Binding of the PH and Polybasic C-Terminal Domains of ARNO to Phosphoinositides and to Acidic Lipids

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ABSTRACT: The activity on ARF of the guanine nucleotide exchange factor ARNO depends on its membrane recruitment, induced by binding of its PH domain to phosphoinositides. A polycationic C-terminal extension to the PH domain might also contribute to its specific binding to phosphatidylinositol 4,5-bisphosphate [(4,5)PIP2] and to phosphatidylinositol 3,4,5-trisphosphate [(3,4,5)PIP3], and to ionic binding to other acidic lipids. We have analyzed in vitro the relative contributions to phospholipid binding of the PH domain and C-terminal extension by cosedimentation of "PH+C domain" and "nominal PH domain" protein constructs including or not including the polycationic C-terminus, with sucrose-loaded unilamellar vesicles made of equal proportions of the neutral lipids phosphatidylcholine and phosphatidylethanolamine, and supplemented or not with 30% acidic phosphatidylserine (PS) and 2% of various phosphoinositides. Binding was measured as a function of the vesicle concentration and of the medium ionic strength. Both proteins bound with higher affinity to (3,4,5)PIP3 than to (4,5)PIP2, the selectivity for (3,4,5)PIP3 being highest for the nominal PH domain. We observed also a clear selectivity of (3,4,5)PIP3 over (4,5)PIP2 for stimulating the activity of ARNO on ARF with vesicles containing 10% PS and 1% PIP2 or PIP3. Our data suggest that the PH domain provides the specific phosphoinositide binding site and some unspecific ionic interaction with acidic PS, whereas the polybasic C domain contributes to binding mainly by unspecific ionic interactions vith PS. Phosphorylation by protein kinase C of a serine in the C domain reduces the ionic affinity of the PH+C domain for PS, but does not affect the phosphoinositide specificity.

Pleckstrin homology (PH)[1] domains are protein modules of about 110 amino acids, which contribute to membrane recruitment of their host protein by binding ionically to the polar head of monoacidic phospholipids, such as phosphatidylserine (PS), and with higher affinities and various specificities to phosphoinositides, such as phosphatidylinositol 4,5-bisphosphate [(4,5)PIP2] and phosphatidylinositol 3,4,5-trisphosphate [(3,4,5)PIP3] (*1*−*4*). With (3,4,5)PIP3 being mostly produced at the plasma membrane by cell surface receptor-activated PI3-kinases, the specificity of a PH domain for (3,4,5)PIP3 suggests its involvement in a signal transduction process at the plasma membrane. Some PH domains bind also to membrane-associated proteins such as the G$\beta\gamma$ subunit of heterotrimeric G-proteins (*5*), and a few have been reported to bind to phosphorylated tyrosine of transmembrane receptor proteins or cytoskeletal proteins (*6*).

PH domains have poorly conserved primary sequences, but keep a highly conserved tridimensional organization (*7*). The core structure is a β sandwich formed by two nearly orthogonal β sheets of three and four antiparallel strands. A C-terminal α helix packs against one edge of this β sheet structure and stabilizes it. The binding site for inositol polyphosphate is within three of the loops connecting the β strands, on one side of the core. These loops have hypervariable sequences, with scattered lysines, arginines, and histidines which form a positively charged area on the opposite side of the domain to that bearing the C-terminal α helix. This α helix contains a characteristic amphipathic motif with a strictly conserved tryptophan and a consensus sequence extending over eight amino acids past this tryptophan, down to the C-terminal end of the nominal PH domain. This corresponds, in X-ray crystal structure, to the ordered α helix packed against the β sheet core. Sequence analogies between the C-terminal α helix of the PH domain and the "α2′" helix of the "switch II" domain of heterotrimeric G-protein Gα subunits have led to the suggest that, like the α2 helix of Gα, the C-terminal α helix of a PH domain could be a binding site for G$\beta\gamma$ (*8*). Evidence from another PH domain-containing protein, the β-adrenergic receptor kinase 1 (βARK1), suggested that the binding site for G$\beta\gamma$ on the PH domain of βARK1 goes beyond the α helix of the nominal PH domain, and encompasses the C-terminal peptide which extends about 40 residues past the conserved tryptophan (*9*). The NMR solution structure of the PH domain of βARK indicated that the α helical structure extends past the nominal PH core domain, becoming more flexible and mobile toward its end, as a "molten helix" (*10*).

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[1] Abbreviations: ARF, ADP-ribosylation factor; ARNO, ARF nucleotide-binding-site opener; GEF, guanine nucleotide exchange factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; (4,5)PIP2, phosphatidylinositol 4,5-bisphosphate; (3,4,5)PIP3, phosphatidylinositol 3,4,5-trisphosphate; PH domain, pleckstrin homology domain; C domain, polycationic C-terminal domain of ARNO; PKC, protein kinase C.

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A patch of 5 basic residues between the 17th and 26th residues past the conserved W in the PH domain of βARK is seen to strongly modify the surface charge polarity of the inositol phosphate binding site, located within the loops connecting the β strands of the PH core domain. This suggested that this polybasic C domain could also participate in the binding of the PH domain to acidic lipids and phosphoinositides.

ARNO (ARF nucleotide-binding-site opener) is a 47 kDa protein recently identified as a guanine nucleotide exchange factor (GEF) for the small ras-like G-proteins of ADP-ribosylation factor (ARF) subtype (*11*). ARNO consists of a central catalytic "Sec 7" domain responsible for the exchange activity on ARF, surrounded on the amino side by a leucine heptad repeat motif forming a short "coiled-coil" domain, and on the carboxylic side by a PH domain followed by a "Polybasic C-domain". This "C-domain" of ARNO, which extends 33 amino acids past the conserved tryptophan in the PH domain α helix, includes near its C-terminus a polybasic motif, RKKRISVKKK. This is quite similar to the C-terminal extension of the PH domain of βARK (Figure 1).

