0.5 mM MgCl_2 overnight, then introduced to CL-, PC- and Ch-coated plates, and bound radioactivity was measured as above.

KCNO treatment [33]

^{125}I -labelled $\beta_2\text{I}$ (1500 μl ; 1.5×10^7 c.p.m.; 10 μg) in 0.5 M potassium phosphate buffer/1 M KCNO, pH 5.7, was incubated for 4 h at 37 °C. A control without KCNO was included. Samples were dialysed against 10 mM potassium phosphate buffer, pH 7.0, containing 1.5 mM CaCl_2 and 0.5 mM MgCl_2 overnight, then loaded on to CL-, PC- or Ch-coated plates, and bound ^{125}I -labelled $\beta_2\text{I}$ was measured as above.

RESULTS

Binding of ^{125}I -labelled $\beta_2\text{I}$ to CL

Microtitre plates were coated with CL, PC or Ch and incubated with different concentrations of radiiodinated $\beta_2\text{I}$ for 2 h at room temperature. Bound radioactivity was eluted and measured as described in the Materials and methods section. Dose-dependent binding of ^{125}I -labelled $\beta_2\text{I}$ to CL was observed, whereas much lower binding to L and Ch occurred and binding to $\alpha_2\text{M}$ was negligible. Binding to CL was saturable at approx. 5 μg (100 pmol) of protein (Figure 1).

Figure 1 Binding of ^{125}I -labelled $\beta_2\text{I}$ to CL

Different dilutions of ^{125}I -labelled $\beta_2\text{I}$ were loaded on to CL- (■), PC- (●) or Ch- (▲) coated microtitre plates, and the bound radioactivity was measured as described in the Materials and methods section. The specific radioactivity of the labelled $\beta_2\text{I}$ was 3.7×10^4 c.p.m./ μg . Results are means of quadruplicate determinations after subtraction of blank values measured without phospholipids ($\alpha_2\text{M}$ -coated wells).

Figure 2 Competition assay with unlabelled $\beta_2\text{I}$, ovalbumin and BSA

A fixed amount of ^{125}I -labelled $\beta_2\text{I}$ (2×10^6 c.p.m.; 1 μg of protein in 50 μl) was preincubated with different concentrations of unlabelled $\beta_2\text{I}$ (—), ovalbumin (····) or BSA (---), and was added to CL- (■), PC- (●) or Ch- (▲) coated microtitre plates. The bound radioactivity was measured as described in the Materials and methods section. The ordinate represents percentage of maximal $\beta_2\text{I}$ binding. Results are means of quadruplicate determinations.

Competition assay with unlabelled $\beta_2\text{I}$

To confirm that binding of $\beta_2\text{I}$ to CL is specific, we inhibited binding of ^{125}I -labelled $\beta_2\text{I}$ with unlabelled $\beta_2\text{I}$, using ovalbumin and BSA as controls. At equimolar amounts of unlabelled to labelled ligand, unlabelled $\beta_2\text{I}$ inhibited binding by 53 % (Figure 2). Ovalbumin did not inhibit binding of ^{125}I -labelled $\beta_2\text{I}$ in a concentration-dependent manner even at 30-fold molar excess. BSA, however, caused some inhibition of binding, although much less effectively than unlabelled $\beta_2\text{I}$. The reason for this is unclear, but may be because of contamination of BSA with $\beta_2\text{I}$ or phospholipids. Inhibition of $\beta_2\text{I}$ binding to phosphatidylserine multilamellar vesicles by BSA has been noted previously [9]. Binding of $\beta_2\text{I}$ to CL is specific, and iodinated $\beta_2\text{I}$ behaves, in this experiment, essentially the same as unlabelled $\beta_2\text{I}$. In other competition assays of this type, however (see Figure 8), unlabelled $\beta_2\text{I}$ was somewhat less effective in inhibiting binding of radio-labelled material, and up to 4-fold molar excess of unlabelled $\beta_2\text{I}$ was required to inhibit binding by 50 %. Thus iodination can apparently affect the binding of $\beta_2\text{I}$ to CL, and we found this effect to be variable.

