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Identification of a region of β_2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity

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ABSTRACT **β_2-Glycoprotein I (β_2-GPI), a phospholipid-binding plasma protein, is an absolute requirement (cofactor) for the binding of autoimmune-type anti-cardiolipin (aCL) antibodies to cardiolipin (CL). The nature of this cofactor activity and the specific regions of the molecule involved have not yet been determined. We have identified a preparation of β_2-GPI that lacks aCL antibody cofactor activity. Analysis of the structural differences between the active and inactive forms enabled identification of the region of β_2-GPI critically important for aCL cofactor activity. The active form of β_2-GPI bound CL and displayed cofactor activity down to 1 μg/ml. The inactive form failed to bind CL and possessed no cofactor activity even at concentrations up to 94 μg/ml, indicating that the ability of β_2-GPI to bind lipid is an absolute requirement for aCL cofactor activity. Both forms possessed identical N-terminal sequences and were recognized as essentially immunoreactively identical by polyclonal antisera to β_2-GPI. However, the inactive form has undergone proteolytic cleavage and exists primarily as a "clipped" molecule, the polypeptide chain being cleaved between Lys-317 and Thr-318 (a potential thrombin cleavage site), with the two cleaved segments linked as a disulfide-bonded complex. This indicates that the C-terminal region is critically important for β_2-GPI to bind lipid and for aCL cofactor activity. The clipped form of β_2-GPI would not be suitable for use as aCL cofactor and its use may have led some investigators to conclude incorrectly that β_2-GPI does not interact with aCL antibodies.**

Anti-cardiolipin (aCL) antibodies are autoantibodies detected in a wide range of conditions including infections (1) and autoimmune disorders (2). However, severe clinical complications such as thrombosis (2) and recurrent spontaneous fetal loss (3) are only reported in the autoimmune group of patients, including those with the primary antiphospholipid syndrome (4). Furthermore, β_2-glycoprotein I (β_2-GPI) plays a critical role (as cofactor) in the recognition of the phospholipid antigen cardiolipin (CL) by aCL antibodies purified from patients with autoimmune disease (5) but not from patients with infection (6). This suggests that the interaction of the autoimmune type of aCL antibodies with β_2-GPI may be associated with the reported thrombotic complications seen in some patients with aCL antibodies.

β_2-GPI was first reported by Shultze *et al.* (7), and the complete amino acid sequence was published by Lozier *et al.* (8). It is a single-chain polypeptide (326 amino acids) with an apparent molecular mass of ≈50 kDa, contains a high proportion of proline and cysteine residues, and is highly glycosylated (8). A number of isoforms have been reported (9).

Although its physiological role remains unclear, β_2-GPI is known to be associated with lipoprotein structures, especially chylomicrons (10), and to bind platelets (11), heparin (12), and negatively charged phospholipids (13). Reports suggest that β_2-GPI may be involved in lipid metabolism (14, 15) and that it possesses anticoagulant properties (16–18).

β_2-GPI is a member of the short consensus repeat or complement control protein repeat superfamily (19). These proteins have in common a repeating motif of ≈60 amino acids, with a highly conserved pattern of cysteine residues. β_2-GPI is composed of five of these repeats (20).

The structural characteristics of β_2-GPI required for aCL antibody cofactor activity, to our knowledge, have not been elucidated. This information may help in identifying the chemical domains that are critical for the interaction(s) among β_2-GPI, phospholipid, and aCL antibody. The identification of a form of β_2-GPI that lacked cofactor activity enabled us to further characterize the interaction(s). Therefore, the aim of this study was to investigate the functional and structural differences between the active and inactive forms of β_2-GPI and to identify the specific regions of this molecule important for binding lipid and for aCL cofactor activity.

METHODS

Purification of β_2-GPI. β_2-GPI was purified from normal human serum as described (5). Briefly, human serum was subjected to phospholipid affinity chromatography (21), followed by gel-filtration (Pharmacia Superose 12/30 column) and ion-exchange chromatography (Pharmacia Mono S HR 5/5 column). Fractions containing β_2-GPI were checked for purity by SDS/PAGE. β_2-GPI prepared using this method in our laboratory will be referred to as β_2-GPI^{LAB}.

