Kinetics of Prothrombin-Mediated Binding of Lupus Anticoagulant Antibodies to Phosphatidylserine-Containing Phospholipid Membranes: An Ellipsometric Study†

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ABSTRACT: Antiphospholipid antibodies interact with phospholipid membranes via lipid binding plasma proteins, mostly, prothrombin and β_2-glycoprotein I. Using ellipsometry, we characterized prothrombin-mediated binding of lupus anticoagulant (LA) positive IgG, isolated from patients with antiphospholipid syndrome, to phosphatidylserine (PS)-containing membranes. LA IgG did not bind to membranes in the absence of prothrombin, but addition of prothrombin resulted in high-affinity binding of prothrombin−LA IgG complexes; half-maximal binding was attained at IgG and prothrombin concentrations of 10 μg/mL and 4 nM, respectively. Adsorption to membranes containing 10−40 mol % PS revealed that membrane-bound rather than solution-phase prothrombin determines the adsorption kinetics. Depletion of prothrombin and LA IgG from the solution results in rapid desorption which is strongly inhibited by addition of prothrombin but not of LA IgG. Prothrombin-mediated adsorption of monovalent Fab1 fragments prepared from patient LA IgG was negligible, indicating that monovalent interaction between prothrombin and LA IgG is weak. The kinetics of adsorption and desorption indicate that divalent binding of LA IgG to prothrombin at the lipid membrane occurs.

Antiphospholipid syndrome is characterized by the presence of antiphospholipid autoantibodies associated with serious clinical conditions such as thrombosis, thrombocytopenia, and recurrent spontaneous abortion. In the past, it has been demonstrated that these antiphospholipid antibodies do not recognize the lipid membrane per se but that the binding to anionic lipid membranes of the antibodies is mediated by lipid binding plasma proteins, of which β_2-glycoprotein I (β_2GPI)[1] (*1*−*3*) and prothrombin (*4*) appear to be the most important representatives. The presence of these antibodies in plasma is commonly detected in lipid-dependent coagulation assays by their capacity to prolong the clotting time in vitro, termed the lupus anticoagulant effect (*5*−*8*). It should be noted, however, that in patients the presence of these antibodies is paradoxically associated with the occurrence of thrombosis.

Anti-prothrombin antibodies have been proposed by Loeliger (*7*) to cause both the LA effect and the prothrombin depletion observed occasionally in these patients. Moreover, this author suggested that these antibodies interfere with the conversion of prothrombin to thrombin via occupation of lipid binding sites by prothrombin−anti-prothrombin complexes. This competition of LA antibodies with coagulation factors for lipid binding sites was confirmed by others (*9*, *10*). High-affinity, non-neutralizing, anti-prothrombin antibodies were detected in the plasma of Lupus patients who had decreased prothrombin levels (*11*). In general, however, patients have normal prothrombin levels, and the anti-prothrombin antibodies appeared to be low-affinity because the anticoagulant was not depleted from plasma simultaneously with the prothrombin upon $BaSO_4$ adsorption of the patient's plasma (*7*, *8*). In addition, the patient's anti-prothrombin antibodies apparently do not bind to prothrombin adsorbed to plain PVC plates. Detection of anti-prothrombin antibodies in enzyme-linked immunosorbent assays (ELISAs) requires the use of high-activated or γ-irradiated plates for presenting the prothrombin, which presumably afford a higher density of the adsorbed antigen. Even more sensitive detection is afforded by prothrombin bound to anionic lipid coated on the plate (reviewed in ref *12*).

Similar observations for anti-β_2GPI antibodies (ACA) from APS patients have recently triggered the characterization of this remarkable binding behavior (*13*, *14*). These studies showed that patient antibodies have low affinity both for β_2GPI in solution and for β_2GPI attached to ELISA plates; high-affinity β_2GPI-mediated binding to ELISA plates or lipid membranes requires divalent attachment to closely juxtaposed β_2GPI molecules on these surfaces. This notion was corroborated by the finding that binding of Fab1 fragments of the patient IgG was negligible. In the latter study, we used ellipsometry to characterize the binding (kinetics) of β_2GPI and ACA IgG to PS-containing lipid

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[1] Abbreviations: ACA, anticardiolipin antibody; APS, antiphospholipid syndrome; β_2GPI, β_2-glycoprotein I; PT, human prothrombin; LA, lupus anticoagulant; PS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid.

