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Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: β_2-Glycoprotein I (apolipoprotein H)

(anti-cardiolipin antibodies/lipid–protein complex/thrombosis)

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ABSTRACT **Anti-phospholipid (aPL) antibodies that exhibit binding in cardiolipin (CL) ELISA can be purified to >95% purity by sequential phospholipid affinity and ion-exchange chromatography. However, these highly purified aPL antibodies do not bind to the CL antigen when assayed by a modified CL ELISA in which the blocking agent does not contain bovine serum, nor do they bind to phospholipid affinity columns. Binding to the phospholipid antigen will only occur if normal human plasma, human serum, or bovine serum is present, suggesting that the binding of aPL antibodies to CL requires the presence of a plasma/serum cofactor. Using sequential phospholipid affinity, gel-filtration, and ion-exchange chromatography, we have purified this cofactor to homogeneity and shown that the binding of aPL antibodies to CL requires the presence of this cofactor in a dose-dependent manner. N-terminal region sequence analysis of the molecule has identified the cofactor as β_2-glycoprotein I (β_2GPI) (apolipoprotein H), a plasma protein known to bind to anionic phospholipids. These findings indicate that the presence of β_2GPI is an absolute requirement for antibody–phospholipid interaction, suggesting that bound β_2GPI forms the antigen to which aPL antibodies are directed. Recent evidence indicates that β_2GPI exerts multiple inhibitory effects on the coagulation pathway and platelet aggregation. Interference with the function of β_2GPI by aPL antibodies could explain the thrombotic diathesis seen in association with these antibodies.**

Anti-phospholipid (aPL) antibodies are autoantibodies that can be detected in plasma or serum in solid-phase immunoassays in which negatively charged phospholipids, most commonly cardiolipin (CL), are used as the antigen (1, 2). We have previously described a simple two-step procedure for purifying aPL antibodies from plasma (or serum) by sequential phospholipid affinity and cation-exchange chromatography, yielding specific immunoglobulin of >95% purity (3). These antibodies exhibit typical binding in CL ELISA but do not possess lupus anticoagulant (LA) activity in phospholipid-dependent clotting tests. Recently, we have also shown that plasma can be resolved by ion-exchange chromatography into fractions containing either anticardiolipin (aCL) antibodies or antibodies with LA activity, strongly suggesting that aCL and LA antibodies represent distinct antibody subgroups (4). Although antibodies binding CL in immunoassays also generally bind all anionic phospholipids (2, 4), and hence are best referred to as aPL antibodies, we hereafter refer to this group as aCL antibodies in distinction to LA, which may also be aPL antibodies but appear to be directed against a different antigen (4).

We have previously noted that when aCL antibody-containing fractions derived from ion-exchange chromatography of plasma were applied to phosphatidylserine or CL affinity columns, there was no binding of the antibody despite the fact that when plasma containing these antibodies was applied to these columns, aCL antibodies could be purified. This suggested that there was a cofactor also present in plasma or serum that was required for aCL antibodies to bind to the affinity columns. Addition of normal (aCL antibody negative) plasma to the ion-exchange fractions resulted in aCL binding to the columns supporting this hypothesis (4).

In this report, we have further investigated this phenomenon. We have found that the plasma cofactor is also required for aCL antibodies to bind CL in a modified immunoassay in which bovine serum (which also contains the cofactor) is excluded. We have been able to purify the cofactor to homogeneity and identify it as β_2-glycoprotein I (β_2GPI), also known as apolipoprotein H. These results may change our understanding of the biology of aCL antibodies.

MATERIALS AND METHODS

Plasma and Patients. Citrated platelet-free plasma was prepared by adding freshly drawn blood from venipuncture into tubes containing 1/10th final vol of 0.11 M trisodium citrate, immediate centrifugation at 2500 × *g* for 15 min, and filtration through a 0.22-μm Millipore Millex filter (4). aCL antibodies were purified from two patients with antiphospholipid syndrome whose plasma contained high levels of aCL antibodies and strong LA activity. Clinical details and complete coagulation screen on these two patients have been previously published (4). Plasmas from two other patients with systemic lupus erythematosus but without histories of thromboses were subjected to ion-exchange chromatography. The plasma cofactor was purified from a normal healthy 31-year-old male whose plasma was negative for aCL antibodies and LA activity.

