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Current Insights into the "Antiphospholipid" Syndrome: Clinical, Immunological, and Molecular Aspects

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I. Introduction

In 1983, a distinct syndrome consisting of vascular thrombosis, livedo reticularis, thrombocytopenia, and movement disorders associated with "antiphospholipid" (aPL) antibodies was first described (Hughes, 1983). Early studies on aPL antibodies were on patients with systemic lupus erythematosus (SLE) and it was in a subset of patients with SLE that the "antiphospholipid syndrome" (APS) came to be recognized (Hughes, 1985). The association of vascular thrombosis and autoimmune disease was found in the 1960s (Bowie *et al.*, 1963; Alarcon-Segovia and Osmundson, 1965) and laid the foundation for studies to discover the pathogenesis and immunological features of this distinct group of individuals.

It was soon noted that a subset of patients had the clinical manifestations of APS without sufficient clinical and immunological criteria to satisfy the 1982 American College of Rheumatology (ACR) diagnostic criteria for SLE (Tan *et al.*, 1982). The definition and criteria for a "primary antiphospholipid syndrome" (PAPS) was first proposed (Asherson, 1988) and the first series was documented the following year (Alarcon-Segovia and Sanchez-Guerrero, 1989). In a 2-year multicenter follow-up study of patients with PAPS and secondary APS (SAPS) in other autoimmune diseases, a lower female/male sex ratio in PAPS was found compared to that in patients with SAPS. Patients with SAPS had more neutropenia, autoimmune hemolytic anemia, endocardial vegetations, and low levels of complements compared to PAPS patients. The incidence of thrombosis was no different in the two groups (Vianna *et al.*, 1994).

Although the original concept of the APS was shown to comprise one or more of the clinical manifestations of venous thrombosis, arterial thrombosis, recurrent fetal loss, and thrombocytopenia, more diverse clinical manifestations are now recognized, such as cardiac valvular lesions, adrenal insufficiency, and multiorgan thrombotic complications known as "catastrophic" APS (Asherson, 1992). Despite better understanding of the target antigens of aPL antibodies, original laboratory criteria of moderate to high

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levels of "anticardiolipin" (aCL) antibodies and/or lupus anticoagulant (LA) antibodies are still being used and have now replaced the ACR criteria listing of the lupus erythematosus (LE) cell in the revised list for the diagnosis of SLE (Hochberg, 1997). Current guidelines for the diagnosis of APS are the presence of at least one of the clinical criteria of venous thrombosis, arterial thrombosis, recurrent pregnancy loss, and thrombocytopenia, with one or more of the laboratory criteria of moderate to high levels of IgG and/or IgM aCL antibodies and detection of LA activity in a clotting assay. Current criteria for the detection of LA activity in plasma will be discussed later in Section XI. In a previous review of this subject, the emphasis was on antibody interactions with phospholipids, and a brief introduction was made on the role of β2-glycoprotein I (β2GPI) (McNeil *et al.*, 1991). This review develops on the current insights on antibody interactions with phospholipid-binding plasma proteins, in particular β2GPI, and covers currently recognized clinical associations, immunological aspects, molecular studies, and therapeutic interventions.

II. "Antiphospholipid" Antibodies

aPL antibodies are a heterogeneous group of autoantibodies that have specificity for a number of phospholipid-binding proteins, phospholipid molecules, and phospholipid–protein complexes. A number of phospholipid-binding proteins have been implicated in APS, including β2GPI, prothrombin, protein C, protein S, kininogens, thrombomodulin, and annexin V. Traditionally, aPL antibodies are detected in LA assays and in solid-phase immunoassays using cardiolipin as the target antigen. The target antigen detected in clotting assays is still unclear, and a number of coagulation proteins have been implicated, particularly β2GPI and prothrombin. It appears, however, that the complexes assembled on the phospholipid surfaces in functional clotting assays are more important than any one protein. The paradoxical phenomenon of prolongation of *in vitro* phospholipid-dependent clotting tests to detect LA antibodies while being associated *in vivo* with vascular thrombosis has intrigued scientists and clinicians for years. Studies of APS may throw some light on the pathophysiology of the mode of action of these autoantibodies.

Because the original immunoassays for the detection of aPL antibodies used coated cardiolipin on microtiter plates, they were called anticardiolipin antibodies (Loizou *et al.*, 1985). It has been shown that the target antigen in this assay is β2GPI (McNeil *et al.*, 1990; Galli *et al.*, 1990), the original nomenclature is a misnomer, and the antibodies should be known as anti-β2GPI antibodies. For the purpose of this review, antibodies detected on assays employing β2GPI as the coated antigen in the absence

of phospholipids are referred to as anti-β2GPI antibodies and antibodies detected in a cardiolipin ELISA are referred to as aCL antibodies. These two populations of antibodies in autoimmune patients are identical with few exceptions. As a generic term for anti-β2GPI antibodies and LA antibodies, the term aPL antibodies will be employed. The units of antibody levels in sera expressed in the standard CL-ELISA have been calibrated to known sera from the Rayne Institute, London. One GPL/MPL unit each represents one microgram of affinity-purified antibody per milliliter of serum.

Antiphospholipid antibodies are found in "normal" individuals. In a population of 499 blood donors, the prevalence of LA antibodies was 8% and anticardiolipin antibodies was 4.6 (IgG aCL), 4.6 (IgM aCL), and 5.6% (for polyvalent aCL antibodies) (Shi *et al.*, 1990). When these samples were stratified and the demographics of the blood donors studied, LA antibodies were found in young females. Prospective studies need to be done on these individuals to detect if any clinical problems had developed in subsequent years. As aPL antibodies are not normally distributed with most individuals having undetectable levels, it is more appropriate to use a nonparametric definition of the normal range, such as the 95% central tendency.

Antiphospholipid antibodies can be divided into two main groups, classified according to their association with autoimmune or infective conditions. Traditionally these antibodies are termed autoimmune and alloimmune, respectively. Until the discovery that autoimmune antibodies are generally directed to the phospholipid-binding protein β2GPI instead of to the phospholipid molecule itself, the difference between these antibodies was unclear. Although there are exceptions to this rule, autoimmune aPL antibodies detected in solid-phase immunoassays with anionic phospholipids, such as cardiolipin as the coated antigen, are directed to β2GPI captured on a negatively charged surface. Alloimmune aPL antibodies found in chronic infections such as malaria, syphilis, leprosy, tuberculosis, and parvovirus infections do not bind β2GPI but are directed against the anionic phospholipid with β2GPI competing for binding with these antibodies (Hunt *et al.*, 1992). The binding of this latter group of antibodies has been shown to be charge dependent as high salt-containing buffers abolish binding to cardiolipin (Monestier *et al.*, 1996).

A. Lupus Anticoagulant Antibodies

Plasma that contained proteins that prolonged phospholipid-dependent *in vitro* clotting assays were first described in SLE in 1952 (Conley and Hartmann, 1952). Increasing the phospholipid in the assay system appeared to neutralize this LA reaction (Yin and Gaston, 1965). LA activity was

found in the IgG fraction of serum. It was shown that immunoglobulins with LA activity react with anionic phospholipid but not with zwitterionic phospholipids. The phospholipid configuration appeared important with LA antibodies directed to the hexagonal phase rather than lamellar-phase phospholipids (Rauch *et al.*, 1989). Current evidence would suggest that the antibodies may actually interfere with the assembly of enzymatic procoagulant and anticoagulant complexes on phospholipid surfaces, resulting in clinical vascular complications. Although these complications are predominantly thromboses, reports of hemorrhagic diatheses in patients with LA activity have been described. One of the probable causes of this is the presence of high-affinity antiprothrombin antibodies that complex with prothrombin, resulting in removal of the immune complexes by the reticuloendothelial system. This creates a situation of functional hypoprothrombinemia and bleeding.

The dilute Russell's viper venom time (dRVVT), the dilute activated partial thromboplastin time (dAPTT), and the dilute kaolin clotting time (dKCT) are the most frequently used tests for the detection of LA antibodies in routine practice and for research papers. However, up to 53% of patients with LA antibodies have a prolonged prothrombin time (Horellou *et al.*,1987). Although this may sometimes be due to low factor II levels, most of the patients studied have been shown to have normal antigenic levels of prothrombin (Horellou *et al.*, 1987; Fleck *et al.*, 1988).

B. Anti-β2 Glycoprotein I Antibodies

Autoantibodies can be produced in response to tissue breakdown as a result of exposure of a target antigen not usually in contact with immune-mediated cells. They can also arise after altered expression of cell surface proteins due to external stimuli or to translocation of intracellular antigens to cell surface membranes. The ability of a particular host to handle specific antibody–antigen complexes also predisposes to tissue and organ damage. These factors may all interact to suggest a pathogenic mechanism for the generation of autoantibodies in APS.

Following purification of aCL antibodies by ion-exchange chromatography or phospholipid-polyacrylamide affinity chromatography, these antibodies failed to bind to the same phospholipid affinity column unless native or bovine plasma was also present (McNeil *et al.*, 1989). Hence a plasma cofactor had been separated from the antibodies during the purification process that formed part of the antigenic target for these antibodies. The purified antibodies were able to bind in a cardiolipin ELISA where bovine serum is used in diluent and blocking buffers (called a standard CL-ELISA). This plasma cofactor was purified to homogeneity, sequenced, and identified as β2GPI (McNeil *et al.*, 1990). This phospholipid-binding

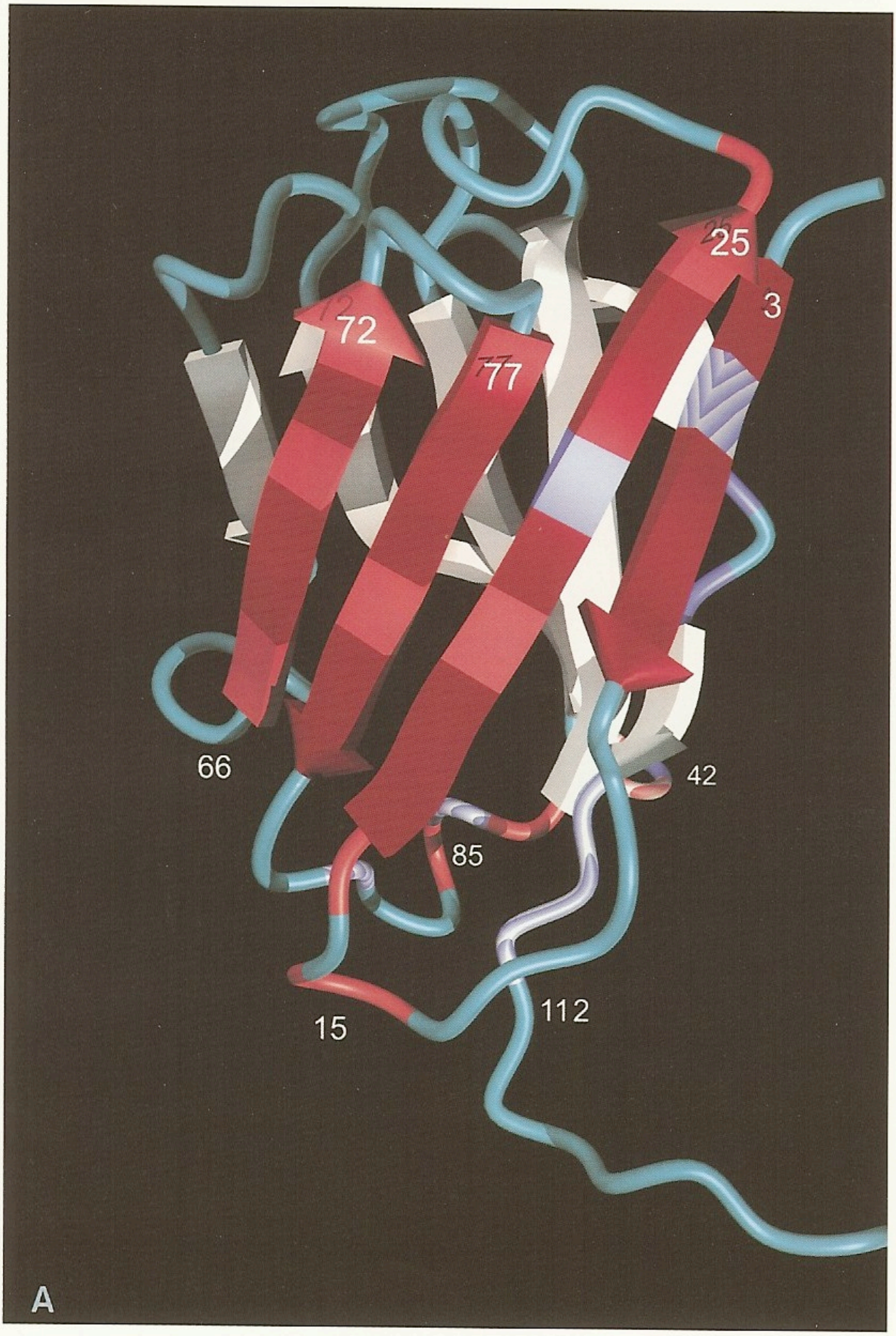

FIG. 7-21A. Four-stranded β-pleated sheet (red arrows) of a V_H domain, taken from the structure of a human Fab with V_H of subgroup III (A. B. Edmundson *et al.*, unpublished data). Totally conserved residue positions are colored blue and those 90% conserved are represented as blue chevrons. Residues conserved at lower levels are designated by pale red (75%) or red striped bands (60%). Strategically located residues are numbered to allow correlation of this model with the sequence presented in Fig. 20. This figure was devised by Benjamin Goldsteen and Allen Edmundson, using the program MOLMOL (Koradi *et al.*, 1996).