We had previously shown that, in vitro, the activity of ARNO on ARF requires the presence of lipid vesicles, is sensitive to the presence in these vesicles of acidic lipids such as PS, and is considerably increased upon the addition in the vesicles of a few percent of (4,5)PIP2 (*11*). This sensitivity to PIP2 of the activity of ARNO on ARF should not be mistaken with the direct effect of cationic lipids on the exchange process in ARF: ARF−GDP interacts hydrophobically with the lipid paraffin chains by its N-terminal myristate, and electrostatically with the cationic lipid polar heads by anionic residues proximal to its N-terminus. These interactions can trigger the release of the N-terminal amphipathic helix of ARF from the protein core, and its flipping toward the lipids into which the hydrophobic residues can insert (*12*). The flipping of the N-terminal helix allows a slow but measurable rate of GDP release, hence of spontaneous GDP/GTP exchange in ARF (*13*). But these direct effects of cationic lipids on the nucleotide exchange process in ARF are not specific for PIP2, which acts significantly on ARF only at very high concentrations at which it indeed denatures the protein (*14*).

The sensitivity to PIP2 of the activity of ARNO on ARF clearly originates from the PH domain of ARNO. When ARNO is deleted of its PH domain, its exchange activity on ARF still depends on the presence of acidic lipid vesicles, but is not very sensitive to PIP2 any more: this reflects only the direct ionic action of acidic lipids on ARF discussed above. But complete ARNO with its PH domain is active in the absence of lipids on the mutant (Δ17)ARF which has been deleted of the N-terminal amphipatic helix and the proximal anionic residues, and hence remains totally soluble in the GTP-bound state. The activity of ARNO on (Δ17)-ARF is unaffected by lipid vesicles, by acidic lipids, and by PIP2. This reflects an absence of direct action of acidic lipids on (Δ17)ARF (*15*).

Altogether these data suggested that the functional activity of ARNO on ARF requires, beside the protein−protein interaction of the catalytic Sec7 domain of ARNO with the core domain of ARF (*16*), two distinct protein−phospholipid interactions: one between hydrophobic residues of the N-terminal domain of ARF and the lipid hydrophobic chains, the other between the PH domain of ARNO and acidic phospholipids and specifically phosphoinositides (*15, 17*). Even though we observed in our initial study a high sensitivity to (4,5)PIP2 of the activity of ARNO on ARF (*11*), we suggested that (3,4,5)PIP3 might well be a more potent activator of ARNO. Indeed, Klarlund et al. (*18, 19*) reported that the close homologues of ARNO, Cytohesin1 and GRP1, bind in vitro with higher affinity to (3,4,5)PIP3 than to (4,5)PIP2. In fact, GRP1 had originally been cloned on the basis of its (3,4,5)PIP3 binding (*18*). Recent in vivo studies in adipocyte (*20*) demonstrated that overexpressed ARNO, which localizes mainly in the cytoplasm, translocates to the plasma membrane upon stimulation of the PI3-kinase with insulin. This suggests that in vivo ARNO binds the PI3-kinase product (3,4,5)PIP3 with higher affinity than for its substrate (4,5)PIP2. Furthermore, it has been proposed (*21*) that in Cytohesin 1 the polybasic C-terminal extension of the PH domain contributes not only to its binding to acidic phospholipids but also to its affinity for (3,4,5)PIP3.

In the present work, we reinvestigated in vitro the binding affinities of the PH domain of ARNO for acidic lipids and for various phosphoinositides, and analyzed the contributions of the polybasic C domain to these affinities. We compared the binding of the short "nominal PH domain" and of a "PH+C domain" protein construct including the polycationic C-terminal extension to artificial unilamellar lipid vesicles made of a basis of the neutral, zwitterionic, phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in equal proportions and supplemented or not with 30% of the acidic phospholipid PS and a small proportion (2%) of phosphoinositides. We analyzed the dependence of the protein binding on the total lipid concentration and on the ionic strength of the sedimentation medium. We attempted to correlate the binding affinities of the PH domain for (4,5)-PIP2-, (3,4,5)PIP3-, and PS-doped vesicles with the exchange activity of full-size ARNO on ARF, measured in the presence of the same phospholipid vesicles and under the same ionic conditions. To further analyze the role of the C domain, we reduced its positive charge by phosphorylating by protein kinase C the serine in the polycationic RKKRISVKKK motif, a process that has been shown to occur in vivo (*22*). We studied the effect of this modification on the lipid binding affinities of the extended PH domain.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), egg phosphatidylserine (PS), and 1-stearoyl-2-arachidonoylglycerol (DAG) were purchased from Sigma. Synthetic dipalmitoyl (3,4)PIP2, (3,5)PIP2, (4,5)PIP2, and (3,4,5)PIP3 were from Echelon. Protein kinase C (rat brain) was from Calbiochem. [^{35}S]GTPγS was from NEN Life Science Products and [γ-^{32}P]ATP from ICN.

Protein Expression and Purification. Myristoylated ARF1 was produced in *E. coli* by coexpression with a yeast *N*-myristoyltransferase and separated from the contaminating nonmyristoylated protein by a precipitation at 35% saturation of ammonium sulfate followed by sequential chromatography on DEAE-Sepharose and Mono-S columns, as described previously (*23*).

ARNO was expressed in *E. coli* and isolated by anion exchange chromatography on QAE-Sepharose and gel filtra-

tion on Sephacryl S-100 HR (Pharmacia Biotech Inc.) as described previously (*11*).

ARNO PH+C domain (a.a. 261−399) was cloned in pet11a vector and expressed in BL21 *E. coli* and purified by SP Sepharose chromatography.

ARNO PH domain (a.a. 261−374) was cloned in pTyB4 vector and expressed in fusion with Intein in BL21 *E. coli* (Impact T7 system from Biolabs). The protein was loaded on a chitin column by binding through the Intein−CBP domain; upon cleavage of the Intein−CBP domain by DTT, the released protein was further purified by Mono S-chromatography.

As judged by Coomassie Blue staining after SDS−polyacrylamide gel electrophoresis, the final purity was between 70 and 90% for ARNO and ARF proteins.

Protein Characterization by Gel Filtration. One hundred microliter samples of purified protein solution were loaded on a Superdex S200 XK10/30 gel filtration column (Pharmacia). The column was equilibrated at room temperature in 20 mM Tris/HCl, pH 7.5, with 1 mM $MgCl_2$ and 50, 120, or 400 mM NaCl, and eluted at a flow rate of 0.5 mL/min. Protein absorbance was monitored at 280 nm. Fractions of 0.3 mL were collected and analyzed by SDS−PAGE.