A preparation of β_2-GPI that was previously commercially available (described as salt free, lyophilized, and of at least 98% purity) was kindly provided by H. G. Schwick (Behringwerke AG Marburg/Lahn, Germany). According to the information provided, the purification procedure started from a Cohn fraction IV with the aid of Rivanol and ammonium sulfate precipitation. This was followed by chromatography on DEAE-cellulose and then gel filtration with Ultrogel AcA 34. This preparation will be referred to as β_2-GPI^{COM}.

SDS/PAGE. Samples were analyzed by SDS/PAGE as described (5).

Modified aCL ELISA. A modification of the standard aCL ELISA, where no serum-derived diluents or blocking agents are used, was performed as described (5). This modified ELISA system can be used for testing samples of β_2-GPI for aCL antibody cofactor activity. Briefly, the sample is incubated in the presence of purified aCL antibodies that only bind CL in the presence of β_2-GPI, and any binding that occurs is evidence of cofactor activity. aCL antibodies used in this assay were purified by CL affinity chromatography

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Abbreviations: β_2-GPI, β_2-glycoprotein I; aCL, anti-cardiolipin; CL, cardiolipin; PC, phosphatidylcholine.

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(21) followed by ion-exchange chromatography on a Pharmacia Mono S HR 5/5 column as described (6).

In the first series of experiments, the cofactor activity of β_2-GPI^{LAB} (0–9.1 μg/ml) and β_2-GPI^{COM} (0–94 μg/ml) was determined when incubated with a constant concentration of purified aCL (1 μg/ml, n = 5).

A further series of experiments were performed with various concentrations of aCL (0.01–4.0 μg/ml) and a constant concentration of β_2-GPI^{LAB} or β_2-GPI^{COM} (8 μg/ml).

Chromatofocusing. Chromatofocusing was performed on a Pharmacia Mono P HR 5/20 column using a Pharmacia FPLC system, a flow rate of 0.8 ml/min, with 0.025 M Tris CH_3COOH (pH = 8.3) as the start buffer and 30% (vol/vol) Polybuffer 96/70% (vol/vol) Polybuffer 74, diluted 1:30 in distilled H_2O and the pH was adjusted to 5.0 with CH_3COOH, as the elution buffer. Absorbance was monitored at 280 nm and 1-min fractions were collected. The pH of the fractions was measured using a mini-pH meter with a small probe (Proxima 2-F; LEM Electronics, Bologna, Italy) and the pH was graphed against fraction number. The pI value estimated using these measurements is referred to as the "apparent pI."

The fractions generated were tested for the presence of cofactor activity (at a 1:5 dilution with aCL at 1 μg/ml) by using the modified aCL ELISA described above.

Binding of ^{125}I-Labeled β_2-GPI^{LAB} and ^{125}I-Labeled β_2-GPI^{COM} to CL and Phosphatidylcholine (PC). β_2-GPI^{LAB} (1.6 μg) and β_2-GPI^{COM} (1.6 μg) were radiolabeled with ^{125}I using the lactoperoxidase method as described (22). For binding experiments, Immulon II microtiter wells (Dynatech) were coated with either 30 μl of CL (a negatively charged phospholipid) at 30 μg/ml in ethanol or 30 μl of PC (a phospholipid with no net charge) at 50 μg/ml in chloroform/methanol [1:3 (vol/vol)], dried under vacuum, and blocked with 200 μl of 1% milk powder/0.3% gelatin in phosphate-buffered saline (PBS) for 1 h at room temperature. The wells were washed three times with PBS, and then 50 μl (5×10^4 cpm) of either ^{125}I-labeled β_2-GPI^{LAB} or ^{125}I-labeled β_2-GPI^{COM} in 0.3% gelatin/PBS was then added to each well (in quadruplicate), incubated at room temperature for 0, 10, 30, 60, 180, or 360 min, and then washed three times with PBS. Radioactivity in individual wells was then measured in a γ counter.

RIA for β_2-GPI. β_2-GPI levels in fractions from the chromatofocusing experiments were measured using a competitive RIA as described (6). Fractions were tested at dilutions of 1:50 and 1:100, 1:100 and 1:200, 1:400 and 1:800, or 1:800 and 1:1600, depending on their protein concentration estimated from the absorbance at 280 nm (A_{280}).

