A new consensus sequence for phosphatidylserine recognition by annexins

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Abstract.

Annexins are abundant and ubiquitous proteins which bind, by their four structurally identical-domain core, to phosphatidylserine-containing membranes in the presence of Ca$^{2+}$. Using molecular simulation and mutagenesis, we have identified a new phosphatidylserine-binding site in annexin V domain 1 and established its structure. The residues involved in this site constitute a consensus sequence highly conserved in all annexins. Remarkably, this consensus sequence is exclusively found in the domains 1 or 2, sometimes in both but never in domain 3 and 4. Such a pattern actually delineates three classes of annexins, shedding new light on the role played by the four-domain core of annexins which could encode specific information discriminating the different annexins that compete within a given cell for membrane binding. Our findings thus provide new strategies for understanding the regulation of annexins cellular functions.
Annexins are cytosolic proteins that bind to negatively charged membranes in response to cellular calcium increase in the micro-molar concentration range (1-3). The membrane binding properties common to these abundant and ubiquitous proteins have suggested their involvement in numerous cellular functions such as membrane trafficking, exocytosis, endocytosis, membrane-cytoskeleton interactions, regulation of membrane protein activity, signal transduction. Some members of the annexin family are also found in the extra-cellular compartment and are thus expected to respond to lipid signaling rather than to calcium signaling since extracellular calcium concentration is in the millimolar range. Exposure of negatively charged lipids, especially phosphatidylserine (PS), at the external surface of cellular plasma membrane constitutes a biologically important ensemble of signaling events. PS exposure at the cellular surface is for instance one of the earliest detectable molecular event in apoptosis (4) and the main event in blood coagulation initiation (5). Indeed, Annexin V (ANX-A5) (35), which binds to PS-containing membrane, has been reported as an anti-coagulant protein (6, 7) probably because it forms a well structured network at the membrane surface (31). ANX-A5 is also used, when conjugated to fluorescent label, for detecting cells undergoing apoptosis (7).

The annexins are composed of a highly conserved four-domain core and a variable N-terminal segment. The N-terminal segment is most probably directly related to the diversity of the functional properties. The four-domain core, which harbors several calcium-binding sites, is responsible for the calcium-mediated membrane recognition and binding (1-3). The structure of an annexin domain is highly conserved and comprises five α-helices named A to E (Fig. 1). The calcium binding sites are well documented as a result of the numerous crystal structures now available (8-16), and are of two main types: type II in the AB loop (AB-site) and type III in the N-terminus of B-helix (B-site) and C-terminus of D-helix (DE-site). The AB loop site has the highest affinity for calcium.

In contrast, and although the annexin family has been known for many years (17), the major PS-binding site has not been reported yet because of the difficulty to obtain crystals of lipid-bound annexin. Nevertheless, using glycerophosphoserine (GPS) as a ligand for the annexin-calcium complex, a first possible PS-binding site could be identified in the ANX-A5 domain 3 (18). This site comprises two calcium ions, one in the AB-site and the other in the B-site. However, domain 3 was latter on shown to be poorly involved in membrane binding while domain 1 was found to play a prominent role in the lipid-binding process of ANX-A5 (19, 20). Consequently, the ANX-A5 domain 3 lipid-binding site may be considered either as a low affinity secondary site or as a hardly accessible site as suggested by a recent work (21). Notwithstanding these binding data, the crystallographic data involving domain 3 can be readily transposed to domain 1 or domain 2 which are identical as they possess exactly the same ligands for the two calcium binding sites and then a priori the same capacity.
of binding PS. These observations emphasize the complexity of annexins and clearly indicate that the properties of the protein-membrane interface must also be considered.

From a general standpoint, how interfacial proteins such as annexins behave at the membrane surface still remains a quite complex matter. This is due to properties of the membrane interface itself which has to be considered as "2-dimensional" intermediate reservoir of a few Å thickness between the bulk solution and the hydrophobic membrane interior. Interaction of the protein moiety with ions, specially divalent cations like calcium, may be considerably different from that in pure bulk solution. This is also true for interactions with lipid polar heads. Several past works have emphasized these aspects (22, 23). As mentioned above, the problem is even more complicated in the case of annexins due to the presence, in these proteins, of numerous potential calcium binding sites with subtle differences. In addition, there is no guarantee that results obtained from bulk solutions will still hold for the membrane interface. It is also worth emphasizing the low intrinsic affinity of annexins for calcium and their apparent absence of binding to isolated phospholipids in the bulk solution. Clearly binding of calcium and phospholipid polar heads have to be understood from properties within the interface.

As soon as a phospholipid bilayer is concerned radiocrystallography as well as NMR hardly provide the essential information at atomic resolution. Thus we turned to molecular modeling in order to take a step further in the understanding of molecular recognition of phosphatidylserine polar head by annexins.

We now describe a new lipid-binding site for phosphatidylserine (PS). The site was first established using molecular simulation and docking of a PS molecule onto the ANX-A5 domain 1. Hence we could identify a consensus sequence which was found in either domain 1 or domain 2 of all known annexins, sometimes simultaneously in both domains, but never in domains 3 or 4. Mutational analysis of this consensus sequence demonstrated its direct involvement in membrane binding and PS recognition. From these results, a more complete picture is now emerging: binding of ANX-A5 to PS containing membranes involves at least two calcium ions and two phosphoserine polar heads in domain 1 acting cooperatively, one bridging the AB and B calcium ions (18) and using only few protein ligands and one using the AB calcium ion and about seven protein ligands. Other secondary sites, may also contribute to membrane binding at high calcium concentration. A direct and precise measurement of the intrinsic affinity of the AB-site for calcium is also presented for the first time.

Fig. 1 near here
EXPERIMENTAL PROCEDURES.

Molecular simulation.