Ion-Exchange Chromatography of Patient Plasma. Patient plasma was extensively dialyzed against 0.05 M acetate/0.05 M NaCl buffer, pH 4.8, centrifuged, then run on a Sepharose fast flow cation-exchange column (Pharmacia) using a Pharmacia fast protein liquid chromatography (FPLC) system with a 1500-ml linear gradient of 0–100% eluting buffer (0.05 M acetate/0.65 M NaCl, pH 5.2) over 5 hr as described (4). Fractions were assayed for aCL antibodies by a standard CL ELISA (5) and aCL antibody-positive fractions were dialyzed against 0.01 M phosphate-buffered 0.15 M saline (pH 7.2) (PBS) (Dulbecco A).

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Abbreviations: β_2GPI, β_2-glycoprotein I; CL, cardiolipin; aCL, anti-CL; aPL, anti-phospholipid; LA, lupus anticoagulant.

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CL Affinity Chromatography. A modification of the previously described method (3) for phosphatidylserine affinity chromatography was used. CL (5.6 mg in ethanol) (Sigma) was placed in a glass scintillation vial and the solvent was evaporated under a stream of nitrogen. Cholesterol (2.32 mg) (BDH) and dicetyl phosphate (0.44 mg) (Sigma) were added and the lipids were redissolved in chloroform. The solvent was again evaporated, 500 μl of ethanol was added, and the vial was capped and immersed in boiling water and swirled until the lipids were dissolved. Five milliliters of a 15% acrylamide/5% bisacrylamide (Bio-Rad) solution was added and the lipid/acrylamide mixture was vigorously mixed in a Vortex, then rapidly polymerized by adding 100 μl of ammonium persulfate (140 mg/ml), 2.5 μl of TEMED (both from Bio-Rad), and then left at room temperature overnight. The rigid gel was homogenized by using a hand-operated loose-fitting Teflon pestle and loaded into an empty Pharmacia FPLC HR 10/10 glass column and equilibrated with 0.01 M phosphate/0.05 M NaCl, pH 7.2. Plasma was diluted 1:5 final volume with this buffer and infused through the column at 0.5 ml/min, after which the column was washed at 0.8 ml/min until the absorbance at 280 nm of the fall-through was <0.01 unit. A 40-ml linear gradient from 0% to 100% of eluting buffer (0.01 M phosphate/1.0 M NaCl, pH 7.2) over 50 min was applied and eluted fractions containing protein were collected. Ion-exchange fractions were applied undiluted and chromatographed as described above.

Standard aCL Antibody Immunoassay. The ELISA for aCL antibodies previously described (2) with minor modifications (5) was used. When using polyvalent second antibody, serial dilutions of a known positive plasma were included on each plate. The aCL antibody level of the test samples was expressed in aCL antibody units with 100 units being the absorbance at 405 nm of the positive sample at 1:50 dilution.

Modified aCL Antibody Immunoassay. The above standard CL ELISA was modified as follows. Milk powder (1%) (Diploma, Unigate Australia, Dandenong, Australia)/0.3% gelatin (Ajax Chemicals, New South Wales, Australia)/PBS was used for the blocking step, samples were added diluted in 0.3% gelatin/PBS, and the second antibody was diluted in 1% bovine serum albumin/PBS instead of 10% adult bovine serum/PBS, which was used in each of the above steps in the standard assay. All other details remained identical to the standard CL ELISA described above.

Affinity Purification of aCL Antibodies. Plasma from patients with high levels of aCL antibodies was chromatographed on the CL affinity column as described above. The protein eluted from this column was dialyzed overnight against 0.05 M acetate/0.05 M NaCl, pH 4.8, then applied to a Mono-S cation-exchange column (Pharmacia) using a FPLC system. A 15-ml linear gradient from 0% to 100% of eluting buffer 0.05 M acetate/0.65 M NaCl, pH 5.2, over 30 min was applied and highly purified aCL antibodies eluted at 30% eluting buffer (3).