This RIA system was also used to examine the antigenic similarity between β_2-GPI^{LAB} and β_2-GPI^{COM}. Four standard curves were constructed with ^{125}I-labeled β_2-GPI^{LAB} as the labeled species and serial dilutions (1750 ng–27.3 ng) of β_2-GPI^{LAB} (curve 1) or β_2-GPI^{COM} (curve 2) as the competitor species or with ^{125}I-labeled β_2-GPI^{COM} as the labeled species and β_2-GPI^{LAB} (curve 3) or β_2-GPI^{COM} (curve 4) as the unlabeled species. The standard curves were constructed using a 4-parameter logistic curve-fitting program (23).

Immunodiffusion. This was performed on glass plates using 1% agarose and PBS by the method of Ouchterlony (24).

Amino Acid Sequence Analysis. β_2-GPI^{LAB} (50 μg) and β_2-GPI^{COM} (50 μg) were subjected to automated Edman degradation using an Applied Biosystems sequencer (model 477A) and an on-line phenylthiohydantoin amino acid derivative analyzer (model 120A) as described (5).

RESULTS

SDS/PAGE. SDS/PAGE revealed that both β_2-GPI^{LAB} and β_2-GPI^{COM} ran as single major bands. However, β_2-GPI^{LAB} ran at a higher molecular mass (48 kDa nonreduced vs. 57.5 kDa reduced) than β_2-GPI^{COM} (46.5 kDa nonreduced vs. 54 kDa reduced), when both samples were electrophoresed on the same gel (Fig. 1).

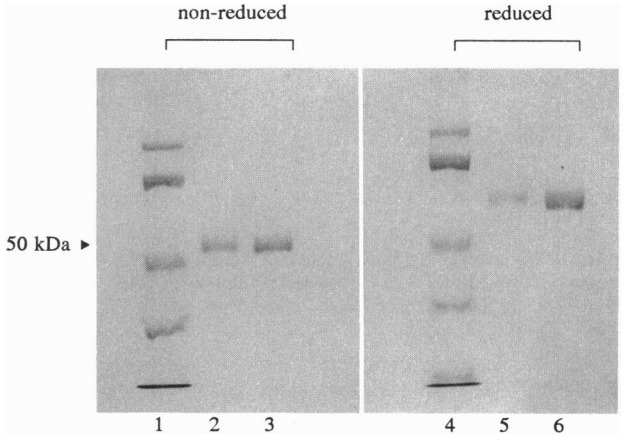

FIG. 1. SDS/PAGE (10% gel) of β_2-GPI^{LAB} and β_2-GPI^{COM}. Lanes: 1–3, nonreduced; 4–6, reduced; 1 and 4, molecular mass markers; 2 and 5, β_2-GPI^{LAB} (5 μg); 3 and 6, β_2-GPI^{COM} (5 μg).

Modified aCL ELISA: Comparison of Cofactor Activity. In the presence of purified aCL antibody at 1 μg/ml, serial dilutions of β_2-GPI^{LAB} resulted in a binding curve typical of that seen in a standard aCL ELISA (5). With increasing dilution of β_2-GPI^{LAB} (9.1 μg/ml to 0.09 μg/ml) binding decreased, with virtually no binding apparent at $\approx$1 μg/ml. In contrast, no cofactor activity was demonstrated with β_2-GPI^{COM} up to a concentration of 94 μg/ml (Fig. 2*A*). In a further series of experiments, we were unable to demonstrate cofactor activity even at β_2-GPI^{COM} concentrations of 256 μg/ml (data not shown).

aCL binding activity increased with increasing concentration of aCL only in the presence of β_2-GPI^{LAB} (Fig. 2*B*). There was no significant binding demonstrated with buffer or β_2-GPI^{COM}.

Chromatofocusing. Chromatofocusing revealed that a number of different isoelectric subspecies were present in the two samples. β_2-GPI^{COM} contained three major peaks (apparent pI values, 6.7, 6.6, and 6.3) and four minor peaks (pI values, 6.9, 6.8, 6.5, and 5.8). Most of the material eluted between fractions 20 and 32 with one peak eluting at fraction 40. β_2-GPI^{LAB}, as well as containing material that eluted after fraction 20 (pI values 6.9, 6.8, 6.7, 6.6, 6.4, and 6.0), contained three isoelectric species that eluted in fractions

