An Electrostatic/Hydrogen Bond Switch as the Basis for the Specific Interaction of Phosphatidic Acid with Proteins

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Phosphatidic acid (PA) is a minor but important phospholipid that, through specific interactions with proteins, plays a central role in several key cellular processes. The simple yet unique structure of PA, carrying just a phosphomonoester head group, suggests an important role for interactions with the positively charged essential residues in these proteins. We analyzed by solid-state magic angle spinning \(^{31}\)P NMR and molecular dynamics simulations the interaction of low concentrations of PA in model membranes with positively charged side chains of membrane-interacting peptides. Surprisingly, lysine and arginine residues increase the charge of PA, predominantly by forming hydrogen bonds with the phosphate of PA, thereby stabilizing the protein-lipid interaction. Our results demonstrate that this electrostatic/hydrogen bond switch turns the phosphate of PA into an effective and preferred docking site for lysine and arginine residues. In combination with the specific packing properties of PA, PA may well be nature’s preferred membrane lipid for interfacial insertion of positively charged membrane protein domains.

Phosphatidic acid (PA) is a minor but important bioactive lipid involved in at least three essential and likely interrelated processes in a typical eukaryotic cell. PA is a key intermediate in the biosynthetic route of the main membrane phospholipids and triglycerides; it is involved in membrane dynamics, i.e. fission and fusion (2–4), and has important signaling functions (5–7). The role of PA in membrane dynamics and signaling is most likely 2-fold, either via an effect on the packing properties of the membrane lipids or via the specific binding of effector proteins (4, 7–9). The origin of the specific binding of effector proteins to PA is not known.

The PA binding domains thus far identified (for review, see Ref. 10) and more recently (7) are diverse and share no apparent sequence homology, in contrast to other lipid binding domains, such as e.g. the PH, PX, FYVE, and C2 domains (11–15). One general feature that the PA binding domains do have in common is the presence of basic amino acids (7). Where examined in detail, these basic amino acids were shown to be essential for the interaction with PA, which underscores the importance of electrostatic interactions. Indeed, the negatively charged phosphomonoester head group of PA would be expected to interact electrostatically with basic amino acids in a lipid binding domain. However, such a simple electrostatic interaction cannot explain the strong preference of PA-binding proteins for PA over other, often more abundant, negatively charged phospholipids.

In a recent study we showed that the phosphomonoester head group of PA has remarkable properties (8). The phosphomonoester head group is able to form an intramolecular hydrogen bond upon initial deprotonation (when its charge is \(-1\)), which stabilizes the second proton against dissociation. We provided evidence that competing hydrogen bonds, e.g. from the primary amine of the head group of phosphatidylethanolamine (PE), can destabilize this intramolecular hydrogen bond and, thus, favor the further deprotonation, i.e. increase the negative charge, of PA (8). These data raised the intriguing hypothesis that a combination of electrostatic and hydrogen bond interactions and not just electrostatic interactions of PA with basic amino acids, i.e. lysine and arginine, forms the basis of the (specific) binding of PA to PA-binding proteins.

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**The abbreviations used are: PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DOPA, 1,2-dioleoyl-sn-glycero-3-phospho (monosodium salt); DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; GST, glutathione S-transferase; LPA, lysophosphatidic acid; MAS, magic-angle spinning; RPA, Raf-1 PA binding region; MD, molecular dynamics.**
We set out to test this hypothesis by determining the ability of lysine and arginine residues in various membrane-interacting polypeptides to form a hydrogen bond with the phosphomonoester head group of PA in a lipid bilayer. Consistent with our hypothesis, we find using magic angle spinning $^{31}$P NMR that the positively charged amino acids lysine and arginine in these peptides are able to increase the charge of PA. We conclude that this increase in charge is due to the formation of hydrogen bonds between PA and the basic amino acids. MD simulations provided a dynamic view on the specific docking of these side chains on the di-anionic form of PA. We conclude that the phosphate of PA is an effective and preferred docking site for lysine and arginine residues in membrane-interacting peptides. In combination with its special packing properties, this turns PA into nature’s preferred membrane lipid to mediate interfacial insertion of positively charged membrane protein domains. This preference for PA and the role of interfacial insertion was confirmed in binding experiments with the PA binding domain of the protein kinase Raf-1, which is one of the best-characterized PA target proteins (16–19). To conclude, we propose that the electrostatic/hydrogen bond switch provides a new mechanism to stabilize the binding of phosphomonoesters to proteins.