Purification of the Plasma Cofactor. Normal plasma was chromatographed on the CL affinity column as described above. Fractions containing the eluted protein were concentrated in an Amicon concentrator with a YM 10 membrane and 200 kPa pressure, then dialyzed against 0.01 M phosphate/1 M NaCl, pH 7.2, and further concentrated to 200 μl in a Micro-Pro-DiCon dialyzing concentrator (Bio-Molecular Dynamics, Beaverton, OR) with a PA-10 membrane. The concentrated CL column eluant was applied to a Pharmacia Superose 12 10/30 gel-filtration column operating with a FPLC system at 0.4 ml/min in a buffer of 0.01 M phosphate/1 M NaCl, pH 7.2. Fractions from this column that were found to have cofactor activity (as described in *Results*) were pooled and dialyzed overnight against 0.05 M acetate/0.05 M NaCl, pH 4.8, then applied to a Pharmacia Mono-S cation-exchange column and eluted with 0.05 M acetate/0.65 M NaCl as described above for the purification of aCL antibodies. Fractions found to have cofactor activity were pooled and dialyzed against PBS and then stored in aliquots at −70°C.

Amino Acid Sequencing. Automated Edman degradation of purified cofactor was performed with an Applied Biosystems sequencer (model 477A) equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120A). Total amino acid derivatives from the sequencer were injected onto the liquid chromatograph by using a modified sample transfer device described elsewhere (6). Polybrene was used as a carrier (7).

Heparin Affinity Chromatography. aCL antibody-positive plasma, or affinity-purified aCL antibody, and/or purified cofactor were applied to a column packed with 40 ml of heparin-Sepharose CL-4B (Pharmacia) in 0.01 M phosphate/0.05 M NaCl buffer, pH 7.2, and bound protein was eluted with 0.01 M phosphate/1 M NaCl, pH 7.2.

Other Methods. Samples for SDS/PAGE were run on a 5–15% linear gradient gel with a 3% stacking gel (8). After electrophoresis, the gel was stained with Coomassie blue. Protein determination was by the method of Lowry *et al.* (9). Vitamin K-dependent coagulation factor-depleted plasma was prepared by mixing 0.1 ml of 50% aluminum hydroxide suspension (BDH) with 1 ml of plasma and inverting the tube every minute for 15 min before centrifuging the precipitate.

RESULTS

CL Affinity Chromatography of aCL Antibody-Containing Ion-Exchange Fractions from Plasma. As previously described (4), plasma containing aCL antibodies and LA activity can be separated into subgroups by cation-exchange chromatography. When aCL antibody-containing fractions were infused through the CL affinity column, there was no binding with the column fall-through containing equivalent amounts of aCL antibody as the applied fraction. When normal plasma was added to the ion-exchange fractions in a ratio of 1:10 (vol/vol) and the mixture was infused through the CL affinity column, ≈30% of the applied aCL antibodies absorbed to and could be eluted from the column (Table 1). Normal plasma that had been mixed with aluminum hydroxide to remove vitamin K-dependent coagulation factors also produced a similar effect. Finally, a fraction derived from normal plasma was purified (see below) and addition of this "cofactor" fraction (protein concentration, 200 μg/ml) in a ratio of 1:5 (vol/vol) resulted in the absorption of 74% of the applied aCL antibodies.

Binding of aCL Antibody-Containing Ion-Exchange Fractions and Affinity-Purified aCL Antibodies in a Modified aCL Antibody Immunoassay. The above results suggested a plasma dependency for the binding of aCL antibodies to CL in the affinity column. However, these aCL antibody-

Table 1. Chromatography of aCL antibody-containing ion-exchange fractions on CL affinity column

Sample	Amount of aCL antibody	
	Total applied, units	Fall-through, units (%)
IE	105	101 (96)
IE + normal Pl*	120	80 (66)
IE + adsorb Pl*	120	90 (75)
IE + β_2GPI†	122	32 (26)

IE, ion-exchange fraction; Pl, plasma; adsorb, $Al(OH)_3$ adsorbed. % of total applied aCL antibody recovered in fall-through is given in parentheses.

*One milliliter of plasma was added to 10 ml of IE.