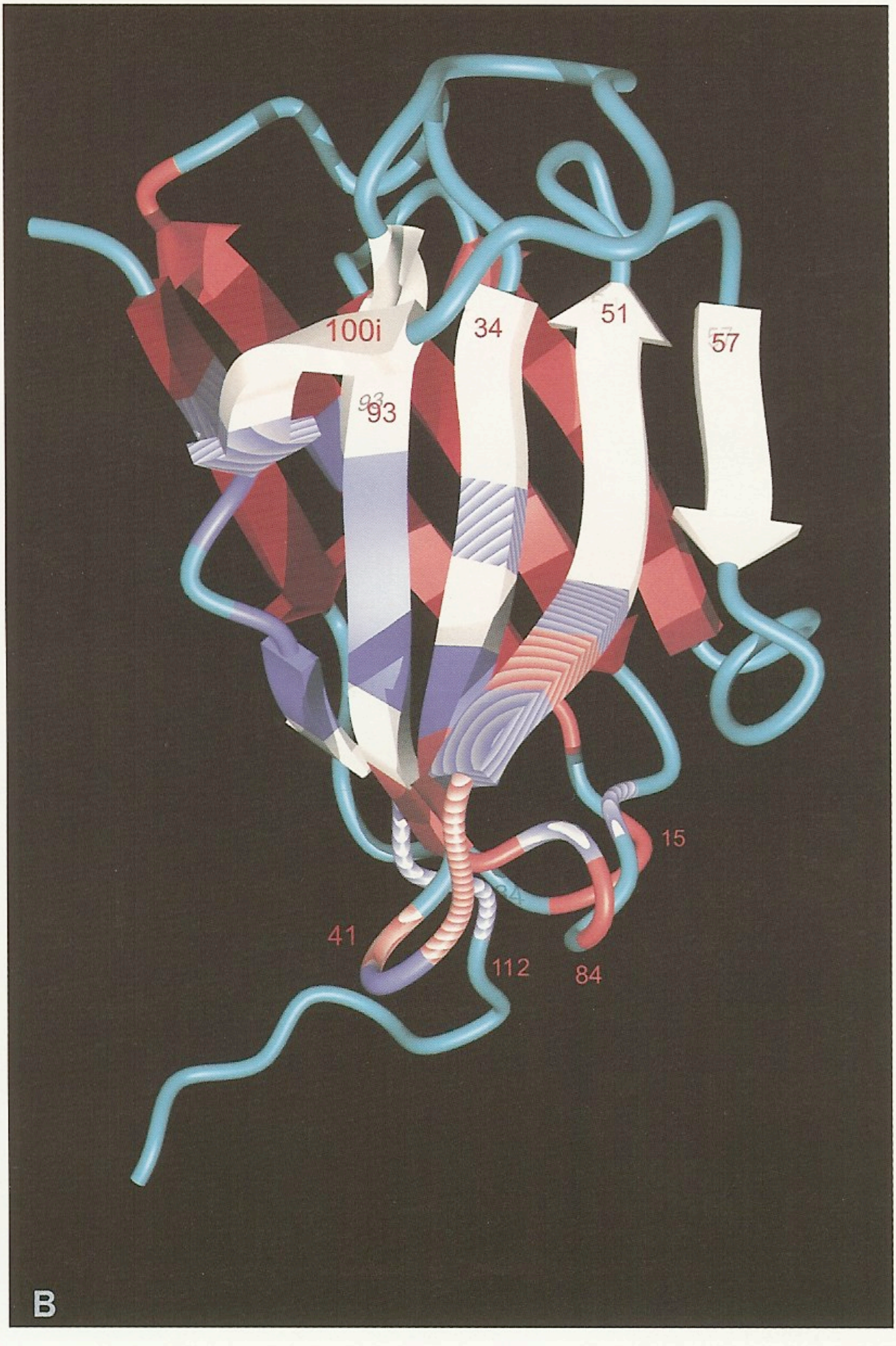

FIG. 7-21B. Five-stranded β-pleated sheet (white arrows) of the same V_H domain. Color coding for the conserved residues and the numbering follow the patterns for A. This figure was devised by Benjamin Goldsteen and Allen Edmundson, using MOLMOL (Koradi *et al.*, 1996).

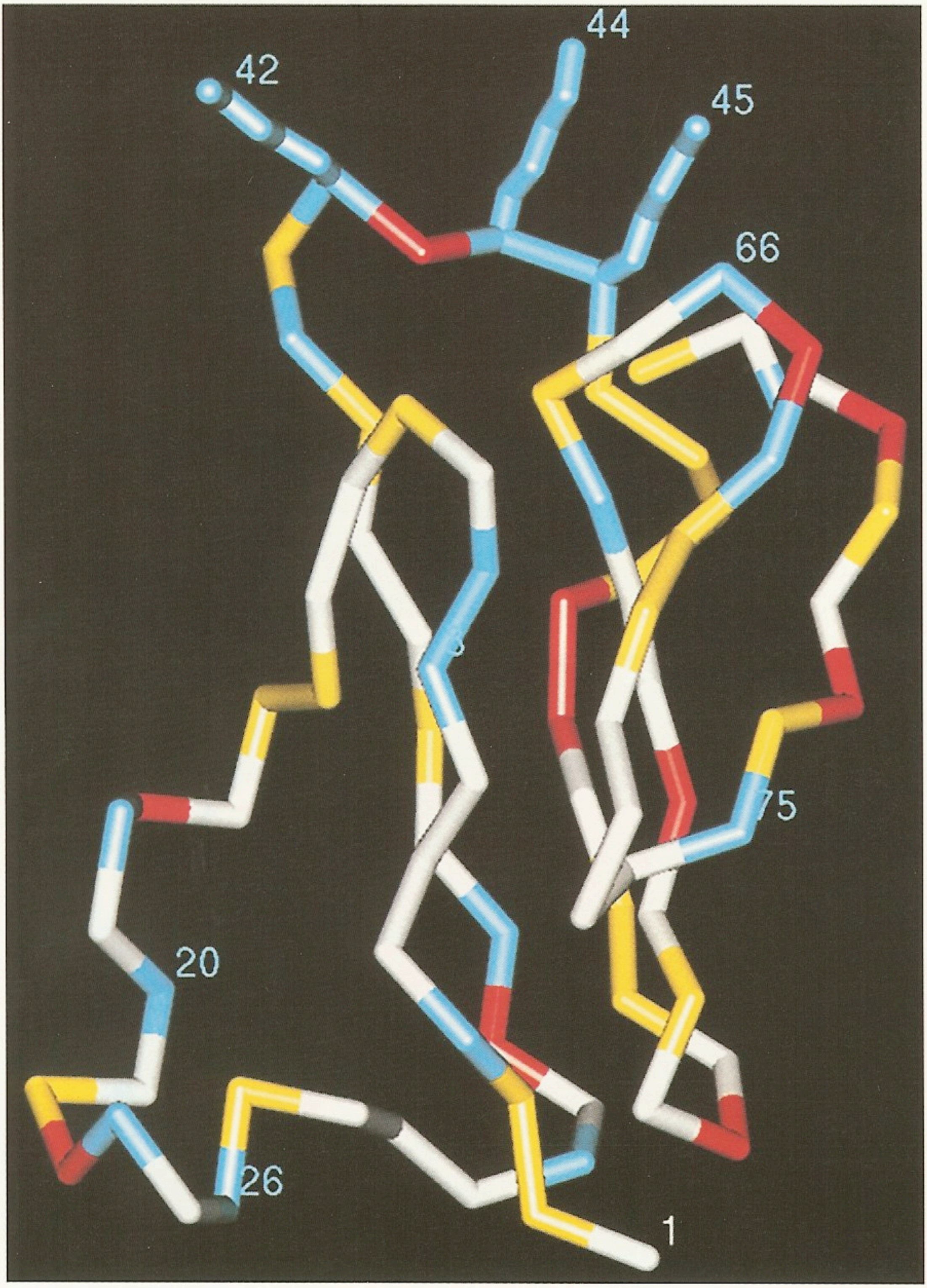

FIG. 8-3. Distribution of charges in the three-dimensional model of human β2GPI-5. Main chain trace of the three-dimensional model of β2GPI-5. The positively charged side chains (*Lys*, *Arg*) are shown in blue. The *His* side chains are not shown, but their main chain is colored blue. The main chains of the negatively charged residues (*Asp*, *Glu*) are shown in red. The phospholipid-binding site is indicated by an arrow. The figure was prepared by program QUANTA (MSI, San Diego, CA). Reproduced with permission from Sheng *et al.* (1996).

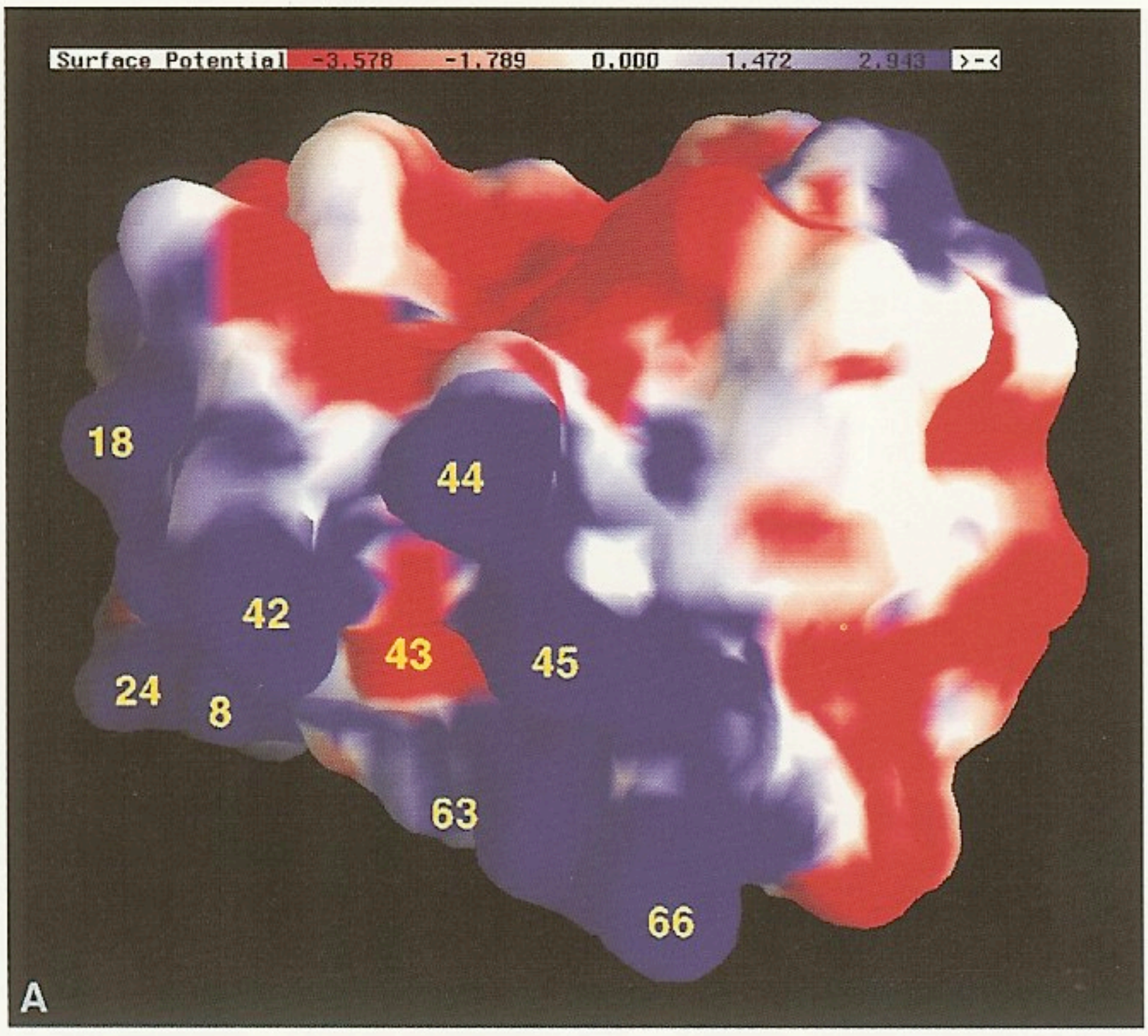

FIG. 8-4. Distribution of charges in the three-dimensional model of human β2GPI-5. Electrostatic potential at the phospholipid-binding region of native and mutant β2GPI-5. (A) Native β2GPI-5 at neutral pH. (B) *Lys* 42/44/45 → *Glu* triple mutant at neutral pH. The molecular surfaces of the models are colored by the electrostatic potential, as shown by the color bar on each panel (in units of kT; 1 kT unit = 0.58 kcal/electron mol). The figures were prepared by program GRASP (Nicholls *et al.*, 1991), using the relative dielectric constants of 2 and 78 for protein and solvent, respectively, and the salt concentration of 150 m*M*. The positively and negatively charged residues are numbered in yellow. Relative to Fig. 3, β2GPI-5 is viewed from the top. Reproduced with permission from Sheng *et al.* (1996). © 1996. The American Association of Immunologists.

plasma protein is found in relatively high concentrations of 4 μM in plasma or sera and appeared to have a role in the coagulation cascade as a natural anticoagulant based on *in vitro* experiments. This has led to the proposed theory that anti-β2GPI antibodies found in APS interfere with the natural procoagulant–anticoagulant homeostatic mechanisms, resulting in a procoagulant tendency and clinical thrombosis and atherogenesis.

The first clinical study to investigate the role of anti-β2GPI antibodies with thrombosis found that 36% of patients with SLE had these antibodies (Viard *et al.*, 1992). If these antibodies were found with LA antibodies, there was a strong association with thrombosis. Other small retrospective studies were performed to confirm an association of anti-β2GPI antibodies, but were fraught with technical problems in patient selection, retrospective analyses, and the ELISA methods used to detect these antibodies (Martinuzzo *et al.*, 1995; Balestrieri *et al.*, 1995; Cabiedes *et al.*, 1995; Pengo *et al.*, 1996). In the last paper the population of patients selected was done on the basis of their reactivity in a cardiolipin ELISA. It is therefore not surprising that anti-β2GPI antibodies were found in all the patients who had thrombosis, as the target antigen in the standard CL-ELISA is bovine β2GPI, which supports the binding of most human aPL antibodies. There is still some controversy as to whether most patients with autoimmune APS have both anti-β2GPI and antibodies that bind anionic phospholipids directly. Examining the sera of patients with both PAPS and SLE/APS, 68% were found to have true aCL antibodies as demonstrated by reactivity to CL on thin-layer chromatography plates, independent of the presence of β2GPI (Sorice *et al.*, 1996). Using delipidated β2GPI as the antigen, 22.6% of the CL-ELISA positive sera bound β2GPI on immunoblotting. There may also be a population of antibodies that require the complex of β2GPI and phospholipid. Sixteen out of 18 patients with SLE and clinical manifestations of APS with negative IgG and IgM standard CL-ELISA reactivity had IgG anti-β2GPI antibodies (Cabiedes *et al.*, 1995). In a study of 97 patients with IgG and/or IgM anti-β2GPI reactivity in a β2GPI-ELISA, 43% of IgM and 8% of IgG antihuman β2GPI antibodies did not bind to purified bovine β2GPI, explaining a negative aCL-ELISA where bovine β2GPI is the major source of β2GPI (Arvieux *et al.*, 1996).

The initial concentration of β2GPI in the patients' sera and the dilution of sera used could determine whether binding occurs in the CL-ELISA. The discovery that β2GPI exhibits genetically determined structural polymorphism with the occurrence of four alleles is another potential source of confusion in the conventional CL-ELISA. β2GPI from certain individuals, homozygous for the APOH*3 allele, is unable to bind anionic phospholipid (Kamboh *et al.*, 1995). This group has also found two structural mutations

at codons 306 and 316 in the fifth domain of β2GPI. These missense mutations affect the structural integrity of the fifth domain of β2GPI affecting phospholipid binding. The authors suggest that there are individuals who are compound heterozygotes for the two mutations, who may be precluded from producing anti-β2GPI autoantibodies (Sanghera *et al.*, 1997). Antibodies with β2GPI reactivity and true CL reactivity exist in the same patient population, with IgG2 subclass restriction of anti-β2GPI antibodies in patients with autoimmune disease (Arvieux *et al.*, 1994). These factors all contribute to the differences in assay results for patients with APS in a conventional CL-ELISA.

III. Clinical Features of the "Antiphospholipid" Syndrome

A. Cardiovascular Manifestations

APS is associated with a number of clinical manifestations affecting multiple organs. Although the common pathophysiological theme for organ damage appears to be thrombotic microangiopathy, there are other clinical manifestations that cannot be explained by this, e.g., cardiac valvular abnormalities. Patients with APS can have both arterial and venous thrombosis, the only clinical condition that predisposes to this without any structural vascular anomalies. Other inherited conditions tend to predispose to thrombosis in one vascular bed, e.g., homocystinemia and arterial thrombosis, and a number of familial protein deficiencies and genetic mutations that predispose to venous thrombosis.