The PS molecule was first drawn, with an approximately correct orientation of the polar head, near the supposed binding site namely the region between the helices A and D of domain 1, where a sulfate group is observed in the crystallographic structure (PDB entry 1avr, (8)). At this step the phosphate group was positioned near the calcium atom. In order to compensate for the absence of an adequate force field, the calcium atoms were maintained by ion-protein constraints derived from the crystallographic data. The lipid and the six residues side-chains located in the space between the helices A and D were allowed to move while the remaining part of the domain were held fixed. A low temperature dynamics (50 K, ≈10 ps) was then run until the PS polar head remained approximately stationary. This allowed to enumerate the polar head-domain interactions described in Fig. 2A and reveal a consensus sequence in the annexin family. The model was subsequently refined. During refinement, again to compensate for the absence of an adequate calcium force field, an additional distance constraints between the calcium ions and the closest PS phosphate oxygen atom was applied to maintain the distance between these two atoms larger than 2.4 Å. The structure of the complex given in Fig. 2B was obtained after a few runs of simulated annealing preceded by a short dynamic at 100 K and final minimization. We utilized the Sybyl program (Tripos Inc.) in this work. The structure of the domain 1 with three PS molecules (Fig. 4) was obtained in the same way.

Mutagenesis procedure.

The wild type, M234, M134 and M1234 mutants of ANX-A5 cDNA were cloned in a pGex 2T expression vector. M1, M2 and M3 mutations correspond to E72Q, D144N and D228A respectively. Site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene). Suppression of consensus sequence in the first domain (R25A, K29S, R65S, D68A) was obtained from M234 ANX-A5. The cDNA of each mutant was sequenced in their full length using the ABI prism sequencing kit.

Expression and purification.

Expression and purification of GST fusion proteins were essentially performed as described elsewhere (20). *E. Coli* strain BL21 Gold (from Stratagene) was used. 100 ml LB medium containing 150 µg/mL ampicillin precultures were incubated at 37 °C overnight. One liter of 150 µg/mL ampicillin LB medium was inoculated with the preculture to get a 0.2 DO600. Bacteria were grown at
37°C until a 0.8 DO$_{600}$ was reached, then IPTG 1mM was added to the medium. The cultures were stopped at DO$_{600}$ level stabilization (around 2.0 DO$_{600}$). Bacteria were pelleted by centrifugation (15 minutes at 3000 g), the pellets were resuspended in a lysis buffer containing Tris 50 mM, NaCl 500 mM, EDTA 1mM, Glycerol 5 %, Triton X100 1 %, PMSF 1 mM, DTT 1 mM, aprotinine 1 µg/ml. After incubation for 1 h with lysozyme (0.5 mg/ml) followed by sonication, lysed bacteria were centrifugated at 10 000 g for 15 min and the supernatant was loaded onto a glutathione-agarose column. Columns were washed with 1 M NaCl and with 50 mM Tris/HCl, pH 8.0, all steps were performed at 4°C. GST-annexin V mutants were cleaved by thrombin (10 units/mg of fusion protein) in 50 mM Tris/HCl, pH 8.0, buffer containing 150 mM NaCl at room temperature. Final purification was performed on a Ressource Q (FPLC system, Pharmacia). Purity and identification of all proteins were checked by SDS-polyacrylamide gel electrophoresis. Proteins were concentrated with an ultrafiltration apparatus (Amicon) with YM10 membranes. Protein concentrations were evaluated using the method of Bradford and UV absorption at 280 nm.

Copelleting assays.

The standard copelleting assays contained 30 mg/ml mixed POPS/POPC (80/20) liposomes equilibrated in a Tris buffer (50 mM Tris, pH 8.0, 150 mM NaCl, EDTA 1 mM, MgCl$_2$ 1 mM), with the indicated concentration of free CaCl$_2$. After a 15 minutes incubation at room temperature with 5 μM of protein, the vesicles were pelleted by centrifugation (270000 g for 30 minutes at 4°C). Supernatant (S1) containing unbound proteins is removed. The pellets containing the bound proteins were resuspended with a Tris buffer (50 mM, pH 8.0) containing EDTA 10 mM. After a 15 minutes incubation, vesicles were pelleted, and supernatants (S2) were collected for SDS PAGE gel analysis.

Surface plasmon resonance binding experiments.

Surface plasmon resonance binding experiments were performed on a Biacore 2000 apparatus, using L1 chips coated with 2 mg/ml mixed POPS/POPC (80/20) size calibrated liposomes using a LiposoFast apparatus (Avestin) with 100 nm pore diameter polycarbonate membranes. Binding experiments were performed in a degassed HBS-N buffer (HEPES 10 mM, pH 7.4, NaCl 150 mM) containing 2 mM CaCl$_2$. This buffer was used during equilibration, association and dissociation phases. Proteins were added to this buffer at the indicated concentrations during association phase. Regeneration of phospholipids bilayer after dissociation phase was performed using EDTA 2 mM in HBS-N buffer.

Typical SPR experiment for each protein concentration proceeds in four steps : the equilibration
of phospholipid bilayer with HBS-N buffer containing 2 mM Ca$^{2+}$ during 60 seconds, the association phase during which protein in a determined concentration is added to this buffer during 60 seconds, the dissociation phase where HBS-N buffer with 2 mM Ca$^{2+}$ is flowed through the detection cell during 400 seconds, and the regeneration phase where 2 mM EDTA in HBS-N buffer is used to remove proteins bound to the bilayer at the end of the dissociation phase.