†Four hundred micrograms of β_2GPI (equivalent to 2 ml of plasma) was added to 10 ml of IE.

containing fractions and affinity-purified aCL antibodies exhibit typical binding in standard solid-phase CL immunoassays in the absence of plasma (3). These assays use bovine serum as the blocking agent and diluent, which could conceivably also contain the plasma factor necessary for antibody binding to CL. To test this, we replaced the bovine serum with gelatin as the diluent and milk powder/gelatin as the blocking agent. When aCL antibody-containing ion-exchange fractions or affinity-purified aCL antibodies were tested in this modified CL immunoassay, there was no binding at all. At 1:50 dilution, aCL antibody-positive plasma exhibited identical binding as in the standard CL ELISA and normal plasma was negative. Binding of antibodies in the ion-exchange fractions or affinity-purified preparations did occur if normal plasma was added at 1:50 dilution. An identical effect was also found if bovine serum was added to these fractions (Fig. 1). Thus, purified aCL fractions will bind in the standard CL ELISA since the cofactor is provided by bovine serum.

This modified CL ELISA provided a convenient method to detect cofactor activity in fractions derived from normal plasma by testing samples containing a mixture of affinity-purified aCL antibodies and the fraction containing an unknown amount of cofactor. In the absence of cofactor, the affinity-purified aCL antibodies would not bind and any binding was evidence of cofactor activity in the test fraction. As described below, we purified the cofactor activity from normal plasma, and the addition of this fraction to affinity-purified aCL antibodies or aCL antibody-containing ion-exchange fractions resulted in binding of these antibodies in the modified CL ELISA (Fig. 1).

Purification and Identification of the Cofactor in Normal Plasma. Normal plasma was chromatographed on the CL affinity column and the fractions were tested for cofactor activity as described above by the modified CL ELISA. Cofactor activity was found in the eluted protein, indicating that the factor bound to anionic phospholipid even in the absence of aCL antibodies. These fractions were concentrated and chromatographed on a Superose 12 gel-filtration column in 1 M NaCl buffer. Cofactor activity was found in a peak eluting at a K_{av} of 0.350, which corresponded to an apparent molecular mass of 67 kDa on this column (Fig. 2). The fractions containing this peak were pooled, dialyzed against the ion-exchange starting buffer, and chromatographed on a Mono-S column. Cofactor activity was found in a late peak eluting at ≈0.5 M NaCl (Fig. 3).

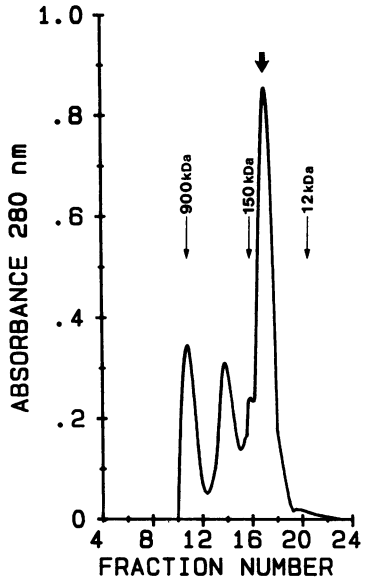

FIG. 2. Gel filtration on a Pharmacia Superose 12 HR 10/30 column of the eluant from CL affinity column chromatography of normal plasma. Buffer was 0.01 M phosphate/1 M NaCl. Cofactor activity was detected in the large peak indicated by the boldface arrow.

The final preparation from the ion-exchange column was found to be highly purified with a single band on SDS/PAGE of apparent molecular mass 50 kDa under both reduced and nonreduced conditions (Fig. 4). This band corresponded to a band previously found in the CL or phosphatidylserine affinity column eluant when aCL antibody-containing plasma was chromatographed on those columns (also shown in Fig. 4). The N-terminal amino acid sequence of the cofactor is shown in Fig. 5. A computer search of current data base releases shows this sequence is identical to the N-terminal sequence of the plasma protein β_2GPI, also known as apolipoprotein H. There were no other proteins that exhibited homology to this sequence except a plasma protein purified by Canfield and Kisiel (10), termed activated protein C