Preparation of Phospholipid Vesicles. Unilamellar phospholipid vesicles were prepared by the extrusion method of Hope et al. (*24*), as described previously (*12*). Solutions of PC, PE, and PS in chloroform, PIP_2 in chloroform/methanol/H_2O (1:1:0.3), and PIP_3 in water were mixed in the required proportion. When phosphoinositides were present, methanol was added to the mixture to 30% of the final volume and ,when required, water did not exceed 15%. The phospholipid concentration of each stock solution was checked by a Bartlett phosphorus assay. A film of phospholipids was formed in a Rotavapor and resupended in 50 mM Hepes, pH 7.5, and 100 mM KCl. The suspension was vortexed for 20 min and then freeze−thawed 5 times. Unilamellar vesicles were produced by extrusion through a 0.1 μm pore size polycarbonate filter (Isopore, Millipore). For sucrose-loaded vesicles to be used at varying external NaCl concentrations, the same procedure was used except that the buffer was 10 mM Tris, pH 7.5, and included 150 mM sucrose, and 0.4 μm pore size polycarbonate filters were used. After extrusion, the sucrose-loaded vesicles were diluted 5 times in 10 mM Tris, pH 7.5, and 50 mM NaCl, centrifuged for 20 min at 400000*g*, and resuspended in the same buffer.

Sedimentation Experiments. ARNO PH+C domain or ARNO PH domain (3 μM) was incubated for 15 min at 25 °C in 75 μL of sucrose-loaded vesicle suspension of the indicated phospholipid composition and concentration, in Tris (10 mM pH 7.5) with NaCl at the concentration indicated, 1 mM DTT, and 1 mM $MgCl_2$ if specified. The tubes were centrifuged for 15 min at 400000*g* and 25 °C. The supernatants were removed, and the pellets were resuspended in 37.5 μL. Both were analyzed by SDS−PAGE and densitometry.

Measurement of the Exchange Activity of ARNO on ARF. The exchange activity of ARNO on ARF was measured as described previously (*15*): 0.1 μM wild-type ARNO was incubated at 37 °C with 1 μM ARF and 10 μM [^{35}S]GTPγS in 50 mM Hepes, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and with 0.4 mg/mL lipid vesicles of the indicated phospholipid composition. At the indicated times, samples of 25 μL (25 pmol of ARF) were removed, diluted into 2 mL of ice-cold stop buffer (20 mM Hepes, pH 7.5, 100 mM NaCl, and 10 mM $MgCl_2$), and filtered on 25 mm BA 85 nitrocellulose filters (Schleicher & Schuell). Filters were washed twice with 2 mL of the same buffer, dried, and counted.

Phosphorylation by PKC. For phosphorylation measurements, ARNO PH domain or PH+C domain (2 μM) was incubated at 37 °C in phosphorylation buffer (50 mM Hepes, pH 7.5, 100 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$) with 20 μM [^{32}P]ATP and 0.4 mg/mL lipid vesicles of the indicated phospholipid composition, including 5% DAG to activate the PKC. The reaction is started upon the addition of 2 nM PKC, and at the indicated times 25 μL samples (50 pmol of protein) were removed, diluted into 2 mL of ice-cold stop buffer, and filtered on 25 mm BA 85 nitrocellulose filters. Filters were washed twice with 2 mL of the same buffer, dried, and counted. For sedimentation experiments with phosphorylated proteins, ARNO PH+C domain (6 μM) was incubated for 15 min at 25 °C in phosphorylation buffer (except that $MgCl_2$ was omitted), with 8 nM PKC, 30 μM [^{32}P]ATP, and lipid vesicles of the indicated compositions and concentrations. The sample was then diluted 2 times in solutions containing various concentrations of NaCl (indicated in figure legends) and treated as described in the sedimentation experiments. The efficiency of phosphorylation was checked by the filtration assay and by autoradiography.

RESULTS

Expression and Purification of ARNO Nominal PH Domain and PH Domain with Its Polybasic C-Terminal Extension (PH+C Domain). To analyze the relative contributions of the PH core domain and of its C-terminal extension to the binding to lipids, we expressed in *E. coli* and purified 2 protein constructs of ARNO PH domain: the nominal PH domain which includes only 8 amino acids past the strictly conserved tryptophan in the C-terminal α helix; it corresponds to the PH domain seen in X-ray crystal structure, with the ordered α helix packed against the β sheet core; the other protein construct, thereafter called PH+C domain, includes the complete 33 amino acid long C-terminal extension of ARNO, with the characteristic polybasic motif RKKRISVKKK (Figure 1). It corresponds roughly to the extended PH domain of βARK whose solution structure has been recently determined by NMR (*10*).

Characterization of the Proteins by Gel Filtration Analysis. To check for possible effects of the NaCl ionic strength, which varied from 50 to 400 mM in our binding studies, on the protein structure, we analyzed the ionic dependence of their elution profiles on a gel filtration column which was calibrated, under the same ionic conditions, with standard globular proteins of approaching molecular masses: cytochrome *c* (12.4 kDa) and carbonic anhydrase (31 kDa).

In 400 mM NaCl, ARNO nominal PH domain eluted in a narrow peak at the elution volume of a 15 ± 1 kDa globular protein. This suggests a compact folded structure for this 13.6 kDa protein. Lowering the NaCl concentration down to 50 mM did not change significantly the elution volume of the PH domain, nor that of the carbonic anhydrase standard, indicating that the PH domain structure is not much

FIGURE 1: PH domain and polybasic C domain of ARNO compared to that of β-adrenergic receptor kinase βARK1. The PH domain folds of ARNO and βARK1 differ only by a large insert in the loop between the sixth and seventh β sheet strands. The PH core domains (PH domain) extend 8 residues past the strictly conserved tryptophan (*) in the C-terminal α helix. The polycationic C extension (C domain) of ARNO is slightly longer than that of βARK1; both include near their C-terminus a cluster of basic residues. In ARNO, a PKC phosphorylatable serine (underlined) is located in the middle of the polybasic cluster. In βARK1, the C-terminal α helix of the PH core domain continues as a molten α helix (*aaaaaa*) in the proximal part of the C domain.

affected by ionic strength in the range from 50 to 400 mM NaCl. However, a significant retardation of the cytochrome *c* standard peak was observable in 50 mM NaCl. It was most probably due to residual ionic interaction of cytochrome *c* with the column support which is recommended by the manufacturer to be used with 150 mM NaCl or KCl.