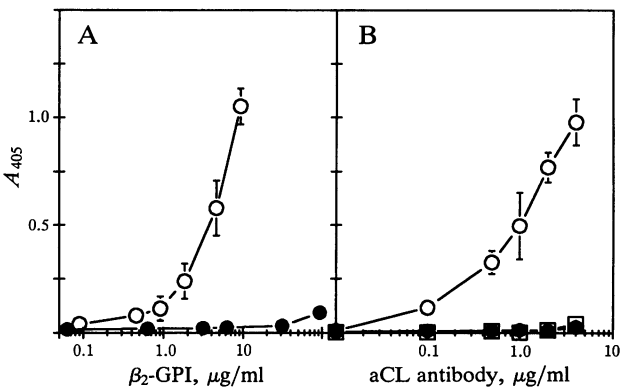

FIG. 2. (*A*) Modified aCL ELISA: Constant aCL antibody concentration (1 μg/ml) and various concentrations of β_2-GPI. ○, β_2-GPI^{LAB} at 0.09–9.13 μg/ml; ●, β_2-GPI^{COM} at 0.06–94 μg/ml. Data are the mean ± SD (n = 5). (*B*) Modified aCL ELISA: various concentrations of aCL antibody (0.01–4 μg/ml) and constant β_2-GPI concentration. □, aCL and buffer only; ●, aCL and β_2-GPI^{COM} (8 μg/ml); ○, aCL and β_2-GPI^{LAB} (8 μg/ml). Data are the mean ± SD.

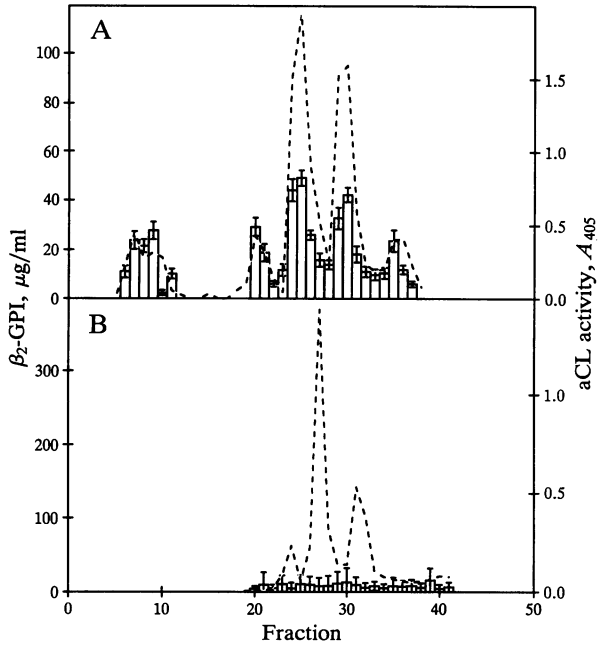

FIG. 3. β_2-GPI RIA results and cofactor activity of chromatofocusing fractions. (*A*) β_2-GPI^{LAB}. (*B*) β_2-GPI^{COM}. Dashed lines, β_2-GPI^{LAB}/β_2-GPI^{COM} concentration (μg/ml); histogram, cofactor activity (A_{405}).

6–12 (pI values, 7.7, 7.6, and 7.5) (according to A_{280}, data not shown).

aCL Cofactor Activity and Immunoreactivity (RIA) of Isoelectric Subspecies. Results of the modified aCL ELISA and the competitive RIA performed on the chromatofocusing fractions are shown in Fig. 3. aCL cofactor activity was demonstrated for all isoelectric species of β_2-GPI^{LAB} (Fig. 3*A*). The level of cofactor activity was proportional to the concentration of β_2-GPI in the fractions as detected by RIA ($r = 0.89$; $P < 0.001$). No cofactor activity above background was detected in any of the eluted fractions from β_2-GPI^{COM} (Fig. 3*B*).