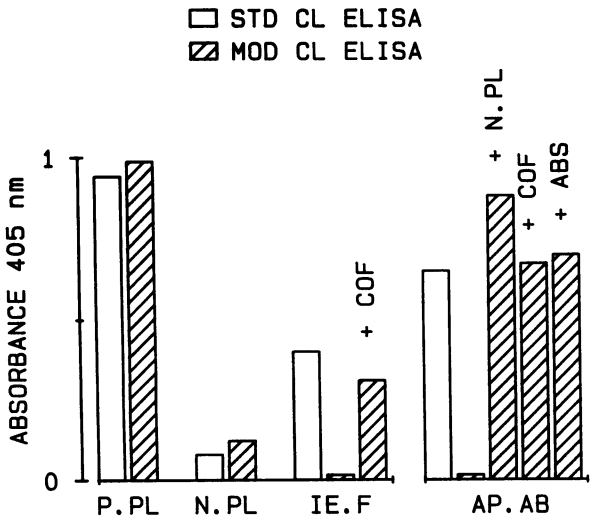

FIG. 1. Binding of various samples in standard and modified CL ELISA. IE.F, aCL antibody-containing ion-exchange fractions; AP.AB, affinity-purified aCL antibodies; N.PL, normal plasma; ABS, adult bovine serum; COF, purified cofactor; P.PL, patient plasma. ABS and COF are negative when tested alone (data not shown).

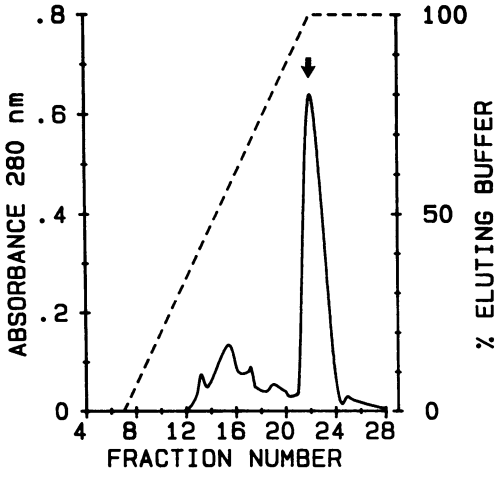

FIG. 3. Cation exchange on a Pharmacia Mono-S HR 5/5 column of the cofactor-containing fractions from Fig. 2. Starting buffer was 0.05 M acetate/0.05 M NaCl, pH 4.8. Eluting buffer was 0.05 M acetate/0.65 M NaCl, pH 5.2. Dashed line, eluting buffer gradient. Cofactor activity was detected in the late peak indicated by the arrow.

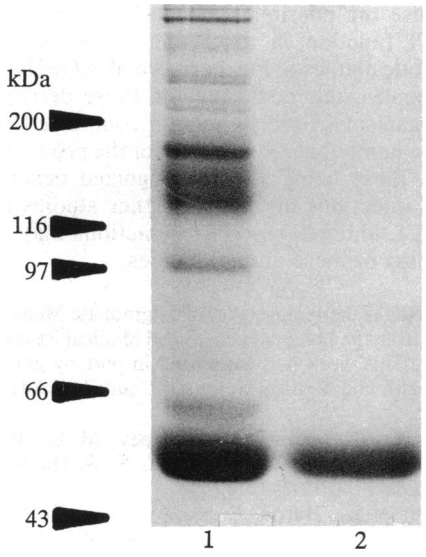

FIG. 4. SDS/PAGE on a 5–15% linear gradient gel under nonreducing conditions stained with Coomassie blue. Lane 1, eluant from CL affinity column chromatography of aCL antibody-positive plasma containing a broad band of IgG aCL (150 kDa) and cofactor (50 kDa). Lane 2, purified cofactor obtained from normal plasma following sequential CL affinity, gel filtration (Fig. 2), and cation-exchange chromatography (Fig. 3).

binding protein, which has subsequently been reported to be identical to β_2GPI (11, 12).