The PH+C domain eluted also as a narrow peak in 400 mM NaCl, but with the apparent size of a 25 ± 1 kDa globular protein, somewhat larger than expected for a 16.4 kDa folded protein. This is probably due to the unfolded C domain extending away from the PH domain core. In 50 mM NaCl, a large retardation was observed for the PH+C domain elution peak, as for the cytochrome *c* standard (see above). This was most probably due to residual ionic interactions of the polycationic C extension with the column support, and precluded a size estimate at this ionic strength. But at intermediate 120 mM NaCl concentration, the elution volumes for the PH+C domain and for the standard proteins were very close to those observed at 400 mM NaCl. Thus, given the limitations of the gel filtration technique at low NaCl concentrations, we are confident that the structure of the PH+C domain is also not much affected in the range of ionic strength used in this study.

Sedimentation Analysis of the Binding of PH Domain and PH+C Domain to Lipid Vesicles: Dependence on Lipid Composition, on Total Lipid Concentration, and on NaCl Concentration in the Medium. We assessed the interaction of the protein domains with lipids by monitoring their cosedimentation with sucrose-loaded vesicles of defined lipid composition. The basal lipid components of the vesicles were the neutral phospholipids PC and PE in equal quantities. Preliminary tests had indicated that the binding of PH domains depended on the presence in the vesicles of monoacidic phospholipids and was very sensitive to small additions of the diphosphoinositide (4,5)PIP2. Thus, for our first assays, the neutral lipids were supplemented with 30% PS, the most common acidic lipid in the cytoplasmic leaflet of cell membranes, and 2% (4,5)PIP2. All the lipids were homogeneously mixed before the formation of unilamellar vesicles by the extrusion technique (see Materials and Methods). The buffer contained only NaCl as monovalent ions, at the indicated concentration, and no divalent ion. Upon sedimentation, the proportions of vesicle-bound protein in the pellet and of soluble protein in the supernatant were determined by SDS−PAGE (Figure 2A).

The binding of PH domain and of PH+C domain to standard vesicles containing 30% PS and 2% (4,5)PIP2 was first studied as a function of the total lipid concentration, at a fixed NaCl concentration in the buffer (Figure 2B). The concentrations of lipids required to ensure 50% binding of the proteins to the vesicles provide estimates of the apparent affinities of PH domain and PH+C domain for membrane lipids of this composition, at this NaCl concentration. Figure 2B shows that, in 120 mM NaCl, the affinity of the PH+C domain for vesicles including 30% PS and 2% (4,5)PIP2 is about 7-fold higher than the affinity for the same vesicles of the nominal PH core domain. The range of lipid concentrations, and thus of affinities of PH domains for lipids, that can be studied with this technique is, however, limited in practice by the size of the lipid pellet to be sedimented and collected, and the distortion of SDS gels by excess lipids.

Alternatively, the binding was studied as a function of the NaCl concentration in the medium, at a fixed total lipid concentration (Figure 2C). Preliminary tests had indicated that the binding of both protein domains to these acidic lipid vesicles was very sensitive to the ionic strength of the suspension medium, and could be totally suppressed by a few hundred millimolar NaCl. This is characteristic of an exclusive contribution to the binding of electrostatic interac-

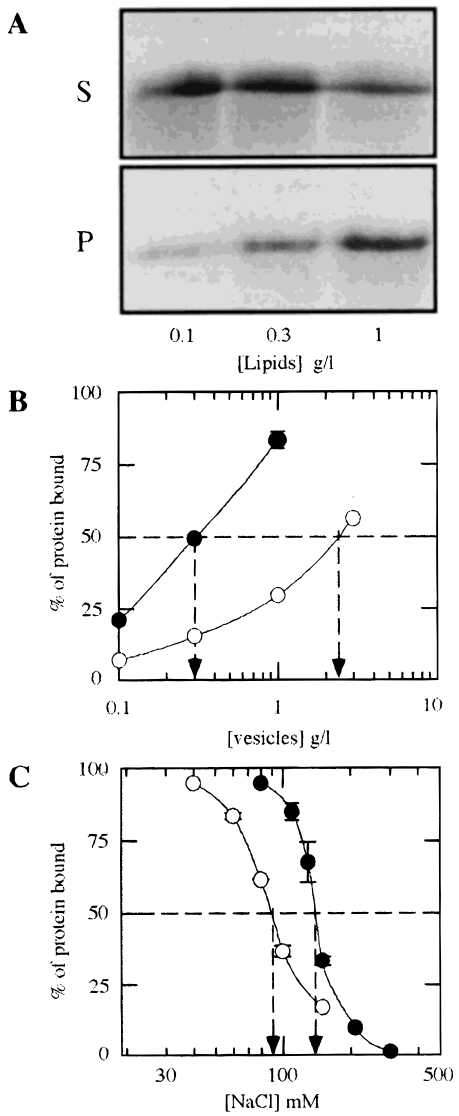

FIGURE 2: Sedimentation analysis of the binding of ARNO PH domain and PH+C domain to phospholipid vesicles. ARNO PH domain or PH+C domain (3 μM) was incubated with sucrose-loaded unilamellar phospholipid vesicles made of equal proportions of the neutral phospholipids PC and PE, 30% PS, and 2% (4,5)-PIP2, suspended in 10 mM Tris buffer (pH 7.5) with 1 mM DTT and supplemented with NaCl as indicated. After sedimentation, the proportions of protein in the lipid pellets and in the supernatants were determined by SDS−PAGE. The binding was analyzed as a function of the concentration of lipids in suspension in a medium containing a fixed NaCl concentration, and as a function of the NaCl concentration at a fixed concentration of lipids in suspension. The same volumes of supernatant and pellet fractions were analyzed, but the pellet fraction was twice as concentrated as the supernatant (see Materials and Methods). (A) SDS−PAGE of PH+C domain in supernatant and pellet of vesicle suspensions at the indicated total lipid concentrations, in buffer with 120 mM NaCl. (B) Binding to the lipid vesicles of PH domain (○) and of PH+C domain (●), as a function of the total lipid concentration, in buffer with a fixed 120 mM NaCl concentration. The binding affinities of PH domain and PH+C domain for lipid vesicles of this composition, in this ionic medium, are related to the lipid vesicle concentrations required to bind 50% of the proteins (arrows). (C) Binding to the lipid vesicles of PH domain (○) and of PH+C domain (●) as a function of NaCl concentration at a fixed (1 g/L) lipid concentration. The experiments have been duplicated; the data points are mean values, and the error bars indicate the two extreme values. The electrostatic binding strengths of PH and PH+C domains for these lipid vesicles can be evaluated from the NaCl concentrations required to dissociate 50% of the proteins from the vesicles at 1 g/L lipid (arrows).