Evaluation of kinetics data were performed using Bialevaluation 3.0 software. Association and dissociation rate constants were independently evaluated. Dissociation rate constants were determined from the 200 last seconds of the dissociation phase to avoid non purely membrane-protein dissociation occurring at the beginning of dissociation phase. Two models were used, simple Langmuir dissociation model and a cooperative dissociation model following the equation:

$$k_{off}^{(app)} = k_{off} (1 + Ad \cdot R / R_{max})$$

where $k_{off}^{(app)}$ is the apparent dissociation rate constant, $k_{off}$ is the dissociation rate constant, $Ad$ is the cooperativity parameter for dissociation, $R$ the response, $R_{max}$ the saturation response. Residuals between experimental values and fit values never exceed 4 response units. Both models led to same dissociation rate values even if the Langmuir model generated non random distributed residuals unlike the cooperative dissociation model. Association phase was fitted with a Langmuir model, excluding the first 10 seconds of association. Residuals never exceeded 10 response units. Association rate constant ($k_{on}$) for each protein concentration was calculated from the kinetic during the association phase, using the dissociation rate constant evaluated for the same protein concentration. Errors on the equilibrium dissociation constant $K_D$, were calculated from fitted $k_{on}$ and $k_{off}$ using the following equation:

$$dK_D = \frac{k_{off}}{k_{on}} \left( \frac{dk_{off}}{k_{off}} + \frac{dk_{on}}{k_{on}} \right)$$

where $dK_D$ is the standard error on $K_D$, $dk_{off}$ is the standard error on $k_{off}$, $dk_{on}$ is the standard error on $k_{on}$.

**NMR experiments.**

Uniformly $^{15}$N labeled proteins were obtained by growing *E Coli* in a minimal medium containing ammonium sulfate as exclusive nitrogen source. The purification protocol was identical to the one described above for unlabeled proteins. The proteins were finally obtained in a Tris-HCl buffer 20 mM, NaCl 150 mM, pH 8.5 containing 10 % D2O.
NMR experiments were carried out on Bruker DRX 800 MHz spectrometer equipped with 5 mm triple-resonance gradient probe with actively shielded three axis gradients. All experiments were performed at 293 K and pH 8.5. The pH was checked after each calcium addition. Standard fHSQC (36) were recorded with a quadrature detection by the TPPI-States method (37). $^{15}$N decoupling during acquisition was performed using a GARP sequence (38). The number of scans was between 8 and 32, the number of points was 2048 in the direct dimension. 200 increments were used in the indirect dimension. The recycling delay was 1.5 s. The spectral width were of 6.2 kHz and 4.1 kHz in the $^1$H and $^{15}$N dimensions respectively.

For each Annexin V mutant, FHSQC spectra were recorded at the following calcium concentrations: 0 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 6 mM, 9 mM, 15 mM and 30 mM. Addition of an excess of EDTA was performed at the end of the titration. The spectrum was superimposable on that obtained at 0 mM calcium. At high calcium concentration, an important reduction of the peak intensities and an increase of the line width showed the presence of protein aggregation. This phenomenon was reversible after addition of EDTA.

NMR spectra were processed using the GIFA software (39). A $(\pi/4)$ shifted squared cosine bell and a $(\pi/4)$ squared cosine bell were applied along $t_1$ and $t_2$, respectively. The data were zero filled to 1024 points along $t_1$ and to 2048 points along $t_2$ prior to Fourier transform. Finally, a baseline correction was applied in both dimensions using the corresponding GIFA baseline routine (40). Difference spectra were performed and allowed to identify most of the correlation peaks that are sensitive to the calcium concentration. The GIFA peak-picking routine was used to determine the chemical shift variation of these correlation peaks.

The titration of M1234 led to the determination of the correlation peaks that are sensitive to secondary calcium sites. By difference it was possible to identify the correlation peaks that are sensitive to the primary calcium site for M234 and for the mutants of M234 with mutated phospholipid equatorial site.

The chemical shift variation against the calcium variation was fitted with the following function:

$$\Delta \delta_{obs} = \Delta \delta_{ppm} \left( \frac{Ca}{Ca + K_D} \right)$$

Where $\Delta \delta_{obs}$ is the observed chemical shift variation, $\Delta \delta_{ppm}$ the difference between the free and bound protein, $Ca$ the bulk calcium concentration and $K_D$ the dissociation constant.

The fitting was achieved using the non-linear Levenburg-Marquardt minimization algorithm (41) implemented in the MATLAB software. The quality of the fits and the uncertainties were
obtained from statistical analysis (42, 43) with an experimental gaussian error set to 0.01 ppm and 0.1 ppm in the $^1$H dimension and in the $^{15}$N dimension respectively and using a set of 500 synthetic data for each data set. A model was considered satisfactory if the optimized $\chi^2_{exp}$ obtained for the experimental data lay within the 95% confidence limit obtained from the 500 Monte Carlo simulations, i.e. $\chi^2_{exp} < \chi^2_{95\%}$. The uncertainties in the values of the calcium affinity were the standard deviation of the 500 simulated calcium affinities.
RESULTS AND DISCUSSION

Docking the PS molecule on the ANX-A5 domain 1 reveals a PS binding consensus sequence.

We started from two observations: i) domain 1 is the unique domain for which removal of the type II calcium-binding site removes almost all the membrane binding property of the whole protein (Ref 19,20, and present data), ii) sulfate ions used to grow annexin crystals are often found bonded to a pair of very close Arg residues, Arg-25 and Arg-63 in the case of ANX-A5 domain 1 suggesting the involvement of these residues in phospholipid binding. Then we docked a PS molecule onto the domain 1 (see "Experimental procedures"). By analogy with phospholipases A2 that possesses a calcium-binding loop very close to that of annexins, we positioned the phosphate group of the PS molecule in contact with the calcium ion in domain 1. We then searched for complementary electrostatic interactions between the PS polar head and annexin residues. This led us to identify a potential binding site as described below. As shown in Fig. 2A, the PS-domain 1 interactions involve:

(i) a subset of positively charged groups comprising the calcium ion in the AB (type II) calcium-binding site, the Gly30 backbone HN, the side-chain NH$_3^+$ of Lys-29, the Arg-25 and Arg-63 side-chains, and:
(ii) a subset of negatively charged groups comprising the Asp-68 carboxyl side-chain, the Ser-71 O$_\gamma$ relayed by a water molecule and one of the side-chain oxygen atom of Glu-72, the so-called bidentate calcium ligand. The presence of a bridging water molecule between the Ser-71 O$_\gamma$ and the polar head amino roup was established by molecular dynamics simulation with explicit solvent (data not shown). In ANX-A5 domain 1 the sequence involved in PS binding can be read as:

R$_{25}$xxxK$_{29}$-----R$_{63}$xxxxD$_{68}$xxS$_{71}$E$_{72}$

The side-chains of this ensemble of residues form a highly polarized pocket well adapted to the binding of the phosphatidylserine polar head, with a negative side, D68-S71-E72, and a positive side Ca$^{2+}$-K29-R25-R63. Using low temperature molecular dynamics simulation as described in “Experimental procedures”, the PS-annexin complex was further refined, leading to the structure given in Fig. 2B.