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membranes. It appeared that the β_2GPI-mediated binding of ACA to PS-containing membranes was of high affinity (apparent $K_d < 1$ nM), despite the low affinity ($K_d \sim 4$ μM) of β_2GPI alone for these membranes. The importance of the divalent interaction has now been confirmed in several studies (*15*−*17*).

In earlier studies, the interaction of prothrombin−antiprothrombin complexes was assessed either indirectly through the effect of these complexes on lipid-dependent coagulation reactions (*4*, *18*−*20*) or by semiquantitative end point determinations carried out by ELISAs (*21*, *22*). In view of the above-mentioned similarities between patient anti-prothrombin and anti-β_2GPI antibodies, we hypothesized that similar mechanisms apply to the lipid binding of patient anti-prothrombin antibodies. This study was undertaken in an effort to provide more quantitative insight into the kinetics and the affinity of binding of patient anti-prothrombin antibodies to PS-containing membranes. More specifically, we wanted to verify the hypothesis suggested by the above-mentioned similarities between anti-prothrombin and anti-β_2GPI antibodies that a similar mechanism of high-affinity divalent binding applies to the interaction of patient anti-prothrombin antibodies with membrane-bound prothrombin. We used ellipsometry to quantitate the binding of proteins at PS-containing planar phospholipid membranes stacked at silicon slides and total, unfractionated, IgG isolated from LA patients as a source of patient antibodies.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin (BSA, essentially fatty acid free) was from Sigma (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Silicon slides were obtained from Aurel GmbH (Landsberg, Germany). Human prothrombin was purified according to the method of ref *23*.

Patients. This study included total IgG preparations isolated from the plasma of 16 patients. All patients had LA, diagnosed according to the criteria proposed by the Subcommittee of the International Society on Thrombosis and Haemostasis (*24*) and anticardiolipin IgG (ACA) above 15 GPL units (*25*). Eleven patients satisfied the criteria for definite antiphospholipid syndrome (*26*); six patients had only deep vein thrombosis, three only arterial thrombosis, and two both venous and arterial thrombosis. Two patients met the revised criteria of the American Rheumatism Association for the diagnosis of systemic lupus erythematosus (*27*).

Antibodies. Total IgG was isolated from patient plasma by affinity chromatography over protein A−Sepharose. Polyclonal antibodies raised in rabbits against human prothrombin were obtained from DAKO (Glostrup, Denmark). Fab1 fragments were produced using immobilized papain (Pierce, Rockford, IL) according to the manufacturer's instructions. Digestion, monitored by SDS−PAGE, was performed to completion. Monovalent Fab1 fragments were subsequently isolated by affinity chromatography using protein A−Sepharose.

Ellipsometric Measurement of Protein Adsorption to Lipid Bilayers. Planar phospholipid bilayers were deposited on silicon slides by adsorption of small unilamellar vesicles (prepared by sonication) to the hydrophilic silicon by immersion of the hydrophilic silicon slide for 5 min in a stirred suspension of small unilamellar lipid vesicles (30 μM) in Tris buffer [50 mM Tris, 120 mM NaCl, and 3 mM $CaCl_2$ (pH 7.5)] (*14*, *28*). Ellipsometry was used to quantitate protein adsorption to planar phospholipid bilayers as described previously (*14*, *29*). Experiments were performed at ambient temperature (20−22 °C) under continuous stirring in a trapezoidal cuvette with Tris buffer [50 mM Tris, 120 mM NaCl, and 0.5 mg/mL bovine serum albumin (pH 7.5)] containing 3 mM $CaCl_2$ unless indicated otherwise.

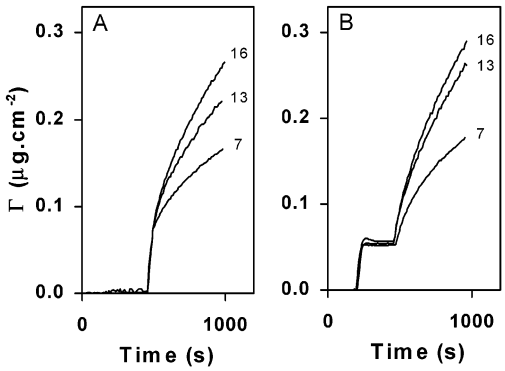

FIGURE 1: Prothrombin-mediated binding of LA patient IgG to PSPC bilayers containing 20 mol % PS. The mass, Γ (micrograms per square centimeter), of adsorbed protein, as measured by ellipsometry, is plotted as a function of time. Experiments were performed with IgGs from three patients (7, 13, and 16). (A) At 200 s, 100 μg/mL LA IgG was added to the cuvette and the adsorption was monitored by ellipsometry for 250 s. Then at 450 s, 25 nM prothrombin was added. (B) Reverse order of addition: first addition of 25 nM prothrombin to the cuvette at 200 s and the adsorption of prothrombin was monitored for 250 s. Then at 450 s, 100 μg/mL LA IgG was added. Experiments were performed at ambient temperature (20−22 °C) in Tris-HCl buffer [120 mM NaCl, 50 mM Tris, and 3 mM $CaCl_2$ (pH 7.5)] containing 0.5 mg/mL BSA.

