Insights into the Complex Association of Bovine Factor V_a with Acidic-Lipid-Containing Synthetic Membranes

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ABSTRACT The mechanism of binding of blood coagulation cofactor factor V_a to acidic-lipid-containing membranes has been addressed. Binding isotherms were generated at room temperature using the change in fluorescence anisotropy of pyrene-labeled bovine factor V_a to detect binding to sonicated membrane vesicles containing either bovine brain phosphatidylserine (PS) or 1,2-dioleoyl-3-*sn*-phosphatidylglycerol (DOPG) in combination with 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC). The composition of the membranes was varied from 0 to 40 mol % for PS/POPC and from 0 to 65 mol % for DOPG/POPC membranes. Fitting the data to a classical Langmuir adsorption model yielded estimates of the dissociation constant (K_d) and the stoichiometry of binding. The values of K_d defined in this way displayed a maximum at low acidic lipid content but were nearly constant at intermediate to high fractions of acidic lipid. Fitting the binding isotherms to a two-process binding model (nonspecific adsorption in addition to binding of acidic lipids to sites on the protein) suggested a significant acidic-lipid-independent binding affinity in addition to occupancy of three protein sites that bind PS in preference to DOPG. Both analyses indicated that interaction of factor V_a with an acidic-lipid-containing membrane is much more complex than those of factor X_a or prothrombin. Furthermore, a change in the conformation of bound pyrene-labeled factor V_a with surface concentration of acidic lipid was implied by variation of both the saturating fluorescence anisotropy and the binding parameters with the acidic lipid content of the membrane. Finally, the results cannot support the contention that binding occurs through nonspecific adsorption to a patch or domain of acidic lipids in the membrane. Factor V_a is suggested to associate with membranes by a complex process that includes both acidic-lipid-specific and acidic-lipid-independent sites and a protein structure change induced by occupancy of acidic-lipid-specific sites on the factor V_a molecule.

INTRODUCTION

Factor V is a vitamin-K-independent pro-cofactor of the coagulation cascade. It circulates in blood plasma as a single polypeptide chain and is activated by thrombin into a heterodimer (factor V_a), the two chains of which are derived from the amino and carboxy terminals of the parent molecule. The two chains are noncovalently associated (Esmon, 1979; Nesheim and Mann, 1979) and may be dissociated in the presence of divalent metal ion chelators, separated, and then reassociated to full activity by the addition of divalent metal ions (Esmon, 1979; Krishnaswamy et al., 1989). The light chain (mol wt = 74,000; Nesheim and Mann, 1979) but not the heavy chain (mol wt = 94,000) of factor V_a will bind to acidic phospholipid membranes in the presence or absence of calcium ions (Bloom et al., 1979; Higgins and Mann, 1983; van de Waart et al., 1983; Pusey and Nelsestuen, 1984). While this work was being prepared for publication, Rosing et al. (1993) reported that two forms of factor V_a were isolated from human plasma. These were reported to differ in the molecular weight of their light chains (74,000 and 71,000), in their functional properties, and dramatically in their binding to supported phospholipid bilayers. We have found similar heterogeneity in bovine factor V_a light chain. However, at the Ca^{2+} concentrations used in these studies, we have found these two forms of factor V_a to bind with quite similar affinities (K_d values within a factor of 2–3) to phosphatidylserine (PS)-containing small, unilamellar vesicles (SUVs) (Koppaka and Lentz, in preparation).

The assembled prothrombinase complex is composed of factor X_a (a serine protease) associated with factor V_a on a negatively charged membrane surface in the presence of calcium. Factor X_a cleaves its substrate, prothrombin, in two places to form another serine protease, thrombin. Thrombin, in addition to cleaving fibrinogen to fibrin, serves as a central control protease in blood coagulation. Although factor X_a alone may activate prothrombin in solution, the assembly of the prothrombinase complex on a membrane surface accelerates the production of thrombin by approximately five orders of magnitude (Nesheim et al., 1979; Rosing et al., 1980). Numerous studies have addressed the issue of how the interaction between the proteins and membrane of this complex might lead to this rate enhancement (e.g., Nesheim et al., 1981; Husten et al., 1987; Boscovic et al., 1990; Walker and Krishnaswamy, 1994). The heavy chain of factor V_a interacts with prothrombin, and this interaction may help direct the sequence of the two proteolytic cleavages required to activate prothrombin so as to

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optimize the rate of thrombin formation (Esmon et al., 1973; Guinto and Esmon, 1984).

The phospholipid membrane may be as important as the protein components of the prothrombinase complex in enhancing the activation of prothrombin. It was concluded in an early study that a negatively charged membrane surface was required for the production of a clot (Papahadjopoulos and Hanahan, 1964); however, only the charge and not the chemical nature of the lipid headgroup was assumed to be important. More recent studies of the activity of the prothrombinase complex assembled on membranes composed of different acidic phospholipids showed greater efficiency of thrombin generation with PS- and phosphatidic-acid-containing membranes than with phosphatidylglycerol-, phosphatidylinositol-, and phosphatidylethanolamine-containing membranes (Jones et al., 1985; Pei et al., 1993). In agreement with our report of PS specificity, even PS-containing membranes with a net positive charge have been shown to retain procoagulant activity (Rosing et al., 1988), whereas membranes containing D-PS do not support thrombin generation (Comfurius et al., 1994). Consistent with this picture, we have observed that prothrombin undergoes conformational changes when bound to PS-containing but not to phosphatidylglycerol-containing membranes (Lentz et al., 1991; Wu and Lentz, 1991) and have noted that these could explain some of the observed PS-specific rate enhancement (Pei et al., 1993). Clearly, an understanding of the specific interactions between different acidic lipid membranes and the component proteins of the prothrombinase complex is needed if we are to decipher the specific role of PS in the functioning of the prothrombinase complex.

In this paper, we report a systematic examination of the binding of pyrene-labeled factor (pyr-V_a) to model membranes composed either of PS/1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) or of 1,2-dioleoyl-3-*sn*-phosphatidylglycerol (DOPG)/POPC. We have analyzed the data both in terms of a traditional Langmuir adsorption model and in terms of a simple, two-process binding model to describe factor V_a interaction with membranes. The results suggest that factor V_a association with an acidic-lipid-containing membrane is a complex process involving a significant acidic-lipid-independent membrane interaction, occupancy of a very few acidic-lipid sites that favor PS over DOPG, and protein conformational changes associated with occupancy of these sites.

MATERIALS AND METHODS

Hepes was purchased from Fisher Scientific (Charlotte, NC). POPC, DOPG, and bovine brain PS were purchased from Avanti Polar Lipids (Birmingham, AL). The *N*-(1-pyrene)maleimide adsorbed to Celite (10%) was from Molecular Probes (Eugene, OR). Polyethyleneglycol, average molecular weight of 15,000, was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade or the best grade available.

Protein purification

Bovine factor V_a was immunopurified and labeled with pyrene maleimide as described previously (Krishnaswamy and Mann, 1988). Such preparations typically show a 55-kDa impurity (10–15%) on Coomassie-stained gels (Odegaard and Mann, 1987). Preparations of the pyr-V_a were evaluated by anisotropy measurements followed by competition binding measurements with unlabeled factor V_a or unlabeled factor V_a light chain (Krishnaswamy and Mann, 1988), by activity in a clotting assay (Nesheim et al., 1980), and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Wycoff et al., 1977) using the Bio-Rad (Richmond, CA) Minigel system. The modified protein was determined to be equivalent to unmodified factor V_a by these criteria. The proteins were stored at −70°C as ammonium sulfate precipitates or in 10 mM Tris, 10 mM borate, 1 mM $CaCl_2$, 50% glycerol buffer, pH 8.3. In preparation for use, the protein was resuspended and exchanged into assay buffer (20 mM Hepes, 150 mM NaCl, 4 mM $CaCl_2$, pH 7.4) in Centricon 10 microconcentrators (Amicon, Danvers, MA) preadsorbed with polyethylene glycol 15,000.

Protein concentration was determined by absorbance at 280 nm, corrected for solution scattering. The extinction coefficient ($E^{1\ mg/ml}_{280\ nm}$) used for factor V_a was 1.74; the molecular weight used was 150,000 (Krishnaswamy and Mann, 1988).