In younger patients and patients with PAPS, the vascular event is often an acute occlusive vascular event instead of being secondary to atherosclerosis. Patients with SLE and secondary APS, however, may have a combination of thrombotic diathesis and atherosclerosis related to other factors, such as long-term steroid administration, hyperlipidemia, and hypertension. Coronary vasculitis is less frequent. The prevalence rates in APS for myocardial infarction have been reported between 0 and 7% (Asherson *et al.*, 1985). A Finnish study showed that the presence of high antibody levels in a standard CL-ELISA was an independent risk factor for myocardial infarction. Subjects with aCL levels in the highest quartile of distribution had a relative risk of myocardial infarction of 2.0 (95% confidence interval, 1.1 to 3.5) independent of confounding factors normally associated with coronary vascular disease, such as smoking, age, systolic blood pressure, and hyperlipidemia (Vaarala *et al.*, 1995). In a series of 83 patients who had undergone coronary artery bypass graft surgery, autoantibodies detected in CL-ELISA were elevated in late bypass graft occlusions (Morton *et al.*, 1986). A placebo group not treated with aspirin with these autoantibodies

had a high rate of coronary artery bypass graft occlusion (Gavaghan *et al.*, 1987).

aPL antibodies may be associated with acute and chronic myocardial dysfunction. The clinical findings of left ventricular isolated and global dysfunction with the presence of insignificant coronary vessel disease as seen on angiography may be associated with aPL antibodies predisposing to coronary microangiopathy (Leung *et al.*, 1990). This can also be found in the context of valvular heart disease in particular mitral regurgitation.

The link between aPL antibodies and aseptic vegetations in patients with autoimmune disease was recognized in the 1980s (Anderson *et al.*, 1987; Ford *et al.*, 1988). Studies using echocardiography have shown that autoimmune patients with aPL antibodies have a higher prevalence of valvular vegetations (Khamashta *et al.*, 1990; Cervera *et al.*, 1992, Roldan *et al.*, 1992). Although patients with SLE, especially if they are immunosuppressed with medication to control the disease activity, may have infective endocarditis, it appears that these patients have a higher prevalence of noninfective, thrombotic endocarditis. Linear deposition of IgG aCL antibodies in the subendothelial layer of heart valves in patients with APS has been demonstrated (Ziporen *et al.*, 1996), suggesting a possible pathogenic role of these antibodies in valvular abnormalities. This would need to be studied more extensively in the future.

B. Neurological Manifestations

The cerebral arterial circulation appears to be the most common site for arterial thrombotic episodes in patients with aPL antibodies (Harris *et al.*, 1984). The Antiphospholipid Antibodies in the Stroke Study Group (APASS) found that the presence of autoantibodies above 10 GPL or MPL units in a standard CL-ELISA to be an independent risk factor for a first ischemic stroke in an elderly population without SLE (APASS, 1993). In a prospective study of stroke in patients below the age of 50 years, the risk of stroke recurrence was eight times higher in patients with aPL antibodies than those without (Brey *et al.*, 1990). It therefore appears that while the presence of aPL antibodies is an important factor to be evaluated for in the context of cerebrovascular events, the positive predictive value is greatest in patients below the age of 50 years. As in other vascular beds, the presence of cigarette smoking and hypercholesterolemia may independently increase the risk of recurrent cerebral ischemia in patients with aPL antibodies (Levine *et al.*, 1990). In another prospective study of patients who presented with focal cerebral ischemia without any prior autoimmune disease, a titer of > 40 GPL units in a standard CL-ELISA conferred a twofold increased risk for a further thromboocclusive event (peripheral or central) or death. This is despite more of these individuals

receiving antiplatelet or anticoagulant therapy or both at the time of follow-up. These results imply that more specific methods need to be derived to stratify these high-risk patients and to maintain them on suitable therapy after a first vascular occlusive event (Levine *et al.*, 1997).

Limited cerebrovascular histopathological data suggest that the vascular abnormality in aPL syndrome is increased fibrin thrombi formation in small- and medium-sized vessels in the absence of vasculitis (Woodard *et al.*,1991). As discussed earlier in the context of cardiac valvular abnormalities, another source of vascular occlusion in patients with aPL antibodies is cardiac emboli, and one-third of the 72 patients studied by the APASS group found cardiac abnormalities in patients with aPL antibodies and cerebral ischemia, predominantly mitral valve abnormalities (APASS, 1990).

Although epilepsy is a recognized clinical event in SLE patients, the etiology of this is multifactorial. Hypertension, infection, cerebral ischemia, and vasculitis have all been implicated in patients who develop epilepsy. Epilepsy as a primary neurological event in SLE patients was associated with a high prevalence of aPL antibodies (Herranz *et al.*, 1994). In a study by Verrot *et al.* (1997), 163 patients with epilepsy were evaluated for autoantibodies in a standard CL-ELISA. The authors found 31 (19%) patients with IgG aCL antibodies of moderate to high titers. None of these patients had any previous clinical events to suggest APS. Brain imagings in these patients showed no significant difference in those who were aCL positive and negative (Verrot *et al.*, 1997). Hence there appears to be a group of patients who have epilepsy as a primary clinical manifestation of the presence of aPL antibodies, independent of possible cerebral ischemia.

Movement disorders have been associated with SLE initially (Lusins and Szilagyi, 1975) and subsequently with aPL antibodies (Asherson and Hughes, 1988). These movement disorders may be brought out in an estrogen-related hormonal environment, e.g., in pregnancy, or if the patient was taking the oral contraceptive pill (Asherson *et al.*, 1986a). Movement disorders in patients with aPL antibodies may also follow cerebral infarctions.

Another neurological manifestation of aPL antibodies is migraine (Hughes *et al.*, 1986), although the association at the moment is considered tenuous (Hering *et al.*, 1991). Transverse myelopathy has also been described in autoimmune patients with aPL antibodies (Adrianakos *et al.*, 1975). Although there have been a number of case reports of aPL antibodies being found in patients with transverse myelitis (Lavalle *et al.*, 1990), the presence of these antibodies in patients with autoimmune disease may just be part of the spectrum of autoantibodies found in these patients and not be directly responsible for the clinical problem.

C. Ocular Ischemia

aPL antibodies may be associated with thromboembolic disease in the visual pathway. A study of patients with cerebrovascular disease and APS found 19% (9/48) had clinical features of amaurosis fugax, ischemic optic neuropathy, and retinal artery occlusions (Levine *et al.*, 1990). Various other studies and case reports have also described patients with aPL antibodies and ocular ischemia, often in the context of more generalized cerebral ischemia (APASS, 1990; Briley *et al.*, 1989). The occurrence of amaurosis fugax in patients under the age of 50 or in patients with frequent episodes ranging from 2 to more than 100 episodes a week may indicate that APS and aPL antibodies should be screened in these patients (APASS, 1993). A prospective study of 550 patients with SLE revealed that 7.5% of these patients had occlusive ocular vascular disease, and 38% of these patients had LA antibodies investigated by one clotting test only (APTT) (Stafford-Brady *et al.*, 1988).

Patients presenting with headaches and found to have papilloedema in the presence of a normal cerebral CT scan may have cerebral venous thrombosis. This condition has been associated with aPL antibodies and should be screened for with multiple sensitive tests (Levine *et al.*, 1987). The true incidence of aPL antibodies in optic ischemia and cerebral venous thrombosis has yet to be clearly defined, as more information is available on the detection methods for aPL antibodies. It appears, however, that in patients below the age of 50 years who present with ocular symptoms and have been found to have vasoocclusive disease should be screened for these antibodies by multiple tests.

D. Pulmonary Manifestations

Recurrent deep venous thromboses are the most common vasoocclusive events that occur in patients with aPL antibodies (Boey *et al.*, 1983). Subsequent pulmonary emboli are not infrequent and often occur in the absence of symptomatic deep venous thromboses (DVTs) (Asherson and Cervera, 1992). Pulmonary hypertension in patients with APS has been documented, but this appears to be mainly associated with SLE and not with thrombotic disease (Asherson, 1990). It is therefore unclear whether SLE patients with pulmonary hypertension and aPL antibodies have the two conditions related or whether the high frequency of aPL antibodies in SLE may mask their true clinical relevance.

Intraalveolar pulmonary hemorrhage has been described in patients with SLE and aPL antibodies (Howe *et al.*, 1988). In a retrospective review of inpatients with intraalveolar pulmonary hemorrhage and SLE, six of the eight patients were found to have aCL antibodies (Schwab *et al.*, 1993).

This study identifies the problems encountered in retrospective clinical studies of patients with aPL antibodies as the current battery of tests for LA and anti-β2GPI antibodies should be performed so as not to miss patients with aPL antibodies. This is particularly true of hemorrhage associated with aPL antibodies where antiprothrombin antibodies should be investigated to detect those high-affinity antibodies that form prothrombin–antiprothrombin complexes that are removed by the reticuloendothelial system. This results in hypoprothrombinemia and hemorrhage in some patients with these antibodies. Hence, pulmonary complications in APS are common but are often related to macrovascular thromboses as part of a systemic hypercoagulable state. Patients may also have adult respiratory distress syndrome as part of multiple organ involvement with extensive microangiopathic thromboses (Ghosh *et al.*, 1993).

E. Renal Manifestations

Primary renal disease is increasingly recognized in APS. Antiphospholipid antibody-related intrarenal thromboses may present with systemic hypertension, proteinuria, hematuria, and progressive renal failure, especially in the context of severe thrombotic microangiopathy in catastrophic APS (Asherson, 1993; Piette *et al.*, 1994). Glomerular capillary thrombosis has been found to have a strong association with LA antibodies and predisposes to glomerular sclerosis independent of immune complex disease (Kant *et al.*, 1981). Renal disease may also arise in the APS with renal artery stenosis. The nature of this vascular occlusion is unclear and may arise as a primary thrombotic phenomenon in the context of aPL antibodies (Ostuni *et al.*, 1990) or may be secondary to previous damage to the renal vessels from atheromatous degeneration in the blood vessel walls or prior renal artery fibromuscular dysplasia (Mandreoli *et al.*, 1992). Renal vein thrombosis may be the cause and result of a thrombotic tendency in the APS. Thrombotic microangiopathy predisposing to the nephrotic syndrome can result in the loss of circulating natural anticoagulants predisposing to vascular thrombosis. However, the thrombophilia associated with aPL antibodies has been shown to occur in the absence of previous renal disease. Comparing two matched groups of SLE patients with renal disease, with and without LA antibodies, no differences were found in their renal biochemical and histological features except in a higher prevalence of intrarenal thromboses in patients with LA antibodies (Farrugia *et al.*, 1992).

F. Adrenal Manifestations

The link between adrenal gland hypofunction and aPL antibodies was first recognized in the late 1980s (Grottolo *et al.*, 1988; Asherson and

Hughes, 1989). A number of possible theories exist for this association, including the primary occlusion of the adrenal veins leading to glandular edema and compression of the arterial blood supply and adrenal infarction. Asherson (1994) reviewed 38 cases with adrenal hypofunction and aPL antibodies and showed that 31 of the 38 patients had PAPS and 7 had SLE-associated APS. In 20 of these patients, vascular occlusive events preceded the adrenal hypofunction, whereas in 10 patients, concurrent events occurred in the context of acute adrenal failure. These events were predominantly venous in particular pulmonary emboli (Asherson, 1994). Therefore, patients with aPL antibodies who suddenly develop circulatory collapse need to be investigated for electrolyte disturbances and adrenal structure and function and treatment with adequate and prompt fluid replacement is essential. Marie *et al.* (1997) identified adrenal failure secondary to bilateral adrenal hemorrhagic infarctions in a 70-year-old patient as a first clinical manifestation of the PAPS who then went on to develop extensive upper limb deep venous thrombosis while on aspirin.

G. Hepatic Manifestations

Structural and functional obstruction of venous blood flow in the liver may lead to Budd–Chiari syndrome. In the context of aPL antibodies, this may be the result of thrombosis in the hepatic veins extending to the inferior vena cava. The occurrence of Budd–Chiari syndrome and aPL antibodies was first reported in 1984 (Pomeroy *et al.*, 1984). The majority of patients described with this syndrome in the presence of aPL antibodies have PAPS and have had previous venous occlusive disease and concurrent thrombocytopenia.

Hepatic venoocclusive disease resulting in hepatomegaly and ascites secondary to central and sublobular vein occlusions occurs with aPL antibodies. The venous occlusions lead to hepatic sinusoidal congestion, hepatocellular necrosis, and finally fibrosis. The liver has a dual blood supply from the systemic and portal circulation. As such, hepatic infarction is rare unless the patient has generalized thrombophilia. The first case of hepatic infarction in association with aPL antibodies was described in 1989 (Mor *et al.*, 1989), and primary portal hypertension has been described in APS (Mackworth-Young *et al.*, 1984).

Thrombosis of mesenteric vessels has been described in APS resulting in intestinal infarction. Patients may have both arterial and venous occlusions. The presentation is usually with acute abdominal pain or "intestinal angina" (Asherson *et al.*, 1986b). Other abdominal organs that have been described with vascular occlusion and infarction include the spleen (Arnold and Schrieber, 1988) and pancreas (Wang *et al.*, 1992).

H. Dermatological Manifestations

In line with the common theme of vascular occlusion and insufficiency in end organ disease in APS, skin changes are fairly common in APS. Livedo reticularis, a mottled violaceous discoloration of the skin in a net-like pattern, is found frequently in APS. In patients with livedo reticularis and aPL antibodies, recurrent episodes of cerebral ischemia have been described (Sneddon, 1965). These patients may have a range of neurological manifestations from headache and dizziness, focal neurological deficits, and progressive cognitive deficits (from loss of concentration and memory loss to severe dementia). Livedo reticularis is found sufficiently frequently to be included in the clinical diagnostic criteria proposed (Alarcon-Segovia *et al.*, 1992). Necrotic skin ulcers have been reported since 1963 in association with circulating LA antibodies (Bowie *et al.*, 1963). Superficial cutaneous necrosis has also been described with aPL antibodies. This condition is also found in deficiencies of natural anticoagulants, e.g., protein C and protein S, or in cryoglobulinemia and cryofibrinogenemia, and these abnormalities may be found concurrently with aPL antibodies, with additive risks for the underlying superficial thrombosis. Digital gangrene may also be seen in APS (Alegre *et al.*, 1989).

The histological features of small vessel disease producing the skin and soft tissue manifestations in APS appear to be a noninflammatory thrombosis of small arteries and veins throughout the dermis and subcutaneous fat tissue, occasionally accompanied by endarteritis obliterans. This condition is characterized by narrowing of the vascular lumen with endothelial cell proliferation and fibrohyalinization of the vessel wall (Alegre and Winkelmann, 1988). In livedo reticularis, skin biopsies rarely reveal thrombosis of the small vessels, and vessel wall hyperplasia may be the only histological feature seen.

These skin lesions may frequently be the first sign of APS; up to 37% of patients with skin lesions and aPL antibodies develop multisystem thrombotic phenomena in the course of their disease (Alegre *et al.*, 1989). This observation, performed before the antigen specificities of aPL antibodies were better defined, should lead to prospective studies investigating this association with multiple screening tests for aPL antibodies. This could determine the subset of patients who present with skin lesions, who could well go on to develop more severe systemic manifestations that may be prevented or diminished with specific antithrombotic therapy.

I. Avascular Necrosis of Bone

aPL antibodies may be associated with clinical avascular necrosis (AVN) of bone. There have been a number of cases in the literature of

patients with PAPS who have developed clinical AVN adjacent to various joints (Vela *et al.*, 1991; Seleznick *et al.*, 1991). The prevalence of AVN in patients with aPL antibodies is difficult to ascertain, as patients with SLE are often on corticosteroid therapy, which predisposes to this condition.