RESULTS

Preliminary experiments showed that binding of human prothrombin to PSPC membranes containing 20 mol % PS could be reasonably well fitted by the Langmuir model with a dissociation constant K_d of 81 nM and a maximal binding Γ_{max} of 0.27 μg/cm^2. For membranes containing 40 mol % PS, a lower K_d of 11 nM is found with a virtually identical Γ_{max} value of 0.29 μg/cm^2 for maximal binding (data not shown).

Figure 1 shows the prothrombin-dependent binding of unfractionated total IgG of three patients to planar bilayers of PSPC containing 20 mol % PS; the total mass (micrograms per square centimeter) of adsorbed protein is plotted as function of time (seconds). Panel A shows that addition of patient IgG alone (100 μg/mL) does not produce measurable adsorption ($\Gamma < 0.003$ μg/cm^2); a significant adsorption is only observed after addition of prothrombin (25 nM). Panel B shows the reverse order of addition of proteins. It is apparent that the addition of 25 nM prothrombin in the absence of LA IgG results in a rapid adsorption with a Γ of 0.05−0.06 μg/cm^2, which is completed within 100 s. Subsequent addition of 100 μg/mL patient LA IgG initiates a gradual but steadily progressing adsorption of prothrombin−LA IgG complexes. Comparison of panels A and B reveals that the biphasic adsorption observed in panel A after addition of prothrombin represents in the initial phase (<100

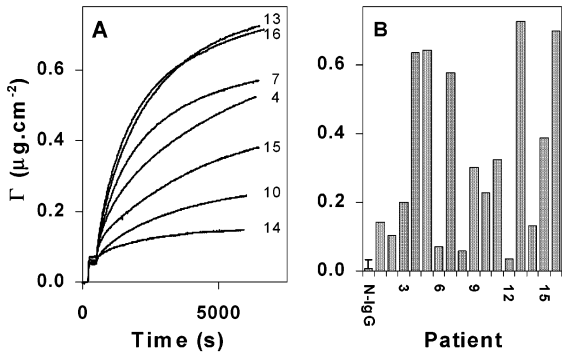

FIGURE 2: Binding of prothrombin−LA IgG complexes to PSPC and the patient-dependent response. Experiments as shown in Figure 1B were performed with LA IgG obtained from 16 patients and for IgG obtained from normal plasma (N-IgG). (A) A representative selection of adsorption experiments for seven patients is shown. At 200 s, 25 nM prothrombin was added to the cuvette, and after completion of the prothrombin adsorption, 100 μg/mL LA IgG was added at 500 s. (B) Additional adsorption caused by N-IgG and LA IgG, i.e., the final binding, estimated by a biexponential fit to the adsorption data shown in panel A diminished with the prothrombin adsorption, for each patient studied. The adsorption for N-IgG is the average of five experiments; the error bar represents three standard deviations.