Membrane preparation

Small unilamellar vesicles (SUVs) were prepared by mixing the appropriate amounts of purified POPC and either PS or DOPG in chloroform/methanol solution, removing the solvent under a stream of argon, and dissolving the resulting residue in benzene or cyclohexane. The dissolved lipids were shell frozen and lyophilized overnight to yield a dry white powder. The dried lipids were suspended in buffer (20 mM Hepes, 150 mM NaCl, pH 7.4) and probe sonicated to clarity at 15°C in a Heat Systems Ultrasonics (Farmingdale, NY) model 350 Sonicator. After sonication, the suspension was centrifuged at 72,000 rpm for 25 min at 15°C in a Beckman TL100 centrifuge (using a TLA100.3 rotor) to obtain a homogeneous suspension of vesicles (Barenholz et al., 1977). The size of the SUVs was estimated by quasi-elastic light scattering (Lentz et al., 1992). All PS/POPC vesicle preparations contained particles with diameters in the range of 400–600 Å (mean, 540 Å), whereas DOPG/POPC vesicles were slightly smaller (350–550 Å; mean, 470 Å). Vesicle size distributions showed no clear trend with lipid composition. POPC concentration was determined by [^{14}C]dimyristoylphosphatidylcholine (Amersham, Arlington Heights, IL) incorporated into POPC stocks. Total phosphate concentration in a vesicle sample was determined using the method of Chen et al. (1956). Comparison of these two concentrations allowed determination of the actual phospholipid composition of the final vesicle sample. In all cases, phospholipid composition, expressed as the mole percent of acidic phospholipids present, was within 2–3 mol % of the target value, this being also the experimental uncertainty in the reported values.

Generation of fluorescence anisotropy binding isotherms

Anisotropy measurements were collected on SLM 8000 photon counting fluorescence spectrophotometers (Krishnaswamy and Mann, 1988). Flu-

orometers were used in the T format with Glan-Thompson polarizing filters in the excitation and two emission pathways (Lakowicz, 1983). Excitation slits were 4 nm, and the excitation wavelength was 330 nm. Long-pass filters (Shott KV389, Duryea, PA) were placed in the emission path to reduce the effects of Raman scatter, and a 6-nm bandwidth, 396-nm bandpass filter (Ealing Optical, South Natick, MA) was used in the measurement emission path. Alternatively, the emission monochromator was set at 396 nm with 8-nm emission slits. All buffers were filtered through 0.2-μm filters (Costar, Cambridge, MA); protein solutions were microfuged for 5 min at 4°C to reduce solution scatter. Fluorescence titrations were performed in a 1 × 1 cm cuvette (initial volume, 2 ml) by adding aliquots of SUVs in microliter volumes, with subsequent stirring for 1 min, to a fixed concentration of pyr-V_a. Protein and phospholipid concentrations were corrected continuously for dilution during the titration so as to accurately reflect actual concentrations in the cuvette. Titrations were performed at a minimum of two protein concentrations from 0.085 to 0.4 μM. Previous measurements (Krishnaswamy and Mann, 1988) employed a comparable concentration range (0.075 to 0.17 μM). For the sake of visual comparison in Fig. 4 of fits of the two-process binding model to experiments performed at different protein concentrations, data at different protein concentrations were normalized to 0.085 μM total pyr-V_a, according to

$$\frac{[P_{total}]_2}{[P_{total}]_1} = \frac{[PL_{total}]_2}{[PL_{total}]_1}$$

Readings (an average of 10 per measurement) were taken approximately 30 s after cessation of stirring by integration of the signal at each position of the excitation polarizer over 10 s. The resulting fluorescence anisotropy values increased saturably, and all isotherms were carried out whenever possible to lipid concentrations at least twofold beyond apparent saturation. Generation and analysis of duplicate isotherms on different instruments demonstrated no variation in the data. Corrections were made for fluorophore dilution but were not made for inner-filter effects because the absorbance of the final titrations was not greater than 0.1 at 280 nm.

Binding parameter analysis: Langmuir adsorption model

Individual isotherms were evaluated to assign binding parameters according to the following expression for the concentration of bound protein (Krishnaswamy et al., 1986; Koppaka and Lentz, 1996):

$$[P_{bound}] = \frac{(i[PL_{tot}]) + [P_{total}] + K_d - \sqrt{((i[PL_{tot}]) + [P_{total}] + K_d)^2 - (4i[P_{total}][PL_{tot}])}}{2} \quad (1)$$

where $1/i$ is the stoichiometry in moles of lipid monomer per mole of protein, $[PL_{tot}]$ is the total phospholipid concentration, $[P_{total}]$ is the total concentration of pyr-V_a, and K_d is the equilibrium dissociation constant. An assumption made in this expression is that the SUVs contain multiple, equivalent, non-interacting sites that may bind pyr-V_a. The concentration of bound pyr-V_a was assumed to be related to the observed fluorescence anisotropy of pyr-V_a by $[P_{bound}] = [P_{total}] \times (\Delta r_{obs}/\Delta r_{sat})$, where Δr_{obs} and Δr_{sat} are the directly observed anisotropy changes at any added membrane concentration and at saturation, respectively. For membranes of high acidic lipid content, Δr_{sat} was determined readily from the initial and saturating fluorescence anisotropy values. For membranes of low acidic lipid content (<15 mol % PS or 35 mol % DOPG), binding was weak, and values of limiting anisotropy could not be clearly defined experimentally. Therefore, unique values of K_d and i could not always be determined for low-acidic-lipid membranes (Koppaka and Lentz, 1996). For this reason, all three parameters were determined from fitting globally at least two data sets to the Langmuir model using Eq. 1 rearranged to express observed fluorescence anisotropy as a function of added lipid concentration (see Eq. 5 of Koppaka and Lentz, 1996). In this analysis, we assigned the dissociation constant K_d and stoichiometry of binding i as global variables and Δr_{sat} values as local variables (i.e., different and individually estimated for each data set). Global analysis was done using SCoP, the Simulation Control Program (Simulation Resources, Berrin Spring, MI). A multifit nonlinear least-squares regression analysis constructed using the algorithms supplied in the SCoP package was then used to vary K_d, i, and Δr_{sat} to fit to the observed data.

Application of a two-process model for protein binding

The binding isotherms were also fit to a model that assumes a simple, general, two-process model for protein-membrane interactions (see Figure A1 in Lentz and Hermans, 1989). No temporal sequence is presumed in this thermodynamic model. In one process, the protein is assumed to adsorb to the membrane surface through an acidic-lipid-independent interaction. No assumptions are made as to the mechanism of this interaction, although nonspecific electrostatic adsorption or partial hydrophobic penetration are possibilities. The other component of the total interaction involves the specific association of acidic lipids (or other special lipids) with equivalent, non-interacting sites on the protein. Filling of these sites is treated as governed by mass action, i.e., by the acidic lipid surface concentration. The acidic lipids are assumed to behave roughly as a two-dimensional ideal mixture with the neutral lipids, thus diffusing freely in the two-dimensional space of the membrane surface. This is clearly an approximation, as it ignores the finite size and structure of lipid polar head regions as well as possible clustering or nonideality of lipid mixtures. However, phase diagrams for mixtures of phosphatidylserine or phosphatidylglycerol with phosphatidylcholine indicate that these lipids mix reasonably ideally within the plane of SUV bilayers (Lentz et al., 1982; Tendian and Lentz, 1990). A recent detailed study of nonideal mixing in PS/phosphatidylcholine membranes confirms that demixing is minimal between species with similar acyl chains and at low (<40%) PS content (Huang et al., 1993). Beyond the potential nonideality of lipid mixtures, the model also ignores the finite size of the bound protein molecules and possible interactions between bound proteins. Alterations in the model to account for nonideal protein mixing in describing the membrane binding of factor V_a, prothrombin, or factor X_a added additional parameters but led to descriptions of the data that were statistically not improved over the simplest treatment (Cutsforth, 1991). In addition, the basic physical picture was unchanged from that provided by the simple model used here (Cutsforth, 1991). This simple form of the model has been used to describe successfully the membrane binding of synthetic charged peptides (Kim et al., 1991) and, with addition of Ca^{2+}-binding sites, the binding of annexin IV to PS-containing membranes (Junker and Creutz, 1994).

Within the context of this model, the observed binding constant for the binding of protein to a membrane surface, $K_{a,obs}$, may be written

$$K_{a,obs} = K_a^0(1 + kx_f)^m, \quad (2)$$

where K_a^0 is the nonspecific binding affinity (i.e., the binding affinity in the absence of specific lipid-protein interactions), k is the acidic-lipid-specific, site-binding constant for m equivalent sites on the protein, and x_f is the mole fraction of free acidic lipid in the membrane. One other parameter is required to describe membrane binding with this model, n, the maximal number of protein molecules per lipid molecule that can fit on a membrane surface.