Searching the whole known annexin sequence family, we found the above sequence or a very closely related sequence, in either domain 1 or domain 2, sometimes in both (ANX-A4 and A7), in all annexins. An example for human annexins is given in Fig. 3. Remarkably, the sequence is never
observed in any domain 3 or 4. From this analysis, the following consensus sequence for PS binding may be proposed:

\[(R/K)xxxK-(B-C helices)-(R/K)xxxxDxxS(D/E) + Ca^{2+}\]

There are few modifications of this sequence in some domains: in ANX-A2 where the third basic residue is shifted one residue upstream, in ANX-A7 domain 2 the “K29” is replaced by a Gln residue and in ANX-A13 where the (R/K)25 residue is also replaced by an uncharged residue Gln. Gln residue is however still able to bind to the PS phosphate group.

The lipid-free annexin calcium complex presents the characteristic pentagonal bipyramid geometry (8) (Fig. 1C): an equatorial plan with five possible coordinations and two possible apical coordinations normal to the equatorial plan. Four equatorial coordinations have protein ligands, namely the G30-G32 backbone carbonyls and the two E72 carboxylic oxygen atoms for domain 1. The fifth equatorial coordination is free i.e. only occupied by a water oxygen atom. One apical coordination points toward the domain core (the M28 backbone carbonyl) and the second points toward the solution and is free.

This geometry allows to define two types of PS binding sites according to which of the two above free coordinations is occupied by a PS phosphate group. We propose, for domain 1, to name these two sites: the “equatorial” site or D1e and the “apical” site or D1a. The D1e site corresponds to the consensus sequence described in this paper with the phosphate group bound to the fifth equatorial coordination. The D1a site would correspond to the site described for GPS in domain 3 (18) with the PS phosphate group bound to the remaining apical coordination and its carboxylic group bound to the Ca^{2+} in the B-site. The calcium B-site is located in the generally conserved sequence TxE at the N-terminus of the B-helix. It comprises one backbone carbonyl oxygen atom from Thr and two oxygen atoms (bidentate) from the Glu side-chain. Docking of a PS molecule on the D1a site shows that the other PS interactions in this site concern the Thr33 and Glu35 side-chains, hydrogen-bonding the PS amino group and the backbone Gly32 HN hydrogen-bonding the two oxygen atoms of the phosphate group one of which being the serine γ-oxygen atom.

Simultaneous binding of two PS molecules to the same AB calcium-binding site i.e. simultaneous occupancy of both D1a and D1e sites, is sterically possible (Fig. 4). Remarkably enough, binding of two PS molecules allows a full coordination of the AB calcium ion, five ligands from the protein and two from the lipid molecules. Hence, while the D3a (GPS site) does not apparently
promote membrane binding as demonstrated below, occupancy of the D1a site most certainly directly contributes to the stabilization of the complex and thus to membrane binding.

Fig. 4 near here

The secondary low affinity calcium binding in the D-helix C-terminus site (DE-site, Fig. 1C) involves two backbone carbonyl oxygen atoms from D-helix and one side-chain ligand from the E-helix Glu78 residue. Docking a PS molecule at this site, with the phosphate group bound to the calcium ion, reveals additional possible interactions of the Glu78 and Arg79 with respectively the ammonium and carboxylic group of the PS polar head. Such interactions may constitute another secondary, low-affinity, PS binding site as shown in Fig. 4. The couple of residues E/(KR) does not present a strict conservation but is more conserved in domains 1 (see Fig. 3) than in domains 2 and is sometime present in other domains. As discussed below, this secondary PS binding site may increase the global effective specificity of domain 1 for PS but is probably not of prominent importance in the binding process.

Finally, it is noteworthy that the lipid density obtained for the three bound lipid molecules is close to the density observed in protein-free membrane. This suggests that annexin binding elicits a minimal perturbation of the phospholipid bilayer despite the partial immobilization of these lipids in well defined sites.

*Domain 1 is essential and sufficient for the binding of annexin V to PS-containing membranes.*

As described in an earlier work (20), several annexin variants were expressed in which all but one of the Asp or Glu bidentate ligand of the AB calcium site (type II calcium sites) was mutated into the corresponding Asn or Gln. Calcium binding in the given AB site is suppressed by the mutation as are suppressed both the apical and equatorial PS binding sites. Four variants with only one intact AB site were produced: M234, M134, M124, M123, the numbers indicating the domains containing the mutations Asp to Asn or Glu to Gln. A variant, M1234, where the four AB calcium sites have been suppressed, was also expressed.

These mutants were first compared on the basis of the amount of calcium needed for binding to PS containing membranes. These membranes are liposomes with a constant molar composition PC:PS = 80:20 prepared and treated as described in “Methods”. The supernatants S2, which contain the protein released from the liposomes by EDTA after binding, were analyzed with SDS-PAGE. Gel
scanning allows a quantitative analysis of the membrane binding properties and the determination of an EC50 for each mutant. Resulting binding curves are gathered in Fig. 5A and the corresponding measurable EC50 values are given in Fig. 5B and Table I. There is obviously a large difference between domains in membrane binding properties. The EC50 index obtained for the M234 (D1 functional) variant is only slightly higher than that of the WT protein indicating that the ANX-A5 membrane binding property is essentially concentrated in domain 1. The M123 (D4 functional) variant could still bind to PS-containing membranes but with a higher EC50 = 1.6 mM. However this value is small enough for binding to extracellular leaflet of PS-containing cell membrane, where calcium concentration is about 1.5 mM, but is out of range for binding to the intracellular membrane leaflet where calcium concentration hardly reaches 0.5 mM. In the same way, neither M124 (D3 functional) nor M134 (D2 functional) could bind to membranes in intracellular conditions. However domain 3 in M124 can still promote cooperative membrane binding at very high, but non-physiological, calcium concentrations. Finally the M1234 variant (no functional domain) does not at all bind at the highest 15 mM calcium concentration explored in this work. This indicates that the DE secondary calcium sites, which were not eliminated by the mutations, are not sufficient to promote insertion of the protein into the membrane interface. These secondary calcium binding sites could however still play an important role once the protein is inserted in the interface by the virtue of the AB calcium sites.