**MATERIALS AND METHODS**

**Sample Preparation**—1,2-Dioleoyl-sn-glycero-3-phosphate (monosodium salt; DOPA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphethanolamine (DOPE), and 1,2-dioleoyl-3-trimethylammonium propanediol (chloride salt) were purchased from Avanti Polar lipids (Birmingham, AL). Dimethyl diacetyladamantium chloride (DOPE) was a kind gift from Dr. M. Scarzello, University of Groningen, The Netherlands. The detergents, dodecylamine and dodecyltrimethylammonium chloride, were purchased from Sigma-Aldrich (Buenos Aires, Argentina). All other reagents were of analytical grade.

**Dry Lipid Films and Lipid Suspensions**—Dry lipid films and lipid suspensions were prepared as described previously (8). Lipid/poly-$\Delta$-lysine samples were prepared by hydrating the lipid film with Hepes buffer (100 mM Hepes, 5 mM acetic acid–NaOH, 100 mM NaCl, and 2 mM EDTA, pH 7.20) containing poly-$\Delta$-lysine. The resulting lipid/poly-$\Delta$-lysine dispersions were freeze-thawed at least once, after which the pH of the dispersions was generally found to be within 0.05 pH units of pH 7.20 (if not, the pH was adjusted to fall within this range). The dispersions were centrifuged at 70,000 rpm for 30 min at room temperature in a Beckman TL-100 ultracentrifuge, and the pellet was transferred to 4 mm TiO$_2$ MAS-NMR sample tubes for $^{31}$P NMR measurement. Lipid/polyethylene-alanine peptide films were prepared as described previously (20). These films were hydrated with Hepes buffer, and the dispersions were centrifuged as described above. The clear supernatant was removed, and the pellet was resuspended in Hepes buffer and centrifuged again. This procedure was repeated until the pH of the supernatant was within 0.05 pH units of pH 7.20, after which the samples were freeze-thawed at least once. When the pH of this dispersion was still within 0.05 pH units of pH 7.20, it was centrifuged, and the pellet was transferred to a 4 mm TiO$_2$ MAS-NMR sample tube; otherwise, the above procedure was repeated until the pH was in the desired range (pH 7.20 ± 0.05).

**NMR Spectroscopy**—$^{31}$P NMR spectra were recorded on a Bruker Avance 500 wide-bore spectrometer (Karlsruhe, Germany) at 202.48 MHz using a 4-mm cross-polarization MAS-NMR probe. Samples were spun at the magic angle (54.7°) at 5 kHz to average the chemical shift anisotropy, and the chemical shift position of PA was recorded relative to 85% H$_3$PO$_4$. Under stable spinning conditions typically 500–3000 scans were recorded. Experiments were carried out at a temperature of 20.0 ± 0.5°C.

**Hydrogen Bond Length Determination**—In the Cambridge Structural Data base (version November 2004, with updates of February and May 2005; total number of entries 347767) 395 crystal structure determinations were found containing 1716 fragments that fit the lysine geometry, i.e. primary amine-to-phosphate hydrogen bond length (see supplemental Fig. 3A). For the arginine geometry, i.e. guanidinium-to-phosphate hydrogen bond length (see supplemental Fig. 3B), 30 crystal structure determinations were found containing 158 fragments. Searches were performed with the program ConQuest (21); for data analysis the program Vista (22) was used.