Binding isotherms were simulated for fixed values of m, K_a^0, and k by taking the total protein concentration as an initial estimate of free protein concentration and the total acidic lipid mole fraction as an initial estimate of x_f. Then, using equations A13 and A14 of Lentz and Hermans (1989), the bound protein concentration and bound acidic lipid surface concentration were calculated and used to update the free protein concentration and x_f. These were then used to recalculate the free protein and x_f. This procedure was iteratively repeated until the change in x_f was <1 ppm

between iterations. Note that a typographical error exists in equation A13 of Lentz and Hermans (1989). The correct form is

$$[P_b] = \frac{K_{a,obs}[P_f][PL^0]}{3N/2n(1 + K_{a,obs}[P_f])}$$

Once the calculation had converged for the lowest phospholipid concentration in an isotherm, the binding equilibrium for the next higher phospholipid concentration was modeled starting with an estimate for free protein concentration equal to that obtained at the lower lipid concentration and with an estimate of the x_f equal to the total acidic lipid concentration in the membrane. This step-wise procedure was repeated until the entire isotherm had been modeled. The summed squares of residuals between calculated and observed bound protein concentrations was computed at the end of each isotherm. This procedure was repeated in a grid search over values of m, K_a^o, and k to obtain parameter values that gave the best fit of the model to experiment.

RESULTS

General description of the binding isotherms

Examples of binding isotherms generated as described under Materials and Methods are shown in Fig. 1. Panels *A* and *B* show isotherms for binding of pyr-V_a to PS-containing membranes, and panels *C* and *D* show isotherms for binding to DOPG-containing membranes. The data are presented as the observed fluorescence anisotropy of pyrene-labeled factor V_a as a function of added membrane concentration. Not surprisingly, binding was much weaker and more difficult to saturate for membranes of low as compared with high PS or DOPG content.

The total change in anisotropy observed at saturation (Δr_{sat}) was estimated directly from titration data. Values were consistent between duplicate or triplicate experiments at high acidic lipid content but were less precisely determined at low acidic lipid content. Within experimental uncertainty, values were independent of protein concentration and did not depend on the fluorescence spectrophotometer used to record the data. Values varied as a function of acidic lipid content, as shown in Fig. 2. The Δr_{sat} values decreased with increasing DOPG content to a minimum at 25 mol % DOPG and then increased slowly with increasing DOPG above this, approaching an asymptotic value of 0.04. For PS-containing membranes, Δr_{sat} increased steadily from 5 mol % PS (the lowest PS content examined) to the same asymptotic value of 0.04 at and above ~12 mol % PS. These data clearly support the contention that there is a qualitative difference between the mechanisms of association of pyr-V_a to membranes of high and low acidic lipid content, with the break in behavior occurring at roughly 12 mol % PS and 25 mol % DOPG. The very different values of Δr_{sat} observed for pyr-V_a bound to low-PS- and low-DOPG-containing membranes also clearly indicate a different behavior of pyr-V_a bound to these two types of membranes.

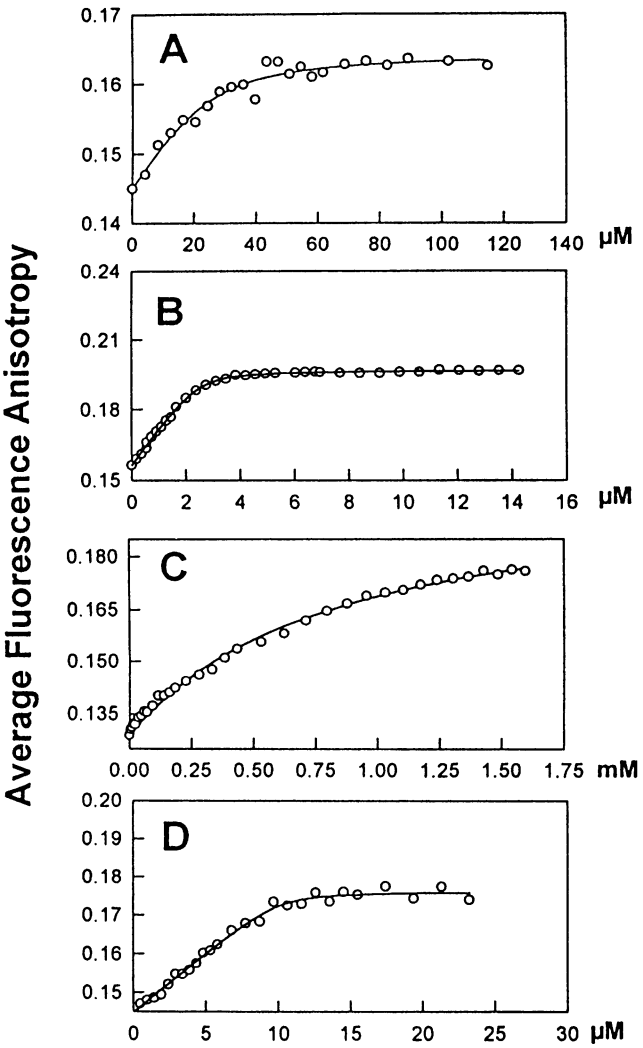

FIGURE 1 Variation in pyrene fluorescence anisotropy as a measure of binding of pyr-V_a to (*A*) 5 mol % PS/POPC, (*B*) 30 mol % PS/POPC, (*C*) 10 mol % DOPG/POPC, and (*D*) 50 mol % DOPG/POPC-containing membranes. The pyr-V_a fluorescence anisotropy values are plotted as a function of concentration of added PS- and DOPG-containing membranes. Lines represent binding isotherms predicted on the basis of the Langmuir model with parameters summarized in Table 1.

Description of the data in terms of a Langmuir adsorption model

Equilibrium dissociation constants (K_d) and stoichiometries ($1/i$ in units of lipids per bound protein) were determined as described in Materials and Methods by fitting our binding isotherms to a classical Langmuir adsorption model. To overcome the indeterminacy of the binding model when applied to weakly binding membranes (Koppaka and Lentz, 1996), we adjusted all three parameters (K_d, stoichiometry, and Δr_{sat}) while fitting the Langmuir adsorption model globally to at least two data sets obtained with membranes having the same acidic lipid content but at different protein concentrations. Stable parameter sets describing the data were obtained by this procedure and are summarized in

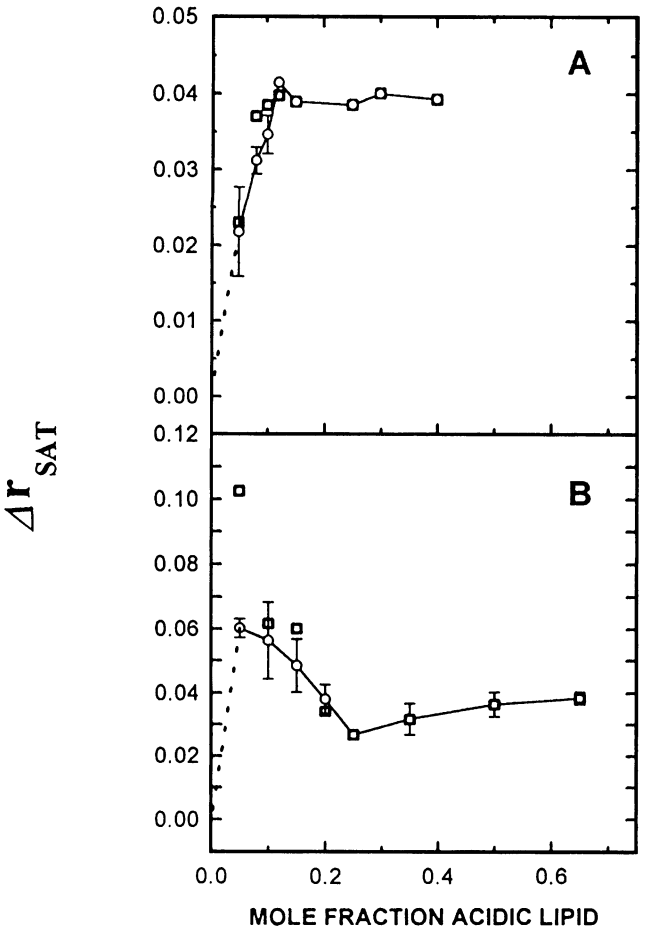

FIGURE 2 Saturating change in anisotropy of isotherms as a function of anionic lipid content. Initial anisotropy values for pyr-V_a in the absence of lipid were subtracted from the saturating anisotropy values in the presence of (*A*) PS-containing membranes or (*B*) DOPG-containing membranes. Values estimated directly from the titration curves (○) represent the average of two to four experiments; error bars indicate the range (minimum/maximum) of values observed. Dashed lines approach zero for POPC membranes, as we could detect no change in fluorescence anisotropy even for vesicle concentrations for which other measurements indicated that an interaction with these membranes does occur (Koppaka and Lentz, 1995). Values of Δr_{sat} obtained by globally fitting the data with K_d, i, and r_{sat} as local variables (□) are also given.