Fig. 5 near here

**Mutational analysis of domain 1.**

To avoid possible interference with lipid-binding sites involving AB-calcium site in the other domains, we used the M234 mutant instead of the wild type protein. Several mutants were constructed: two single mutants M234-K29S and M234-D68A, the double mutant M234-R25A-R63S and the triple mutant M234R25A-R63S-K29S (M234-TM). In the triple mutant, the three basic residues of the consensus sequence were replaced by uncharged residues. These mutants were also first compared on the basis of the amount of calcium needed for membrane binding. The membranes were still liposomes with a constant molar composition PC:PS = 80:20. The SDS-PAGE analysis of the supernatant S2, which contains the protein released from the liposomes by EDTA after binding, is shown in Fig. 5C and the corresponding EC50 are given in Fig. 5D and Table I. The progressive removal of the basic residues up to the M234-TM mutant clearly shifts membrane binding toward non-physiological calcium concentrations for intracellular environment. The upward shift is still increased by the additional removal of the acidic residue D68 (Table I). Provided the effective calcium binding is not
affected by the mutations, these experiments demonstrate the direct involvement of the basic residues in PS recognition by domain 1. This is shown in the following section.

To which extent mutations affect calcium binding in the bulk solution?

Because of the complex relationships between calcium binding and lipid binding to the protein, it is important to verify that the EC50 increase resulting from the mutations in the equatorial binding site is not the result of an alteration of the intrinsic protein affinity for calcium. Calcium affinity for the protein in the bulk solution can be measured using NMR performed on the $^{15}$N labeled protein and mutants. HSQC spectra such as that given in Fig. 6A for the M234 variant are recorded for increasing calcium concentrations. A few resonance lines are sensitive to calcium and can be detected as shown in Fig. 6B. Although resonance assignment of the protein is not available, comparison between the two mutants M234 and M1234 allows a clear identification of the spectral changes associated with calcium binding in the domain 1 AB-site. Chemical shift variation with calcium concentration is given in Fig. 6C for the M234 (open symbols, domain 1 AB site, not observed in M1234) and M1234 mutants (filled symbols, not an AB site, observed in both variants). The M1234 response corresponds to yet undetermined secondary calcium binding sites such as DE-sites left unchanged by the mutations. Affinity for calcium of the different variants can be calculated from the binding isotherms and are shown in Fig. 6D. Clearly the suppression of the three basic residues of the consensus sequence, variant M234-TM, does not affect calcium binding. The dissociation constant remains equal to 1 mM which confirms the low affinity of annexins for calcium in the absence of membrane. However suppression of the acidic residue, D68A mutant, increases by a factor two the calcium dissociation constant.

We thus conclude that the mutation of the basic residues of the consensus sequence has no effect on the protein affinity for calcium thus confirming their direct involvement in PS binding. For the D68A mutation the result is less clear cut.

Surface Plasmon Resonance experiments.

The SDS-PAGE data were confirmed by surface plasmon resonance (SPR) experiments. The main results are gathered in Fig. 7 which compares the ANX-A5 mutants M234 and M234-TM and clearly demonstrates the decrease of the protein affinity for the PS-containing membrane due to the mutations.
It is also important to observe that, during the dissociation phase, the SPR signal of the M234 variant (Fig. 7A), of the M234-TM mutant (Fig. 7B) and of the wild type annexin (data not shown), does not return to zero as it should be expected for a complete dissociation of the protein from the membrane. This points out the important persistent binding that is most probably due to the very slow disruption of the annexin network (31) formed during the association phase. Importantly, such a slow annexin unloading is not abolished by the mutations in domain 1 suggesting that the mutations did not affect the protein network formation. The fast and small amplitude signal decrease, observed at the beginning of the dissociation phase, probably results from the release of annexin molecules bound with a low affinity to the annexin molecules of the network and not directly bound to phospholipids. We already observed such low affinity annexin-annexin interaction in solution at high calcium concentration (25). These interactions are also observed in the present NMR experiment as described in "experimental procedures". This binding complicates the quantitative kinetic analysis of the annexin membrane binding and the determination of an equilibrium constant. Nevertheless quantitative analysis could be made by considering the unaffected part of the kinetics. Results are given in Fig. 7C. Although a clear dependence of the $K_D$'s on protein concentration probably due to protein-protein interaction is still observed, it does not affect the main result i.e. the mutations increases the dissociation constant by up to two order of magnitude when considering the lowest protein concentrations.

**Comparison with other annexins.**

There are only few data concerning the relative importance of the domains in other annexins for membrane binding. To our knowledge, apart from ANX-A5, data exist only for annexin A1, A2 and A4. For ANX-A1 it has been clearly demonstrated (28) that the main capacity of binding to PS-containing vesicles is concentrated in domain 2 which is the domain that contains the equatorial site. Other domains of this annexin seem to play a role in vesicle aggregation which is a different phenomenon, not completely elucidated yet as far as structural requirements are concerned (16). For ANX-A2, the domain 2, where the consensus sequence is found, was shown to play a direct role in membrane binding (29). This set of data provides an additional evidence that the sequence we described, is a consensus sequence that ensures molecular recognition of PS.