Binding of human serum proteins to CL

To confirm that the binding to CL is not a common property shared by many serum proteins, human serum was loaded on to a CL-coated plate as described in the Materials and methods section, then the CL-bound material was analysed by SDS/PAGE. As shown in Figure 3(a), there are only two major components in the CL-binding fraction together with some minor components. One of the major ones is $\beta_2\text{I}$, as shown by its co-migration with a $\beta_2\text{I}$ standard, and the other, confirmed by submitting reduced samples to SDS/PAGE (not shown), is IgG. The IgG in the sample may bind directly to CL or may be binding via $\beta_2\text{I}$; this was not investigated further. It was known that

Figure 3 (a) Binding of total human serum proteins to CL and (b) confirmation, by Western blotting, that factor H in serum binds to CL

(a) Human serum was incubated on a CL-coated 24-well plate. Proteins that remained bound after thorough washing were eluted and analysed by SDS/PAGE unreduced on a 7.5% (w/v) acrylamide gel and stained with Coomassie Brilliant Blue. Lane A, CL-bound material; lane B, molecular mass standards; lane C, purified β_2 I. (b) Isolated human factor H (0.65 μ g) and CL-bound material from human serum (2 μ g) were submitted to SDS/PAGE [7.5% (w/v) acrylamide gel] and proteins were transferred to nitrocellulose. The non-specific binding sites were blocked with 1% (w/v) dried milk in PBS containing 0.1% Tween 20 for 30 min at room temperature. Then the blot was incubated with rabbit anti-(human factor H) antibodies (diluted 10000-fold in the same buffer; 3.15 μ g/ml total immunoglobulin) for 1 h at room temperature then for 1 h at 4 °C. The blot was washed in PBS/0.1% Tween 20 and incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antibodies (diluted 1000-fold in PBS/0.1% Tween 20; 1.27 μ g/ml total immunoglobulin) for 1 h at room temperature. After extensive washing in the same buffer, protein bands were visualized using SIGMA Fast BCIP/NBT substrate. Lane A, isolated factor H; lane B, CL-bound material from human serum; the positions of molecular-mass standards were determined by SDS/PAGE.

isolated complement factor H competes with isolated β_2 I for binding to CL (see Figure 8), and so we investigated whether factor H bound to CL from serum. Factor H is a minor component in the CL-binding fraction of approx. 150 kDa (non-reduced; Figure 3a) and is readily identifiable by Western-blot analysis (Figure 3b).

Effect of pH on binding

In Figure 4 is shown the binding of 125 I-labelled β_2 I to CL in the pH range 4.3–10.3. The binding was found to be pH-dependent; it was greatest at the lowest pH tested (pH 4.3) and decreased by 8-fold at pH 10.3. Binding to PC and Ch was low, and not dependent on pH. The effect of pH on binding to CL over this pH range was surprising, as it does not suggest participation of basic amino side chains in the protein. The pK values for the phosphate ionization in CL are below pH 2, and so the curve does not reflect protonation of CL. The binding at low pH and at neutral pH are qualitatively different, as discussed below. The curve in Figure 4 therefore represents a transition between two different types of binding, which may obscure information on the pK values of the side chains involved.

Figure 4 Effect of pH on binding of β_2 I to CL

CL- (■), PC- (●) and Ch- (▲) coated microtitre plates were incubated with 125 I-labelled β_2 I at different pH values, and bound radioactivity was measured as described in the Materials and methods section. Results are means of quadruplicate determinations after subtraction of blank values measured without phospholipids (α_2 M-coated wells).

Effect of ionic strength on binding

Binding of 125 I-labelled β_2 I to CL at NaCl concentrations of 5–200 mM was studied. At pH 7, maximum binding was observed at 5 mM and binding had decreased by 6-fold at 200 mM. Binding was still considerably above background at physiological salt strength, as confirmed by the binding of β_2 I from serum shown in Figure 3(a). Background binding to PC and Ch was not sensitive to ionic strength (Figure 5). However, binding of β_2 I to CL at pH 3 was not dependent on salt (not shown) and remained

Figure 5 Effect of increasing NaCl concentration on the binding of β_2 I to CL at neutral pH

125 I-labelled β_2 I containing different concentrations of NaCl was added to CL- (■), PC- (●) or Ch- (▲) coated plates. Bound radioactivity was measured as described in the Materials and methods section. Results are means of quadruplicate determinations after subtraction of blank values measured without phospholipids (α_2 M-coated wells).