TABLE 1 Empirical parameters for binding of pyrene-V_a to PS- and DOPG-containing membranes

Membrane composition	K_d (nM)	$1/i$
5/95 PS/POPC	7.04 ± 1.0	283 ± 59
8/92 PS/POPC	143.4 ± 0.6	106 ± 6
10/90 PS/POPC	51.3 ± 9.2	42 ± 10
12/88 PS/POPC	9.1 ± 1.3	73 ± 26
15/85 PS/POPC	2.3 ± 0.3	72 ± 5
25/75 PS/POPC	2.2 ± 0.4	42 ± 5
30/70 PS/POPC	2.6 ± 0.2	35 ± 2
40/60 PS/POPC	6.6 ± 1.0	19 ± 3
5/95 DOPG/POPC	422 ± 60	1116 ± 770
10/90 DOPG/POPC	332 ± 19	1064 ± 560
15/85 DOPG/POPC	1281 ± 21	63 ± 2
20/80 DOPG/POPC	662 ± 2	22 ± 2
25/75 DOPG/POPC	41.2 ± 14	40 ± 10
35/65 DOPG/POPC	7.9 ± 2.1	61 ± 17
50/50 DOPG/POPC	2.9 ± 0.4	77 ± 18
65/35 DOPG/POPC	1.1 ± 0.4	92 ± 28

K_d and stoichiometry values (lipids per binding site) were obtained by adjusting both parameters so as to globally fit binding data (two or more sets in each case) to Eq. 1, with K_d and i being global variables and the values of Δr_{sat} (best fit values shown in Fig. 2) as local variables. Global fitting was accomplished using the ScOP simulation package (see Materials and Methods), from which was obtained estimates of the standard deviations in the best fit parameters.

Table 1 and then displayed as a function of acidic lipid content in Fig. 3. The lines drawn through the data shown in Fig. 1 were calculated using the Langmuir adsorption model and the best fit parameters given in Table 1. Limiting anisotropy changes (Δr_{sat}) estimated by these curve-fitting procedures are plotted versus membrane acidic lipid content in Fig. 2. These Δr_{sat} values agreed well with estimates made directly from titration curves except for membranes of low acidic lipid content for which Δr_{sat} values estimated experimentally were somewhat lower than those estimated by fitting. Only for two experiments (10 mol % PS and 5 mol % DOPG) was there a significant difference between these estimates of Δr_{sat}.

Although uncertainty still exists in these binding parameters (especially the stoichiometry) at low acidic lipid content, there are two important observations that are clear despite this uncertainty. The first and most remarkable is that the parameter values seem to display very different behaviors below and above 10–12 mol % PS and 15–25 mol % DOPG. K_d values did not decrease monotonically with acidic lipid content (Fig. 3 *A*). Rather, a maximum was observed at low acidic lipid for both PS and DOPG, with the K_d dropping dramatically and then changing very little at higher acidic lipid content. A similar dramatic change takes place in the stoichiometry parameter at the same acidic lipid contents. We noted above a similar discontinuity in the Δr_{sat} values for bound factor V_a at 12 mol % PS and 25 mol % DOPG. These observations suggest that the association of factor V_a with membranes may be somehow different above and below 10–12 mol % PS or 15–25 mol % DOPG.

A second important observation is that the variations of factor V_a binding parameters with membrane acidic lipid content are unlike the behavior of prothrombin and factor X/X_a binding parameters, for which K_d decreased monotonically with acidic lipid content (Cutsforth et al., 1989). This is the behavior to be expected when binding is dominated by association of a few acidic lipid molecules to a limited number of sites on the protein (Cutsforth et al., 1989). That this simple model may not apply to the binding of factor V_a is argued by the fact that the variation of K_d with acidic lipid content is so dramatically different from that seen with prothrombin or factor X_a.

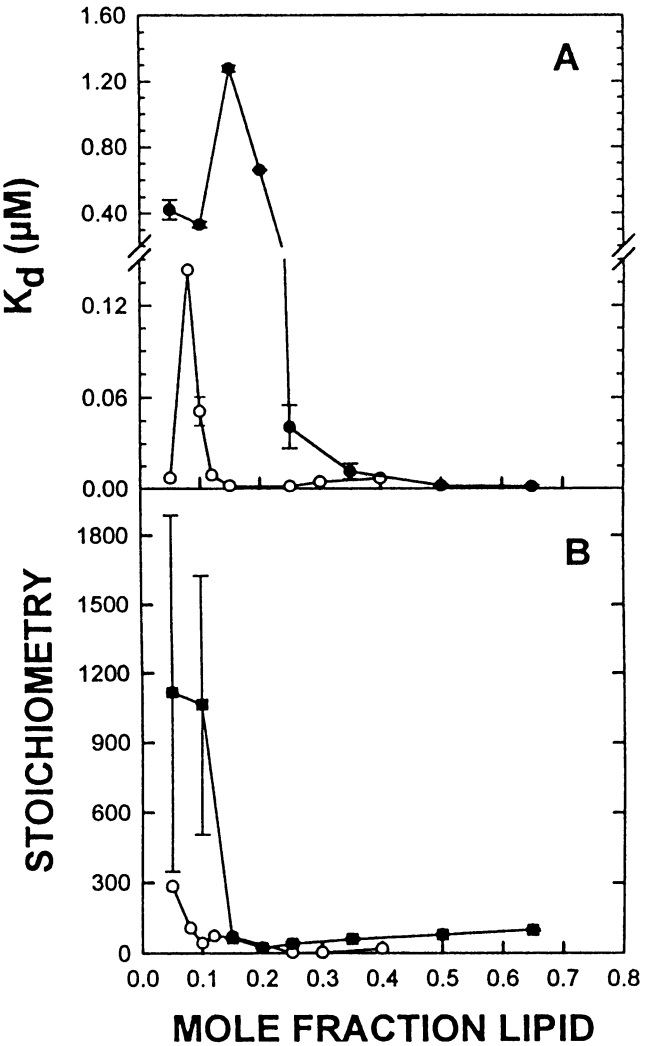

FIGURE 3 (*A*) Variation of empirical dissociation constants with acidic lipid content. Values for DOPG vesicles (●) or PS vesicles (○) were obtained by global fitting of at least two independent titrations performed at different total protein concentrations (see Materials and Methods). (*B*) Acidic lipid dependence of the apparent binding stoichiometry. Stoichiometry values (phospholipids per bound protein) for PS-containing (○) and DOPG-containing (●) membranes were determined as in *A*.

Application of a simple two-process model for extrinsic protein binding

To contrast more precisely the membrane binding of factor V_a with that of prothrombin and factor X/X_a, the simple two-process binding model described by Lentz and Hermans (1989) was applied to the binding of pyr-V_a. Application of this binding model involves the evaluation of four parameters: m, n, K_a^o, and k (see Materials and Methods for more detail). The maximal number of bound protein molecules per phospholipid molecule, n, was fixed based on the experimentally determined minimal or asymptotic number of phospholipids per bound factor V_a molecule (see Fig. 3 *B*). For PS-containing membranes, $n = 0.0526$ proteins per phospholipid was used; for DOPG-containing membranes, n was fixed at 0.018 proteins per phospholipid. It is worth commenting that the number of phospholipids needed to accommodate one molecule of factor V_a on PS-containing membranes (19–20; derived from data obtained with 40 mol % PS membranes; see Fig. 3 *B*) was surprisingly low, as other, lower molecular weight coagulation proteins appear to require much larger numbers of phospholipids per bound protein (40 for factor X/X_a or 50 for prothrombin; Cutsforth et al., 1989). We note that attempts to fit the data for binding to PS-containing membranes with smaller values of n (greater numbers of phospholipids per bound protein) generated measurably poorer fits. A recent attempt at defining the structure of membrane-bound factor V_a (Stoylova et al., 1994) estimates that the membrane-contacting region of factor V_a is quite small and covers only approximately 10 phospholipids in the outer leaflet of a PS-rich membrane, consistent with our observations and with the value of n we used.

Parameter values for m, K_a^o, and k were determined by a grid search to minimize the sum of squared residuals. Briefly, a range of values for each of the parameters was defined and a grid was designed to test all combinations of the parameters. The value for m was fixed and systematically varied from 1 to 20. For each integer value of m, the values for K_a and k were varied to determine the best fit parameter values. For both PS- and DOPG-containing membrane data sets, $m = 3$ provided the best fit to the data (lowest sum of squared residuals). Large values of m (>6–10) were not tenable. This small value of m argues against a lipid pool or lipid domain binding model, as discussed below.