In the case of ANX-A4, domain 1 was shown to be directly involved in binding to PS-containing membrane (30) in agreement with the presence of the consensus sequence in this domain while domain 2 was found to be minimally involved. The domain 4 was also shown to be involved in membrane binding. The results concerning the domains 2 and 4 of ANX-A4 resemble those obtained
for ANX-A5. It may be that, likewise ANX-A5 D2, ANX-A4 D2 has such a low intrinsic affinity for calcium that PS cannot bind despite the presence of the consensus sequence for an equatorial binding site. As for ANX-A5, we have no direct explanation for the domain 4. However, it is noteworthy that the calcium requirement for membrane binding of ANX-A5 D4 in M123 falls in the same range as the residual requirement of the M234-TM and it may be that binding occurs by the D4 apical site (see discussion below).

We may also observe that, when binding to the pure lipid part of a cell membrane is considered, the ANX-A5 domain 4 should not present binding in the intracellular space where calcium concentration never reaches the required value, excepted perhaps transiently, in the close surrounding of for instance an active calcium channel. When in vivo experiments are considered (19) domain 4 does show a detectable biological effect. These ANX-A5 (20) and ANX-A4 (30) in vivo experiments concern membranes of complex compositions, containing other negatively charged lipids than PS. Thus the domain 4 of annexins ANX-A4 and A5 could bind nonspecifically any negatively charged lipids which would explain membrane binding by this domain while domain 1 would bind PS more specifically. A second possibility could be a bivalent annexin-lipid complexation, associating one lipid and two annexin molecules as a result of the protein oligomerization on membrane surface. Last, domain 4 could simply bind to a particular membrane protein in a calcium and lipid-dependant manner. Clearly, understanding domain 4 behavior needs additional work.

Discussion.

The aim of the present work was to proceed a step further in the understanding, at the molecular level, of membrane recognition by annexins. Several lines of evidence (19,20) and data presented here clearly indicated that domain 1 of ANX-A5 was mainly responsible for the effective binding of the protein to PS-containing membrane. Because X-ray diffraction was unable to provide information on domain 1 we turned to molecular simulation. Hence, docking of a PS molecule onto the ANX-A5 domain 1 allowed the discovery of a new PS binding site in ANX-A5 D1 to which corresponds a consensus sequence present in the whole annexin family. As it is summarizes in Figure 2A, the domain 1 provides two possible PS binding sites: (i) an "apical" binding site which corresponds to the GPS binding site found in the domain 3 of ANX-A5 crystal (18) and involves the two calcium ions present in the main AB-site and in the B-site, and (ii) an "equatorial" site which involves only the calcium ion of the AB-site, and six or seven protein ligands corresponding to the above consensus sequence.

Mutational analysis of the consensus sequence demonstrated its direct involvement in membrane
binding and PS recognition. However, multiple mutations in part of this consensus sequence (M234-TM) do not abolish binding to PC:PS membrane as it was observed for the M1234 mutant where the binding of calcium in the AB-site was suppressed. As a matter of fact these mutations do not act on calcium binding in the AB-site which is not suppressed. This was demonstrated by the absence of variation of the intrinsic protein affinity for calcium in the AB-site, i.e. the affinity in the absence of membrane, as it was shown by direct NMR measurement. Hence the above triple mutations only affect the phospholipid binding to domain 1 in the membrane interface which simply requires more calcium to compensate for the loss of PS binding in the equatorial site due to the suppression of protein ligands.

The affinity of the protein for calcium in the AB-site thus crucially depends on the presence and number of lipid phosphate groups in the calcium ligand shell. The same conclusion can be drawn from the data provided in reference (24). As described above (see also fig 2A), there are two possible lipid phosphate groups in the calcium ligand shell in the AB-site: one apical and one equatorial. The apical phosphate ligand is distant enough (fig 2B and 4) from the points of mutation introduced in the M234-TM and is most probably not directly affected by these mutations. Hence, a PS molecule can still intrinsically bind to the apical site of the M234-TM. In contrast, the equatorial phosphate ligand is directly affected by the mutations because it depends directly on the interactions of the corresponding polar head with purely protein basic ligands that were mutated. However, when these latter protein ligands are suppressed, the equatorial site can be still occupied by a phosphate group because three ligands of the PS polar head are always potentially present: the bidentate calcium ligand E72, which binds the serine ammonium group, the G30 backbone HN and the calcium ion itself which directly binds to the phosphate group. Hence, the protein can still bind to the membrane but, because the affinity of the protein for the equatorial lipid is lowered as a result of the mutations the affinity of protein for calcium in the membrane interface is also lowered. Thus, the binding equilibrium of PS to both apical and equatorial sites of the annexin variant is shifted to higher calcium concentration. In conclusion, the EC50 values effectively reflect the protein binding properties to the PS-containing membrane and more precisely the modulation of this binding by the affinity for a phospholipid in the equatorial site. Two mechanisms may account for the increase in the calcium affinity induced, in the membrane interface, by the equatorial PS. First, the equatorial PS obviously closes the ligand sphere around calcium ion which may be simply viewed as an effect on the k_{off} rate constant. Second and in a more subtle way, binding of an equatorial lipid polar head which involves the bidentate ligand Glu72 and the calcium ion itself, could increase the partial electrostatic charge born by all the atoms precisely involved in the calcium binding due to change in bond polarization. As a consequence, the binding of the apical phosphate may be strengthened. This may be viewed as a possible cooperative effect of the
Taken altogether, our experiments allow to conclude that the consensus sequence described above directly contributes to the effective PS molecular recognition by annexins. We can also conclude that the apical site does not sustain, alone, membrane binding at low calcium concentration. Both the apical and equatorial lipid-binding sites are necessary, and act cooperatively, for a substantial membrane binding at the relevant calcium concentration of the intracellular space.

Furthermore, because the mutant M1234 does not at all bind to membrane, the secondary DE calcium-binding sites in the domain 1 as well as those in other domains cannot contribute to the main binding process of ANX-A5 to membranes. These secondary DE calcium-binding sites could play however a functional role once the protein is bound to the membrane interface by transiently binding calcium and phospholipids and thus could contribute to reduce the global lateral diffusion of these two species (26, 27). The consensus sequence for the equatorial PS binding site was found exclusively in the domain 1 or domain 2 but not in the other two domains. This has some possible biological consequences that may be now developed.