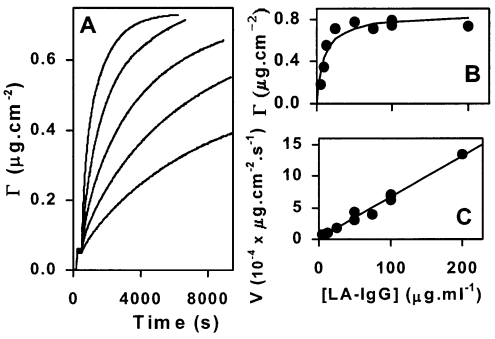

FIGURE 3: Dependence of the binding of LA IgG−prothrombin complexes on the LA IgG concentration. Experiments were performed at planar PSPC bilayers containing 20 mol % PS. (A) At 200 s, 25 nM prothrombin was added followed by 12.5, 25, 50, 100, or 200 μg/mL LA IgG (lower to upper curve) of patient 16 at 500 s, and the adsorption was monitored by ellipsometry until equilibrium was approached. (B) The final equilibrium adsorption from experiments as shown in panel A was estimated from a biexponential fit to the adsorption data and is plotted vs the concentration of LA IgG. The solid line represents the best fit of the formula $\Gamma = \Gamma_{max}C/(C + C_{0.5})$, with values for the maximal binding Γ_{max} and apparent dissociation constant $C_{0.5}$ of 0.85 μg/cm^2 and 9.8 μg/mL, respectively. (C) The initial adsorption rates, as estimated from linear regression to the adsorption measurements as shown in panel A between 30 and 130 s after addition of LA IgG, are plotted vs the LA IgG concentration. The solid line represents the least-squares fit to the line $V_{ads} = a + bC$, where C is the concentration of LA IgG. The estimated values of the intercept and slope are 18×10^{-6} μg cm^{-2} s^{-1} and 6.5×10^{-6} cm/s, respectively.

s after addition of prothrombin) mainly prothrombin adsorption, whereas the second, slower, phase represents the adsorption of LA IgG−prothrombin complexes. Although experiments were performed with total patient IgG, which may contain other "anti-phospholipid" antibodies in addition to anti-prothrombin, e.g., anti-β_2GPI, only anti-prothrombin antibodies contribute to the measured adsorption because no other lipid binding proteins such as β_2GPI are present. An obvious consequence of the use of total IgG preparations is the requirement of considerably higher IgG concentrations compared to those in the experiments performed with affinity-purified antibodies as reported previously (*14*, *30*, *31*). For reasons of clarity, only the initial phase of the adsorption is shown in Figure 1.

The complete adsorption profiles of the prothrombin-mediated adsorption of LA IgG are shown in Figure 2A for IgGs obtained from seven patients. Apparent is the slow evolution of the adsorption, which even after 1.5 h is not entirely completed. Therefore, the final additional adsorption caused by addition of patient IgG was estimated by a biexponential fit to the adsorption data measured for at least 90 min after addition of the IgG. Figure 2B shows these additional adsorptions caused by the prothrombin−LA IgG complexes for all (*16*) patients tested and for IgG isolated from the plasma of healthy subjects (N-IgG). N-IgG exhibited a negligible adsorption ($\Gamma = 0.007 \pm 0.007$ μg/cm^2; mean $\pm$ standard deviation). IgGs from all patients exhibit a response exceeding the mean value of N-IgG + three standard deviations, although the responses of different patients vary widely, presumably reflecting different titers of anti-prothrombin antibodies in the total IgGs. A common feature of all experiments is that the adsorption proceeds rather slowly; more than 1 h is required to reach equilibrium. It should, however, be emphasized that these experiments are performed at concentrations of IgG (100 μg/mL) and prothrombin (25 nM) that are 100- and 60-fold lower than the plasma concentration of IgG (10 mg/mL) and prothrombin (1.5 μM), respectively.

This raises the question of how equilibrium binding and adsorption kinetics of LA IgG−prothrombin complexes depend on prothrombin and LA IgG concentrations. Experiments were performed with total IgG from patients 7, 13, and 16. First, we studied the adsorption of prothrombin−LA IgG complexes to PSPC bilayers containing 20 mol % PS at a fixed prothrombin concentration of 25 nM and IgG concentrations varying from 12.5 to 200 μg/mL as shown in Figure 3A for LA IgG of patient 16. It is apparent that increasing the LA IgG concentration indeed results in an increase in the adsorption rate. Final binding, as estimated by biexponential extrapolation of these adsorption curves, is much less sensitive to the IgG concentration. Figure 3B shows that equilibrium binding is half-maximal at 9.8 μg/mL LA IgG, and maximal binding (Γ_{max}) is estimated to be 0.85 μg/cm^2. For patients 7 and 13, the values of $C_{0.5}$ were 2.9 and 9.5 at 9.8 μg/mL LA IgG, respectively, with Γ_{max} values of 0.70 and 0.86 μg/cm^2, respectively. Figure 3C shows that the initial rate of adsorption of prothrombin−LA IgG complexes increases linearly with increasing IgG concentration, presumably reflecting transport-limited transfer of the LA IgG from bulk solution to the macroscopic surface of the lipid-coated silicon slide, as described before for ACA binding (*14*). As described in that study on β_2GPI-mediated binding of ACA, this linearity enables the estimation of the concentration of anti-prothrombin in the total IgG preparation. Using a Δ value of 10^{-3} cm/s (*14*) for the mass transfer coefficient Δ of transport of IgG from bulk solution to the surface of the slide and the slope b of the adsorption rate as a function of the LA IgG concentration ($b = 6.5$, 6.6, and 4.9×10^{-6} cm/s for patients 16, 7, and 13, respectively), we estimate that less than 0.6% of total IgG is anti-prothrombin. Therefore, the $C_{0.5}$ values present an ~100-