tions of cationic residues of the protein with the anionic lipid polar heads. McLaughlin and colleagues (*25*) have analyzed experimentally and modelized the binding of basic peptides to membranes containing acidic lipids; they observed that the electrostatic binding energy decreases approximately linearly with the log of the NaCl ionic strength of the suspension medium. Thus, the analysis of the dependence of binding of the proteins to the vesicles on the NaCl concentration at a fixed lipid concentration is another way to assess the strength of their electrostatic interactions with these lipids, and thus their apparent affinities for these lipids. The values of [NaCl] that ensure 50% dissociation of PH domain and PH+C domain from the vesicles provide estimates of their binding energy that are equivalent to estimates of their binding affinities. As shown in Figure 2C, at a fixed lipid concentration of 1 mg/mL, the NaCl concentration required to dissociate 50% of the protein is higher for the PH+C domain than for the nominal PH domain. This increase of electrostatic binding to lipids for the PH+C domain compared to the nominal PH domain correlates well with the increase in lipid affinity observed for the same PH+C domain, compared to the PH domain alone, by varying the lipid concentration, in Figure 2B. As in vesicle sedimentation studies, varying the NaCl concentration over a wide range was practically much easier than varying the lipid concentration; this technique of [NaCl] variation was mostly used in subsequent studies of lipid binding specificities.

Phosphoinositide Binding Specificities of PH+C Domain to Lipid Vesicles. We first assessed the phosphoinositide binding specificity of the extended PH+C domain by monitoring its binding, as a function of NaCl concentration, to acidic lipid vesicles made of neutral PC and PE in equal amounts, 30% PS and supplemented or not with 2% of the different tested phosphoinositides: (3,4)PIP2, (3,5)PIP2, (4,5)PIP2, or (3,4,5)PIP3 (Figure 3A). The values of [NaCl] that induced 50% dissociation from the various vesicles with the same percentage of the different phosphoinositides provide a scale of relative electrostatic binding strength, and thus of phosphoinositide binding specificity of the PH+C domain (Figure 3B). The highest increment of electrostatic binding was observed with (3,4,5)PIP3. Among the diphosphoinositide isomers, (3,4)PIP2 and (4,5)PIP2 both induced the same significant increase of electrostatic binding; by contrast, at the same 2% concentration, (3,5)PIP2 did not contribute a significant increase of electrostatic interaction above that already observed with only 30% acidic PS in the vesicles. Thus, in the presence of PS (normally present in the cytoplasmic leaflet of membranes), the extended PH+C domain shows a clear binding specificity for (3,4,5)PIP3 over (4,5)PIP2 or (3,4)PIP2.

Comparison of the Binding of Nominal PH Domain and of PH+C Domain to Lipid Vesicles: Dependences on PS and on (4,5)PIP2 or (3,4,5)PIP3. We next studied, by the same technique of [NaCl] variation at a fixed total lipid concentration, the binding of PH domain and PH+C domain to vesicles of various lipid compositions: neutral lipids PE and PC, in equal amounts, supplemented or not with 30% PS, and 2% of (4,5)PIP2 or (3,4,5)PIP3 (Figure 4). The comparison of the electrostatic binding strengths, that, is the [NaCl] for 50% dissociation of PH domain and PH+C domain, respectively, for the same vesicle composition,

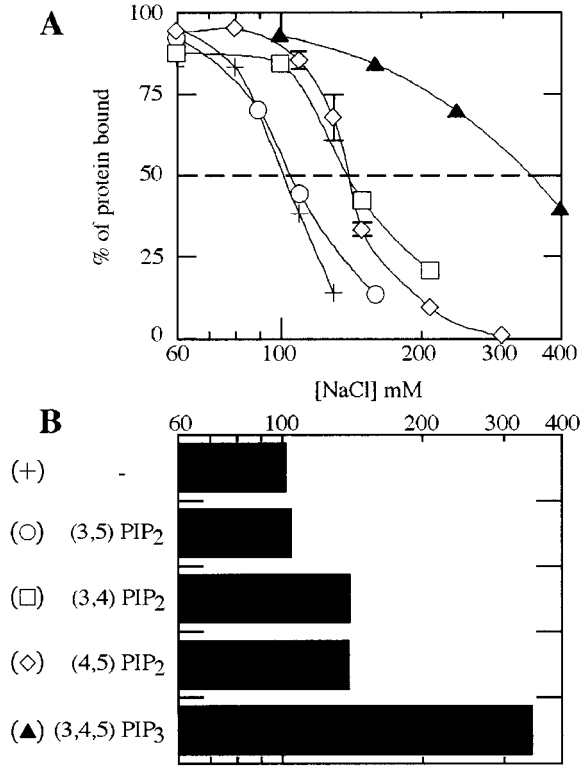

FIGURE 3: Phosphoinositide binding specificity of complete PH+C domain. Binding of complete PH+C domain (3 μM) to vesicles made of equal proportions of neutral phospholipids PC and PE, 30% PS, and supplemented with 2% of various phosphoinositides, as indicated. The vesicles were in suspension in 10 mM Tris buffer (pH 7.5), 1 mM DTT, and increasing concentrations of NaCl. (A) Binding as a function of NaCl concentration at a fixed (1 g/L) total lipid concentration, as in Figure 2C. Error bars (see Figure 2C) are shown only for (4,5)PIP2, not to confuse the picture. (B) Electrostatic binding affinities of PH+C domain for the various phosphoinositides, expressed as the NaCl concentrations inducing 50% dissociation of PH+C domain from the vesicle pellet, as estimated from panel A.