Interaction Between Purified β_2GPI and Affinity-Purified aCL Antibodies in the Modified CL ELISA. Since affinity-purified aCL antibodies do not bind CL in the modified CL ELISA unless β_2GPI is present (Fig. 1), we examined the binding of sequential dilutions of the antibody in the presence of sequential dilutions of glycoprotein purified as described above. These results are shown in Fig. 6*a*. In the presence of β_2GPI (8 μg/ml), serial dilutions of aCL antibody show a binding curve typical of that found with the standard CL ELISA. With increasing dilutions of β_2GPI, binding decreases dramatically at all dilutions of antibody with virtually no binding when the glycoprotein is reduced to 1 μg/ml. The dashed line in Fig. 6*a* accounts for serial dilution of both factors. Dilution of aCL antibody-positive plasma is shown in Fig. 6*b*. The solid line shows the familiar binding curve in a standard CL ELISA in which the presence of adult bovine serum ensures adequate cofactor at all antibody dilutions. The dashed line shows the binding curve when aCL antibody-positive plasma is diluted in the modified CL ELISA. There is virtually no binding of antibody once plasma is diluted to 1:200, corresponding to a β_2GPI level of $\approx$1 μg/ml.

Heparin Affinity Chromatography. Affinity-purified aCL antibodies did not bind in a heparin-Sepharose affinity column with $\approx$60% of the applied aCL antibody recovered in the fall-through fractions, but no antibody was eluted with 1 M NaCl. Purified β_2GPI bound to the heparin-Sepharose when applied to the column. When a mixture of affinity-purified aCL antibodies and purified β_2GPI was infused through the heparin-Sepharose column, the fall-through contained aCL but not β_2GPI and the eluted protein contained β_2GPI but not aCL. When plasma containing aCL antibodies was infused through heparin-Sepharose, the fall-through fractions contained equivalent quantities of aCL antibody to the applied plasma, but the eluted protein contained β_2GPI and not aCL antibody.

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     1                5                   10
NH2-Gly-Arg-Thr-X-Pro-Lys-Pro-Asp-Asp-Leu-Pro-Phe-
          15                  20                  24
Ser-Thr-Val-Val-Pro-Leu-X-Thr-Phe-Tyr-Glu-Pro-
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FIG. 5. N-terminal amino acid sequence of the purified cofactor. X, residues cannot be identified unambiguously.

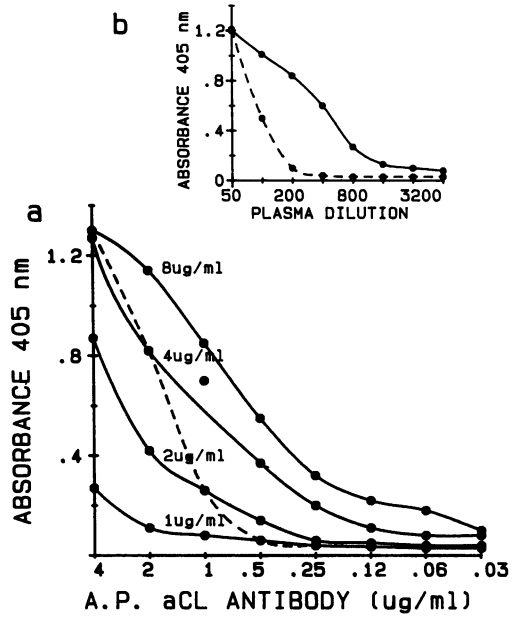

FIG. 6. (*a*) Binding in the modified CL ELISA of serial dilutions of affinity-purified (A.P.) aCL antibodies in the presence of serial dilutions of cofactor. Dashed line, extrapolated binding curve based on dilution of both antibody and cofactor. (*b*) Binding in the standard CL ELISA (solid line) and modified CL ELISA (dashed line) of serial dilutions of aCL antibody-positive plasma.

DISCUSSION

We have clearly demonstrated in two systems that a plasma cofactor is required for aCL antibodies to bind CL. We have purified this cofactor and identified it as a known plasma protein, β_2GPI.