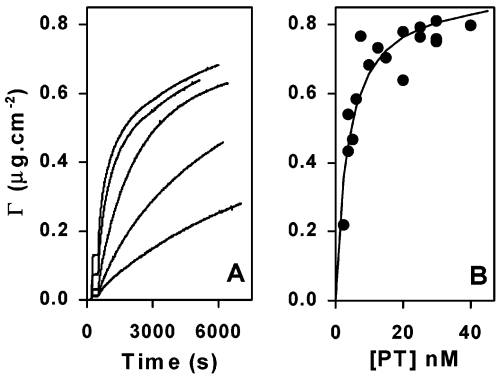

FIGURE 4: Dependence of the kinetics of adsorption of LA IgG−prothrombin complexes on the prothrombin concentration. Experiments were performed at planar PSPC bilayers containing 20 mol % PS. (A) At 200 s, prothrombin, 5, 7.5, 10, 20, or 40 nM (lower to upper curve), was added followed by 100 μg/mL LA IgG of patient 16 at 500 s, and the adsorption was monitored by ellipsometry until equilibrium was approached. (B) The final equilibrium adsorption from experiments as shown in panel A was estimated from a biexponential fit to the adsorption data and is plotted vs the prothrombin concentration. The solid line represents the best fit of the formula $\Gamma = \Gamma_{max}C/(C + C_{0.5})$, with values for the maximal binding Γ_{max} and apparent dissociation constant $C_{0.5}$ of 0.91 μg/cm^2 and 3.9 nM, respectively.

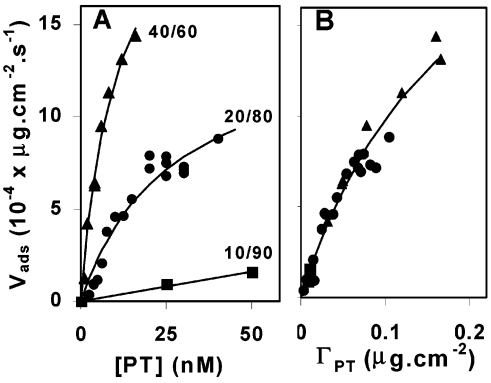

FIGURE 5: Initial rate of adsorption of LA IgG−prothrombin complexes depending on the prothrombin concentration, prothrombin surface coverage, and PS content of the lipid bilayers. Experiments as shown in Figure 4A were also performed at PSPC membranes containing 10 or 40 mol % PS for several prothrombin concentrations and 100 μg/mL LA IgG of patient 16. (A) The initial adsorption rate, estimated as in Figure 3C, is plotted as a function of the prothrombin concentration. The solid lines represent the best fits of the equation $V = V_{max}C/(C + C_{0.5})$, where V_{max} is the maximal adsorption rate at saturating prothrombin concentrations, C is the concentration of prothrombin, and $C_{0.5}$ is the concentration of prothrombin resulting in half-maximal adsorption velocity to the 20 and 40 mol % PS data sets. The estimated values of the parameters were as follows: $V_{max} = 1.5 \times 10^{-3}$ μg cm^{-2} s^{-1} and $C_{0.5} = 27$ nM prothrombin at 20% PS and $V_{max} = 2.4 \times 10^{-3}$ μg cm^{-2} s^{-1} and $C_{0.5} = 10$ nM prothrombin at 40% PS. (B) Replot of the data in panel A. The initial adsorption rate is now plotted vs the amount of prothrombin (Γ_{PT}) adsorbed to the lipid membrane prior to the addition of the LA IgG. The solid line represents the best fit of the equation $V = V_{max}\Gamma/(\Gamma + \Gamma_{0.5})$, where V_{max} is the maximal adsorption rate at saturating prothrombin concentrations, Γ is the surface concentration of prothrombin, and $\Gamma_{0.5}$ is the surface concentration of prothrombin resulting in half-maximal adsorption velocity to the combined data sets. The estimated values of the parameters were as follows: $V_{max} = 2.8 \times 10^{-3}$ μg cm^{-2} s^{-1} and $\Gamma_{0.5} = 0.18$ μg/cm^2.

fold overestimation of the apparent dissociation constant, and the apparent dissociation constant for binding of LA IgG to membrane-associated prothrombin is well below 0.1 μg/mL (0.6 nM).