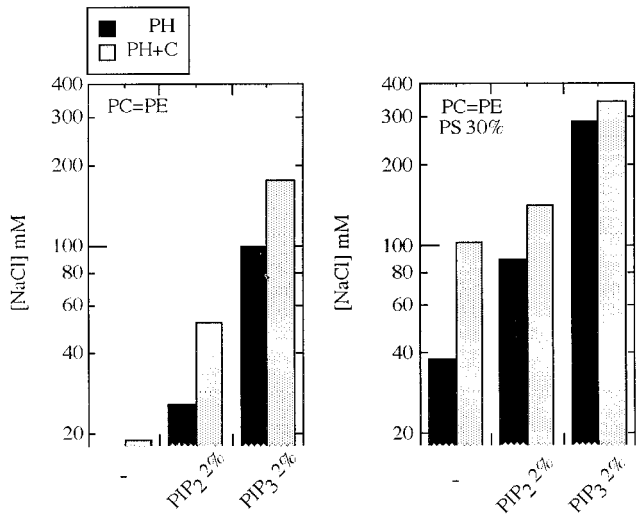

FIGURE 4: Contributions of PS, PIP2, and PIP3 to the [NaCl]-dependent electrostatic binding of PH domain and PH+C domain to vesicles. Same conditions as in Figure 3. The binding of PH core domain and of PH+C domain was analyzed as a function of NaCl concentration at a fixed (1 g/L) concentration of lipid vesicles in suspension in 10 mM Tris buffer (pH 7.5), 1 mM DTT, and increasing concentrations of NaCl. The vesicles were made of equal proportions of the neutral phospholipids PC and PE and supplemented with 2% (4,5)PIP2 or 2% (3,4,5)PIP3 (left panel), or further supplemented with 30% PS (right panel). The NaCl concentrations inducing 50% dissociation of PH domain and of PH+C domain from the same vesicles monitor the relative strengths of their electrostatic binding to the given lipid mixture (see Materials and Methods and Figure 2 legend). Comparisons of all these data suggest that the PH domain core has high affinity for phosphoinositides and binds tighter to PIP3 than to PIP2 and that the C domain provides mainly affinity for PS. Contributions of the 10 mM Tris buffer to the ionic strength limit the significance of the data below 20 mM NaCl concentration. See text for discussion.

provides estimates of the relative contributions of the PH domain and C domain to the affinity of the complete PH+C domain for vesicles with this lipid composition. The variation of binding with the lipid composition provides estimates of the relative contributions to the binding of the different types of lipids. For example, Figure 4 shows that the contribution of PS to the binding of the PH+C domain may be important, as compared to that of PIP2, taking into account the relative natural abundance of these two phospholipids: the addition of 30% PS to neutral (PC+PE) lipid vesicles increases the NaCl concentration required for 50% dissociation of the PH+C domain from less than 20 mM up to above 100 mM; by comparison, the addition of 2% PIP2 increases the 50% dissociating NaCl concentration only up to 50 mM.

The binding of PH+C domain to PS is mostly due to the C domain, as shown by the ~2.5-fold difference in binding strength to PS between PH and PH+C domains (two first bars in Figure 4, right panel). Replacement of PIP2 by PIP3 increased the binding strength of the PH domain by a factor of 4 in the absence of PS (Figure 4, left panel) and only 3 in the presence of 30% PS (Figure 4, right panel). For the complete PH+C domain, the increase was 3.5-fold in the absence of PS and 2.5-fold in the presence of PS.

Thus, the highest selectivity for PIP3 over PIP2 was observed with the nominal PH domain in the absence of PS. Both the addition of PS and the addition of the C domain attenuated this selectivity. These results therefore strongly suggest that the specific binding site for phosphoinositides is in the nominal PH domain. The polybasic C domain makes a major contribution to unspecific electrostatic interactions of the PH+C domain with acidic phospholipids, but does not contribute to the specific binding to PIP2 or PIP3.

Phosphoinositide Binding Specificity of PH Domain and PH+C Domain in the Presence of Magnesium. The above studies of binding of PH domain and PH+C domain of ARNO to acidic lipids and phophoinositides had been performed in buffers devoid of magnesium and other divalent cations, by analogy with previously published studies of binding of basic peptides to acidic lipid vesicles (*25*, *26*) and of binding of PH domains to phosphoinositides (*27*, *28*). A major goal of the present study was to correlate the phosphoinositide-induced membrane binding of the PH domain of ARNO to the phosphoinositide stimulation of the GDP/GTP exchange activity of ARNO on its natural substrate ARF. But ARF is unstable in the absence of magnesium, which is required at about 1 mM concentration, close to the cytosolic concentration in situ, to stabilize the binding of GDP or GTP in the nucleotide site. We thus had to check whether the presence of 1 mM Mg^{2+} affected notably the [NaCl] dependence of the binding of PH domain and PH+C domain of ARNO to acidic lipids and phosphoinositides. To our surprise, these effects were found important (Figure 5): The addition of 1 mM magnesium reduced about

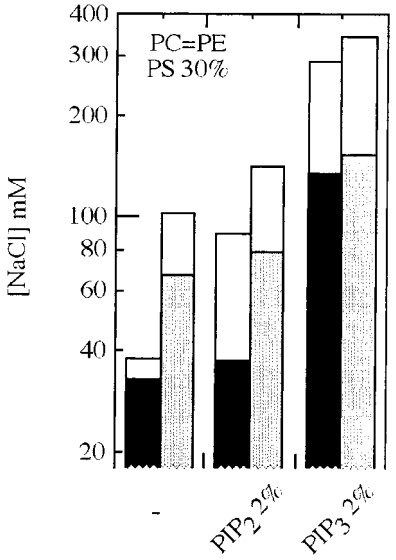

FIGURE 5: Effect of magnesium on the [NaCl]-dependent binding of PH domain and PH+C domain to PS, PIP2, and PIP3. Same experiments as in Figure 4, right panel, but with 1 mM $MgCl_2$ added in the buffer. The blank bars reproduce the data without magnesium of Figure 4, right panel. The presence of 1 mM does not affect much the binding of the PH core domain to vesicles containing only PS, but by contrast reduces notably the binding of the PH core domain to vesicles supplemented with PIP2 or PIP3; this latter effect can account entirely for the comparable reduction observed for PH+C domain binding to PIP2 and PIP3. Thus, 1 mM $MgCl_2$ inhibits notably the specific binding of the PH core domain to phosphoinositides, and less its unspecific electrostatic binding to PS. The apparent selectivity of the complete PH+C domain for PIP3 over PIP2 is conserved.