**MD Simulations**—Starting structures for molecular dynamics simulations were based on previous simulations of DOPC-peptide interactions and used the same exact protocols (23). The original DOPC bilayer consists of 64 lipids, 32 in each membrane leaflet. In each leaflet 3 randomly chosen DOPC molecules were changed into DOPA (−1) or DOPA (−2) by removing the choline head group. Head group charges and geometries for DOPA (−1) and DOPA (−2) lipids were calculated using density functional theory calculations and charge-fitting. The resulting charges were close to the phosphate group charges in DOPC. 0.2 M salt was added to match the experimental conditions. The DOPA (−1) simulation system contained 58 DOPC and 6 DOPA (−1) lipids, 2791 water molecules, 11 Na$^{+}$ ions, and 5 Cl$^{-}$ ions. The DOPA (−2) simulation system contained 58 DOPC, 6 DOPA (−2) lipids, 2785 water molecules, 17 Na$^{+}$ ions, and 5 Cl$^{-}$ ions. These two bilayers were simulated for 25 ns for equilibration, as determined by changes in the area per lipid and hydrogen bonding, and 25 ns for production.

Lys$_8$ and Arg$_8$$_8$ peptides were built in an extended configuration with uncapped termini. Two peptides were added to the water phase of the pre-equilibrated DOPC/DOPA bilayers above by removing overlapping water molecules and adding additional counter ions to maintain a zero net charge in the simulations. Initially the peptides were placed such that they had no contact with lipids at all, but in all cases the peptides rapidly bound at the water/lipid interface and showed little

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**Results**

Basic Amino Acids in Membrane-interacting Peptides Increase the Charge of PA—The $^{31}$P chemical shift of PA was previously found to be very sensitive to the ionization (charge) state of the phosphate head group. A change in chemical shift to downfield values, e.g. induced by an increase in pH, corresponds to an increase in negative charge observed either via high resolution NMR in small membrane vesicles (28, 29) or by MAS NMR in physiologically more relevant extended bilayers (8). In an earlier study we observed that inclusion of PE in a PA-containing PC bilayer resulted in a profound downfield shift of the $^{31}$P NMR peak of PA (8). This is exemplified in Fig. 1A (compare curves 1 and 2). Curve 1 shows the MAS $^{31}$P NMR spectrum of a PC/PA (9:1) bilayer with the minor downfield peak (to the left) corresponding to PA and the major upfield peak (to the right) to PC. Inclusion of 10 mol % PE (PC/PE/PA 8:1:1, curve 2) results in a large shift of the PA peak to downfield values due to an increase in the charge of PA. This increase in negative charge of PA induced by PE is caused by hydrogen bonding between the primary amine in the head group of PE and the phosphomonoester head group of PA (see Ref. 8).

To determine whether the basic amino acid lysine, which contains a primary amine, is also able to form a hydrogen bond with PA, we first determined the effect of polylysine$_{20}$ on the chemical shift of PA at neutral pH. Polylysine$_{20}$ with a polyamionization degree of 20 was chosen because it binds with high affinity to membranes containing negatively charged phospholipid (30, 31). Fig. 1A (curve 3) shows that an equimolar amount of lysine residues with respect to PA causes the majority of the PA peak to shift to downfield values. The minor part of the PA peak that does not shift upon polylysine addition most likely represents a small pool of PA in the multilamellar vesicles that is not reached by polylysine. In the presence of an excess of lysine residues with respect to PA (Fig. 1A, curve 4), maximizing the PA-polylysine interaction, the PA peak shifts even further...
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The Increase in Charge Is Largely Due to Hydrogen Bonding—Apart from the possibility of forming a hydrogen bond with the phosphomonoester head group of PA, lysine and arginine also introduce a net positive charge at the membrane surface. This will decrease the local proton concentration (due to charge repulsion) and, thus, increase the local (interfacial) pH. An increase in the interfacial pH will result in an increase in the negative charge of PA and is accompanied by a downfield shift of the PA peak. To distinguish between positive charge and hydrogen bond effects, we sought a simpler system in which to address this issue. We chose the positively charged amphiphiles dodecyltrimethylammonium chloride and dodecylamine, since these amphiphiles carry the same positive charge but differ in the ability to form hydrogen bonds because the quaternary amine cannot act as a hydrogen bond donor. Dodecyltrimethylammonium chloride and dodecylamine were included in the PC/PA bilayer at a lipid-amphiphile molar ratio of 12.5 and 6.25 to be able to compare the positive charge effects induced by these amphiphiles with those induced by the transmembrane peptides (each KALP and RALP peptide contains 4 positive charges); the results are shown in Fig. 3. As expected, the positively charged dodecyltrimethylammonium chloride increased the charge of PA due to the interaction of a positive charge (Fig. 3, white bars; control, cross-hatched bar). Similar results were obtained for the structurally very different, quaternary amine containing, diacyl amphiphiles, 1,2-dioleoyl-3-trimethylammonium propanediol and dimethyl dioctadecylammonium, which also only caused a small increase in the chemical shift and, thus, the negative charge of PA (data not shown). However, dodecylamine, which in addition to being positively charged can also form a hydrogen bond, induced a substantially larger increase in the negative charge of PA (Fig. 3, striped bars). Quantitation of the change in charge induced by primary and quaternary amines requires full titration curves, which are shown in supplemental Fig. 1. These data show that at pH 7.2 primary amines induce an ~60% larger increase in the negative charge when compared with quaternary amines. These results clearly demonstrate that hydrogen bond-forming primary amines are able to further increase the negative charge of PA beyond the increase induced by quaternary amines and indicate that the extra increase in negative charge induced by the primary amine is due to the formation of a hydrogen bond with the phosphomonoester head group of PA.