Next we addressed the dependence of binding kinetics of anti-prothrombin−prothrombin complexes on the prothrombin concentration. Figure 4A shows the prothrombin-dependent adsorption to PSPC bilayers containing 20 mol % PS for several prothrombin concentrations ranging from 5 to 40 nM and a fixed IgG concentration of 100 μg/mL. Again final adsorptions are extrapolated by a biexponential fit. Figure 4B shows the equilibrium adsorptions as a function of the prothrombin concentration. A hyperbolic dependence is observed, and half-maximal adsorption is attained at a prothrombin concentration of 4 nM, i.e., 400-fold lower than the concentration of prothrombin in plasma. It is evident that the initial adsorption rates increase with increasing prothrombin concentrations, but the amount of prothrombin bound to the membrane in the absence of LA IgG also increases with the concentration of (free) prothrombin.

This raises the question of whether membrane-bound or free prothrombin regulates the adsorption rate. To find an answer, we performed similar experiments as shown in Figure 4A with PSPC membranes containing 10−40% PS as the amount of bound prothrombin at a fixed concentration of free prothrombin can be increased by increasing the PS content of the membrane. Figure 5A shows the initial adsorption rates as a function of the prothrombin concentration. It is apparent that for fixed prothrombin concentrations, an increase of the percentage of PS in the membrane produces a higher adsorption rate. Figure 5B presents a replot of the adsorption rate versus the density of bound prothrombin and shows that the adsorption rate is completely determined by the amount of bound prothrombin. This finding strongly indicates that LA IgG−prothrombin interaction in solution is weak, suggesting that the observed high-affinity binding is the result of divalent interaction with membrane-bound prothrombin.

To verify this notion, we studied the desorption kinetics of LA IgG−prothrombin complexes accumulated at the PSPC membrane by exposure to 25 nM prothrombin and 100 μg/mL LA IgG for ~30 min. Then, desorption of adsorbed LA IgG−prothrombin complexes is initiated by flushing the cuvette (4 mL) with fresh buffer (30 mL), causing a rapid desorption (Figure 6A). Strikingly, the rate of desorption is strongly inhibited by low concentrations of prothrombin, whereas addition of LA IgG does not influence the desorption rate. To determine the desorption rate as function of the prothrombin concentration, we performed experiments as shown in Figure 6A using LA IgG from two patients, 13 and 16, and several prothrombin concentrations, 0−10 nM, were added during the desorption. The desorption rate was estimated from the linear regression to the measured adsorbed mass between 100 and 200 s after addition of prothrombin, and Figure 6B shows a plot of the desorption rate as a function of the prothrombin concentration that was added during the desorption. The hyperbolic equation $V_{des} = V_o/(1 + [PT]/[PT]_{0.5})$ allows a good approximation as the fit to the data in Figure 6B shows. It is estimated that half-maximal desorption occurs at a prothrombin concentration of 1−2 nM. These results are highly suggestive for divalent binding of LA IgG, as it is inconceivable that the desorption of a monovalent prothrombin−anti-prothrombin complex, which is singly attached to the lipid membrane, can be