Binding of ^{125}I-Labeled β_2-GPI^{LAB} and ^{125}I-Labeled β_2-GPI^{COM} to CL and PC. ^{125}I-labeled β_2-GPI^{LAB} was able to bind to wells coated with negatively charged phospholipid (CL)-coated wells but not to PC-coated wells (Fig. 4). In contrast, ^{125}I-labeled β_2-GPI^{COM} showed no significant binding to CL- or PC-coated wells. In a further set of experiments

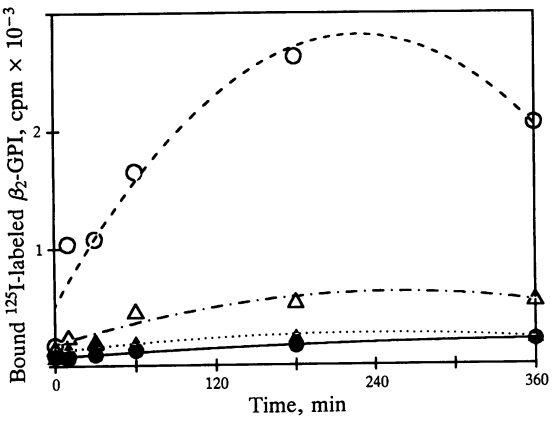

FIG. 4. Binding of ^{125}I-labeled β_2-GPI^{LAB} and ^{125}I-labeled β_2-GPI^{COM} to CL and PC. ○, β_2-GPI^{LAB} binding to CL; △, β_2-GPI^{LAB} binding to PC; ●, β_2-GPI^{COM} binding to CL; ▲, β_2-GPI^{COM} binding to PC.

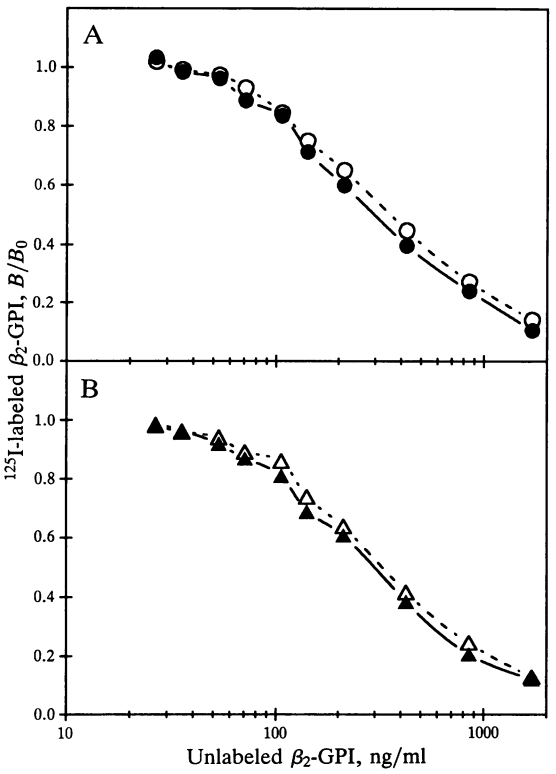

FIG. 5. Standard curves for β_2-GPI RIA. (*A*) ^{125}I-labeled β_2-GPI^{LAB}. Unlabeled competitor species are as follows. ○, β_2-GPI^{LAB}; ●, β_2-GPI^{COM}. (*B*) ^{125}I-labeled β_2-GPI^{COM}. Unlabeled competitor species are as follows. △, β_2-GPI^{LAB}; ▲, β_2-GPI^{COM}.

with various concentrations of ^{125}I-labeled β_2-GPI^{COM} (up to 300,000 cpm), there was minimal binding to CL (640 cpm).

RIA Experiments. The four standard curves generated for the RIA were of identical slope (Fig. 5) and consistent with the standard curve of the competitive RIA for β_2-GPI (6). When using ^{125}I-labeled β_2-GPI^{LAB}, 50% B/B_0 was 270.8 ng/ml for β_2-GPI^{LAB} and 236.8 ng/ml for β_2-GPI^{COM}. Similarly, with ^{125}I-labeled β_2-GPI^{COM}, 50% B/B_0 was 260.2 ng/ml for β_2-GPI^{LAB} and 232.6 ng/ml for β_2-GPI^{COM}.

Immunodiffusion. Rabbit anti-β_2-GPI antibody recognizes both forms of β_2-GPI, and a lack of spurs indicated total immunoreactive identity (data not shown).

Amino Acid Analysis. β_2-GPI^{LAB} and β_2-GPI^{COM} were found to have an identical amino acid composition (data not shown) consistent with that published for β_2-GPI (8, 20).