Importantly, the effects induced by the quaternary amines are substantially less than those induced by KALP and RALP.
peptides, whereas the effect of the primary amine dodecylam- 
ione is essentially identical to that of KALP (compare Figs. 2B 
and 3). Therefore, we conclude that the interaction of KALP 
and RALP peptides with PA involves the formation of hydrogen 
bonds between the basic amino acid residues and the 
phosphomonoester head group of PA.

Basic Amino Acids Have a Preferential Interaction with Di-
anionic PA—To get a broader insight into the interaction of 
lysine and arginine containing membrane active peptides with a 
PA-containing PC bilayer, we carried out MD simulations. 
These simulations were performed with PC bilayers containing 
10 mol % of either a mono-anionic or di-anionic PA and con-
tained either polylysine₈ or polyarginine₈ peptides, one on 
either side of the lipid bilayer. The length of these peptides is 
sufficient to assure an efficient interaction with an anionic 
bilayer (30). The simulations were started with the peptides in 
solution, and we monitored the interaction between lysine or 
arginine side chains and phosphates in the head group of PA 
and PC. The peptides quickly bound to the lipid bilayer (within 
10 ns) and stayed bound for the duration of the simulation (50 
ns). A two-dimensional snapshot of the simulation of a PC 
bi layer containing di-anionic-PA in the presence of polylsine₈ is 
shown in Fig. 4. As predicted by the MAS-NMR experiments, 
hydrogen bond interactions between the di-anionic PA and 
lysine side chains are clearly visible (shaded in yellow). How-
ever, hydrogen bonds between the phosphate of PC and lysine 
side chains were also present. To get an estimate of the relative 
preference or accumulation between/around mono-anionic PA 
and PC, respectively. These normalized histograms show that it is much 
more likely for a lysine side chain to form a hydrogen bond with 
the phosphate of di-anionic PA than with the phosphate of PC; 
in contrast, there is no apparent difference between mono-an-
ionic PA and PC, i.e. the histograms overlap. These histograms 
also indicate that over time PA-2 is clearly accumulated around 
the polylsine peptide(s), information not readily available from 
the two-dimensional snapshot of the hydrogen bond 
interactions of Fig. 4. To provide a visualization of the simul-
ations, a movie of the polylsine, PA-2/PC MD simulation is 
included in the supplemental information. The hydrogen bond 
interaction between lysine side chains and di-anionic PA is very 
stable, with some hydrogen bond lifetimes lasting well over 10 
ns (data not shown). Identical simulations were performed with 
the polyarginine₈ peptide, and an identical hydrogen bond 
analysis was carried out. The normalized histograms for the 
mono- and di-anionic PA simulations are shown in Fig. 5, C and 
D, respectively. These indicate also that arginine side chains are 
more likely to form a hydrogen bond with the phosphate of 
di-anionic PA than PC, indicating an accumulation of PA-2 
around the polyarginine peptide and that there is no relative 
preference or accumulation between/around mono-anionic PA 
and PC.