With the value for m fixed at 3, K_a^o and k were varied to optimize globally the fit of all the calculated isotherms to all the experimental data (fits not shown). Although the isotherms could be fit adequately for either the PS membranes, the low DOPG membranes, or the high DOPG-containing membranes, it was not possible to obtain an adequate fit of all the data with a single set of parameters. Attempts to fit individual isotherms further supported the conclusion that it would be impossible to obtain a global fit of all the isotherms with the same set of parameters. Fits to PS membrane isotherms could be obtained with a narrow range of values for the nonspecific association constant ($K_a^o = 2200$ to 2500 M^{-1}), but for DOPG membranes very different values were needed (4500–5500 M^{-1} for 5, 15, and 20 mol % DOPG membranes and 3700 M^{-1} for >20 mol % DOPG membranes). In addition, the specific site affinity constants had to be increased with acidic lipid content to optimize the fits of calculated isotherms to individual experimental isotherms (see Table 2). Fig. 4 shows the fits of the simulated curves generated by the parameters given in Table 2 to semi-logarithmic plots of the data. The failure of the model, under near saturating conditions, for low-acidic-lipid-containing membranes seems to reflect uncertainty in the parameter Δr_{sat}, as discussed in reference to Fig. 2. As k and K_a^o are determined mainly by the rising portion of the

TABLE 2 Parameter values used to obtain a best fit of simulations to semilogarithmic isotherms

Lipid	m*	Stoichiometry‡	ka§	K_a^0 (M^{-1})*	s^2¶, χ^2‖
8% PS	3	19	26 (18–35)	2200 (800–5400)	1.8×10^{-16}, 0.72
10% PS	3	19	28 (17–46)	2200 (1800–3900)	3.2×10^{-16}, 1.26
>10% PS	3	19	40 (30–55)	2500 (1800–3900)	0.6×10^{-16}, 0.22
<20% DOPG	3	60	3 (0–6)	5000 (3000–7600)	1.6×10^{-16}, 0.6
20% DOPG	3	60	8 (4–10)	4500 (3200–6700)	6.2×10^{-17}, 0.23
>20% DOPG	3	60	16 (10–30)	3700 (2500–8100)	5.5×10^{-17}, 0.21

*Only integer values were considered. Small values of m (2–5) all gave comparable values of s^2 in a grid search for optimal parameters (see Materials and Methods); $m = 3$ gave slightly lower values of s^2.

‡Fixed according to minimal values of the stoichiometry obtained from fitting isotherms to a classical surface adsorption model (Table 1). Values given have units of phospholipids per bound protein, i.e., $1/n$.

§The uncertainty ranges of k and K_a^0 were defined by values that resulted in an increase in s^2 by a factor of two relative to the minimized s^2.

¶The variance of the fit (s^2) was calculated as the mean square deviation of the calculated from the observed concentration of bound protein divided by the number of degrees of freedom for the data set.

‖The reduced χ^2 of the fit is defined as $s^2/\langle\sigma^2\rangle$, where $\langle\sigma^2\rangle$ is the mean square standard deviation of all points in a data set. This was estimated from uncertainties in anisotropy measurements. The reduced χ^2 is a measure of goodness of fit; a value close to 1 theoretically indicates that the model provides a good description of the data (Bevington, 1969). A value less than 1 can mean that the errors associated with individual data points were overestimated.

binding isotherms, this uncertainty in Δr_{sat} should not affect our overall conclusions.

DISCUSSION

Difficulties in defining the mechanism of factor V_a binding to membranes

The mechanism by which factor V_a binds to acidic lipid membranes is a matter of controversy. The acidic lipid requirement for binding as well as the ionic strength dependence of the binding has led to the proposal that binding may occur via an electrostatic adsorption mechanism (Pusey et al., 1982; Pusey and Nelsestuen, 1984), with little if any penetration of the bilayer (Mayer et al., 1983). However, there is disagreement over this issue and even over whether binding is indeed ionic strength dependent (Bloom et al., 1979; Higgins and Mann, 1983). Thus, some have suggested that factor V_a binding is not electrostatic in nature but may involve hydrophobic penetration into the contacted monolayer (Lecompte et al., 1987, 1994; Kalafatis et al., 1990). Others suggest that both types of interaction, electrostatic and hydrophobic, are present (Pusey et al., 1982) but occur in different regions of the factor V_a light chain (Kalafatis et al., 1994) involved in factor V_a binding.

In addition to disagreement as to the binding mechanism, there is also controversy about the nature of the acidic-lipid-binding site for factor V_a. The condensation of the prothrombinase complex on an acidic lipid membrane has often been discussed in terms of existence of acidic-lipid-rich binding sites on the membrane surface. This acidic lipid domain model was originally proposed for cytochrome c binding to mixed cardiolipin/spin-labeled cholesterol membranes (Birrell and Griffith, 1976) and then applied to the binding of several extrinsic proteins, including prothrombin (Lim et al., 1977; Mayer and Nelsestuen, 1981) and factors X_a and V_a (Bloom et al., 1979; Mayer and Nelsestuen, 1981, 1983; Pusey et al., 1982). The domain or cluster model of extrinsic protein interaction has been questioned even for cytochrome c (Reitveld et al., 1986) and has also been challenged with regard to the prothrombinase components (Lentz et al., 1985; Jones and Lentz, 1986; van de Waart et al., 1987; Tendian and Lentz, 1990; Bazzi and Nelsestuen, 1991).

One reason for the controversies over binding mechanism has been the difficulty associated with measuring factor V_a binding to membranes. The problems in evaluating factor V_a binding have been, first, in obtaining an adequate protein preparation and, second, in identifying a detection system sensitive enough to report incomplete binding for such a tightly bound protein. The development and application of a pyrene-labeled, antibody-purified bovine factor V_a molecule by Krishnaswamy and Mann (1988) has provided a methodology of sufficient sensitivity to resolve the tight binding of an immunopurified factor V_a preparation. The fluorescent signal is sensitive to binding at submicromolar concentrations of pyr-V_a, and the anisotropy change is saturable upon association of the protein molecules with the membrane surface. It is worth noting that dissociation constants obtained using the labeled factor V_a agree with dissociation constants obtained as the ratio of off to on rate constants obtained using light-scattering techniques (Krishnaswamy et al., 1988).

Insights into the factor V_a binding mechanism

Our results with pyrene-labeled factor V_a extend the work of Krishnaswamy and Mann (1988) and provide five new insights into the binding of factor V_a to acidic-lipid-containing membranes: 1) behavior depends on membrane acidic lipid content, 2) behavior is different for PS- versus DOPG-containing membranes, 3) two types of interactions are involved, 4) a small number of acidic lipids are involved, and 5) binding appears to cause acidic-lipid-dependent conformational changes in factor V_a. First, the essential difference between the interaction of factor V_a with acidic-

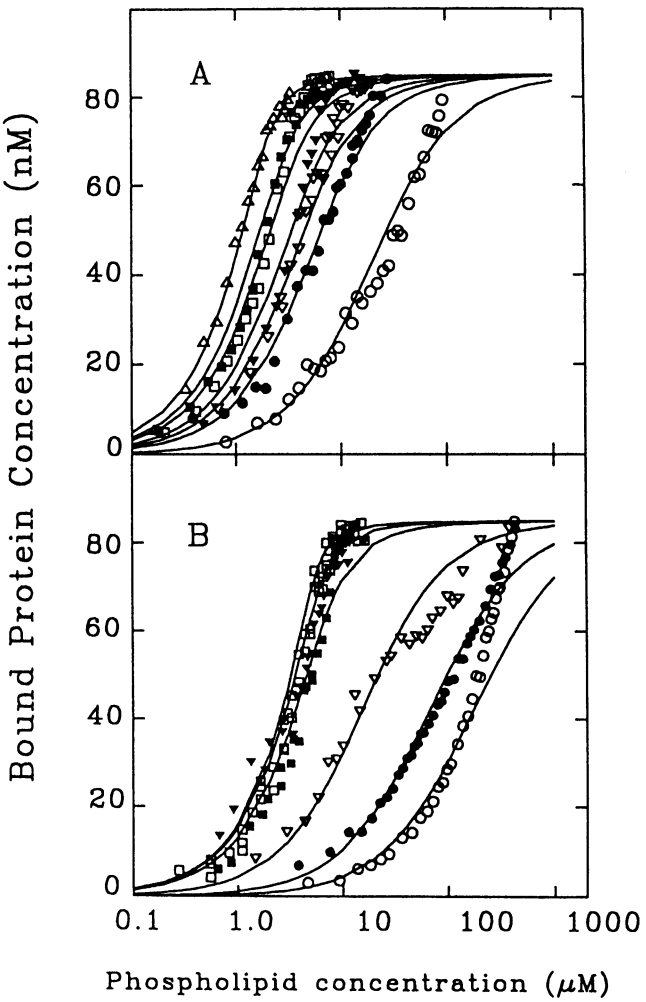

FIGURE 4 Semilogarithmic binding isotherms of pyr-V_a to (*A*) PS- and (*B*) DOPG-containing membranes. (*A*) ○, 8 mol % PS; ●, 10 mol % PS; ▽, 12 mol % PS; ▼, 15 mol % PS; □, 25 mol % PS; ■, 30 mol % PS; △, 40 mol % PS. Data were also obtained for 5 mol % PS membranes but are not shown due to their similarity to the 8 mol % PS data. (*B*) ○, 5 mol % DOPG; ●, 15 mol % DOPG; ▽, 20 mol % DOPG; ▼, 35 mol % DOPG; □, 50 mol % DOPG; ■, 65 mol % DOPG. The ability of the two-process binding model to describe the binding isotherms is demonstrated by the simulated isotherms (lines) generated using the values summarized in Table 2.

lipid-rich versus acidic-lipid-poor membranes is evident from at least three features of our results. As indicated by Fig. 3, very different empirical binding parameters were obtained in these two regimes, with a dramatic drop and then little variation with increasing acidic lipid content. This contrasts with the monotonic decrease of K_d from low to high acidic lipid content for prothrombin and factor X_a binding (Cutsforth et al., 1989). Similarly, the parameters providing description by the two-site binding model also differed according to acidic lipid content (Table 2). Finally, the observed values of Δr_{sat} were very different for binding to membranes of low and high acidic lipid content (Fig. 2). Not only must the binding be described differently but the nature of the bound protein also appears to be different for membranes that are either poor or rich in acidic lipids. This dependence on acidic lipid content correlates with the difference in k_{cat} for prothrombin activation by prothrombinase assembled on PS- or DOPG-rich versus PS- or DOPG-poor membranes (Pei et al., 1993).