J. Obstetric Manifestations

Lupus anticoagulant antibodies have been associated with pregnancy loss and intrauterine death (Nilsson *et al.*, 1975). Others have shown the same association with aCL antibodies (Lockshin *et al.*, 1985; Harris *et al.*, 1986). It is often suggested that patients with aPL antibodies exist in a prothrombotic state that need some other trigger to precipitate a clinical event. This could well include surgery, oral contraceptive use, and pregnancy. Previous pregnancy failures are an important feature for predicting subsequent pregnancy failure. Autoimmune disease has a variable effect on pregnancy. It has been suggested that the underlying immune abnormality that permitted the development of the autoimmune disease or the autoantibodies that arise may be directly responsible for the fetal loss (Gleicher, 1994). The maternal effects of aPL antibodies in pregnancy are uncommon, but have been reported, including preeclampsia (Scott, 1987), chorea gravidarum (Lubbe and Walker, 1983), and cardiopulmonary distress (Branch, 1990). Severe early onset preeclampsia and abruptio placentae may predispose to fetal complications in late pregnancy. However, the fetus appears to be at risk throughout the pregnancy, and detection of aPL antibodies appears to be a useful test in the investigation of autoimmune reproductive failure (Aoki *et al.*, 1995).

Abnormal uterine artery flow velocity may predict a poor outcome in cases of aPL antibodies (Caruso *et al.*, 1993). The most common cause of pregnancy loss in the first trimester is chromosomal abnormalities, and this has not been adequately studied in patients with aPL antibodies. Cross-reactivity of aPL antibodies with villous trophoblast cell membrane phospholipids may expose these cells to cytotoxic maternal immune effector cells (Hasegawa *et al.*, 1990; McCrae *et al.*, 1993). One of the essential considerations in pregnancy loss associated with aPL antibodies is whether the aPL-related pregnancy loss(es) may have been triggered by a nonrelated earlier miscarriage in an immunologically susceptible individual. There may be a biphasic pattern of pregnancy loss with embryonal death by 8.5 weeks and fetal complications from week 14 (Goldstein, 1994). Animal models have suggested that aPL antibodies per se appear to predispose to increased fetal resorption (Blank *et al.*, 1991). However, the results of these studies are not conclusive (Blank *et al.*, 1994a and Silver *et al.*, 1997).

Late fetal death is the most commonly found obstetric complication in

APS. This has been attributed by early (Nilsson *et al.*, 1975) and later studies (de Wolf *et al.*, 1982) to placental infarction. Immune complex deposition on the trophoblast basement membrane has also been implicated in SLE-related fetal loss (Grennan *et al.*, 1978). Elevated aPL levels have been associated with chronic uteroplacental vasculitis in the placental bed (Erlendsson *et al.*, 1993). The occurrence of thrombosis and infarction in non-aPL fetal death, as well as inflammatory changes in aPL-related fetal death, suggests that the end-organ damage (placenta) in aPL disease is multifactorial and may have complex humoral and cellular interactions together with coagulation pathway abnormalities in the maternal circulation, villous trophoblast surface, and within the fetoplacental circulation. Antitrophoblast cytotoxicity initiated by maternally derived aPL antibodies cross-reactive with fetal trophoblast phospholipid epitopes and phosphatidylserine may induce chronic inflammation in the villi (Hasegawa *et al.*, 1990).

IV. β2-Glycoprotein I

β2GPI, a plasma protein,was first described in 1961 (Schultze *et al.*, 1961) and has been the subject of extensive research in autoimmune disease. β2GPI is associated with different lipoprotein fractions in plasma and is also designated apolipoprotein H (Lee *et al.*, 1983). β2GPI is a single-chain polypeptide of 326 amino acids with an apparent molecular mass of 50 kDa and is highly glycosylated (Lozier *et al.*, 1984). The carbohydrate content of β2GPI has been reported as being approximately 18% of its molecular mass (Schultze *et al.*, 1961) and, when tested in phosphate buffer at pH 7.4, exists as 40% β sheet, 30% β turn, and 30% random coil (Walsh *et al.*, 1990). β2GPI is a member of the complement control protein repeat (CCP) or short consensus repeat (SCR) superfamily (Reid and Day, 1989). The SCR is found in proteins involved in the regulation of the complement system (e.g., C4b-binding protein and factor H) and in some noncomplement proteins (selectin family and factor XIII). Although the first four of the five domains are typical examples of this CCP superfamily, the fifth domain is aberrant, containing an additional disulfide bond and a long C-terminal tail. β2GPI is highly conserved among mammalian species, suggesting that it plays an important physiological role (Kandiah and Krilis, 1994).

Haptoglobin and factor H, two other members of this superfamily, are not bound by anti-β2GPI antibodies. Haptoglobin is used routinely as a control protein for antibody binding to β2GPI, as nonspecific binding can be detected when compared to β2GPI. β2GPI could not bind complement C3b coated on both activator (zymosan) and nonactivator (sheep

erythrocytes) of the alternative complement pathway, whereas factor H bound to both surfaces coated with C3b, suggesting that despite structural similarities, these proteins had distinct nonoverlapping functions (Puurunen *et al.*, 1995).

Although β2GPI has been characterized structurally, the tertiary structure of β2GPI and its biological function are not clear. β2GPI has a highly conserved pattern of cysteine residues (Steinkasserer *et al.*, 1991). Molecular modeling has suggested that a highly positively charged sequence in the fifth domain of β2GPI is surface exposed (Steinkasserer *et al.*, 1992; Sheng *et al.*, 1996). This had been predicted previously using the known tertiary structure of factor H, another CCP protein. This surface-exposed net positive charge could well explain the binding of β2GPI to negatively charged surfaces, e.g., anionic phospholipids (Wurm, 1984), heparin (Polz, 1979), and DNA (Kroll *et al.*, 1976). Although β2GPI has been shown to be an absolute requirement for autoimmune aPL antibodies to bind in CL-ELISA, a preparation of β2GPI, proteolytically cleaved predominately between Lys317 and Thr318 in the fifth domain, lacked binding to anionic phospholipid (Hunt *et al.*, 1993). This led to further work to map the major phospholipid-binding site on β2GPI initially with peptide inhibition studies (Hunt and Krilis, 1994) and then with site-directed mutagenesis (Sheng *et al.*, 1996).The lysine-rich segment in the fifth domain (Lys^{282}–Lys^{287}) has been shown to be the major phospholipid-binding site on β2GPI. Modification of amino acid residues on β2GPI by potassium thiocyanate treatment completely destroys binding capacity, indicating the crucial involvement of lysine residues in the binding of β2GPI to anionic phospholipids (Kertesz *et al.*, 1995).

Many of the proposed physiological functions of β2GPI involve its phospholipid-binding properties. The binding of β2GPI to anionic phospholipids had been assessed using multilamellar, predominantly anionic phospholipid vesicles under nonequilibrium conditions (Wurm, 1984). Data suggested a high-affinity interaction of β2GPI with phospholipid in the 10–20 n*M* range. *In vivo*, however, physiological membranes contain significantly lower concentrations of anionic phospholipids, and normal plasma levels of β2GPI could easily displace Gla-containing proteins from cell membranes disrupting normal homeostatic mechanisms. Moreover, normal plasma concentrations of sodium and divalent cations would markedly inhibit this charge-dependent interaction. Physiological concentrations of β2GPI do not have much effect in *in vitro* coagulation tests unless anti-β2GPI antibodies are also present (Oosting *et al.*, 1992; Roubey *et al.*, 1992; Galli *et al.*, 1992; Matsuda *et al.*, 1993). Extrapolation of the calculation of the apparent dissociation constant for β2GPI binding with physiological anionic phospholipid membranes, to 10^{-6} *M*, suggests that

β2GPI alone may not be able to displace other coagulation proteins from these membranes (Willems *et al.*, 1996). However, the binding of a complex of an IgG anti-β2GPI molecule bivalently to two β2GPI molecules could have a markedly higher affinity for that anionic membrane *in vivo* and hence displace other coagulation proteins. *In vitro*, the presence of less procoagulant proteins will result in a delay in the clotting time, a plausible explanation for the lupus anticoagulant phenomenon. Excess phospholipid will allow the capture of other coagulation proteins again and restore the clotting time back to normal levels.

As the target antigen of pathogenic antibodies in APS, much research has gone into studying the interaction of these antibodies with β2GPI. Three hypotheses have been proposed to explain the interactions between β2GPI and anionic phospholipids, allowing subsequent binding of aPL antibodies. They are (1) a shared epitope on the phospholipid–β2GPI complex (McNeil *et al.*, 1990), (2) a cryptic epitope exposed on β2GPI when it interacts with anionic phospholipids (McNeil *et al.*, 1990), and (3) increased density of β2GPI captured on the anionic phospholipid (Roubey *et al.*, 1995).

There has been increasing evidence to suggest that aPL antibodies bind preferentially to β2GPI immobilized on anionic phospholipids or certain synthetic surfaces (irradiated plates), whereas binding in the fluid phase is weak and often nondetectable. It is unlikely that the same epitope on β2GPI is exposed by different surface interactions, as shown by work done with monoclonal antibodies that are immunoreactive with β2GPI bound to anionic phospholipids and to irradiated plastic wells (Wang *et al.*, 1995). The intrinsic low affinity of anti-β2GPI antibodies in APS is significantly enhanced when there is an increased density of β2GPI bound to a negatively charged surface. It has been found that the binding of purified monoclonal anti-β2GPI antibodies on γ-irradiated polystyrene wells was higher than on untreated wells. This binding is most likely due to increased density of β2GPI, as the amount of iodinated β2GPI retained on irradiated wells after the same amount of protein was coated overnight was 200% higher than untreated wells (Kandiah and Krilis, 1996a).

Matsuura and colleagues (1994) have reported that polyclonal human aCL antibodies and a monoclonal murine aCL antibody bound β2GPI coated on electron or γ-irradiated microtiter wells but not on untreated wells. The degree of binding depended on the irradiation dose, and aCL binding to β2GPI adsorbed to these wells correlated well with that of β2GPI complexed to solid-phase CL. Antibodies binding to β2GPI on these irradiated wells were only competitively inhibited by the simultaneous addition of CL-coated latex beads mixed together with β2GPI, but were

unaffected by the addition of excess β2GPI, CL micelles, or CL-coated latex beads.

These findings again support the hypothesis that aCL antibodies are low-affinity antibodies and that interaction of antibodies to β2GPI, the target antigen, requires capture of this protein to an appropriately charged surface. Roubey *et al.* (1995) have has shown that Fab′ fragments of patient IgG demonstrated little or no binding to β2GPI on γ-irradiated polystyrene wells, whereas the whole molecule bound to β2GPI, suggesting a critical role for antibody bivalency.

A. Epitope Mapping of Phospholipid- and Antibody-Binding Sites on β2-Glycoprotein I

Using synthetic peptides spanning the fifth domain of β2GPI, the peptide sequence Cys281-Lys-Asn-Lys-Asp-Lys-Lys-Cys288 inhibited binding of β2GPI to anionic phospholipid in a dose-dependent manner (Hunt and Krilis, 1994). Removal of the flanking cysteines abolished the ability of the peptide to inhibit phospholipid binding of native β2GPI, suggesting that the tertiary structure of β2GPI is important for phospholipid binding. By site-directed mutagenesis of the Lys residues in this amino acid sequence, binding of β2GPI to anionic phospholipid was reduced to about 50% by substituting one Lys residue with an Asp residue and abolished binding with two and three substitutions in this amino acid sequence (Sheng *et al.*, 1996). Using monoclonal antibodies derived from patients with APS and peptide inhibition studies, linear epitopes in the C-terminal end of the fifth domain of β2GPI were recognized by these antibodies (Wang *et al.*, 1995). Constructing two kinds of plasmid expression vectors that express β2GPI and the fifth domain of β2GPI only, polyclonal human anti-β2GPI antibodies were shown to bind the fifth domain of β2GPI directly and could inhibit, in a dose-dependent manner, the binding of these polyclonal antibodies to whole molecule β2GPI coated on irradiated microtiter wells (Yang *et al.*, 1997). Their results suggest that the antigenic epitope for antibody binding is in the fifth domain.

B. Molecular Modeling of β2GPI Modules

1. Introduction

To determine and understand the function of β2GPI, it would be useful to know its three-dimensional (3D) structure. Regrettably, no experimentally determined structure of β2GPI is available. However, it has been shown that the 3D structure of a protein may be calculated with useful accuracy if its amino acid sequence is sufficiently similar to that of a protein with a known 3D structure (Sanchez and Sali, 1997b). This comparative or homology modeling technique is particularly

useful when only low to medium resolution results are required, such as prediction of exposed regions that may interact with antibodies (de la Paz *et al.*, 1986; Sali *et al.*, 1993) and models of interaction based on electrostatic complementarity (Salemme, 1976; Sali *et al.*, 1993). This review describes a comparative modeling study of the "sushi" domains of β2GPIs from five mammalian species. In particular, the authors review their previously published model of the fifth module in human β2GPI (β2GPI-5) and data on the cardiolipin (CL)-binding site on its surface (Sheng *et al.*, 1996). The relationship between the various β2GPI modules is also discussed.

2. *Alignment of SCR Modules in β2GPI and Factor H*

Amino acid sequences are known for 24 modules in β2GPIs from five mammalian species, including human, bovine, dog, mouse, and rat (Table I). Each β2GPI consists of 5 modules, except for rat β2GPI, which consists of only 4 modules. It has been suggested that the β2GPI modules are related to the SCR modules of factor H (Reid and Day, 1989). Four medium-resolution 3D structures of two different SCR modules from factor H (Table I) have been determined by solution nuclear magnetic resonance (NMR) and deposited in the Brookhaven Protein Databank (PDB) (Abola *et al.*, 1987). Structures of the factor H modules and sequences of the 24 β2GPI modules were compared manually to obtain the

TABLE I
SOURCES OF STRUCTURAL AND SEQUENCE DATA USED IN COMPARATIVE MODELING OF β2GPI MODULES[a]

Name	PDB Code	Reference
A. Factor H modules with 3D structures determined by NMR		
Factor H, module 16	1HCC	Norman *et al.* (1991)
Factor H, module 15	1HFI	Barlow *et al.* (1993)
Factor H, modules 15–16	1HFH	Barlow *et al.* (1993)
B. β2GPI sequence		
Human		Kristensen *et al.* (1991)
Bovine		Bendixen *et al.* (1992)
Rat		Aoyama *et al.* (1989)
Mouse		Nonaka *et al.* (1992)
Dog		Sellar *et al.* (1985)

[a] Structures were obtained from the summer 1993 release of the Brookhaven Protein Databank (Abola *et al.*, 1987). The deduced amino acid sequences of β2GPIs were obtained from the GenBank database (Bilofsky and Burks, 1988), except for dog β2GPI, which was obtained from the original paper.

alignment in Fig. 1. Even though sequence identity between factor H modules and β2GPI modules 1–4 is only approximately 20%, their alignment appears to be relatively accurate because there are few gaps and because of the invariability of the two disulfide bonds. Similarly, the alignment of the core regions of factor H and the fifth modules in β2GPIs is strongly determined by the assumption that disulfide bonds 5–50 and 36–61 in H-15 are equivalent to disulfide bonds 3–54 and 39–64 in β2 GPI-5 (Steinkasserer *et al.*, 1993), even though only 9–13 residues out of 62 are identical between factor H modules and β2GPI-5s. The only major ambiguity arises around the 5-residue insertion at residue 21 in human β2GPI-5. There are also three single residue insertions in human β2GPI-5 relative to factor H-15 at positions 47 (loop), 51 (loop), and 57 (extended chain). A major difference between factor H modules and β2GPI-5 modules is that the latter has a 19 residue addition at the C terminus.