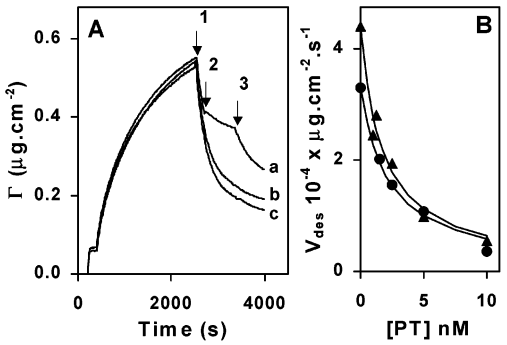

FIGURE 6: Prothrombin-dependent desorption of LA IgG−prothrombin complexes. (A) LA IgG−prothrombin complexes were adsorbed to PSPC membranes containing 20 mol % PS by addition of 25 nM LA IgG at 200 s followed at 400 s by addition of 100 μg/mL LA IgG (patient 16). At 2530 s, all proteins were depleted from the solution by flushing the cuvette (4 mL) with 30 mL of buffer (arrow 1), and the resulting desorption of LA IgG−prothrombin complexes was followed for 180 s by ellipsometry. Subsequently, either 5 nM prothrombin (curve a), 15 μL of buffer (curve b), or 100 μg/mL LA IgG (curve c) was added at 2710 s (arrow 2), and the desorption was followed for 660 s. Finally, proteins were depleted once more by flushing the cuvette at 3370 s (arrow 3). (B) Experiments as shown in panel A were repeated with LA IgG of patients 13 and 16 for several prothrombin concentrations, 0−10 nM, added during the desorption. The rate of prothrombin-dependent desorption was estimated by the slope of the best fitting line to the adsorbed mass as measured by ellipsometry every 10 s between 100 and 200 s after addition of prothrombin. Panel B shows a plot of this desorption rate as a function of the prothrombin concentration.

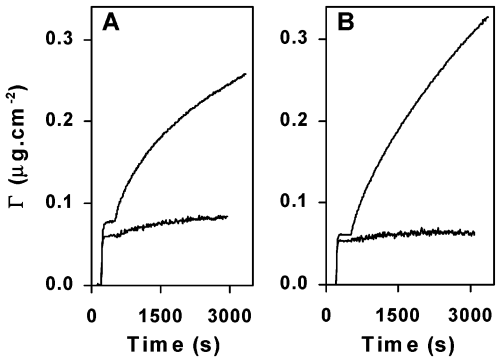

FIGURE 7: Adsorption of LA IgG−prothrombin complexes requires divalent antibodies. Fab1 fragments of the IgG of patients 5 and 16 were produced by papain digestion. Prothrombin complexes were bound to PSPC membranes containing 20 mol % PS. Prothrombin (25 nM) was added to the cuvette at 200 s followed at 500 s by 25 μg/mL LA IgG (upper curves), or the corresponding Fab1 fragment (25 μg/mL) (lower curves) was added and the ensuing adsorption measured: (A) patient 5 and (B) patient 16.

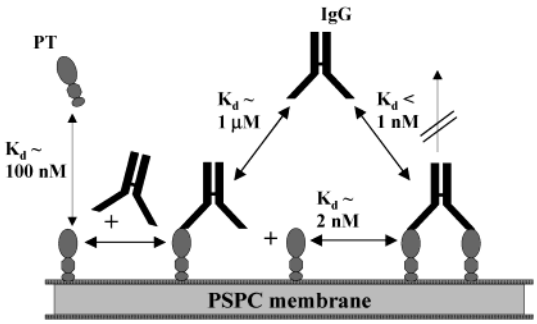

FIGURE 8: Proposed model for prothrombin-mediated binding of LA IgG to PS-containing phospholipid membranes.

affected by the presence of additional prothrombin at the membrane.

These results clearly suggest that LA anti-prothrombin antibodies, like anti-β_2GPI antibodies, bind to the antigen−lipid membrane complex via divalent interaction. This notion, that the high-affinity binding of LA IgG−prothrombin complexes reflects divalent interaction of the antibody with membrane-bound prothrombin, was tested by using monovalent Fab1 fragments produced by papain digestion of the LA IgG. In Figure 7, prothrombin-mediated binding of the monovalent Fab1 fragments is compared to that of intact antibodies. It is evident that adsorption of Fab1 fragments is negligible compared to the binding found for intact IgG.

DISCUSSION

Our data demonstrate the potency of patient anti-prothrombin antibodies, circulating in APS patients, to form high-affinity anti-prothrombin−prothrombin complexes at PS-containing phospholipid membranes. Half-maximal binding is attained at concentrations of (total) IgG and prothrombin that are 300−1000-fold lower than the concentrations of these proteins in plasma. Analysis of desorption kinetics revealed that the high affinity of the antibodies of these patients was due to formation of divalent (or multivalent) IgG−prothrombin complexes at the lipid membrane. The minimal binding found for monovalent Fab1 fragments corroborated this notion.

The high affinity of the prothrombin−LA IgG complexes for PS-containing phospholipid bilayers is apparent from Figures 3 and 4, which show a steep increase in the amount of binding with an increase in both the prothrombin concentration and the IgG concentration with half-maximal equilibrium binding attained at a concentration of 10 μg/mL patient IgG and 4 nM prothrombin, respectively. Thus, the binding of patient anti-prothrombin−prothrombin complexes is 20-fold higher in affinity than the binding of prothrombin alone, which suggests multivalent interaction with the lipid membrane. On the other hand, the enhanced affinity of monovalent anti-prothrombin−prothrombin complexes for lipid membranes could arise from an altered conformation of the prothrombin moiety interacting with the membrane. Our data cannot rule out a change in the conformation of prothrombin upon lipid binding, but the experiments with Fab1 fragments (Figure 8) indicate that such a putative conformational change does not cause the high affinity of the anti-prothrombin−prothrombin complexes for the lipid membrane. Alternatively, the high-affinity binding as observed in our experiments can be explained by multiple prothrombin−membrane interactions of multivalent anti-prothrombin antibody−prothrombin complexes. The high-affinity binding fits well with the model (*7*) in which anti-prothrombin−prothrombin complexes inhibit lipid-dependent reactions of blood coagulation as observed previously (*4*, *18*−*20*, *31*).