Second, it is evident from our results that binding of factor V_a was qualitatively different for the two species of acidic lipids employed in these studies. We have chosen to contrast binding to membranes containing PS and DOPG, as these two acidic lipids will produce membranes with very similar surface potentials (Winiski et al., 1986) that nonetheless support prothrombinase complexes with very different activities (Pei et al., 1993). The apparent dissociation constants for binding to PS-containing membranes were between 3.5- and 78-fold lower than for binding to membranes containing comparable surface concentrations of DOPG. The difference was greatest for 15 mol % and decreased at high and low acidic lipid surface concentrations. A paper that appeared since this work was completed reports that the binding of factor V_a is 25-fold tighter to PS-containing membranes prepared from the natural L-stereoisomer rather than the D-stereoisomer (Comfurius et al., 1994). It is unclear at this point whether this specificity in factor V_a binding can account for the PS specificity we have recorded for activation of prothrombin by the assembled prothrombinase (Pei et al., 1993) as both factor X_a binding (Cutsforth et al., 1989) and prothrombin structural changes also show PS specificity (Lentz et al., 1991; Wu and Lentz, 1991).

A third insight offered by analysis of our data in terms of the two-process binding model is that two types of interactions seem necessary to describe the membrane binding of factor V_a. In contrast to prothrombin and factor X/X_a binding, for which the acidic-lipid-independent surface adsorption constant (K_a^o) was insignificant (1 M^{-1}; Cutsforth et al., 1989), the values of this constant needed to describe factor V_a binding are substantial (Table 2). This prediction of a significant acidic-lipid-independent interaction is supported by our observation by four different measurements of factor V_a binding to neutral membranes (Koppaka and Lentz, 1996). Binding to neutral membranes appears to occur with a K_d of roughly 3.0 μM and stoichiometry of approximately 200 lipids per site (Koppaka and Lentz, 1996). This interaction is stronger than the acidic-lipid-independent interaction observed with PS- or DOPG-containing membranes (K_a^o in Table 2). This means that the acidic-lipid-independent interaction predicted by our two-process binding model is not truly independent of acidic lipid content but is stronger for neutral than for charged membranes. In fact, K_a^o decreased as acidic lipid content increased, a situation illustrated most dramatically by the binding to DOPG-containing membranes (Table 2). This linkage between k and K_a^o violates one of the assumptions made in our two-process binding model as applied to factor V_a binding and therefore forces us to conclude that the membrane-binding mechanism of factor V_a may be more complex than the simple, two-process, independent-site, binding mechanism that is

capable of describing the membrane binding of prothrombin and factor X/X_a.

Our data do not address the nature of the acidic-lipid-independent interaction. It could be hydrophobic or electrostatic in nature. In either case, the strength of the interaction appears to depend on the chemical nature of acidic lipids in the membrane (Table 2). As membranes containing comparable surface concentrations of PS and DOPG have comparable surface potentials (Winiski et al., 1986), the observed species specificity of K_a^o means that acidic-lipid-independent binding is not likely to occur by adsorption to a diffusely charged surface. Also, if K_a^o were due solely to electrostatic attraction, it should increase with acidic lipid content rather than decrease or remain constant as observed (Table 2). At this point, we speculate, on the basis of other reports (Kalafatis et al., 1990, 1994; Lecompte et al., 1987, 1994), that the acidic-lipid-independent interaction is hydrophobic in nature.

The fourth insight offered by our results is that factor V_a binding is not likely to occur by a nonspecific electrostatic adsorption to a pool or domain of acidic lipids. This is based on the observation of acidic lipid specificity, as discussed above, and on the value of m required to fit the data. For both types of membranes examined, the assumption of the existence of three such sites ($m = 3$) provided the best fit to our data. The assumption of an acidic lipid cluster ($m = 10$ or greater) as a binding site could not provide a fit to our data because it results in a more dramatic decrease in K_d with acidic lipid content than observed.

The fifth and most intriguing insight offered by our results is that membrane binding of factor V_a appears to alter the protein in a significant way, perhaps via protein conformational changes induced by binding of acidic lipids to specific sites on the protein. This derives primarily from the variation of the saturating anisotropy change, Δr_{sat}, with acidic lipid content (see Fig. 2).

There are three effects that can account for the observed shift in the value of Δr_{sat}. First, there should be an increase in anisotropy due to decreased rotational freedom of the whole factor V_a molecule when bound to a membrane. This effect is evidenced by a changed slope of a Perrin plot (Lakowicz, 1983). Second, there may be a change in the intrinsic anisotropy (r_0) of the pyrene probe bound to the free protein versus bound to the membrane-bound protein. This would have to arise from a change in the electronic distribution within pyrene in these two states and so this effect should be insignificant. The third effect reflected in Δr_{sat} will be a change in the local or segmental motion of the probe attached to the protein. This effect is revealed in the limiting or apparent r_0 ($r_{0,app}$) obtained from measurements extrapolated to infinite viscosity by means of a Perrin plot (Lakowicz, 1983). This contribution would reflect a change in the local (i.e., in the region of the probe) dynamics of the protein in the membrane-bound versus free state. As Perrin plots for free and membrane-bound (25% PS) pyr-V_a were nearly parallel (Krishnaswamy and Mann, 1988), this clearly indicates that the major influence of membrane binding is to effect a substantial change in $r_{0,app}$. This is verified by comparing Δr_{sat} (0.038) to $\Delta r_{0,app}$ (0.034) obtained from the Perrin plot (Krishnaswamy and Mann, 1988). We conclude that the variation in Δr_{sat} values with PS or DOPG membrane content probably reflects a change in the local dynamics of pyr-V_a bound to membranes rich in these lipids as compared with membranes with low acidic lipid content.

The Δr_{sat} values, to a first approximation, follow two behaviors. For membranes of high acidic lipid content, Δr_{sat} was reasonably invariant or approached a limiting value of roughly 0.04. For membranes of lower acidic lipid content, however, Δr_{sat} varied substantially with changes in acidic lipid content. This indicates that the conformational state of pyr-V_a was quite different when bound to acidic-lipid-poor membranes as compared with the conformational state on acidic-lipid-rich membranes. This shift in conformation could reflect the effect of surface charge or could result from binding of acidic lipids to specific acidic lipid sites on the protein. Given the very different effects of PS and DOPG membranes, which have comparable surface potentials (Winiski et al., 1986), we prefer the latter interpretation. This argues in favor of changes in protein dynamics or conformation being triggered by occupancy of acidic-lipid-specific sites on the protein. An acidic-lipid-induced protein conformational change would be consistent with the observation that the acidic-lipid site binding constants (k values) and acidic-lipid-independent binding constants (K_a^o) derived from the two-process model varied with acidic lipid species and acidic lipid membrane content (Table 2). We suggest that occupancy of acidic-lipid-binding sites alters the configuration of the protein such that both the acidic lipid sites and the acidic-lipid-independent interaction are altered.