In the affinity chromatography system, aCL antibodies contained in fractions derived from ion-exchange of plasma did not bind to immobilized CL despite doing so when present in plasma, but binding did occur if normal plasma was added to the ion-exchange fraction. This was the first suggestion that a plasma cofactor was involved in aCL antibody–CL interactions, and we have confirmed this by isolating this cofactor activity to a single plasma protein, β_2GPI (Table 1). Having demonstrated this requirement, why these ion-exchange fractions bound CL in the standard CL ELISA was problematic. One explanation was that the cofactor or a similar molecule was also present in the bovine serum diluent used in the immunoassay. We confirmed this by modifying the CL ELISA, omitting bovine serum and using gelatin as the diluent. In this assay, aCL antibody-containing ion-exchange fractions and affinity-purified aCL antibodies did not bind CL unless normal plasma or bovine serum was added (Fig. 1). Once again, in this system, the cofactor activity was isolated to β_2GPI. This was a consistent finding in 11 separate aCL antibody-containing ion-exchange fractions from four patients consecutively studied, and three affinity-purified aCL antibody preparations [two IgG and one IgM] (data not shown).

The modified CL ELISA proved to be a convenient system to examine the cofactor effect in more detail. We found the action of β_2GPI on aCL antibody binding to CL was dose dependent with a steep dilution effect, such that there was virtually no antibody binding at any dilution when β_2GPI was

present at 1 μg/ml or less (Fig. 6*a*). Since the normal plasma level of β_2GPI is 200 μg/ml, this figure corresponded exactly to the results of diluting aCL antibody-positive plasma in the modified CL ELISA, where there was a rapid drop-off in binding, which became negative at 1:200 dilution, or at a β_2GPI level of 1 μg/ml, despite the presence of appreciable amounts of aCL antibody (Fig. 6*b*).

When plasma containing IgG aCL antibodies is chromatographed on CL or PS affinity columns, two major protein bands are eluted (3). One of these is IgG, and the other is β_2GPI (Fig. 4). Since we purified β_2GPI from normal plasma by using the CL affinity column, it is clear that β_2GPI binds to anionic phospholipid columns in both the presence and absence of aCL antibodies. Yet these antibodies do not bind CL in the absence of β_2GPI. Purified β_2GPI but not aCL bound to a heparin-Sepharose column, but aCL antibodies did not bind to this column even in the presence of bound β_2GPI. Thus, aCL antibodies recognized β_2GPI bound to anionic phospholipid, but not β_2GPI bound to heparin, indicating that both phospholipid and glycoprotein comprise the epitope to which these antibodies are directed. These results suggest that aCL antibodies are directed against either a complex consisting of β_2GPI bound to anionic phospholipid, or a cryptic epitope formed during the interaction of β_2GPI with phospholipid, but not β_2GPI independent of the presence of phospholipid.

β_2GPI is a plasma β_2-globulin first described in 1961 (13). The complete amino acid sequence was determined in 1984 (11). It is composed of 326 amino acids and has a unique sequence with abundant proline residues, multiple disulfide bridges, and a high carbohydrate content (19%) resulting in an apparent molecular mass of 50 kDa (11). The physiologic function of this glycoprotein is uncertain, but it is known to bind to lipoproteins (14), anionic phospholipids (15), platelets (16), heparin (17), DNA (18), and mitochondria (19). The concentration of β_2GPI in plasma is ≈200 μg/ml and 40% is associated with lipoproteins of various classes (14). Because of this, β_2GPI has been designated apolipoprotein H (20). In recent years, β_2GPI has been found to inhibit the intrinsic coagulation pathway (12, 21) and to bind to platelets (16) and inhibit ADP-mediated platelet aggregation (22). Schousboe (12) has suggested that one function of β_2GPI is to bind to and neutralize negatively charged macromolecules that might enter the bloodstream and thus diminish unwanted activation of blood coagulation.

The finding that aCL antibodies are directed against an antigen that includes β_2GPI opens more avenues to our understanding of these autoantibodies. It provides an explanation for aCL antibodies binding equally well to all anionic phospholipids despite various structures (2, 4). In addition, since β_2GPI appears to inhibit the intrinsic pathway of coagulation and ADP-dependent platelet aggregation, these findings raise the possibility that aCL antibodies interfere with β_2GPI function *in vivo*, thereby predisposing to a prothrombotic diathesis. Furthermore, if β_2GPI binds anionic macromolecules, this could include those derived from infectious organisms. Foreign antigen complexed with β_2GPI could be the immunogenic stimulus for the production of aCL antibodies, these being a well-recognized occurrence in a number of infectious diseases. Further studies directed at studying aCL antibody–β_2GPI interactions may add to our understanding of these autoantibodies.

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