Figure 6 Effect of Ca^{2+} and Mg^{2+} on the binding of $\beta_2\text{I}$ to CL

A fixed amount of ^{125}I -labelled $\beta_2\text{I}$ containing different concentrations of CaCl_2 (—) or MgCl_2 (---) was loaded on to CL- (■), PC- (●) or Ch- (▲) coated microtitre plates, and bound radioactivity was measured as described in the Materials and methods section. The ordinate represents percentage of maximal $\beta_2\text{I}$ binding. Results are means of quadruplicate determinations after subtraction of blank values measured without phospholipids ($\alpha_2\text{M}$ -coated wells).

constant over NaCl concentrations of 0–300 mM, which indicates that different mechanisms of binding occur at pH 3 and pH 7.

Effect of bivalent cations on binding

^{125}I -labelled $\beta_2\text{I}$ was added to CL-, PC- or Ch-coated plates in the presence of different concentrations of CaCl_2 or MgCl_2 as described in the Material and methods section. An increase in either CaCl_2 or MgCl_2 decreased the binding of $\beta_2\text{I}$, but at physiological concentrations of CaCl_2 (1.5 mM) or MgCl_2 (0.5 mM), the decrease was small (Figure 6). Part of the effect of CaCl_2 and MgCl_2 can be attributed to the increase in ionic strength (see Figure 5). Background binding to PC and Ch was not affected by CaCl_2 or MgCl_2 . EDTA (0–10 mM) added to a Mops/ CaCl_2 / MgCl_2 buffer as described in the Materials and methods section caused first a slight increase in binding (up to 2.5 mM EDTA), then a small gradual decrease up to 10 mM EDTA (not shown). These effects were consistent with, first, negation of the effect of CaCl_2 and MgCl_2 , followed by a slight decrease in binding due to increase in salt strength. These results indicate that binding of $\beta_2\text{I}$ to CL is not dependent on bivalent cations.

Sensitivity of binding to protein denaturation or modification

Heat treatment

In order to study the effects of heat treatment on binding of ^{125}I -labelled $\beta_2\text{I}$ to CL, radiolabelled $\beta_2\text{I}$ was incubated for 30 min at different temperatures from 56 °C to 100 °C, then the binding assay was performed. No regular effect on binding was observed in this temperature range (not shown). The melting temperature of the CCP repeat is in the range 60–65 °C [34]. Our results suggest that binding of $\beta_2\text{I}$ to CL is not dependent on the three-dimensional structure of the CCP modules, but is instead a feature of a heat-stable motif, possibly a short linear sequence.

Reduction/alkylation

To study the effect of reduction of disulphide bonds followed by

Figure 7 Effect of reduction/alkylation and KCNO treatment of $\beta_2\text{I}$ on binding CL

Reduction/alkylation (▨) and KCNO treatment (▤) were carried out as described in the Materials and methods section and samples were loaded on to CL-, PC- or Ch-coated microtitre plates. Binding of untreated $\beta_2\text{I}$ (■) and control [incubated in guanidinium chloride/Tris buffer without dithiothreitol or iodacetamide (□)] are shown. The control for KCNO treatment was similar (not shown). The ordinate represents percentage of applied $\beta_2\text{I}$ that bound. Results are means of quadruplicate determinations.

alkylation of free -SH groups on binding, ^{125}I -labelled $\beta_2\text{I}$ was reduced and alkylated as described in the Materials and methods section. Binding of reduced/alkylated $\beta_2\text{I}$ to CL was increased, but an increase in binding to PC and Ch was also observed (Figure 7), suggesting that reduction/alkylation of $\beta_2\text{I}$ results in an increase in non-specific binding, but does not abolish specific binding.

KCNO treatment

KCNO reacts with -NH₂ and -SH groups forming stable carbamyl derivatives, and unstable or reversible derivatives are formed with histidine, tyrosine -OH and -COOH groups [33]. To study whether modification of amino acids altered binding of $\beta_2\text{I}$ to CL, ^{125}I -labelled $\beta_2\text{I}$ was treated with 1 M KCNO at pH 5.7; binding to CL was completely abolished. No binding was observed to PC or Ch either (Figure 7). $\beta_2\text{I}$ has no free -SH groups, and it is likely that this procedure leads to stable modification of free amino groups only, although modification of histidine cannot be discounted.