Our data extend earlier reports on prothrombin-mediated binding of LA antibodies to phospholipid membranes. Rao et al. (*21*), using [^{125}I]prothrombin to detect prothrombin bound at microtiter plate wells coated with PSPC (40:60), showed that LA enhances prothrombin binding to this PSPC coating, reducing the dissociation constant 3−4-fold from

~800 to 200−300 nM. Recently, we cooperated in a study on the lupus anticoagulant effect of affinity-purified anti-prothrombin antibodies (*31*). In this study, we showed enhanced binding of anti-prothrombin−prothrombin complexes to planar lipid bilayers. Enhanced binding was further shown in a recent, elaborate, study (*32*) by a variety of techniques. Remarkably, the data of this last study suggested a rather low affinity not only of prothrombin itself but also of anti-prothrombin−prothrombin complexes for lipid membranes: half-maximal binding requiring ~500 nM prothrombin and 30 μM (~4.5 mg/mL) LA IgG, a concentration which is 100-fold higher than that observed in the present study. Such a low affinity is difficult to reconcile with the inhibition of lipid-dependent coagulation reactions.

The desorption rate of the patient's anti-human prothrombin antibodies ($k_{off} = 0.7 \times 10^{-3}$ s^{-1}) is remarkably high in view of the high affinity of these complexes, but can be strongly reduced by the addition of prothrombin during the desorption. This indicates that anti-prothrombin−prothrombin complexes are mainly adsorbed as divalent IgG−(prothrombin)$_2$ complexes (Figure 8).

It should be noted that these desorption rates concern initial rates. It appears that in experiments as shown in Figure 6, a fraction of approximately 15−25% of the bound LA IgG−prothrombin complexes remains irreversibly attached to the lipid membrane, even after desorption for 2 h. During the desorption, the cuvette solution (4 mL) was continuously refreshed at a rate of 60 mL of buffer per hour to prevent accumulation of desorbed proteins. The adsorption period in Figure 6 was short, namely, 2100 s; for more prolonged adsorptions, 11 000 vs 2100 s, this nondesorbing fraction increases to 60% (data not shown). Slow formation of multivalent LA IgG−prothrombin complexes at the membrane would offer an obvious explanation for this observation.

Binding similar to that for patient LA IgG was found for anti-(human)prothrombin antibodies raised in rabbits, although half-maximal binding required 4-fold less prothrombin. In addition, desorption of the rabbit anti-prothrombin−prothrombin complexes was 30-fold slower than for patient antibodies, presumably reflecting a higher affinity of the monovalent anti-prothrombin−prothrombin interaction of the rabbit antibodies than of the patient's antibodies (data not shown).

In Figure 8, we propose a model for prothrombin-mediated binding of LA IgG, which is similar to the previously proposed model for β_2GPI-mediated binding of ACA (*14*). Prothrombin binds with a rather low affinity to the lipid membrane with a dissociation constant of ~100 nM. Association of IgG and prothrombin in solution is unimportant (cf. Figure 4), presumably reflecting a low affinity of LA IgG for free prothrombin. Also, monovalent binding to lipid-bound prothrombin is minimal, but the small fraction of lipid-bound prothrombin occupied by LA IgG rapidly interacts with a second membrane-bound prothrombin forming a trimolecular complex with the IgG doubly attached to the membrane via two prothrombin molecules. Because of the divalent lipid attachment of this complex, the rate of desorption is extremely slow. Only dissociation to the monovalently bound bimolecular IgG−prothrombin complex is feasible. This monovalent IgG−prothrombin complex can then rapidly dissociate at least in the absence of free prothrombin. The presence of prothrombin, however, results in a practically complete shift in the equilibrium toward the trimolecular complex, with an overall result that LA IgG binds with high affinity (dissociation constant ~ 1 nM) in the presence of prothrombin.

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