3. Overall Similarities among Factor H and β2GPI Modules

To find the clustering of the modules in factor H and the five β2GPIs, the table of percentage sequence identities for all pairs of the modules was calculated from their alignment (Fig. 2). This matrix was used with the Kitsch computer program (Felsenstein, 1985) to calculate a tree that expresses the relationships among the sequences of the modules, similar to the trees used to deduce the evolution of protein families. In this tree, differences between two groups of sequences are approximated by a vertical distance from the top of the tree to the highest node from which the two groups of sequences branch off. The sequences cluster in six groups. There is one group with the factor H modules H-15 and H-16 and five groups each containing the modules with the same relative position in the five β2GPI sequences. This arrangement suggests that the missing module in rat β2GPI is probably the first module because the group of the first modules does not contain a member from the rat β2GPI. The tree indicates that the first event in evolution of a multidomain β2GPI was gene duplication, which separated β2GPI module 5 from the predecessor of the rest of the modules. This may have been followed by the consecutive appearances of modules 1 and 3, with the final duplication resulting in modules 2 and 4.

4. The Three-Dimensional Model of the Fifth Domain of Human β2GPI

The template structure for comparative modeling of β2GPI-5 was that of the 15th domain of human factor H. H-15 conformation has been determined by solution NMR (Barlow *et al.*, 1993) (PDB code 1HFH). The alignment between β2GPI-5 and H-15 (Fig. 1) was used as input for MODELLER-11 (Sali and Blundell, 1993; Sanchez and Sali, 1997a), which


1HFI EKIPCSQPPQIEHGTINSSRSSQ-----ESYAHGTKLSYTCEGGFR-ISEENETTCYM-GKWSS-PPQCE
1HCC EGLPCKSPPEISHGVVAHM--SD------SYQYGEEVTYKCFEGFG-IDGPAIAKCLG-EKWSH-PPSCI
Human-1 GRTCPKPDDLPFSTVVPLKT--------FYEPGEEITYSCKPGYVSRGGMRKFICPLTGLWPINTLKCT
Bovine-1 GRTCPKPDELPFSTVVPLKR--------TYEPGEQIVFSCQPGYVSRGGIRRFTCPLTGLWPINTLKCM
Dog-1 GRTCPKPDDIPFATVVPLKT--------FYDPGEQIAYTCQPGYVFRGLTRRFTCPLTGVWPTNTVRCE
Mouse-1 GRICPKPDDLPFATVVPLKT--------SYDPGEQIVYSCKPGYVSRGGMRRFTCPLTGMWPINTLRCV
Human-2 PRVCPFAGILENGAVRYT----------TFEYPNTISFSCNTGFYLNG-ADSAKCTEEGKWSPELPVCA
Bovine-2 PRVCPFAGILENGTVRYT----------TFEYPNTISFSCHTGFYLKG-ASSAKCTEEGKWSPDLPVCA
Dog-2 PRVCPFAGILENGAVRYT----------TFEYPNTISFACNTGFYLNG-SSSAKCTEEGKWSVDLPVCT
Rat-2 PRVCPFAGILENGVVRYT----------TFEYPNTIGFACNPGYYLNG-TSSSKCTEEGKWSE-LPVCA
Mouse-2 PRVCPFAGILENGIVRYT----------SFEYPKNISFACNPGFFLNG-TSSSKCTEEGKWSPDIPACA
Human-3 PITCPPPSIPTFATLRVYKPSAGN----NSLYRDTAVFECLPQHAMFG-NDTITCTTHGNWTK-LPECR
Bovine-3 PITCPPPPIPKFASLSVYKPLAGN----NSFYGSKAVFKCLPHHAMFG-NDTVTCTEHGNWTQ-LPECR
Dog-3 RVTCPPPSVPKFATLSVFKPLATN----NSLYGNKAVFECLPHYAMFG-NDTITCTAHGNWTT-LPECR
Rat-3 RITCPPPPIPKFAALKEYKTSVGN----SSFYQDTVVFKCLPHFAMFG-NDTVTCTAHGNWTQ-LPECR
Mouse-3 RITCPPPPVPKFALLKDYRPSAGN----NSLYQDTVVFKCLPHFAMIG-NDTVMCTEQGNWTR-LPECL
Human-4 EVKCPFPSRPDNGFVNYPAKP-------TLYYKDKATFGCHDGYSLDG-PEEIECTKLGNWSA-MPSCK
Bovine-4 EVRCPFPSRPDNGFVNHPANP-------VLYYKDTATFGCHETYSLDG-PEEVECSKFGNWSA-QPSCK
Dog-4 EVKCPFPSRPDNGFVNYPAKQ-------ILYYKDKAMYGCHDTYTLDG-PEVVECNKFGNWSA-QPSCK
Rat-4 EVKCPFPSRPDNGFVNYPAKP-------VLSYKDKAVFGCHETYKLDG-PEEVECTKTGNWSA-LPSCK
Mouse-4 EVKCPFPPRPENGYVNYPAKP-------VLLYKDKATFGCHETYKLDG-PEEAECTKTGAWSF-LPTCR
Human-5 ASCKLPVKKATVVYQGERVKIQEKFKNGMLHGDKVSFFCKNKEKKCSYTEDAQCID-GTIE--VPKCFKEHSSLAFWKTDASDVKPC
Bovine-5 ASCKLSIKRATVIYEGERVAIQNKFKNGMLHGQKVSFFCKHKEKKCSYTEDAQCID-GTIE--IPKCFKEHSSLAFWKTDASDVKPC
Dog-5 ASCKLSVKKATVLYQGERVKLQEKFKDGMLHGQKVSFYCKNKEKKCSYTEDAECID-GTIE--IPKCFKEHSSLAFWKTDASDVKPC
Rat-5 ASCKLSVKKATVLYQGQRVKIQDQFKNGMMHGDKVHFYCKNKEKKCSYTEEAQCID-GTIE--IPKCFKEHSSLAFWKTDASDVTPC
Mouse-5 ESCKLPVKKATVLYQGNRVKIQEQFKNGMMHGDKIHFYCKNKEKKCSYTVEAHCRD-GTIE--IPSCFKEHSSLAFWKTDASELTPC
PDB-1HCC BBB BBBBBBBB B BB BBBBBB BBB BBB
AF BBBB BBBBB BBB B BBBB
PsiD BBBBBBBBBBB BBBBBBBBB BBBBB HHHHH BB BHHHHHH BBBBBB
JMC BBBB HHHHHHHHHH HHHHH HHHH
Homolog B BBBB S H HHH BBB BB BB
GOR HH H BBB HHHHHHHHHHH H HH H H HHHHHH

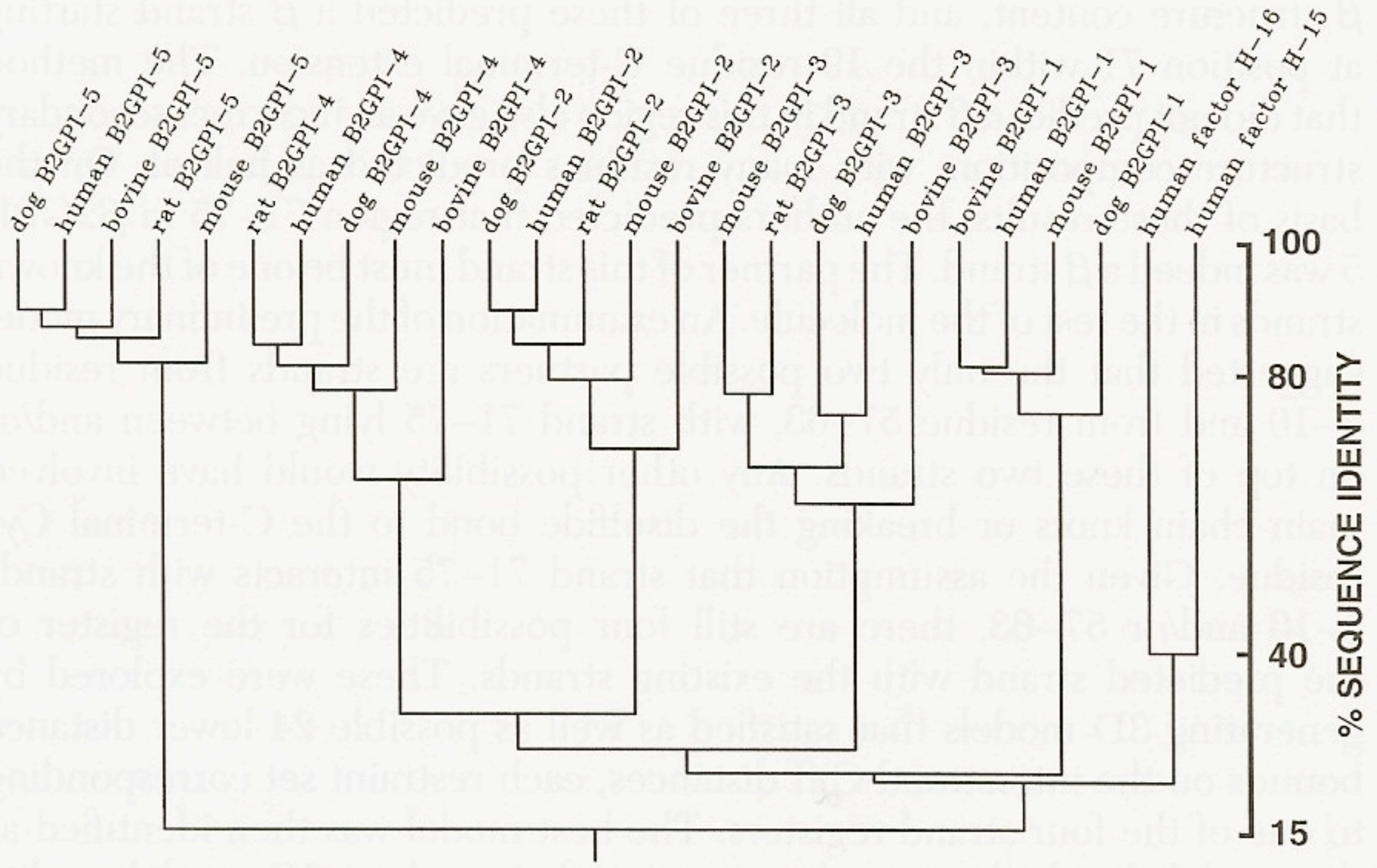

FIG. 2. Clustering of the SCR modules from human factor H and five mammalian β2GPIs.

produced a model of β2GPI-5 containing all main chain and side chain nonhydrogen atoms. [Modeller is available at URLhttp://guitar.rockefeller.edu:pub/modeller and also as part of Quanta, InsightII, and GeneExplorer (MSI, San Diego, CA, USA; e-mail blp@msi.com)].

The standard automated modeling procedure was used, except that additional distance restraints were imposed on the 19 residue extension at the C terminus. These restraints were obtained as follows. First, four secondary structure prediction methods were applied to human β2GPI-5 (Fig. 1). Three of the four methods resulted in an approximately correct

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FIG. 1. Alignment of the amino acid sequences of the modules from five β2GPIs and modules 15 and 16 from human factor H. The top line refers to the residues in 1HFI. The stars indicate the Lys residues that were mutated to the Glu residues. The line PDB-1HFI contains the secondary structure assignments for 1HCC from the corresponding PDB (protein data bank) file. The predictions by the following secondary structure prediction methods are shown: AF, a method based on the physicochemical properties of the residues (Ptitsyn and Finkelstein, 1983); PhD, a neural network method (Rost and Sander, 1993); JMC, a neural network method (Chandonia and Karplus, 1996); Homolog, a method based on residue statistics (Biou *et al.*, 1988); and GOR, a method based on the residue statistics (Biou *et al.*, 1988). The secondary structure predictions are indicated by H for helix and B for β strand.

β structure content, and all three of these predicted a β strand starting at position 71 within the 19 residue C-terminal extension. The method that did not predict a β strand in this region also gave an incorrect secondary structure composition, with many residues predicted as helical. On the basis of these results, the authors predicted that region 71–75 in β2GPI-5 was indeed a β strand. The partner of this strand must be one of the known strands in the rest of the molecule. An examination of the preliminary model suggested that the only two possible partners are strands from residue 4–10 and from residue 57–63, with strand 71–75 lying between and/or on top of these two strands. Any other possibility would have involved main chain knots or breaking the disulfide bond to the C-terminal Cys residue. Given the assumption that strand 71–75 interacts with strands 4–10 and/or 57–63, there are still four possibilities for the register of the predicted strand with the existing strands. These were explored by generating 3D models that satisfied as well as possible 24 lower distance bounds on the interstrand C_{α}ff distances, each restraint set corresponding to one of the four strand registers. The best model was then identified as the model that had minimal restraint violations, best 3D model quality index of Eisenberg and co-workers (Luthy *et al.*, 1992), and the smallest number of residues other than Gly and Asn that had positive ϕ angles (Fig. 8-3, see color insert). The 3D_PROFILES quality index of this representative β2GPI-5 model is 21.5, which is within the allowed range for the protein of the same size as β2GPI (Luthy *et al.*, 1992).This quality index can be compared with the quality indices for the experimental structures of the H-15 and H-16 modules in PDB files 1HFI, 1HCC, 1HFH-15, and 1HFH-16, which are generally, but not always, higher at 30.3, 21.0, 30.1, and 24.4, respectively. The fold of the β2GPI-5 model consists of eight strands, organized in two distorted β sheets with long coiled regions connecting the strands (Fig. 3). There are no helices.

5. *Electrostatic Properties of Human β2GPI-5*

Electrostatic terms in the potential energy often give rise to specific interactions in complexes (e.g., that between a Lys and a sulfate at contact distance). However, in order to understand or to predict the nature of a complex between two molecules, it is often useful to look at their global electrostatic potential. If the structure of only one ligand is known, it is particularly helpful to examine its electrostatic potential for possible binding sites of the other ligand. This is true in the present case where the interaction between a positive (the protein) and a negative (cardiolipin) ligand is considered and the detailed structure of cardiolipin is not available. Thus, to investigate more closely which particular amino acid residues are critical for phospholipid binding by the intact fifth domain of β2GPI,

electrostatic properties of the 3D model of the fifth domain of human β2GPI (Fig. 8-4, see color insert) were examined.

The electrostatic potential on the surface of β2GPI-5 and its mutants was calculated with GRASP (Nicholls *et al.*, 1991), a computer program that uses the finite difference method to solve the linearized Poisson–Boltzmann equation. A net charge of −1 was assigned to each Asp and Glu residue and a net charge of +1 to each Lys and Arg residue. Each His was assigned a neutral charge because β2GPI is active in plasma at a pH of about 7.2. Models with all hydrogen atoms and partial charges from the CHARMM-22 force field were used for electrostatic calculations. Although there are considerable uncertainties in the positions of positive charges at the end of long Lys and Arg side chains on the protein surface, these have a small effect on the global features of the electrostatic potential considered below (Sali *et al.*, 1993).

Most of the positively charged side chains (14 out of 16) are located on the surface of two regions. The first of these regions is defined by segments 40–46, 63–66, and 81–84 (top face of the module in Fig. 4A). The second region is defined by one long and wide omega loop 3–28 (left face in Fig. 4A). Most of the negatively charged residues (8 out of 11) are located in segments 33, 50–62, and 67–80 (right face in Fig. 4A). The pronounced positive electrostatic potential above the top region in β2GPI-5 is predicted to be significantly reduced if any one of the three Lys residues in the center of the top region is mutated to the glutamic acid residue (Fig. 4B).

The sequences of the fifth modules from the five species are highly similar (~ 80%), which is reflected in the similarities of the charge distribution and of the electrostatic potentials. For example, the central segment Cys39–Cys46 of the top region is identical in all five species, as is Lys66, whereas Lys82 is present in three of the five species.