Inhibition of binding of ^{125}I -labelled $\beta_2\text{I}$ to CL by complement factor H

Factor H and $\beta_2\text{I}$ are structurally similar: factor H is built up from 20 CCP repeats, whereas $\beta_2\text{I}$ has five. The 13th domain of factor H contains a highly charged sequence, KNKKEF, similar to the site on $\beta_2\text{I}$ posulated to interact with negatively charged phospholipids (KNKEKK) [4,25,35]. It was of interest to study whether these structural similarities would translate into functional similarities in binding to CL. As shown in Figure 8, factor H does inhibit binding of ^{125}I -labelled $\beta_2\text{I}$ to CL, supporting the

Figure 8 Competition assay with unlabelled β_2 I and factor H

A fixed amount of 125 I-labelled β_2 I (1.5×10^6 c.p.m.; $0.8 \mu\text{g}$ of protein in $50 \mu\text{l}$) was preincubated with $50 \mu\text{l}$ of different concentrations of unlabelled β_2 I (■) and factor H (□), and was added to CL-coated plates as described in the Materials and methods section. The ordinate represents percentage of β_2 I bound relative to uninhibited control. Results are means of quadruplicate determinations.

suggestion that the positively charged region present in both proteins might be involved in binding to CL.

DISCUSSION

We investigated the binding characteristics of human β_2 I to CL. Dose-dependent binding of 125 I-labelled β_2 I to CL was observed, whereas binding to PC and Ch was negligible. Binding to CL was saturable, whereas no saturation was obtained with PC or Ch.

The binding of 125 I-labelled β_2 I to CL is specific as shown by a competition assay with unlabelled β_2 I (Figure 2) and by binding of total serum proteins to CL under physiological conditions (Figure 3a). Only two serum proteins, β_2 I and IgG, bound in high quantity to CL. Complement factor H was also shown to bind to CL (Figure 3b), but binding of factor H from serum was quantitatively less than the binding of β_2 I. At low ionic strength, however, the binding affinities of factor H and β_2 I appeared similar (Figure 8).

Binding of β_2 I to CL was pH-dependent (Figure 4). It is notable that in the normal physiological experimental range (pH 7–8) considerable alteration in binding is observed. Binding of 125 I-labelled β_2 I decreased with increasing salt concentration from 5 to 200 mM at pH 7 (Figure 5), whereas the effect of salt was negligible at pH 3. This observation shows that, at pH 7, interaction is mainly ionic, whereas, at pH 3, it is probably mainly hydrophobic. The results of Wurm [9] show a similar dependence on ionic strength of binding of β_2 I to phosphatidylserine multilamellar vesicles at pH 7.4.

In the presence of physiological concentrations of CaCl_2 or MgCl_2 at pH 7, binding of 125 I-labelled β_2 I to CL was slightly decreased (Figure 6). However, EDTA had no significant effect on binding. These results indicate that binding of β_2 I to CL is independent of bivalent cations. The decrease in binding in the presence of $\text{CaCl}_2/\text{MgCl}_2$ is partially due to increased salt concentration. Wurm [9] also found that CaCl_2 and MgCl_2 in the physiological range slightly reduced the interaction of β_2 I with phosphatidylserine, and Nimpf et al. [36] showed that CaCl_2 decreased the binding of β_2 I to platelets.

Heat treatment of β_2 I (temperatures from 56°C to 100°C) had no effect on binding of β_2 I to CL, indicating that it is independent of the three-dimensional or domain structure, but depends on the primary structure of β_2 I or on a heat-stable tertiary-structure motif. Further evidence that native three-dimensional structure of β_2 I is not required in binding was obtained from the failure of reduction/alkylation to abolish binding of β_2 I to CL (Figure 7).