A model for factor V_a-membrane association

Although our data and analysis do not provide sufficient information to define the mechanism of factor V_a binding, they do suggest some insights that can be summarized in terms of a model that is at least consistent with available data (see Fig. 5). We propose that factor V_a interacts with PS- or DOPG-containing membranes by both acidic-lipid-dependent and acidic-lipid-independent mechanisms. Our current bias is that the acidic-lipid-independent binding is hydrophobic in nature. The existence of two types of interactions presumably involving at least two sites on factor V_a is consistent with the fact that a membrane-associated photoactivatable reagent labels two regions of factor V_a light chain (Kalafatis et al., 1994). We propose further that a small number of acidic-lipid-specific sites on factor V_a can account for the acidic-lipid-dependent interaction. Occupancy of these sites seems also to induce a substantial change in the conformation or molecular dynamics of membrane-bound factor V_a, which, in turn, alters the affinity of these sites and the strength of the acidic-lipid-independent interaction. The cooperative rearrangement of protein and

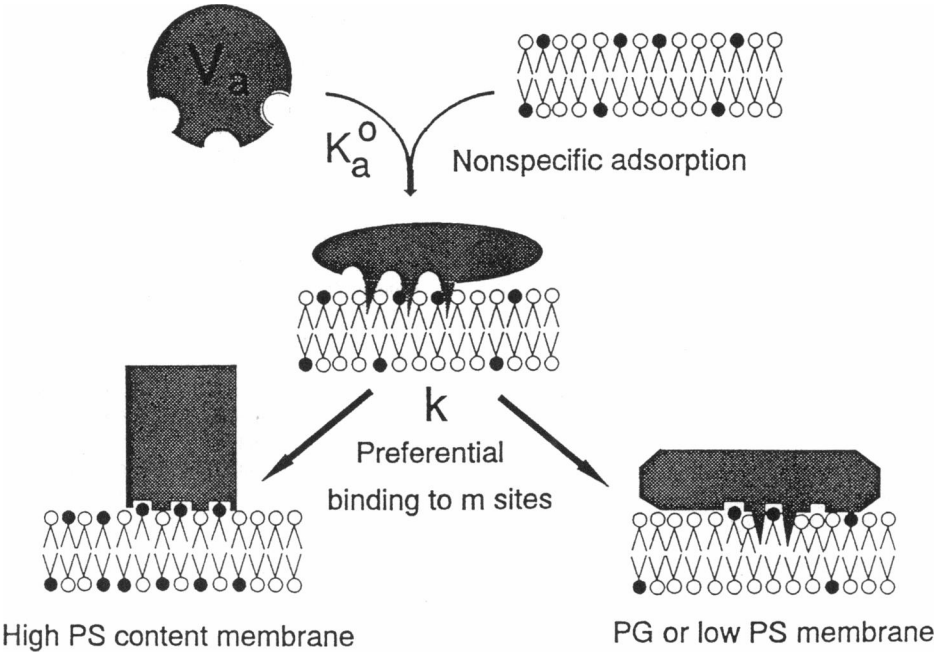

FIGURE 5 A proposed model for the binding of factor V_a to a membrane surface. This model summarizes the features of factor V_a binding that have been revealed by the analysis presented here as follows: 1) binding behavior depends on membrane acidic lipid content, 2) behavior is different for PS- versus DOPG-containing membranes, 3) two types of factor V_a-membrane interactions are involved, 4) a small number of acidic lipids are involved, and 5) binding appears to cause acidic-lipid-dependent conformational changes in factor V_a. The first type of interaction is an acidic-lipid-independent adsorption, which for factor V_a is significant and is represented by hydrophobic penetration, although our data cannot demonstrate this mechanism. The second type of interaction involves binding of negatively charged lipid molecules in the membrane to a few discrete sites on the protein. There is no significance to the order in which these steps are represented in the drawing. Acidic-lipid-induced changes in the dynamics or conformation of factor V_a implied by our results are illustrated as changes in the shape of the molecule.

lipid implied by this picture suggests that factor V_a binding might better be viewed as formation of a protein-lipid complex rather than as a simple surface binding phenomenon.

This model implies that factor V_a exists in different bound forms depending on the occupancy of its acidic-lipid-binding sites. On DOPG-containing or PS-poor membranes, factor V_a would be expected to exist in a form that covers a large number of phospholipids, whereas, on PS-rich membranes, it might exist in a form that extends from the membrane surface and covers only a small number of phospholipids. Such a picture would be consistent with apparently conflicting reports offering evidence in favor of both types of structures (Pusey et al., 1982; Lampe et al., 1984; Isaacs et al., 1986; Stoylova et al., 1994). Such a conformational equilibrium might also be influenced by interaction with other components of the prothrombinase complex, an interesting possibility that will have to be tested independently as more evidence is sought to test this picture.

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REFERENCES

Barenholz, Y., D. Gibbs, B. J. Litman, J. Goll, T. E. Thompson, and F. D. Carlson. 1977. A simple method for the preparation of homogeneous phospholipid. *Biochemistry.* 16:2806–2810.

Bazzi, M. D., and G. L. Nelsestuen. 1991. Extensive segregation of acidic phospholipids in membranes induced by protein kinase C and related proteins. *Biochemistry.* 30:7961–7969.

Boscovic, D. S., A. L. Giles, and M. E. Nesheim. 1990. Studies of the role of factor V_a in the factor X_a-catalyzed activation of prothrombin, fragment 1.2-prethrombin-2, and dansyl-L-glutamyl-glycyl-L-arginine-meizothrombin in the absence of phospholipid. *J. Biol. Chem.* 265: 10497–10505.

Bevington, P. R. 1969. Data Reduction and Error Analysis for the Physical Sciences. McGraw-Hill, New York. Chap. 10.

Birrell, G. B., and O. H. Griffith. 1976. Cytochrome c induces phase separation in diphosphatidylglycerol-phosphatidylcholine suspensions. *Biochemistry.* 15:2925–2929.

Bloom, J. W., M. E. Nesheim, and K. G. Mann. 1979. phospholipid binding properties of bovine factor V and factor V_a. *Biochemistry.* 18: 4419–4425.

Chen, P. S., T. Y. Toribara and H. Warner. 1956. Microdetermination of phosphorous. *Anal. Chem.* 28:1756–1758.

Comfurius, P., E. F. Smeets, G. M. Willems, E. M. Bevers, and R. F. A. Zwaal. 1994. Assembly of the prothrombinase complex on lipid vesicles depends on the stereochemical configuration of the polar headgroup of phosphatidylserine. *Biochemistry.* 33:10319–10324.

Cutsforth, G. A., R. N. Whitaker, J. Hermans, and B. R. Lentz. 1989. A new model to describe extrinsic protein binding to phospholipid membranes of varying composition: application to human prothrombin and factor X. *Biochemistry.* 28:7453–7461.

Cutsforth, G. A. 1991. A binding model for the proteins of the prothrombinase complex. Ph.D. thesis. University of North Carolina at Chapel Hill, Chapel Hill, NC. 122 pp.

Esmon, C. T. 1979. The subunit structure of thrombin-activated factor V_a. *J. Biol. Chem.* 254:964–973.

Esmon, C. T., W. G. Owen, D. L. Duiguid, and C. M. Jackson. 1973. The action of thrombin on blood clotting factor V: conversion of factor V to a prothrombin-binding protein. *Biochim. Biophys. Acta.* 310:289–294.

Guinto, E. R., and C. T. Esmon. 1984. Loss of prothrombin and factor X_a-factor V_a interactions upon inactivation of factor V_a by activated protein C. *J. Biol. Chem.* 259:13986–13992.

Higgins, D. L., and K.. Mann. 1983. The interaction of bovine factor V and factor V-derived peptides with phospholipid vesicles. *J. Biol. Chem.* 258:6503–6508.

Huang, J., Swanson, J. E., Dibble, A. R. G., Hinderliter, A. K., and G. W. Feigenson. 1993. Nonideal mixing of phosphatidylserine and phosphatidylcholine in the fluid lamellar phase. *Biophys. J.* 64, 413–425.

Husten, E. J., C. T. Esmon, and A. E. Johnson. 1987. The active site of blood coagulation factor X_a: its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex. *J. Biol. Chem.* 262:12953–12961.

Isaacs, B. S., E. J. Husten, C. T. Esmon, and A. E. Johnson. 1986. A domain of membrane-bound coagulation factor V_a is located far from the phospholipid surface: a fluorescence energy transfer measurement. *Biochemistry.* 25:4959–4969.

Jones, M. E., B. R. Lentz, and F. A. Dombrose. 1985. Comparison of the abilities of synthetic and platelet-derived membranes to enhance thrombin formation. *Thromb. Res.* 39:711–724.

Jones, M. E., and B. R. Lentz. 1986. Phospholipid lateral organization in synthetic membranes as monitored by pyrene-labeled phospholipids: effects of temperature and prothrombin fragment 1 binding. *Biochemistry.* 25:567–574.

Junker, M., and C. E. Creutz. 1994. Calcium-dependent binding of endoexin (annexin IV) to membranes: analysis of the effects of lipid composition and development of a predictive model for the binding interaction. *Biochemistry.* 33:8930–8940.

Kalafatis, M., R. J. Jenny, and K. G. Mann. 1990. Identification and characterization of a phospholipid-binding site on bovine factor V_a. *J. Biol. Chem.* 265:21580–21589.

Kalafatis, M., M. D. Rand, and K. G. Mann. 1994. Factor V_a-membrane interaction is mediated by two regions located on the light chain of the cofactor. *Biochemistry.* 33:486–493.

Kim, J., M. Mosier, L. A. Chung, H. Wu, and S. McLaughlin. 1991. Binding of peptides with basic residues to membranes containing acidic phospholipids. *Biophys. J.* 60:135–148.