N-terminal sequencing revealed a single sequence for β_2-GPI^{LAB}. However, each cycle of the Edman degradation revealed three phenylthiohydantoin amino acid derivatives for β_2-GPI^{COM}, the relative proportions of each indicating the presence of two major sequences and one minor (Table 1). The result is consistent with β_2-GPI^{COM} existing predominantly as a molecule cleaved between Lys-317 and Thr-318 (domain 5) and the cleaved segment remaining attached to the parent molecule by disulfide bonding (Fig. 6). A smaller proportion of the preparation exists as a similar structure but is cleaved between Ala-314 and Phe-315. This resulted in

Table 1. N-terminal sequencing of β_2-GPI^{COM}

		Residue									
Sequence	Type	1	2	3	4	5	6	7	8	9	10
i	Major	G	R	T	–	P	K	P	D	D	L
ii	Major	T	D	A	S	D	V	K	P		
iii	Minor	F	W	K	–	–	A	S	–	V	K

Residues from cycles 1 to 10 of the Edman degradation are shown.

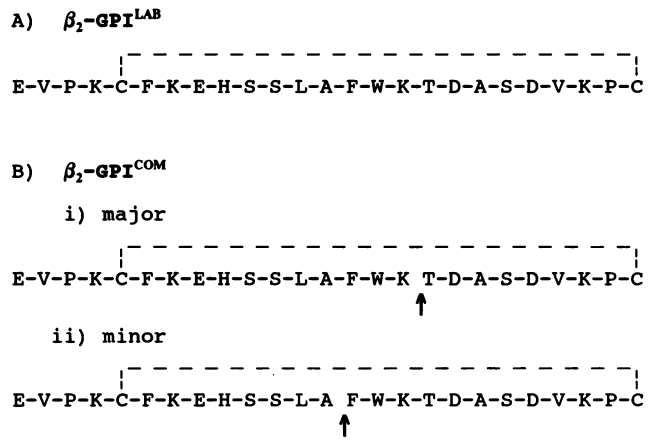

FIG. 6. C-terminal sequence of β_2-GPI^{LAB} and β_2-GPI^{COM}. Dashed line, predicted disulfide bond (20); arrow, position of clip.

three distinct N termini being detected for β_2-GPI^{COM}, one from the true N terminus, one from the cleaved segment beginning at Thr-318, and another from the cleaved segment beginning at Phe-315. The disulfide bonds shown are speculative (20) based on the incomplete series determined by Lozier *et al.* (8).

DISCUSSION

In this study we have identified a region of the β_2-GPI molecule critical for lipid binding and aCL antibody cofactor activity. This finding has allowed us to further characterize the nature of the interaction of β_2-GPI, aCL antibodies, and lipid.

Native β_2-GPI was shown to bind negatively charged phospholipid and displayed a dose-dependent aCL cofactor activity. We have demonstrated that when cleaved ("clipped") between Lys-317 and Thr-318 (the two fragments existing as a disulfide-linked complex), β_2-GPI completely lost its ability to bind negatively charged phospholipid and to act as aCL cofactor. The lower apparent molecule mass by SDS/PAGE of β_2-GPI^{COM} under reducing conditions is consistent with the presence of the C-terminal clip.

One of the previously reported characteristics of β_2-GPI is its ability to bind negatively charged phospholipids (13), and this is likely to be critical to its role as aCL antibody cofactor. The precise regions of β_2-GPI involved in its interaction with negatively charged phospholipids and with other structures, to our knowledge, have not previously been determined. Prior to the publication of the amino acid sequence of β_2-GPI, Schousboe (25) suggested that the lipid-binding activity of β_2-GPI was due to a hydrophilic interaction between a histidine-rich region of the molecule and the negatively charged phospholipids. However, the published sequence (20) reveals that β_2-GPI has only five histidine residues; two in the fifth domain, one in the fourth domain, and two in the third domain. Wurm (13) proposed that ionic and hydrophobic interactions were important in the binding of lipids by β_2-GPI but did not indicate a specific binding site. Recently, it has been suggested that a sequence of highly positively charged amino acids, Lys-Asn-Lys-Glu-Lys-Lys (residues 281–288), predicted to be a surface-exposed turn, is a likely lipid-binding site (20). However, our results suggest that lipid binding is dependent on the integrity of the region that includes Lys-317 and Thr-318. This region is probably surface-exposed as it was susceptible to enzymatic cleavage (clipped), and the resulting disruption to this sequence resulted in complete loss of lipid-binding activity. Interestingly, the C-terminal clip is in the vicinity of a histidine residue (His-310).