KCNO treatment of β_2 I abolished binding to CL completely. No binding to PC or Ch was observed either. Since KCNO reacts stably only with $-\text{NH}_2$ and $-\text{SH}$ groups, it is likely that the reaction results in modification of lysine residues, as β_2 I has no free $-\text{SH}$ groups. This is consistent with the suggested CL-binding site on β_2 I. The involvement of histidine residues in binding of β_2 I to negatively charged phospholipid liposomes has been suggested by Schousboe [37], who found that β_2 I with histidine residues modified by treatment with diethyl pyrocarbonate exhibited decreased binding to phosphatidic acid liposomes. The diethyl pyrocarbonate modification was not, however, completely reversed by hydroxylamine treatment, suggesting that residues other than histidine may have been modified [33].

The possible role of histidine residues in the binding of the CL- β_2 I complex to anti-CL antibodies was also highlighted by Lauer et al. [26]. These authors used an assay system that depended on both binding of β_2 I to CL and subsequent binding of anti-CL antibodies. They used oligopeptides representing different segments of the fifth domain of human β_2 I, and found that a peptide representing amino acids at positions 268–278 (KNGMLHGDKVS) showed about the same binding activity as intact β_2 I. Replacement of the histidine residue in this peptide with alanine, or blocking it with a benzyl residue, decreased the binding by about 30% and 50% respectively. This suggests a role, but not a crucial one, for histidine in both binding processes. It was noted that lysine in the peptide was probably also important for the binding requirements of the assay. With lysine-rich peptides representing amino acids at positions 281–288 (CKNKEKKC) and 282–287 (KNKEKK), however, a lower degree of activity was found (49% and 11% respectively of maximal binding mediated by β_2 I).

Surprisingly, substantial binding activity was also found with a third peptide, KCFKEHSSLAF (305–315). These results are not directly comparable with our own study, as they rely not only on binding of β_2 I to CL but also binding of antibodies to the β_2 I-CL complex. However, they suggest that, if the KNKEKK sequence binds to CL, it does not also mediate antibody binding [38].

Hunt et al. [39] have shown that forms of β_2 I cleaved at residues 317–318 (K–T) or 314–315 (A–F) are unable to bind to CL. This suggests that a region very close to the C-terminus of β_2 I mediates CL binding. The precise region was not defined, as it was not determined whether short segments of β_2 I on the N-terminal side of the cleavage points were still present, i.e. it was not clarified whether loss of activity was due to cleavage of a single peptide bond or to the excision of a short peptide.

Comparison of available sequences of β_2 I from five species [20–24] (human, mouse, rat, cow, dog) shows that two segments (a) CKNKEKKCSYT (281–291) and (b) KCFKEHSSLAF-WKTDAS (305–322) are absolutely conserved. The region indicated by Lauer et al. [26] as being of most interest (KNG-MLHGDKVS) is not absolutely conserved, and D/Q substitutions occur in cow and dog and N/D in dog. Both of the conserved regions (a) and (b) were discussed as possible CL-binding sites by Steinkasserer et al. [25]. As there is considerable cross-reactivity among β_2 I from different species [40], and CL does not vary between species, the absolutely conserved regions

remain the best candidates for sites of interaction with CL. The binding interaction is ionic in nature and is clearly principally mediated by lysine residues, as indicated by the effects of KCNO. It is quite probable that more than one segment of the fifth domain may be involved, as suggested by the reports cited above. Multiple ionic interactions with groups of lysines in the three sequence segments discussed above may be required to provide an overall interaction of reasonably high affinity.

Structural similarities between β_2 I and factor H led us to study whether factor H had similar characteristics to those of β_2 I with regard to binding to CL. Factor H does inhibit binding of 125 I-labelled β_2 I to CL, and again an equimolar quantity of unlabelled factor H inhibits binding of radiolabelled β_2 I by 50 %. It is not known whether binding of factor H to CL has any physiological role, but it does bind to CL from serum (Figure 3b). Sequence similarities of factor H to β_2 I include the high-lysine regions DKLKKCKS (residues 748–755) and KNKKEFDH (residues 766–773) [35]. Both are within CCP 13 of factor H, and resemble the β_2 I sequence KNKEKK; however, the other β_2 I segments discussed above are also lysine-rich, and the relationship of the factor H sequences to the different β_2 I segments should not be overinterpreted.

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