Binding of a PA Binding Domain Is Enhanced by Negative 
Membrane Curvature—The unique position of the phosphate 
head group of PA, very close to the acyl chain region of the lipid 
bilayer, turns PA into an attractive partner for hydrophobic 
interactions. Moreover, PA is a type II lipid, i.e. has a cone shape 
(4, 8), and this cone shape of PA will also favor hydrophobic 
interactions between PA and PA binding domains (33). Indeed, 
most PA binding domains contain essential hydrophobic resi-
dues, typically tryptophans and/or phenylalanines, to allow for 
such interactions. We investigated the effect of negative curva-
ture on the binding of the well known PA binding domain of the
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hydrophobic core of the dioleoyl-
lipid bilayer (32) and thereby posi-
tions the lysine residues in the acyl
chain/head group interface where
the phosphate group of PA is
located. The position of the phos-
phate of PA was determined by MD
simulation of a peptide free PA/PC
(~1:10 molar ratio) bilayer (see
“Materials and Methods”). The
results indicate that both the mono-
anionic (PA −1) and di-anionic (PA
−2) phosphate of PA is located at
the same depth as the phosphates
of PC in the head group interface (see
supplemental Fig. 2).

Interestingly, the lysine residues
in KALP23 and KALP31 induced an
identical shift of the PA peak, indi-
cating an equal effect on the nega-
tive charge of PA. This is despite
the fact that KALP31 is considerably
longer than KALP23, with a hydro-
phobic segment that is ~10 Å lon-
ger than the hydrophobic thickness
of a DOPC bilayer (32). Earlier stu-
dies on KALP peptides in PC bilayers
indicate that the lysine residues in
KALP are flexibly anchored in the head
group region of the lipid bilayer
and effectively modulate the hydrophobic length of the
peptide to “match” that of the PC bilayer (snorkeling effect, see
Ref. 20). Apparently, in the presence of PA, the ly-sines localize
to the PA phosphate instead of throughout the head group
region, indicating that the phosphomonoester head group of
PA acts as an effective docking site for the lysine residues.
Importantly, the formation of hydrogen bonds between lysine
residues and PA, as shown by the amphiphile experiments and
MD simulation with polylysine, implies direct docking of the
membrane-interacting peptides on the phosphomonoester
head group of PA and not merely an electrostatic attraction into
the electric layer over the lipid head groups. Furthermore, MD
simulation showed that the most effective docking occurs on a
di-anionic PA, consistent with our 31P NMR finding that the
interaction of lysine residues with the phosphate of PA
increases its charge from −1 to −2 at pH 7.2. The interaction
between PA and lysine (or arginine) side chains is substantially
strengthened by removing a proton (i.e. transition from mono-
to a di-anionic PA species) from the phosphomonoester head
group of PA, which appears energetically favorable in the pres-
ence of a positively charged side chain.

Arginine Side Chains Are Weaker Hydrogen Bond Donors
than Lysine Side Chains—The increase in PA charge induced by
arginine residues in RALP23 is less than that induced by the
lysine residues in KALP23. The exact nature of this difference is
unclear but may be related to the substantial delocalization of
charge in the guanidinium group of arginine when compared
with the primary amine of lysine, potentially reducing its
hydrogen bond donating capacity. This suggestion is supported

DISCUSSION

The Phosphomonoester Head Group of PA Is an Effective
Docking Site for Basic Amino Acids—We observed that the posi-
tively charged amino acids lysine and arginine in the mem-
brane-interacting peptides polylysine, KALP, and RALP caused
downfield shift in the 31P NMR peak of PA at constant pH. We
subsequently showed that the main cause behind this increase
in negative charge of PA is hydrogen bond formation between
the lysine or arginine side chains and the phosphomonoester
head group of PA. The existence of hydrogen bonds between
the mono- and di-anionic phosphate of PA and lysine and argi-
nine side chains was further confirmed by MD simulation.
Interestingly, a similar effect on PA charge is observed irrespec-
tive of whether the lysine residues are added from the outside as
polylysine or are already stably anchored in the membrane
water interface as is the case in the KALP experiments (com-
pare Figs. 1 and 2). The hydrophobic core of the KALP23 (and
consequently RALP23 and WALP23) peptide matches the