**Biological implications.**

The present results constitute an important step in the description of the membrane binding of annexins that suggest a range of fine-tuned in vivo membrane properties. As a whole, the four-domain core of annexins was generally considered as having merely a membrane-anchoring function, the effective function being under the control of the variable N-terminal segment. Thus, different membrane binding properties distinguishing the various annexins could be ascribed to their different apparent calcium requirement. In this context, finding of a second lipid-binding site that can be localized either in domain 1 or in domain 2 and only in these domains, reveals new potentialities that may be hypothesized.

There are generally different annexins in the same cell. All annexins bind to PS-containing membrane after a signaling calcium flux therefore they could compete for the same membrane or protein-membrane environment. The fact that an equatorial site is located either in domain 1 or in domain 2 ipso facto creates at least two classes of annexins and this may encode specific information concerning annexin interactions with other proteins, including annexins.

First we observe that, in the initial step of annexin membrane binding, the equatorial site in domain 1 can be more accessible to membrane lipids than when located in domain 2, this domain being somewhat sheltered by domain 1. This may contribute to differential kinetic effects (kon) and thus to different apparent affinities between annexins, although domain 1 and domain 2 may have the
same intrinsic binding energy for lipids (≈ same $k_{\text{off}}$) giving rise to additional threshold effects in response to cellular calcium increase.

A second possibility is a differential involvement, according to which domain has the equatorial lipid binding site, in annexin-annexin interactions as well as in annexin interactions with other proteins that annexins are expected to regulate. Concerning annexin-annexin interactions we observe that “domain 2” annexins do not form networks at membrane surface while some “domain 1” annexins, ANX-A4, A5 and A6, do form networks (31, 32). An example of proteins that interact with annexins is the PKC, which also needs PS and calcium for activity and does phosphorylate some annexins on their N-terminal segment (33, 34). Thus the presence-or the absence-of an equatorial PS binding site in a precise domain may dictate which annexin is devoted to productively interact with a given target protein.

Another point to be considered concerns the low affinity DE PS-binding sites. Although these secondary sites do not substantially contribute to effective protein binding to PS-containing membrane, as mentioned above, they can however transiently trap negatively charged lipids and calcium, increasing their local concentration and contributing to reduce their global lateral diffusion in the membrane. The effect is all the more efficient that the proteins oligomerize to form a network at the membrane surface. The Arg, Lys and Gln residues located on top of the annexin proteins and pointing toward the membrane interface could also have the same effect on PS and more generally on negatively charged phospholipids. Considering the intracellular situation, annexin networks could thus constitute bidimensional conducting bridges between calcium-providing membrane proteins (calcium channel for instance) on the one hand and proteins with calcium and PS dependent activities on the other hand (interfacial proteins like PKC for instance). The resulting effect would be a more efficient, i.e. less dissipative or more vectored communication between both kinds of proteins.

As a conclusion we would like to emphasize that the four-domain core common to all annexins is more than a mere membrane binding module and harbors more biological functions than previously estimated.

Acknowledgment:

The 800 MHz NMR spectra were run on the spectrometer of the “Institut de Chimie des Substances Naturelles du CNRS”. The spectrometer was procured with the help of the région Ile de France and the Association pour la Recherche contre le Cancer.
FIGURE CAPTIONS

**Figure 1.** ANX-A5 structure (8) (PDB accession number 1avr): (A) Top view (convex face), calcium atoms are represented as magenta spheres; (B) side view; (C) close view of domain 1 showing the five helices, the three bound calcium atoms and the pentagonal bipyramid geometry of the type II AB-site (blue solid lines); red balls correspond to the backbone calcium ligands and the green balls are the apical (a) and equatorial (e) free calcium ligands occupied by water molecules in the absence of lipid. The three “ball&stick” side-chains correspond to the three bidentate calcium ligands E35 (B site), E72 (AB site) and E78 (DE site).

**Figure 2A.** Mapping the interactions of the equatorial PS polar head with the different components of the protein binding site. The equatorial calcium ligands are indicated as e1 to e5 and the two axial ligands as a (exposed) and a' (buried). Hydrogen bonds are indicated as cyan lines and calcium ligands as green lines. All protein interactions with equatorial PS polar head are with side-chain except for the G30 backbone HN. The axial PS bridging the two calcium ions is also indicated.

**Figure 2B.** Close view of the 3D model showing the geometry of the different interactions of PS with the ANX-A5 domain 1. Hydrogen bonds between the serine polar head and the protein are indicated by doted lines.

**Figure 3.** Aligned sequences of domains 1 and 2 for several human ANX. Residues defining the AB (type II) calcium-binding sites are in red. The set of residues defining the consensus sequence of the main PS binding site is in green and also includes the bidentate calcium ligand indicated by a green arrow. The residues defining the calcium-binding sites (type III) are indicated by blue letters for the B-site and pink or violet letters for the DE-site, with a “b” for backbone and an “s” for side-chain. The residues defining the putative complementary PS binding site in E-helix, using a DE calcium, are in violet. The residue numbering (first digit indicating the residue) corresponds to ANX-A5 numbering. Residues in helices are indicated by a “H”.

**Figure 4.** Model building of ANX-A5 domain 1 with the three PS-binding sites occupied. The same docking procedure described in “methods” was used for the apical site and accessory DE-site. The average distance between glycerol C2 atoms is approximately 8 Å.
Figure 5. Ca$^{2+}$-dependency of POPC/POPS (80:20) liposomes binding of annexin V domains and mutants that affect the hypothesized PS binding site. (A) Binding assays of annexin V domains, wild type annexin V (circles), M234 (squares), M123 (up triangles), M124 (down triangles), M134 (diamonds), and M1234 (hexagons). The amount of bound protein is determined by densitometric analysis of Coomassie blue-stained SDS PAGE gels. The error, not shown for clarity is about 10%. (B) EC50 values for annexin V, M234 and M123 mutants. Since M124, M134, and M1234 do not reach maximal binding for the highest calcium concentration tested, no accurate EC50 value could be obtained for these mutants (see table I). (C) Binding assays of M234 (up triangles) and M234 R25A K29S R63S (down triangles). No significant binding could be detected below 0.1 mM Ca$^{2+}$ for all the proteins tested.