6. Location of the Cardiolipin-Binding Region in Human β2GPI-5

Both positively charged faces on β2GPI-5 are likely to attract negatively charged ligands such as cardiolipin (Fig. 4A). However, because the top positively charged face contains peptide 40–46, which is known to bind cardiolipin (Hunt and Krilis, 1994), the three central charges in this particular region are predicted to be part of the binding site for cardiolipin in the intact β2GPI-5 domain. Moreover, the mutation of these three Lys residues to Glu residues is predicted to prevent the interaction between CL and β2GPI-5 (Fig. 4B). These predictions are similar to those made based on homology modeling alone (Steinkasserer *et al.*, 1991, 1992). To test this prediction, the cDNA for human β2GPI was inserted into the baculovirus viral DNA BacPAK 6 for expression in insect cells (Sf21) (Sheng *et al.*, 1996). As discussed previously, site-directed mutagenesis

was then performed to assess the role of the individual amino acids in the Lys40–Lys45 loop in phospholipid binding and anti-β2GPI activity. It was found that residues Lys42, Lys44, and Lys45 were indeed critical for β2GPI binding to anionic phospholipids, but not crucial for direct binding of β2GPI by anti-β2GPI antibodies.

As mentioned earlier, it has been shown that cardiolipin binds to an isolated peptide Cys39–Cys46 with the two flanking Cys residues, but not to Lys40–Lys45 or to Ser39–Ser46, where the flanking Cys residues were replaced by Ser (Hunt and Krilis, 1994). Thus, the conformation of segment 40–46 is likely to be critical for phospholipid binding. It appears that the flanking Cys residues form a disulfide bond that favors the peptide conformation in the peptide–phospholipid complex, thus increasing the free energy of binding via reducing its entropy. This is explained by the 3D model of β2GPI-5 as follows. Even though the two flanking Cys residues are not disulfide bonded to each other in the native molecule, their relative position in the model is consistent with such a bond (Fig. 3). As a consequence, a nonnative disulfide bond between Cys 39 and Cys 47 is expected to favor the native conformation for the intervening peptide segment. The model for interaction is not sufficiently detailed to distinguish between a specific electrostatic interaction that requires a certain peptide sequence and an interaction that relies on charge density without many steric restrictions. Nevertheless, the model did serve as the basis for informed site-directed mutagenesis experiments that provided more information on the binding of phospholipids to β2GPI.

C. Cloning and Characterization of the Gene Encoding Mouse β2GPI

A mouse ES genomic library in the bacteriophage P1 cloning system was screened using polymerase chain reaction (PCR). A *Hind*III fragment was shown to contain the entire mouse β2GPI gene and was ligated into the pBluescript SK vector for further analysis and sequencing. This plasmid clone was digested with different restriction enzymes, and some fragments were further subcloned into pBluescript SK vectors for sequencing. The mouse β2GPI gene was subsequently found to be encoded by eight exons spread over about 18 kb of genomic DNA. Exon 1 contained the 5′-untranslated region, the 19 amino acid long signal peptide, and the first 2 amino acids of the mature β2GPI protein. Exons 2–7 contain the rest of the protein-coding sequences. Exon 8 contained the last 19 codons and the entire 3′-untranslated region. The exons correlate well with the structural domains. CCP I is encoded by exon II, CCP II by exons III and IV, CCP III by exon V, CCP IV by exon VI, and CCP V by exons VII and

VIII (Sheng *et al.*, 1997) (Fig. 5). The mouse β2GPI gene has been localized to distal chromosome 11 (Nonaka *et al.*, 1992), whereas the human β2GPI gene has been assigned to chromosome 17 (Haagerup *et al.*, 1991). However, comparative mapping of human and mouse genomes has shown that the mouse distal chromosome 11 has extensive homology with human chromosome 17 (Buchberg *et al.*, 1989). The amino acid sequence of β2GPI for mammalian species discovered so far reveals a large degree of homology to the human sequence: mouse (76.1%), bovine (83%), and rat (80%). Alignment of these sequences shows that the fifth domain is the most highly conserved, suggesting that the main functional activities of the protein are present here.

V. Immunogenicity and Animal Models

In order to determine if autoantibodies are pathogenic *in vivo*, suitable animal models need to be studied. A murine model of autoimmune vascular disease (NZW × BXSB/F1) was first described (Hang *et al.*, 1981). Other autoimmune strains predisposed to lupus were studied and showed autoantibodies reactive in a standard CL-ELISA (Gharavi *et al.*, 1989). NZW × BXSB/F1 mice also have thrombocytopenia and were found to have anti-

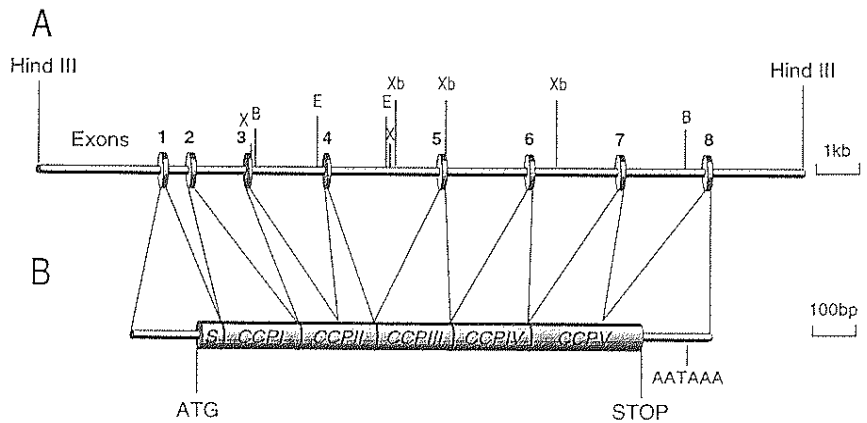

FIG. 5. Organization of the mouse β2GPI gene. (A) The structure of the mouse β2GPI gene is shown with restriction enzyme sites. The positions of exons are shown as boxes, and the introns are shown as lines connecting the exons. Restriction sites indicated: X (*Xho*1), B (*Bam*HI), E (*Eco*RV), and Xb (*Xba*I). (B) SCR repeat domain structure of β2GPI. The positions of the translation initiation site (ATG), the polyadenylation site (AATAAA), and the termination codon are indicated. S, signal peptide (Sheng *et al.*, 1997)

bodies to β2GPI similar to that seen in autoimmune human APS patients (Hashimoto *et al.*, 1992).

Two groups have suggested that aPL antibodies have a direct role in causing pregnancy loss *in vivo*. Passive immunization of normal pregnant mice with human polyclonal (Branch *et al.*, 1990; Blank *et al.*, 1991) antibodies or monoclonal aPL antibodies (Bakimer *et al.*, 1992) have been shown to result in increased fetal loss and fetal resorption and lower mean weights of embryos and placentas compared with mice immunized with normal immunoglobulins. However, one of these groups have since suggested that passive immunization with human IgG polyclonal aPL antibodies had variable effects on murine pregnancy outcome. The rate of fetal death did not increase uniformly with increasing doses of IgG and was unrelated to the individual patient's medical history (Silver *et al.*, 1997). BALB/c mice immunized with CL mixed with β2GPI, CL alone, β2GPI alone, or buffer alone were studied. Mice immunized with CL mixed with β2GPI produced high levels of anti-β2GPI antibodies and antibodies reactive in a standard CL-ELISA. Mice immunized with CL alone did not produce aPL antibodies, and mice immunized with β2GPI alone produced anti-β2GPI antibodies (Rauch and Janoff, 1992). Another group suggested that immunization of mice with β2GPI produced a high percentage of fetal resorption *in utero* when the mice were mated, suggesting an induced model of the APS (Blank *et al.*, 1994b). To study the issue of pathogenesis and thrombosis in an animal model, mechanical stimulus of exposed femoral veins in CD-1 mice was used to promote clot formation. Mice actively immunized with β2GPI and human IgG aPL antibodies from patients with APS developed propagation of the clot and slower dissolution (Pierangeli *et al.*, 1996). Immunization of MLR/++ mice with β2GPI produced aPL antibodies. The development of neurological dysfunction and production of antinuclear and anti-DNA antibodies was controversial, with two opposing conclusions (Cote *et al.*, 1994; Aron *et al.*, 1995). Further research in this area needs to be done as another study has suggested that immunization of BALB/c mice with a monoclonal human aPL antibody (H-3) induces neurological and behavioral defects (Ziporen *et al.*, 1997).

Polyclonal antibodies purified from patients with APS, with a β2GPI affinity column, have binding characteristics similar to anti-β2GPI antibodies induced by immunization of a rabbit with human β2GPI. Some of the polyclonal human autoantibodies bound both β2GPI and anionic phospholipids. The binding to anionic phospholipids involves ionic interactions as the binding was reduced significantly in the presence of high ionic strength buffers (Kouts *et al.*, 1995). Nine monoclonal antibodies derived from NZW × BXSB/F1 mice had two populations of antibodies with β2GPI reactivity and anionic phospholipid reactivity in the absence of β2GPI.

These latter antibodies, as with the polyclonal human antibodies, had charge-dependent binding to the anionic phospholipid with abolishment of binding in the presence of high ionic strength buffers (Monestier *et al.*, 1996). Anti-β2GPI antibodies had a clear preference for purified murine β2GPI in a fashion similar to the preference human polyclonal antibodies from patients with APS had for purified human β2GPI. The analysis of the V region sequences of these antibodies suggest that cationic residues in the H chain CDR3 are important for their charge-dependent phospholipid reactivity. Sequence analysis of one of the monoclonal antibodies that recognized β2GPI in a phospholipid-free system, with little change in binding in the presence of high ionic strength buffers, did not reveal any cationic amino acid residues. The structural features of the V_H-D-J_H junctions of these monoclonal autoantibodies further support the view that an increased frequency of unusual V(D)J rearrangements contribute directly to the development of murine autoimmunity (Monestier *et al.*, 1996).

The presence of these animal models of APS allow for the comparative study of the mechanisms of action of autoantibodies and induced antibodies. This will allow the continuing study of therapeutic interventions that may prevent the clinical manifestations of APS.

VI. Prothrombin

Lupus anticoagulant antibodies could potentially inhibit any of four procoagulant phospholipid complexes or two anticoagulant phospholipid reactions. A number of early reports suggested a role for plasma proteins in the activity of lupus anticoagulants. The lupus anticoagulant "cofactor" phenomenon, i.e., the addition of normal plasma to patient plasma, increasing the inhibition of coagulation, was first attributed to the presence of prothrombin (Loeliger, 1959). Autoantibodies to prothrombin were shown in two patients with the lupus anticoagulant-hypoprothrombinemia syndrome (Bajaj *et al.*, 1983). Circulating prothrombin complexes were found in 74% of patients with LA antibodies and normal prothrombin levels (Fleck *et al.*, 1988). The authors and others have shown that LA can react with human prothrombin directly on phospholipid-free, high-binding (irradiated) ELISA plates (Arvieux *et al.*, 1995, Kandiah and Krilis, 1997a), phospholipid-bound prothrombin (Bevers *et al.*, 1991), and phospholipid alone (McNeil *et al.*, 1989; Pierangeli *et al.*, 1993; Kandiah and Krilis, 1997b).

Antiprothrombin antibodies have been found to have immunological prediction of myocardial infarction in men (Vaarala *et al.*, 1996). In a study of 233 patients with aPL antibodies, 26% had IgG and/or IgM

antiprothrombin antibodies. There was poor correlation between antiprothrombin and anticardiolipin and anti-β2GPI antibodies in this same patient population. Univariate analysis suggested that antiprothrombin IgG correlated well with a history of venous thrombosis, but this effect was lost in the multivariate analysis, whereas anti-β2GPI IgG was the only variable that showed statistical significance (Forastiero *et al.*, 1997). In another retrospective study of SLE patients with aPL antibodies, the presence of LA antibodies was the only variable that had statistical significance in the multivariate analysis of association with venous thrombosis (Horbach *et al.*, 1996). The varying results obtained by different groups on the prevalence and pathological links of antiprothrombin antibodies cannot be explained by the patient population studied alone. The method of performance of the antiprothrombin-ELISA is different in different studies, varying from the buffers used to dilute the samples and block the prothrombin-coated wells, to the cutoff levels determined. Some investigators use buffer-only wells as controls, which may be important, as it deducts nonspecific binding that can occur with the high binding plates used. If any meta-analysis is to be performed on these studies, to make generalizable deductions on the role of antiprothrombin antibodies, this important variable would need to be considered.

It has been shown that LA antibodies in some patients with APS can be separated into antibodies positive in the dRVVT clotting assay and the dKCT. The immunoreactivity of these separate populations of autoantibodies cannot be explained by their immunoreactivity to β2GPI or prothrombin (Kandiah and Krilis, 1997b). Affinity-purified antiprothrombin antibodies from different patients had different reactivities in these two clotting assays (Kandiah and Krilis, 1997a). This observation was in variance with indirect studies on plasma reactivities, which suggested that anti-β2GPI reactivity corresponded to a prolongation in the dRVVT assay and antiprothrombin reactivity with prolongation in the dKCT assay (Galli *et al.*, 1995), but was supported by another study on a large population of patients that did not find a difference in the plasma reactivities in the dRVVT and dKCT clotting assays and their anti-β2GPI and antiprothrombin reactivities (Forastiero *et al.*, 1997).

The anticoagulant activity of antiprothrombin antibodies appears to be dependent on their recognition of a phospholipid–human prothrombin complex that inhibits both the conversion of prothrombin into thrombin in the prothrombinase complex (Bevers *et al.*, 1991) and the tenase complex (Permpikul *et al.*, 1994). There appears to be a high species specificity to human prothrombin in the functional assays (Bevers *et al.*, 1991; Rao *et al.*, 1995). LA IgG from two patients inhibited the activation of human but not bovine prothrombin in a purified prothrombin activation system

(Bevers *et al.*, 1991), whereas LA IgG from a third patient inhibited the activation of both human and bovine prothrombin (Galli *et al.*, 1993). This is in variance to the immunoreactivity of the purified antibodies that recognized both human and bovine prothrombin coated on microtiter wells and on a Western blot, although the binding to human prothrombin was substantially higher in 6 of the 14 preparations studied. Twelve of the preparations showed a significantly increased binding to human prothrombin and 9 to bovine prothrombin in the presence of phosphatidylserine and calcium ions. Further experiments with phosphatidylserine/phosphatidylcholine (PS/PC) vesicles, soluble prothrombin, and LA IgG failed to explain why LA IgG inhibits human prothrombin activation more effectively than it inhibits bovine prothrombin activation (Rao *et al.*, 1995).

In a study of 59 patient plasmas with aPL antibodies, 90% showed reactivity to prothrombin bound to phosphatidylserine in the presence of calcium, whereas only 58% of these plasmas had reactivity to prothrombin coated directly on high binding wells (Galli *et al.*, 1997). These authors suggested that the mode of presentation of prothrombin in solid-phase influenced its recognition by antiprothrombin antibodies. They postulated that these differences were produced either due to clustering and conformational orientation of the prothrombin bound to phosphatidylserine, allowing better capture of the antibodies, or that the capture of prothrombin–antiprothrombin complexes may be better in the presence of calcium ions. This may also be due to the patient population studied, as in patients with antiprothrombin antibodies, the binding to prothrombin coated on irradiated surfaces in the absence of calcium ions was significantly higher than for prothrombin bound to phosphatidylserine in the presence of calcium. This applied to both plasma samples, as in the previous study as well as to affinity-purified antiprothrombin antibodies through a prothrombin column. The dissociation constant calculated for these antibodies was in the region of 200 n*M*, which showed about 10 times higher affinity than anti-β2GPI antibodies purified from a β2GPI column (Kandiah and Krilis, 1997a). Both studies, however, confirmed the heterogeneity of antiprothrombin antibodies in coagulation assays with no one assay detecting these antibodies consistently.