FIGURE 5. Histograms of the total number of hydrogen bonds between lysine side chains and the phos-
phate of PC and PA in the DOPC/DOPA(−1)/polylysine8 (A) and DOPC/DOPA(−2)/polylysine8 (B) simu-
lations and between arginine side chains and the phosphate of PC and PA in the DOPC/DOPA(−1)/
polyarginine8 (C) and DOPC/DOPA(−2)/polyarginine8 (D) simulations, normalized to the PC/PA ratio in
the lipid bilayer. Hydrogen bonds to PC are indicated in white and dashed (differentiating between the two
leaflets of the bilayer) and to PA in black and gray (differentiating between the two leaflets of the bilayer).

protein kinase Raf-1 (16–19). To this end PC in mixed PC/PA
model membranes was progressively replaced by PE, and
protein binding was monitored. The results (Fig. 6A) clearly
show that inclusion of PE greatly increases vesicle binding of
the Raf-1 PA domain. We not only find, in analogy with litera-
ture data, that binding is specific for PA but also that PA spec-
cificity is preserved even in the presence of high concentrations
of PE (Fig. 6B). Similar results were obtained for the plant PA-
binding proteins AtPDK1 and AtCTR1.9

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by a search of the Cambridge Structural data base for the hydrogen bond length of lysine (primary amine)- and arginine (guanidinium group)-like geometries to a phosphate oxygen, which shows that the mean hydrogen bond length for the lysine like compounds (2.813 Å) is ~0.1 Å shorter than that of the arginine-like compounds (2.919 Å, see supplemental Fig. 3). The MD simulations of polylysine_{\text{e}} and polyarginine_{\text{e}} peptides with a PC/PA bilayer further substantiated these results (see supplemental Fig. 4) and showed that indeed the mean hydrogen bond length between arginine and the PA phosphate is ~0.1 Å longer than that for lysine to PA phosphate. These results indicate that indeed the guanidinium group of arginine is a weaker hydrogen bond donor than the primary amine of lysine.

Molecular Model, the Electrostatic/Hydrogen Bond Switch—Collectively our data point to the following mechanism for the interaction of lysine and arginine residues in membrane-interacting peptides with low concentrations of PA (Fig. 7A) in a PC bilayer. The positive charge of lysine and arginine side chains in these peptides first results in an electrostatic attraction to the negatively charged phospholipid bilayer. Upon binding to the membrane, the positively charged side chains are able to randomly sample their surroundings, i.e. interact electrostatically and form hydrogen bonds with negatively charged (−1) phosphates. However, as soon as the side chain of lysine or arginine encounters the phosphomonoester head group of PA and comes into close enough proximity (<3.5 Å) to form a hydrogen bond, the negative charge of PA increases (to −2) due to the further deprotonation of its phosphomonoester head group. We coin this mechanism the electrostatic/hydrogen bond switch. The increase in negative charge, enhancing the electrostatic attraction, coupled to the formation of hydrogen bonds now locks the positively charged lysine or arginine side chains on the head group of PA and results in a docking of the membrane-interacting peptide on a di-anionic PA molecule. The difference in hydrogen bond length and effect on the negative charge of PA observed between lysine and arginine residues would suggest that lysine residues are more effective in docking on the phosphomonoester head group of PA. We propose that the electrostatic/hydrogen bond switch is a key element of the specific recognition of PA by PA-binding proteins.

Further support for our model comes from the interaction of basic residues in transmembrane proteins with anionic phospholipids and from the interaction of lysophosphatidic acid (LPA) with its G-protein-coupled receptors. An important feature of many transmembrane proteins is that they are flanked on the cytosolic side of the membrane by basic amino acids. These basic residues are thought to stabilize the transmembrane orientation of the protein and/or regulate its activity (34–37). Clusters of these basic amino acids may specifically bind to PA. Indeed, recent evidence suggests that this is the case for the mechanosensitive channel of large conductance MscL. MscL carries a cluster of three basic amino acids (Arg-98, Lys-99, and Lys-100) on its cytosolic face and has a specific, high affinity interaction with PA (38). LPA-like PA has a phosphomonoester head group, and binding of LPA to its G-protein-coupled receptors is likely to depend on similar principles as those discussed above for PA. Indeed, the binding of LPA (and coincidentally also its sphingolipid counterpart sphingosine 1-phosphate) to its receptors critically depends on the phosphomonoester head group (39, 40), and the phosphate binding region of the LPA receptors all contain two conserved basic residues (one arginine and one lysine residue (40)). Interestingly, our experimental results and the discussion above would suggest that LPA binds to its receptors in a di-anionic form.