Lozier *et al.* (8) mapped only 6 of the 11 disulfide bonds for human β_2-GPI, and only one of these, Cys-281 to Cys-288, was in the fifth domain. In contrast, Kato and Enjyoji (26) have determined all disulfide bonds for bovine β_2-GPI, and the linkages in the fifth domain are Cys-245 to Cys-296, Cys-281 to Cys-306, and Cys-288 to Cys-326. This model results in a more conserved disulfide bond pattern for the fifth domain (i.e., similar to domains 1 to 4). If these assignments are used for human β_2-GPI, then the sequence disrupted by the clip would be linked by a disulfide bond to Cys-288, immediately adjacent to the highly positive charged sequence Lys-Asn-Lys-Glu-Lys-Lys, a possible lipid binding site (20). Consequently, it is possible that a clip between residues Lys-317 and Thr-318 could result in a conformational change in this or other lipid-binding sites and alter the ability of β_2-GPI to bind lipid. However, it is not yet known whether the clip causes such a conformational change.

The cause of the alteration to β_2-GPI^{COM} is not known but is likely to be an artefact of the purification procedure. The major clip is a trypsin-like cleavage (i.e., C-terminal to lysine) and such a protease may have been copurified with β_2-GPI^{COM} or introduced as a contaminant. There are reports of this type of clipped molecule occurring naturally. For example, the membrane-fusing glycoproteins of most enveloped viruses exist as cleaved disulfide-linked complexes (27). Nevertheless, it is unlikely that β_2-GPI^{COM} is a naturally occurring form of the molecule, as it is doubtful that the purification technique employed could selectively purify only the clipped form of β_2-GPI. However, it is interesting to note that sequence that includes Lys-317 and Thr-318 satisfies the criteria for a thrombin cleavage site as proposed by Chang (28). It is possible that cleavage of β_2-GPI by thrombin may operate as a regulatory mechanism to modify its biological activity.

The microheterogeneity of β_2-GPI is well-established and a number of isoelectric subspecies have been reported (9). There have been no reports as to whether all such forms possess aCL cofactor activity or bind lipid. If these activities of β_2-GPI are due to simple charge interactions with negatively charged phospholipid, it may be hypothesized that the presence or extent of activity might be correlated with the charge of the subspecies. However, our results indicate that all isoelectric subspecies of the native molecule possessed significant cofactor activity. Despite having subspecies with pI values that are similar to the pI value of the active form, the clipped form of β_2-GPI possessed no subspecies with any discernible cofactor activity. Our results indicate that the factors responsible for the isoelectric microheterogeneity of the β_2-GPI molecule are not important determinants for aCL cofactor (and presumably lipid binding) activity.

These results have important implications for researchers investigating the properties and function of β_2-GPI. It is evident that the clipped form of β_2-GPI, previously commercially available, would be unsuitable for experiments designed to test whether native β_2-GPI possesses aCL cofactor activity. Recently, there has been a report (29) by investigators who were unable to demonstrate any aCL cofactor activity by β_2-GPI. A number of purification procedures exist for purifying β_2-GPI (5, 30) and some of these may be more likely to produce β_2-GPI with this or similar alterations. These negative findings may be due to the use of the clipped, and hence inactive, form of β_2-GPI. We have recently purified a preparation of β_2-GPI with properties similar to those of β_2-GPI^{COM} (unpublished observations). The use of CL affinity chromatography as the final step ensures that only β_2-GPI with lipid-binding activity is purified.

Although we have detected one significant structural difference between the two forms of β_2-GPI, we have not excluded other possible causes such as differences in carbohydrate content or amino acid substitutions or deletions in

other regions of the molecule. However, both forms show essentially identical immunoreactivity, when tested with polyclonal antisera to native β_2-GPI, and have identical amino acid composition, suggesting that no major differences exist.

In summary, we have identified a region of the β_2-GPI molecule, residues 317–318 (a potential thrombin cleavage site), whose integrity is critical for lipid binding and aCL antibody cofactor activity. Our results indicate that the ability to bind negatively charged phospholipid is a prerequisite for β_2-GPI to act as a cofactor for aCL antibodies. These results may also prove to be of benefit in the study of β_2-GPI's anti-coagulant properties and its role in lipid metabolism.

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