VII. Lupus Anticoagulant Antibodies and Protein C Activation

LA antibodies have been shown to have multiple effects on protein C. Results in the literature have been contradictory, with some researchers finding a significant inhibition on the rate of activation of protein C by thrombin on endothelial cells by purified LA IgG (Cariou *et al.*, 1988), whereas others could not confirm this (Oosting *et al.*, 1991; Keeling *et al.*,

1993). LA antibodies have also been shown to prevent the inactivation of factor Va by protein C. These appeared to be IgGs directed against negatively charged phospholipid–protein complexes of either protein C or protein S (Oosting *et al.*, 1993). APC resistance (i.e., the association of dysfunctional APC with a venous thrombotic tendency) predominates in LA plasma, but is not restricted to the presence of the Arg506-Gln point mutation on factor V (Bokarewa *et al.*, 1995).

VIII. Lupus Anticoagulant Antibodies and Phosphatidylethanolamine

The presence of phosphatidylethanolamine (PE) has been shown to augment LA activity and inhibit the anticoagulant effect of activated protein C (APC) *in vitro*. This effect appeared to arise from interference of LA antibodies with APC activity by binding to PE or the complex of APC and PE (Smirnov *et al.*, 1995). aPE antibodies have also been shown to require plasma cofactors in their binding to PE, including high and low molecular weight kininogens (HMWK and LMWK) and, less frequently, prekallikrein and factor XI (Sugi and McIntyre, 1995).

Kininogens inhibit thrombin-induced platelet aggregation. Kininogen-dependent IgG aPE markedly increased thrombin-induced platelet aggregation *in vitro*, whereas kininogen-independent IgG aPE did not (Sugi and McIntyre, 1996). Hence, kininogen-dependent aPE could cause thrombosis *in vivo* by disrupting the antithrombotic effects of kininogen. As PE can increase the procoagulant activity of vesicles containing PS and PC, and aPE has been associated with thromboembolic events, the pathogenesis of the thrombosis in these patients is multifactorial.

PE undergoes the transition from lamellar to hexagonal II phase under certain physiological conditions, and mice immunized with hexagonal PE develop phospholipid-dependent inhibitors of coagulation (LA antibodies) (Rauch and Janoff, 1990). Preincubation of aPL-positive plasma with hexagonal-phase II PE has been shown to reduce or even abolish the prolongation of clotting times in phospholipid-dependent coagulation assays, suggesting that this phospholipid is the target antigen for some LA antibodies (Rauch *et al.*, 1989). These authors have suggested that the target antigen for these antibodies may be a complex of PE and human prothrombin (Rauch *et al.*, 1997), although these experiments have been performed in clotting assays and not in a purified system.

IX. Antiphospholipid Antibodies and Endothelial Cells

When a more physiological surface, such as endothelial cells, is used for the assembly of the prothrombinase complex, only 18% of IgG fractions

with LA activity were able to inhibit prothrombinase activity (Oosting *et al.*, 1993). Endothelial cells play a central role in the prevention of unwanted activation of the coagulation cascade in intact vessels. This antithrombogenic property of endothelial cell surfaces responds to physiological stimuli and is therefore susceptible to injury. aPL antibody-positive SLE sera, but not purified antibody, in the presence of low doses of tumor necrosis factor (TNF) stimulated procoagulant activity by cultured endothelial cells. However, no association was found with clinical thrombotic events (Hasselaar *et al.*, 1989). A high prevalence of antiendothelial cell (AECA)-binding activity is found in sera from patients with APS (Hasselaar *et al.*, 1990; Del Papa *et al.*, 1992). β2GPI is able to bind resting endothelial cells and be recognized by monoclonal and polyclonal anti-β2GPI antibodies (Del Papa *et al.*, 1995; Le Tonqueze *et al.*, 1995). Although platelet binding has been related to the expression of anionic phospholipids on their cell membranes after activation, a comparable phenomenon is unlikely on resting endothelial cells that do not display such phospholipid distribution changes (Del Papa *et al.*, 1992). Both polyclonal and monoclonal anti-β2GPI antibodies can upregulate adhesion molecule expression after endothelial cell binding (Del Papa *et al.*, 1995; Del Papa *et al.*, 1997). Del Papa *et al.* (1998) showed that β2GPI binds endothelial cell membranes through its fifth domain. The major phospholipid-binding site that mediates the binding of β2GPI to anionic phospholipids is also involved in endothelial binding (Fig. 6). Human umbilical vein endothelial cell (HUVEC) monolayers provide a suitable surface for β2GPI binding comparable to that displayed by anionic phospholipids dried on microtiter wells. The formation of β2GPI and anti-β2GPI complexes induces endothelial activation as supported by E-selectin expression and IL-6 secretion (Del Papa *et al.*, 1998) (Fig. 6).

X. Pathogenesis of the Antiphospholipid Syndrome

Patients with APS have a tendency to atherogenesis that is likely related to the multiple immunological abnormalities that occur in this condition. The oxidation of plastic microtiter plates that increases the capture of the target antigens for aPL antibodies may be an *in vitro* model of the vascular inflammatory processes that result in a high oxidative capacity in vascular walls. Oxidation of plasma proteins and oxygen-mediated endothelial injury decrease the physiological anticoagulant function of endothelium (Vaarala, 1997).

Antibodies to oxidized low density lipoproteins (LDL) are associated with carotid atherosclerosis (Salonen *et al.*, 1992) and myocardial infarction (Puurunen *et al.*, 1994). In patients with SLE, these antibodies have been

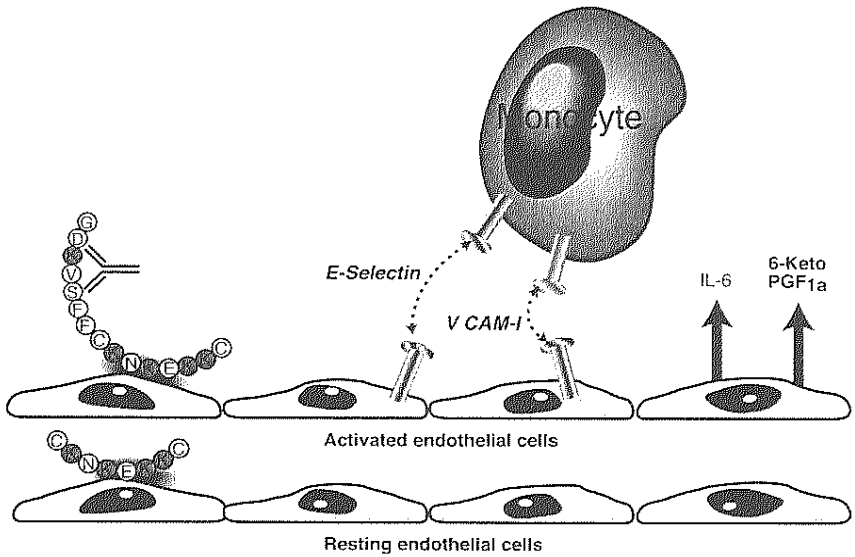

FIG. 6. β2GPI, anti-β2GPI, and endothelial cells. Anti-β2GPI antibodies bind a synthetic peptide spanning the fifth domain of β2GPI after capture on activated endothelial cells. β2GPI binds activated endothelial cells through the major phospholipid-binding site, KNKEKK (Del Papa *et al.*, 1998). Human polyclonal anti-β2GPI antibodies bind to the same peptide sequence previously shown to support human monoclonal antibody binding after β2GPI bound to a negatively charged surface (Wang *et al.*, 1995). Antibody binding upregulates adhesion molecule (E-selectin) expression and IL-6 secretion (Del Papa *et al.*, 1995, 1998).

shown to cross-react with autoantibodies detected in the standard cardiolipin ELISA to β2GPI (Vaarala *et al.*, 1993). Monoclonal anti-β2GPI antibodies derived from NZW x BXSB/F_1 mice also cross-react with oxidized LDL (Mizutani *et al.*, 1995).

Lipids are transported in blood as lipoproteins, macromolecular complexes of lipids and proteins (apolipoproteins). The properties and functions of apolipoproteins include being structural components of lipoproteins, the regulation of enzyme activity, and binding of lipoproteins to cell surface receptors for internalization and catabolism (Laker and Evans, 1996). Lipoprotein(a), which consists of an LDL particle and apolipoprotein(a), has been shown to be a strong independent risk factor for coronary heart disease (Mbewu and Durrington, 1990; Scott, 1991). Lipoprotein(a) has physiological interactions with coagulation and fibrinolytic systems (Hajjar *et al.*, 1989; Miles *et al.*, 1989). By studying its cDNA sequence, apolipoprotein(a) has been shown to have marked similarities in structure with plas-

minogen (McLean *et al.*, 1987). Elevated levels of lipoprotein(a) have been reported in patients with APS, with significantly higher levels in patients with arterial than venous thrombosis (Yamazaki *et al.*, 1994). It has been shown that a protein ligand for apolipoprotein(a) is β2GPI. Using the repetitive apolipoprotein(a) kringle IV type 2 domain as bait to screen a human liver cDNA library by the yeast two-hybrid interaction trap system, 11 clones were identified, of which 8 were β2GPI (Kochl *et al.*, 1997). Coimmunoprecipitation experiments showed specific binding of β2GPI to immobilized apolipoprotein(a), lipoprotein(a), and low density lipoproteins (which had been shown previously). The binding of β2GPI to lipoprotein(a) is via domains 2–4. These observations will lead to further investigations into the role of this β2GPI–apolipoprotein(a) interaction and its role in a prothrombotic tendency. Apolipoprotein(a) may form a multimeric complex with β2GPI, which would be cleared from the circulation by macrophages, a process that could be affected by anti-β2GPI antibodies.

In a study of middle-aged men with elevated lipids but no autoimmune disease or history of thrombosis, antiprothrombin antibodies were significantly higher in men who developed myocardial infarctions or cardiac deaths than in controls. When all variables were analyzed, there was an interactive effect of antiprothrombin antibodies with smoking and triglyceride levels independently. Autoantibodies detected in the standard CL-ELISA and antibodies to oxidized low-density lipoproteins had an additive effect with antiprothrombin antibodies to the risk of cardiac events (Vaarala *et al.*, 1996).

The possible link between antibodies to β2GPI and the pathogenesis of thrombosis has been studied extensively. Anionic PLs promote initiation of the contact activation system in blood coagulation, which is inhibited *in vitro* by physiological concentrations of β2GPI (Schousboe, 1988). The autoactivation of factor XII in prekallikrein-deficient plasma in the presence of anionic PLs and cationic zinc is inhibited by β2GPI and the anti-β2GPI antibodies/β2GPI complex, which could behave as a LA (Schousboe and Rasmussen, 1995). One group classified aPLs into two types, depending on their sedimentation characteristics after adsorption with cardiolipin liposomes (Galli *et al.*, 1992). If LA activity cosedimented with the liposomal pellet, antibodies eluted from the liposomes were β2GPI dependent in prolonging dRVVT. However, the LA antibody present in the supernatant prolonged dRVVT, irrespective of the presence of β2GPI. This group subsequently suggested that antibodies positive in a dRVVT clotting assay were more likely to be associated with clinical thrombosis. This study was done indirectly with patient plasma and retrospective analysis of the clinical histories (Galli *et al.*, 1995). β2GPI at physiological concentrations has been shown to inhibit the generation of factor Xa in the presence of

activated gel-filtered platelets (Shi *et al.*, 1993). aCL antibodies interfered with this inhibition, whereas LA antibodies inhibited this process in a manner similar to β2GPI without any additive effect shown. β2GPI also appears to inhibit the prothrombinase activity of resting nonactivated platelets, lysed platelets, and phosphatidylserine/phosphatidylcholine vesicles (Nimpf *et al.*, 1986), although it is unclear whether it requires small amounts of anti-β2GPI antibodies for this activity (Galli *et al.*, 1993). β2GPI at physiological levels inhibits the factor Va-dependent prothrombinase complex (Mori *et al.*, 1996). However, in the same system, it potentiates thrombin generation in the presence of activated protein C (APC). This inhibitory effect was diminished by the addition of increasing concentrations of cephalin, suggesting that β2GPI competitively inhibits the binding of APC to the phospholipid surface. This group also showed that the anticoagulant activity of APC was significantly potentiated in β2GPI-depleted plasma, an effect that was reduced with the addition of increasing concentrations of β2GPI. (These *in vitro* reactions are illustrated in Fig. 7.)

The affinity of β2GPI for phospholipid is increased in the presence of anti-β2GPI antibodies (Willems *et al.*, 1996). Hence in individuals with these antibodies, the β2GPI–anti-β2GPI complex may well displace other coagulation proteins affecting homeostatic mechanisms. Although the affinity constant for polyclonal anti-β2GPI to β2GPI is low at 3.4–7.2 μM (Tincani *et al.*, 1996), this affinity constant is increased with dimerization of β2GPI (Sheng *et al.*, 1998).

In a study of 46 patients with SLE and autoantibodies detected in a standard CL-ELISA, comparisons of binding specificities and avidity of binding of the 22 patients with APS and the 24 patients without clinical manifestations of the APS were made. The authors found that while all patients had a positive result in the standard CL-ELISA, the absorbance values were higher in the group with APS. Reactivity in an anti-β2GPI-ELISA was significantly more in patients with APS. Urea, a chaotropic agent interfering with electrostatic interactions, was able to reduce binding of the antibodies to β2GPI in both the standard CL-ELISA and the anti-β2GPI-ELISA, mainly in the group without clinical manifestations (Vlachoyiannopoulos *et al.*, 1998). This suggested that patients with APS have a significant increase in autoantibodies detected in an anti-β2GPI ELISA and that these antibodies are generally of high avidity as compared to the autoantibodies found in individuals without clinical manifestations.

Auger *et al.* (1995) reported that heparin-induced thrombocytopenia developed in 56.5% of patients with LA antibodies with an increased occurrence of thrombotic events. Although the mechanism is not clear, this could be due to heparin binding to β2GPI, enhancing the antibody binding to anionic phospholipids, e.g., activated platelets. Unfractionated

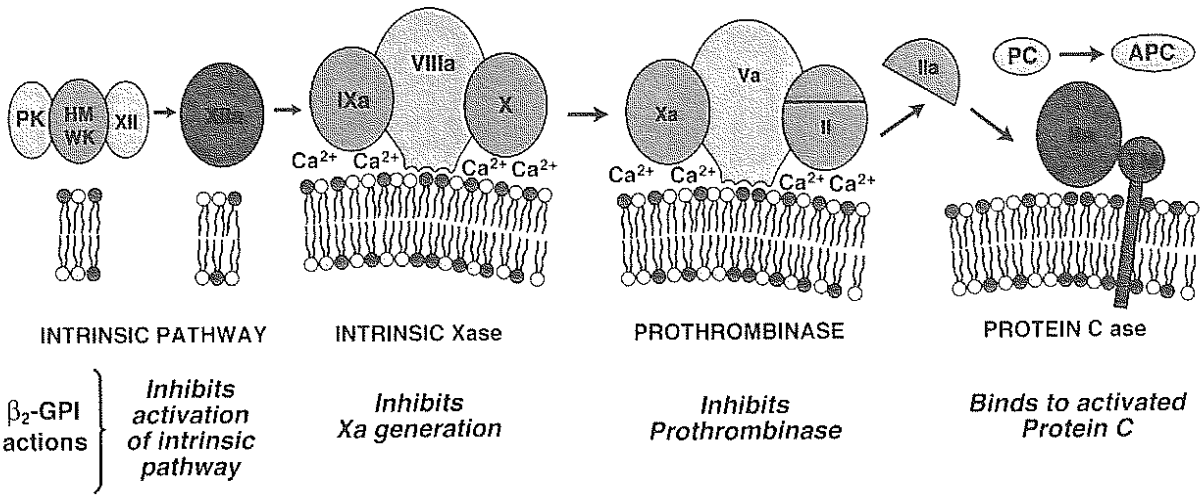

FIG. 7. β2GPI effects in coagulation reactions. *In vitro* experiments with β2GPI suggest that it inhibits activation of the intrinsic pathway (Schousboe, 1988); inhibits Xa generation (Shi *et al.*, 1993), inhibits prothrombinase activity of human platelets (Nimpf *et al.*, 1986), and modulates the anticoagulant activity of activated protein C on phospholipid (Mori *et al.*, 1995).

heparin has been shown to enhance aPL binding to β2GPI in the presence of phosphatidylserine as well as changing the electrophoretic mobility of β2GPI, which did not occur with low molecular weight heparin (McNally *et al.*, 1994). Hence the perceived anticoagulant properties of β2GPI may be altered by unfractionated heparin and may influence its use in clinical practice for the prophylaxis and treatment of thrombosis associated with these autoantibodies. Another group suggested that heparin reversibly bound aPL antibodies *in vitro* as shown by depletion of aPL antibodies after passage through a heparin affinity column and by dose–response heparin inhibition of aPL to CL in the presence of β2GPI (standard CL-ELISA) (Ermel *et al.*, 1995). This effect would be mediated by the binding of β2GPI to heparin.