Phosphomonoesters are ubiquitous in nature, occurring not only in lipids such as PA and LPA but also in many proteins, where (de)phosphorylation often switches enzymes between active and nonactive states as well as in many small bioactive molecules, such as glucose 6-phosphate. Recognition and binding to the phosphomonoester moiety(ies) of these compounds is likely regulated by similar principles as those presented for PA (see Fig. 7A). Indeed a comparison of the crystal structure of 12 randomly selected proteins binding a phosphomonoester moiety or moieties shows that multiple hydrogen bonds are present between amino acid side chains and the phosphomonoester, which inevitably carries two negative charges (41–53). Lysine and arginine residues form the positively charged binding pocket and often form the hydrogen bond donors as well, but other residues donating side chain hydrogen bonds also.

FIGURE 6. Binding of the RPA to PA-containing liposomes. Large unilamellar vesicles of different phospholipid composition (250 nmol) were incubated with 300 ng of GST-tagged protein. Bound protein was analyzed on SDSPAGE and visualized by silver staining. A, effect of increasing PE concentration in the liposomes on PA binding. Lipid mixtures consisted of the indicated amounts of PE and PC supplemented with 20% PA, except for the control, an equimolar mixture of PC and PE without PA. I is 50% of the input material. B, specificity of GST-RPA binding to PA. Lipid mixtures consisted of 40% PC, 40% PE, and 20% PA or 20% phosphatidylserine (PS) as indicated. I is 50% input. HIS-GST was used as a negative control. The input material shows an additional band underneath RPA-GST, a breakdown product, most likely GST alone, that does not bind to the liposomes.
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regularly occur. We propose that the electrostatic/hydrogen bond switch is the mechanism by which phosphomonoesters are recognized and bound by proteins.

**PA Is the Preferred Anionic Lipid for the Interfacial Insertion of Proteins**—The docking of basic protein domains on PA may be followed by insertion of hydrophobic protein domains into the hydrophobic interior of the lipid bilayer (see Fig. 7B). One example of such a favorable hydrophobic interaction has been described in vitro for the GTPase dynamin. Dynamin, which binds to negatively charged membranes, shows considerably more insertion in mixed-lipid monolayers containing PA instead of other negatively charged phospholipids (the molecular area of insertion is highest in the presence of PA; see Ref. 54). How can we understand these hydrophobic interactions? On top of its high charge and capacity to form hydrogen bonds, (unsaturated) PA also has a special molecular shape (Refs. 4 and 9; see Fig. 6B). PA is the only anionic phospholipid with a pronounced cone shape under physiological conditions (37). Cone-shaped lipids facilitate protein penetration into the membrane by forming favorable insertion sites in the head group region of the lipid bilayer (33). We further confirmed the effect of cone shape lipids on the binding of PA binding proteins to PA in experiments with the well known PA binding domain of the protein kinase Raf-1 (RPA). The cone-shaped lipid PE was found to strongly increase PA binding of the PA binding domain of Raf-1. This binding depended critically on the presence of PA. Only hydrophobic insertion sites did not lead to a binding of the RPA domain, and RPA bound at least 2.5-fold more to PA when compared with phosphatidylserine, even in the presence of high concentrations of PE.

Our observation that PA may act as a docking site for membrane-interacting peptides very close to the hydrophobic interior of the lipid bilayer together with the cone shape of PA turns PA into a very effective insertion site for positively charged membrane-active proteins. We propose that the electrostatic/hydrogen bond switch in the phosphate head group of PA coupled to the location of the phosphate head group close to the hydrophobic interior of the lipid bilayer sets PA apart from all the other anionic membrane lipids.

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