by half the NaCl concentrations that are required to dissociate PH+C domains from vesicles containing 30% PS and 2% (4,5)PIP2 or 2% (3,4,5)PIP3. The same effect of magnesium was observed on the binding to these vesicles of PH domain alone. This suggested that the main effect of magnesium was on the nominal PH domain and was a reduction of the ionic interaction of the PH domain specific binding sites for the inositol polyphosphates. Indeed, in the absence of phosphoinositides, millimolar magnesium hardly affected the binding of PH domain alone to vesicles containing only PS, and diminished only by 30% the electrostatic binding of PH+C domains to PS. These results are consistent with the old observation of Toner et al. (*29*) that Mg^{2+} significantly binds to PIP2 but not to PS. Overall, the addition of 1 mM magnesium reduced notably the binding affinities of the complete PH+C domain for the inositol polyphosphate heads of phosphoinositides, down to a point where the contribution of 2% (4,5)PIP2 binding specifically to the PH domain became barely detectable over that of 30% PS binding unspecifically to the PH and C domains.

Phosphoinositide Specificity of the Exchange Activity of ARNO on ARF. The exchange activity of ARNO on ARF was analyzed by monitoring the rate of ARNO-catalyzed [^{35}S]GTPγS binding to ARF_{GDP} in the presence of lipid vesicles under conditions similar or comparable to those of the binding studies, that is, with vesicles made of equal quantities of neutral PC and PE, and supplemented or not with PS and PIP2 or PIP3, in a medium containing 100 mM KCl and 1 mM $MgCl_2$. As observed in our previous work (*11*), addition of 2% (4,5)PIP2 to the vesicle lipids already increased considerably the activity of ARNO on ARF.

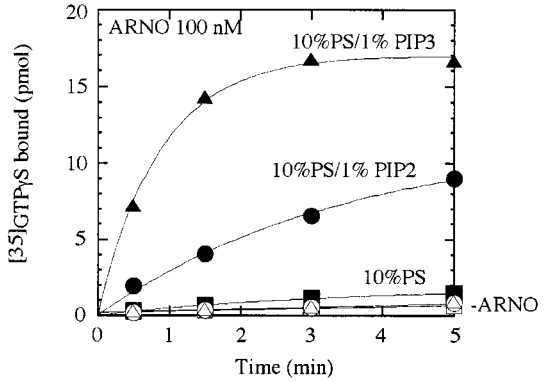

FIGURE 6: Stimulation by PS, PIP2, and PIP3 of the activity of ARNO on ARF. Stimulation by ARNO of [^{35}S]GTPγS binding in ARF was measured as indicated under Materials and Methods, in the presence of phospholipid vesicles made of equal proportions of PC and PE and supplemented with the indicated proportions of PS, PIP2, or PIP3. In this assay, stimulation by 1% PIP3 was 6 times that observed by 1% PIP2.

Indeed, under our standard conditions for the binding assays, that is, 1 mg/mL lipids with 30% PS and 2% phosphoinositides, the activity of ARNO was found nearly as high with (4,5)PIP2 as with (3,4,5)PIP3 (data not shown). It is only with reduced proportions of PS and phosphoinositides (e.g., 10% PS and 1% phosphoinositides) that a clear specificity for PIP3 over PIP2 could be observed for the activity of ARNO (Figure 6). Klarlund et al. (*19*) similarly observed that PIP3 does not cause preferential increase of the activity of GRP1, a close homologue of ARNO, in vesicles with a high negative charge.

Reduction of the Binding Affinity of ARNO PH+C Domain for Acidic Lipids upon Phosphorylation by PKC of a Serine in the C Domain. PH+C and nominal PH domains of ARNO were incubated with PKC in the presence of lipid vesicles made of equal quantities of PC and PE, 20% PS, 5% PIP2, and 5% diacylglycerol as required to activate the PKC. Insertion of approximately one phosphate per PH+C domain was obtained, and no phosphorylation was detected under the same conditions on the PH domain alone (Figure 7A). This confirms that the phosphorylation site is in the C domain; it is most probably the serine that is located in the middle of the polycationic motif RKKRISVKKK, as previously reported by Frank et al. (*22*). We studied the effect of this phosphorylation on the binding of PH+C domain to lipids: Phosphorylation reduced very significantly the strength of electrostatic binding of PH+C domain to vesicles with PS and PIP2, but reduced by approximately the same factor the binding to vesicles with only PS (Figure 7B). Thus, the phosphorylation perturbs only the ionic interaction of the polycationic C domain with PS. This further suggested that the polycationic C domain contributes mainly to unspecific ionic interaction with PS, and not to specific interactions with phosphoinositides. In our hands, phosphorylation of ARNO by PKC did not affect significantly its activity on ARF in vitro, in vesicles containing 20% PS and 2 or 5% (4,5)PIP2 (data not shown).

DISCUSSION

We originally observed (*11*) that in vitro the activity of ARNO on ARF1 was dependent on the presence of acidic lipid vesicles and was greatly enhanced upon addition in