Figure 6: Measurement of the calcium affinity in the bulk solution. (A) HSQC spectrum of uniformly labeled annexin V mutant M234. (B) Part of the HSQC spectrum delimited by a box in panel A. Eight HSQC spectra recorded at increasing calcium concentrations are superimposed, each spectrum is represented with a different color: black 0 mM, blue 0.5 mM, cyan 1 mM, green 2 mM, magenta 4 mM, brown 6 mM, orange 9 mM, yellow 15 mM of CaCl$_2$. The arrow indicates the increasing calcium concentrations. (C) Relative variation of the chemical shifts versus the calcium concentration for two correlation peaks. The experimental data are indicated with a symbol and the fitted curve is shown with a line. Filled circles correspond to a correlation peak sensitive to a secondary calcium site (observed in the M234 and M1234 spectra). Open circles correspond to a correlation peak sensitive to the primary site AB site (observed in the M234 spectra but not in the M1234 spectra). (D) Affinity for calcium of the primary binding site (M234), the triple mutant M234R25AR63SK29S, the single mutant M234D68A and the secondary calcium sites of M234/M1234.

Figure 7. Surface plasmon resonance experiments: (A) Overlay of M234 binding kinetic curves on POPC/POPS (80:20) bilayer at various protein concentrations (varying from 20 to 0.37 µM). Association phase occurs from 0 to 60 seconds, dissociation phase occurs between 60 and 400 seconds. (B) Binding kinetics of M234 R25A K29S R63S with experimental conditions similar to A). (C) Plot of the dissociation constant (K_D) calculated from SPR experiments, versus M234 (squares) and M234 R25A K29S R63S (open circles) concentrations.
REFERENCES.

236, 199-208
Table I

EC50 values for the annexin V domains and mutants that affect the hypothesised PS binding site. EC50 and dEC50 values were obtained by applying a Hill 3-parameters model to the curves from POPS binding assays. No precise values could be obtained for M124 and M134 mutants, their maximal binding abilities exceeding the highest calcium concentration used for the binding assays (see Figure 5). No significant POPS binding was observed under these conditions for M1234.

<table>
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<tr>
<th>Protein</th>
<th>EC50 (mM)</th>
<th>dEC50 (mM)</th>
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<tr>
<td>ANX-A5</td>
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</tr>
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<tr>
<td>M124</td>
<td>~ 6.5</td>
<td>nd</td>
</tr>
<tr>
<td>M134</td>
<td>&gt; 15.5</td>
<td>nd</td>
</tr>
<tr>
<td>M1234</td>
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</tr>
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<tr>
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<tr>
<td>M234-R25A-K29S-R63S-D68A</td>
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Fig. 1A
Fig. 1C
Fig. 2A
Fig. 2B
### Domain 1

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<th>B</th>
<th>C</th>
<th>D</th>
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<tr>
<td>20</td>
<td>25</td>
<td>30</td>
<td>35</td>
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- ax 4---NAMEDAQTLRKAMGLTDEDAIISVLAYRTAQREIERTAYKSTIGRDLIDDILKSELSGNEEQVIVGMT
- ax 5---DERADAETLRKAMGLTDESSILLLTSSRSAQRQEISAAAFKLTFGRDLLDILKSELTGKEKRLIVALMK
- ax 62--NPADAKALRKAMGLTDEEDIIDIIIRSNVQQIRRQTFKSHFGRDLMDDILKSEIGDALARILGLMM
- ax 7---DAIIRDAILRKAMGLTDEQAIVDVVANRNSNDQQRKIAAFKTSYKLKDLIKLSLGNSNEELILALFM
- ax 11--DPLRDAEVLRKAMGLTDEQAIIIDCLGSRSNKQRQQILLSFKTAYGKLKDLSLGNSFEKTLALMK

### Domain 2

<table>
<thead>
<tr>
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<td>1--</td>
<td>2--</td>
<td>3--</td>
<td>4--</td>
<td>5--</td>
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- ax 1--TPAQFDADELRAAMGLTDEDLIEILASRTNKEIRDINRVYREELRDLAKDITSDTSGDFRNALLSLAKG
- ax 2--TPAQYDAESLKAAMGLTDEDSLIEICSRTQIQELQEINRVYKEMYTDLEKIDISDTSGDFRKLVALAKG
- ax 3--PTAVFDAQHLIKSKGACTMDALIEILTITRFSQMKDQAYTVYKSLGDDISSETSGDFRALALLDG
- ax 4--PTVLYDQVAILRAAMGLTDEEGLIEILASRTPEEIRISQTYQQYGRSLEDIRRSDKSFMFQVRVLVLSAG
- ax 7--PPTYDASWLRAAMGQGTERVLIILCCTRNTQUEIREIVRCYQSEFGRDLEKDISHEDRGSHFERLVLVSMCQG
- ax 13--RPSEYAARQLRAAMGLTDESVLIEFLCTRTNKENIAIKEAYQRLDFSLESDVKGDTSGNLKILVSLLAQ

Fig. 3
Fig. 5

A

B

C

D

---

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Fig. 6
Fig. 7

A

B

C

Response (RU)

Time (Sec)

Protein concentration (M)

KD (M)

K_D (M)

Protein concentration (M)

2.5 µM

1.25 µM

0.63 µM

0.37 µM

10 µM

5 µM

20 µM

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A new consensus sequence for phosphatidylserine recognition by annexins
Pierre MONTAVILLE, Jean-Michel NEUMANN, Françoise RUSSO-MARIE, Françoise OCHSENBEIN and Alain SANSON

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