Koppaka, V., and B. R. Lentz. 1996. Binding of bovine factor V_a to phosphatidylcholine membranes. *Biophys. J.* 70.

Krieg, U. C., B. S. Isaacs, S. S. Yemul, C. T. Esmon, H. Bayley, and A. E. Johnson. 1987. Interaction of blood coagulation factor V_a with phospholipid vesicles examined by using lipophilic photoreagents. *Biochemistry.* 26:103–109.

Krishnaswamy, S., K. C. Jones, and K. G. Mann. 1988. Prothrombin complex assembly: kinetic mechanism of enzyme assembly on phospholipid vesicles. *J. Biol. Chem.* 263:3823–3834.

Krishnaswamy, S., and K. G. Mann. 1988. The binding of factor V_a to phospholipid vesicles. *J. Biol. Chem.* 263:5714–5723.

Krishnaswamy, S., G. D. Russell, and K. G. Mann. 1989. The reassociation of factor V_a from its isolated subunits. *J. Biol. Chem.* 264:3160–3168.

Krishnaswamy, S., E. B. Williams, and K. G. Mann. 1986. The binding of activated protein C to factors V and V_a. *J. Biol. Chem.* 261:9684–9693.

Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York. 111–150.

Lampe, P. D., M. L. Pusey, G. J. Wei, and G. L. Nelsestuen. 1984. Electron microscopy and hydrodynamic properties of blood clotting factor V and activation fragments of factor V with phospholipid vesicles. *J. Biol. Chem.* 259:9959–9964.

Lecompte, M. F., S. Krishnaswamy, K. G. Mann, M. E. Nesheim, and C. Gitler. 1987. Membrane penetration of bovine factor V and V_a detected labelling with 5-iodonaphthalene-1-azide. *J. Biol. Chem.* 262: 1935–1937.

Lecompte, M. F., G. Bouix, and K. G. Mann. 1994. Electrostatic and hydrophobic interactions are involved in factor V_a binding to membranes containing acidic phospholipids. *J. Biol. Chem.* 269:1905–1910.

Lentz, B. R., D. R. Alford, M. Hoeckli, and F. A. Dombrose. 1982. Phase behavior of mixed phosphatidylglycerol/phosphatidylcholine multilamellar vesicles. *Biochemistry.* 21:4212–4219.

Lentz, B. R., D. R. Alford, M. E. Jones, and F. A. Dombrose. 1985. Calcium-dependent and independent interactions of prothrombin fragment 1 with phosphatidylglycerol/phosphatidylcholine unilamellar vesicles. *Biochemistry.* 24:6997–7005.

Lentz, B. R., and J. Hermans. 1989. A new model to describe extrinsic protein binding to phospholipid membranes of varying composition: quantitative treatment. *Biochemistry.* 28:7459–7461.

Lentz, B. R., G. F. McIntyre, D. J. Parks, J. C. Yates, and D. Massenburg. 1992. Bilayer curvature and certain amphipaths promote poly(ethyleneglycol)-induced fusion of dipalmitoyl phosphatidylcholine unilamellar vesicles. *Biochemistry.* 31:2643–2653.

Lentz, B. R., J. R. Wu, A. M. Sorrentino, and J. N. Carleton. 1991. Membrane binding induces lipid-specific changes in the denaturation profile of bovine prothrombin. *Biophys. J.* 60:942–951.

Lim, T. K., V. A. Bloomfield, and G. L. Nelsestuen. 1977. Structure of the prothrombin- and blood clotting factor X-membrane complexes. *Biochemistry.* 16:4177–4181.

Mayer, L. D., and G. L. Nelsestuen. 1981. Calcium- and prothrombin-induced lateral phase separation in membranes. *Biochemistry.* 20: 2457–2463.

Mayer, L. D., and G. L. Nelsestuen. 1983. Membrane lateral phase separation induced by proteins of the prothrombinase complex. *Biochim. Biophys. Acta.* 734:48–53

Mayer, L. D., M. L. Pusey, M. A. Griep, and G. L. Nelsestuen. 1983. Association of blood coagulation factors V and X with phospholipid monolayers. *Biochemistry.* 22:6226–6232.

Nesheim, M. E., and K. G. Mann. 1979. Thrombin-catalyzed activation of single chain bovine factor V. *J. Biol. Chem.* 254:1326–1334.

Nesheim, M. E., K. B. Taswell, and K. G. Mann. 1979. The contribution of bovine factor V and factor V_a to the activity of prothrombinase. *J. Biol. Chem.* 254:10952–10962.

Nesheim, M.E., J.A. Katzmann, P.B. Tracy, and K.G. Mann. 1980. Factor V. *Methods Enzymol.* 80:249–275.

Nesheim, M. E., C. Kettner, E. Shaw, and K. G. Mann. 1981. Cofactor dependence of factor X_a incorporation into the prothrombinase complex. *J. Biol. Chem.* 256:6537–6540.

Odegaard, B., and K. G. Mann. 1987. Proteolysis of factor V_a by factor X_a and activated protein C. *J. Biol. Chem.* 262:11233–11238.

Papahadjopoulos, D., and D. J. Hanahan. 1964. Observation on the interaction of phospholipids and certain clotting factors in prothrombin activator formation. *Biochim. Biophys. Acta.* 90:436–439.

Pei, G., D. D. Powers, and B. R. Lentz. 1993. Specific contribution of different phospholipid surfaces to the activation of prothrombin by the fully assembled prothrombinase. *J. Biol. Chem.* 268:3226–3233.

Pusey, M. L., L. D. Mayer, G. J. Wei, V. A. Bloomfield, and G. L. Nelsestuen. 1982. Kinetics of hydrodynamic analysis of blood clotting factor V-membrane binding. *Biochemistry.* 21:5262–5269.

Pusey, M. L., and G. L. Nelsestuen. 1984. Membrane binding properties of blood coagulation factor V and derived peptides. *Biochemistry.* 23: 6202–6210.

Rietveld, A., T. A. Berkhout, A. Roenhorst, A. D. Marsh, and B. de Kruiff. 1986. Preferential association of apocytochrome c with negatively charged phospholipids in mixed model membranes. *Biochim. Biophys. Acta.* 858:38–46.

Rosing, J., G. Tans, J. W. P. Govers-Riemslag, R. F. A. Zwaal, and H. C. Hemker. 1980. The role of phospholipids and factor V_a in the prothrombinase complex. *J. Biol. Chem.* 255:274–283.

Rosing, J., G. Tans, H. Speijer, and R. F. A. Zwaal. 1988. Prothrombin activation on phospholipid membranes with positive electrostatic potential. *Biochemistry.* 27:9048–9055.

Rosing, J., H. M. Bakker, M. C. L. G. D. Thomassen, H. C. Hemker, and G. Tans. 1993. Characterization of two forms of human factor V_a with different cofactor activities. *J. Biol. Chem.* 268:21130–21136.

Stoylova, S., K. G. Mann, and A. Brisson. 1994. Structure of membrane-bound human factor V_a. FEBS Lett. 351:330–334.

Tendian, S. W., and B. R. Lentz. 1990. Evaluation of membrane phase behavior as a tool to detect extrinsic protein-induced domain formation: binding of prothrombin to phosphatidylserine/phosphatidylcholine vesicles. *Biochemistry.* 29:6720–6729.

van de Waart, P., H. Bruls, C. Hemker, and T. Lindhout. 1983. Interaction of bovine blood clotting factor V_a and its subunits with phospholipid vesicles. *Biochemistry.* 22:2427-2432.

van de Waart, P., A. J. W. G. Visser, H. C. Hemker, and T. Lindhout. 1987. The effect of factor V_a on lipid dynamics in mixed phospholipid vesicles as detected by steady-state and time-resolved fluorescence depolarization of diphenylhexatriene. *Eur. J. Biochem.* 164:337–343.

Walker, R. K., and S. Krishnaswamy. 1994. The activation of prothrombin by prothrombinase complex: the contribution of the substrate-membrane interaction to catalysis. *J. Biol. Chem.* 269:27441–27450.

Winiski, A. P., A. C. McLaughlin, R. V. McDaniel, M. Eisenberg, and S. McLaughlin. 1986. An experimental test of the discreteness-of-charge effect in positive and negative lipid bilayers. *Biochemistry.* 25: 8206–8214.

Wu, J. R., and B. R. Lentz. 1991. Fourier transform infrared spectroscopic study of Ca^{2+} and membrane-induced secondary structural changes in bovine prothrombin and prothrombin fragment 1. *Biophys. J.* 60: 70–80.

Wycoff, M., D. Rodbard, and A. Chrambach. 1977. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate-containing buffers using multiphasic buffer systems: properties of the stack, valid R_f measurement, and optimized procedure. *Anal. Biochem.* 78:459–482.