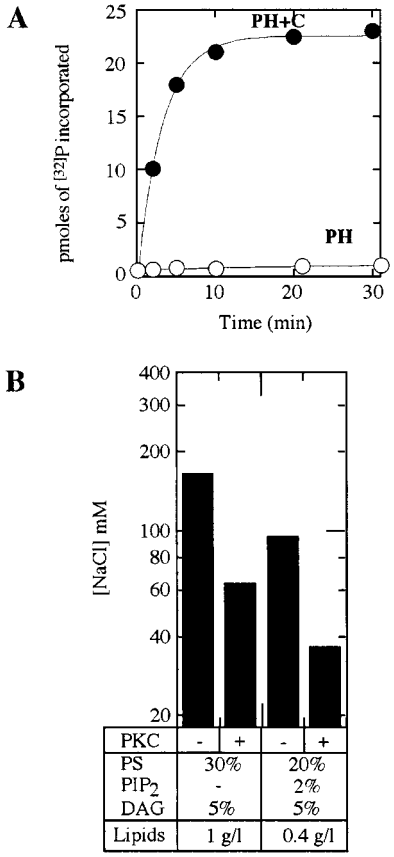

FIGURE 7: Reduction of the binding of PH+C domain to PS upon phosphorylation by PKC of a serine in the C domain. (A) Phosphorylation by PKC of one serine in the PH+C domain, and none in the PH core domain, in the presence of lipid vesicles (35% PC, 35% PE, 20% PS, 5% PIP2, and 5% DAG) required to activate the PKC. If PIP2 was omitted, the time course of the phosphorylation was slowed by ~3-fold, but the plateau was unchanged. (B) Reduction by serine phosphorylation by PKC of the [NaCl]-dependent electrostatic binding (see Figure 4) of PH+C domains to lipid vesicles made of equal proportions of PC and PE, 5% DAG with 30% PS (left, 1 mg/mL total lipids), or with 20% PS and 2% PIP2 (right, 0.4 mg/mL total lipids).

these vesicles of a few percent (4,5)PIP2. Later works indicated that in vitro close homologues of ARNO bind with higher affinity to (3,4,5)PIP3 than to (4,5)PIP2 (*18*, *19*) and in vivo a GFP−ARNO fusion protein translocates to the plasma membrane upon the activation of PI3-kinase (*20*). Another work suggested that the adjacent polybasic C domain cooperates with the nominal PH domain to increase its affinity for (3,4,5)PIP3 (*21*). Here we have reanalyzed in vitro the binding of the nominal PH domain and of the PH+C domain, that is, the PH domain supplemented with the polybasic C domain, to vesicles made of equal amounts of neutral phospholipids, PC and PE, and supplemented or not with a large proportion, 30%, of the acidic phospholipid PS, and small proportions, 2%, of various phosphoinositides. Having observed that in all cases the binding was very sensitive to the ionic strength of the medium and could be totally suppressed by high concentrations of NaCl, we assessed the strength of this purely electrostatic binding from its resistance to dissociation by increasing NaCl concentrations in the suspension medium, keeping the vesicle lipid concentration constant, according to the technique introduced by S. McLaughlin (*25*). Given that the binding is strictly electrostatic, this is equivalent to the classical affinity measurement that would be obtained by monitoring the binding as a function of the lipid concentration in a medium of fixed ionic strength.

Our results first confirm that the complete PH+C domain of ARNO binds with higher affinity to (3,4,5)PIP3 than to (4,5) PIP2. We observed a stereospecificity among the PIP2 isomers: (3,4)PIP2 binds with the same affinity as (4,5)-PIP2, but (3,5)PIP2 binds with a much lower affinity, of the same order of magnitude as that for an acidic lipid like PS (Figure 3). This indicates that the phosphoinositol binding site of the PH domain of ARNO specifically recognizes two neighbor monoesterified phosphates, but not two distant monoesterified phosphates. Accordingly, (3,5)PIP2 should bind only by unspecific electrostatic interactions such as any acidic phospholipid. Moreover, our results clearly show that the PH domain of ARNO does not exclusively recognize 3-phosphorylated inositol derivatives, in contrast to the PH domains of protein kinase B and of its activating kinase PDK1 (*30*).

Our results further demonstrate that the specific binding site for phosphoinositides is located exclusively on the nominal PH domain. The polybasic C domain largely contributes to electrostatic interactions between the PH+C domain and negative phospholipids, but in an unspecific manner. It reinforces the binding of the PH domain as well to PS as to PIP2 or PIP3, with no contribution to phosphoinositide selectivity. In fact, the nonspecific interactions between PH and C domains with PS tend to attenuate the apparent selectivity of the PH domain for PIP3 over PIP2. The importance of these nonspecific interactions with PS should not be underestimated, given the abundance of this acidic phospholipid in eukaryotic cell membranes (it may represent up to 30% of the phospholipids on the cytoplasmic leaflet of membranes).

Thus, our conclusions slightly differ from those of Nagel et al. (*21*), who interpreted their observation that the C domain of cytohesin-1 cooperates with the PH domain for binding PIP3 as evidence for a stabilizing role of the C domain on the phosphoinositide binding site.

It should be noted that in our studies the selectivity for PIP3 over PIP2 is at best by a factor of 4. A much higher selectivity ($\geq$100) has been observed for the PH domain of ARNO or close homologues, when using an assay measuring the binding of soluble inositol phosphates, corresponding to the polar heads of the various phosphoinositides (*20*, *28*). This emphasizes the importance of the membrane surface for the binding of PH domains, in addition to the major role played by surrounding acidic phospholipids such as PS. But it would be hazardous to try to correlate quantitatively the affinities of the PH domains for inositol phosphates in solution with their binding to membrane vesicles into which the corresponding phosphoinositides are integrated.

We also observed a specificity for PIP3 over PIP2 for the nucleotide exchange activity for full-length ARNO on ARF, but this difference was most obvious at low PS and phosphoinositide concentrations. The selectivity was greatly attenuated at 30% PS.

Thus, given the possible high local concentrations of PS in membranes, and the relative abundance of (4,5)PIP2 as compared to the transient signaling species (3,4,5)PIP3, our results altogether question the absolute dependence of ARNO activation on PI3-kinase signaling, that has been inferred

from a number of recent studies on ARNO and homologues (*19*, *20*, *28*). It should be noted, however, that, in contrast to our artificial vesicles, natural membranes may contain other PIP2 binding proteins that could compete with ARNO for the binding to PIP2 more than to PIP3.

The observation that phosphorylation by PKC of the serine in the polycationic motif RKKRISVKKK of the C domain decreases the electrostatic binding of the PH+C domain to PS, but does not seem to perturb its specific binding to phosphoinositides, further strenghtens the suggestion that the C domain contributes mainly to the unspecific ionic binding of ARNO to constitutive acidic phospholipids such as PS rather than to its specific binding to phosphoinositides. Whether this phosphorylation regulates the activity of ARNO remains an open question because Santy et al. (*31*) recently reported an inhibitory effect of PKC-mediated phosphorylation on ARNO activity, whereas we have been unable to detect a significant effect.

Obviously, a clear definition of the exact roles of PI3-kinase and PKC in activation and regulation of ARNO must await the development of an in vivo assay for ARNO activity.

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