The activation of human umbilical vein endothelial cells by IgG autoantibodies from patients with APS, as measured by increased monocyte adhesion, has been shown to be β2GPI dependent. Interestingly, rabbit polyclonal anti-β2GPI antibodies also activated endothelial cells (Simantov *et al.*, 1995). β2GPI adhesion to endothelium has been described in normal placental vessels. In the placental samples studied, increased β2GPI deposition was found by indirect immunofluorescence on the trophoblast surfaces of placentas from patients with persistently raised titers of aPL antibodies (La Rosa *et al.*, 1994). aPL antibodies have been eluted from placentas of women with elevated serum aPL antibodies. β2GPI was present in placental eluates from both control and aPL affected pregnancies (Chamley *et al.*, 1993). Using reverse transcriptase polymerase chain reaction (RT-PCR), placental cells were shown to synthesize β2GPI transcripts. Immunoblotting experiments suggested that β2GPI is localized in syncytiotrophoblast and extravillous cytotrophoblast. Anti-β2GPI antibodies may therefore bind placental β2GPI, inhibiting their function *in vivo* (Chamley *et al.*, 1997). The function of placental β2GPI is unclear at the moment, but may have an effect on placental circulatory hemostatic mechanisms.

The binding of β2GPI to endothelial cells, via the cluster of Lys residues (Del Papa *et al.*, 1998), suggests the same possibilities as for anionic phospholipid binding. This binding may increase antigen density and/or induce conformational changes, allowing for the capture of circulating anti-β2GPI antibodies. Anti-β2GPI monoclonal antibodies have been shown to exert LA activity *in vitro* by enhancing the binding of β2GPI to phospholipids (Takeya *et al.*, 1997). This clustering may hinder the lateral mobility of coagulation proteins, affecting the fine balance between their procoagulant and anticoagulant activities. This effect may also occur in β2GPI binding to endothelial cell membranes perturbing endothelial function. It is therefore possible *in vivo* that autoantibodies directed against β2GPI induce endothelial cell activation, which in the presence of some other

insult may trigger a thrombotic event. Hence aPL antibodies may independently influence atherogenesis by moderating coagulation reactions toward hypercoagulation by an as yet unexplained mechanism.

XI. Laboratory Investigations of the Antiphospholipid Syndrome

As aPL antibodies are increasingly shown to be a heterogeneous group of autoantibodies, the need to perform multiple immunoassays and coagulation tests has become imperative. This is especially important in prospective studies of patients with APS as subsets of patients may be identified who are at particular risk of clinical events and may be identified by certain laboratory tests or a combination of tests. Standardization of assays for anticardiolipin antibodies and lupus anticoagulants have been fraught with difficulty, despite numerous attempts to perform this by international standardization workshops and committees.

A. Anticardiolipin Antibodies

At the third international workshop held in 1992, the delegates confirmed that bovine β2 glycoprotein I (β2GPI) supported the binding of purified aPL antibodies to CL (Harris *et al.*, 1994). The authors and others have now found that there are patients who have selective binding to human β2GPI and may therefore have persistent negative aCL titers in conventional immunoassays. The workshop delegates also found binding of purified antibodies to ovalbumin and casein used in the blocking and diluting buffers, which suggest that any protein solutions used in an ELISA system need to be checked for contamination with β2GPI, even in small concentrations (Harris *et al.*, 1994). It has also been found that non-fatty acid-free bovine serum albumin used in a number of laboratory experiments contains sufficient β2GPI to support binding of anti-β2GPI antibodies.

The current standard ELISA kits are increasingly using β2GPI as a discriminator of autoimmune aCL antibodies from true aCL antibodies that do not require β2GPI for direct binding to CL. Nine commonly used commercial kits for measuring aCL antibodies were compared with a standardized in-house method. The authors found marked differences in the positivity rate between kits ranging from 31 to 60% for IgG and from 6 to 50% for IgM (Reber *et al.*, 1995). The β2GPI content of the dilution buffers and the wells supplied with the kits were significantly different. Despite extensive efforts over the years to achieve standardization, these results suggest that some technical aspects need to be reevaluated, including cutoff points and the use of controlled amounts of β2GPI or incubation times. β2GPI has been shown to be inhibitory to the binding of antibodies from patients with chronic infections (Hunt *et al.*, 1992). This is due to

the competition of β2GPI with the positively charged antibodies for binding to anionic phospholipids (Monestier *et al.*, 1996). Hence high positivity of aCL titer may be found in some patients with chronic infections, and in the right clinical setting, further tests need to be performed to identify the binding specificities of these antibodies.

SLE patients with clinical manifestations of APS but negative for conventional tests in the aCL assay and LA clotting tests were tested for immunoreactivity to various phospholipids, including phosphatidylserine, phosphatidylinositol, phosphatic acid, phosphatidylcholine and phosphatidylethanolamine (Roch *et al.*, 1997). No correlation was found between detected antibodies to phosphatidylethanolamine and clinical manifestations of APS. Overall, in all patient groups, the authors found no additional benefit from testing for immunoreactivity to other phospholipids other than CL (Roch *et al.*, 1997).

The use of these new β2GPI immunoassays may supplant the conventional aCL immunoassays in use because of their improved specificities but not completely replace them because of their relative costs. Clinical studies highlight the importance of detecting aPL antibodies and quantitating their levels and therefore stratifying the risk for each patient so that optimum treatments could be developed.

B. Lupus Anticoagulant Antibodies

The LA/aPL antibody subcommittee has met annually since 1988 to update the nomenclature, methods, and standardization practices of LA testing. Screening tests for LA need to be sensitive, and the amount of phospholipid in the test system is a critical determinant of sensitivity. The reactivity of a particular patient LA antibody is also important. Hence the use of at least two sensitive screening tests is important in detecting the LA antibody. The combination of tests also needs to detect reactivity to different parts of the clotting cascade (Kandiah and Krilis, 1996; Triplett, 1995).

Current criteria for the diagnosis of LA antibodies are:

1. Prolongation of at least one PL-dependent clotting test.
2. Evidence of inhibitory activity shown by the effect of patient plasma on pooled normal plasma.
3. Evidence that the inhibitory activity is dependent on PL by the addition or alteration of PL, hexagonal-phase PL, platelets, or platelet vesicles in the test system originally used.
4. LAs must be carefully distinguished from other coagulopathies. Specific factor assays and the clinical history may be helpful in these situations (Brandt *et al.*, 1995).

Predictive tests that identify patients most at risk of the clinical manifestations of APS have not yet been developed. More recent test systems have looked at dot blots to various protein antigens, *in vitro* thrombin generation, functional assays to detect acquired APC resistance, and inhibition of downregulation of factors Va and VIIIa (Triplett, 1996). Laboratory tests for the detection of aPL antibodies have become more specialized. Hence, clinical and research studies looking at aPL antibodies and clinical features need to be precise in the performance and reporting of the methodology of their tests, which will promote the reproducibility of results across different population groups and allow accurate interpretation of data obtained.

XII. Antiphospholipid Syndrome and Future Therapies

Antiphospholipid syndrome belongs to a wide spectrum of clinical disorders that are categorized as autoimmune disorders based on the presence of autoantibodies and/or the finding of lymphocytic infiltrates in the target organs. As in most autoimmune disease, the particular role of the immune system in the initiation and progression of the disease remains uncertain. The finding that plasma proteins are the target antigens for some of the aPL antibodies has gone a long way in investigating the potential pathogenesis in APS, especially in relation to thrombosis and atherogenesis. Some patients respond well to aspirin alone, whereas other patients require high doses of anticoagulation to prevent recurrent vascular events. Autoantibodies in APS have increasingly been shown to be heterogeneous, and the combination of antibodies may be the precipitant for the diverse clinical manifestations of these patients. Not only does the combination of binding specificities of the antibodies need to be identified, but also those patients who respond to existing therapies.

In disease in general, and autoimmune disease in particular, the nature of the inciting antigen is central to the definition of the disease and therapeutic interventions. In APS, as in other autoimmune diseases, the three processes that interact to produce disease need to be studied. These are (a) selection in the thymus of a repertoire of T cells that discriminates self from nonself, i.e., tolerance; (b) lymphocyte activation after a potential autoreactive T cell has emerged from the thymus and entered the peripheral circulation; and (c) preprogrammed cell death that eliminates T and B cells with particular autoreactive properties, i.e., apoptosis.

In PAPS and APS associated with SLE, there is considerable diversity in the spectrum of autoantibodies and severity of disease among affected individuals. One theory proposed for the diversity of autoantibodies is the presence of common structural motifs found in many diverse implicated

molecules, e.g., phosphodiester groups found in single-stranded DNA and phospholipids, and β2GPI- and DR9-binding motif (Fujisao *et al.*, 1996). "Antigenic spreading," in which the T-cell response to a particular peptide antigen leads to the involvement of other T cells with a progressively wider spectrum of activity (Lehmann *et al.*, 1993), has also been proposed. Thymic tolerance failure may also explain the development of autoimmune disease. This may arise by (a) anatomical sequestration of self-antigens, not exposing developing T cells in the thymus to these self-antigens during their maturation; (b) formation of neoantigens or cryptic antigens when the target antigen is conformationally changed, thus not being recognized by T cells (Fatenejad *et al.*, 1993); and (c) failure to suppress autoreactive T cells in the periphery by some as yet unexplained mechanism (Clark and Ledbetter, 1994).

The ability of B cells to internalize antigens and present them as peptide/class II complexes on their cell surface may play a critical role in "antigenic spreading," promoting a progressively more polyclonal T-cell response against an autoantigen (Mamula and Janeway, 1993).

In addition to the crucial processes generating autoreactive T cells, apoptosis and factors leading to the death of cells and their removal in the thymus and the periphery may be important in pathogenesis. It has been shown that MRL/lpr autoimmune mice with T-cell antigen receptor α chain knockout (which lack αβ T cells) lack IgG and IgM aCL antibodies compared to their wild-type controls and that CD40 ligand-deficient MRL/lpr mice lack IgG aCL antibodies compared to their wild-type controls. These observations suggest that the development of aCL antibodies in these autoimmune mice is dependent on cognate T/B-cell interaction (augmented by the presence of αβ T cells) and is facilitated by the binding of CD40 ligand on activated T cells to CD40 on B cells (Kang *et al.*, 1997).

Multiple genes are involved in autoimmune disease pathogenesis. The strongest argument for genes predisposing to disease is increased disease frequency in monozygotic twins. Disease concordance in this twin population, however, is generally less than 30%, and other factors such as random events occurring during the maturation of antigen receptors on T and B cells and environmental factors must also play a significant part. The detection of viral protein epitopes similar to that found in β2GPI may promote further insights into the mechanism of initiation of pathological events and clinical manifestations in APS (Celli *et al.*, 1997).

β2GPI is in the CCP family. The complement system and its receptors play an important role in immune defense, linking humoral and cell-mediated responses. Complement receptor (CR1), through its binding to activation products of complement C3, can activate cells and induce chemotaxis (Fearon, 1991). B lymphocytes have complement receptor

CR2, which may form complexes with CD19 that are important in B-cell activation (Matsumoto *et al.*, 1991). Complement receptors also serve to concentrate immune complexes on the surface of B cells, leading to antigen presentation by the B cell (Tuveson *et al.*, 1991). In deriving therapy for an autoimmune disease such as APS, the immune system needs to be modulated so as not to result in total immunosuppression. This modulation may be performed at the level of autoimmune T-cell generation in the thymus or their clonal expansion in the periphery. Current treatment appears to only address the end result of the autoimmune process, i.e., treatment of the thrombosis.

Synthetic mimotope peptides, characterized by (a) the inability to activate T cells while (b) retaining the ability to bind immune B cells, may be used to tolerize B cells in an antigen-specific manner. B-cell tolerance entails administering such peptides conjugated to multivalent, stable, nonimmunogenic valency platforms in order to abrogate antibody production via B-cell anergy or clonal deletion after the cross-linking of surface immunoglobulin. The ability to modulate B-cell activity in humans on an antigen-specific basis, using single signal inactivation of target B cells, has been identified as a means of pharmacological intervention in antibody-mediated pathologies (Coutts *et al.*, 1996). Although the exact molecular nature of the target epitopes recognized by aPL antibodies is unknown, the use of peptides derived from epitope libraries may allow for the construction of successful tolerogens.

XIII. Summary and Conclusions

Advances in defining the target antigen(s) for the autoantibodies in the APS highlight the inadequacies of the current classification of these autoantibodies into anticardiolipin and LA antibodies. The discovery that β2GPI is the target antigen for the autoantibodies detected in solid-phase immunoassays has opened a number of areas of research linking these autoantibodies to atherogenesis and thrombus formation. Although the role of β2GPI in the regulation of blood coagulation in unclear, current evidence suggests that anti-β2GPI antibodies interfere with its "normal" role and appear to promote a procoagulant tendency.

The expansion of research in this area and the diversity of the clinical manifestations of patients with APS have resulted in the inclusion of molecular biologists and pharmaceutical companies joining immunologists, hematologists, rheumatologists, obstetricians, neurologists, vascular surgeons, and protein and lipid biochemists in attempting to understand the pathophysiology of this condition. Although the published literature may result in conflicting results and introduce new controversies, developing standard-

TABLE II
VASCULAR THROMBOSIS

Presence of	Arterial Thrombosis	Venous Thrombosis
Antiphospholipid antibodies	Yes	Yes
Homocystinemia	Yes	
Protein deficiencies		
Protein C		Yes
Protein S		Yes
Antithrombin III		Yes
APC resistance		Yes
Genetic point mutations		Yes

ized laboratory methods and extrapolation of *in vitro* experimental results to the *in vivo* situation will advance our understanding of the regulation of the immune system and its interaction with normal hemostatic mechanisms. Since the authors' last review in 1991, the study and understanding of the pathophysiology of APS have evolved from lipid biochemistry to molecular techniques that may eventually provide specific therapies for the clinical manifestations of this condition. Although current treatment has improved the morbidity associated with this condition, especially in improving pregnancy outcomes, future therapies, as outlined in this review, may specifically address the biological abnormalities and have fewer side effects. Better diagnostic tools, such as magnetic resonance imaging with perfusion studies, will allow the study of the true incidence and prevalence of vascular flow changes/tissue ischemia and infarction associated with aPL antibodies and help determine treatment and prophylaxis for APS patients. APS is still the only hypercoagulable condition where both arterial and venous beds can be affected independently or in the same individual (Table II).

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